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Diversity and Phylogenetic Position of *Bothrostoma* Stokes, 1887 (Ciliophora: Metopida), with Description of Four New Species



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Bothrostoma is a genus of anaerobic ciliates in family Metopidae comprising four species, all described based solely on the morphology of living and fixed cells. Unlike other metopids, cells of *Bothrostoma* are not twisted anteriorly, have a flattened preoral dome, a very prominent sail-like paroral membrane, and an adoral zone of distinctive, very narrow, curved membranelles confined to a wide, non-spiraling peristome on the ventral side. We examined 20 populations of *Bothrostoma* from hypoxic freshwater sediments. We provide morphological characterization and 18S rRNA gene sequences of four new species, namely *B. bimicronucleatum* sp. nov., *B. boreale* sp. nov., *B. kovalyovi* sp. nov., and *B. robustum* sp. nov., as well as *B. undulans* (type species), *B. nasutum*, and *B. ovale* comb. nov. (original combination *Metopus undulans* var. *ovalis* Kahl, 1932). Except for *B. nasutum*, *Bothrostoma* species show low genetic variability among geographically distant populations. Intraspecific phenotypic variability might be driven by environmental conditions. In phylogenetic analyses, *Bothrostoma* is not closely related to *Metopus* sensu stricto and forms a moderately supported clade with *Planometopus*, here referred to as BoPI clade. The anterior axial torsion of the body, typical of other Metopidae, appears to have been lost in the last common ancestor of the BoPI clade.

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Introduction

The order Metopida Jankowski, 1980 consists of a diverse group of mainly free-living ciliates thriving in anoxic/hypoxic environments. Although many metopid species have been known for more than a century, the majority were studied based solely on observations of living or unstained-fixed cells, and most were assigned to the genus *Metopus* Claparède and Lachmann, 1858 (Jankowski 1964a, b, 2007; Kahl 1927, 1932). However, the morphological heterogeneity of *Metopus* species led to the establishment of several new genera (Bourland et al. 2020; Jankowski 1964a, 2007; Lynn 2008). The application of protargol impregnation, scanning electron microscopy (SEM), and methods of molecular phylogenetic (primarily based on the 18S rRNA gene) have clarified the status of some metopid species during the last decade. This has resulted in the erection of multiple new species, genera, and families in Metopida (Bourland et al. 2014, 2017a, b, 2018a, b, 2020; Bourland and Wendell 2014; Foissner 2016a, b; Li et al. 2021; Omar et al. 2017; da Paiva et al. 2013; Rotterová et al. 2018a, b; Silva-Neto et al. 2016; Vd'áčný and Foissner 2017a, b; Zhuang et al. 2021).

Currently, Metopida comprises three families: Metopidae Kahl, 1927, Apometopidae Foissner, 2016, and Tropidoatractidae Rotterová, Bourland, and Čepička, 2018. Genera of the largest family, Metopidae, are mainly characterized by leftward axial torsion (with respect to the cell) of the anterior body part and a five-rowed perizonal stripe of dikinetids following the margin of the preoral dome (Bourland et al. 2017a; Jankowski 2007; Lynn 2008; Rotterová et al. 2018a). Metopidae contains several genera and species with uncertain phylogenetic positions and poorly characterized morphology (Adl et al. 2019; Bourland and Wendell 2014; Bourland et al. 2014, 2017a, b, 2018a, b, 2020; Foissner 2016b; Li et al. 2021; Rotterová et al. 2018a). One such genus is *Bothrostoma* Stokes, 1887.

The original description of the genus *Bothrostoma* was based solely on observations of living cells of the type species, *B. undulans* Stokes, 1887. Mermod (1914) transferred *Bothrostoma undulans* to the genus *Blepharisma* Perty, 1852, and subsequently, Kahl (1927) assigned the species to *Metopus*. Jankowski (1964a) partially revised the taxonomy of metopids, resurrecting the genus *Bothrostoma*, based on morphological differences between *Bothrostoma* and *Metopus* species.

Bothrostoma is not twisted anteriorly, has a more or less flattened preoral dome, possesses a very prominent paroral membrane, and has an adoral zone, comprising long slender membranelles (polykinetids), in a wide, shallow peristomial area confined to the ventral side of the cell (Foissner et al. 1992; Jankowski 1964a, b, 2007; Kahl 1927, 1932; Stokes 1887). Subsequently, three additional *Bothrostoma* species were described based exclusively on living specimens: *B. nasutum* (Cunha, 1915) Jankowski, 1964 (specific name suffix changed to agree in gender with the genus name, which is neuter, Art. 30.1.1 [International Commission on Zoological Nomenclature 1999]), *B. extantum* (Kahl, 1926) Jankowski, 1964, and *B. mirabilis* (Kahl, 1927) Jankowski, 1964 (Cunha 1915, 1916; Jankowski 1964a, b; Kahl 1926, 1927, 1932). No sequence data for any *Bothrostoma* species have been previously published and the phylogenetic position of the genus has been unknown until now.

This study aims to reveal the morphological and molecular characterization of *Bothrostoma* and to elucidate its phylogenetic position within Metopida.

Results

General Morphological Features of *Bothrostoma* Stokes, 1887

We studied 20 populations that belong to the genus *Bothrostoma* based on morphology (Figs 1–14; Table 1). Morphological features shared by all *Bothrostoma* species include the following: a narrow to broadly elliptical cell shape, anterior end terminating in a “proboscis” or “snout” of varying shape and length (absent in *B. kovalyovi* sp. nov., see below), and a truncate to broadly rounded posterior end. Leftward anterior axial torsion of the cell, so typical of other metopids, is absent and the preoral dome is flattened as opposed to convex or vaulted. Cells are usually colorless but, may appear brownish depending on the content in the food vacuoles. All species have a single ellipsoidal to reniform macronucleus, often irregular in protargol preparations, always confined to the anterior half of the cell, and usually with numerous prominent nucleoli in vivo. Except for *B. bimicronuclatum* sp. nov., there is a single globular micronucleus either adjacent to the ellipsoidal macronucleus or in the hilum of the reniform macronucleus. The contractile vacuole is terminal, rounded rectangular to trapezoidal, and large relative to the cell size. The terminal slit-like cytoproct (usually seen only in SEM preparations;

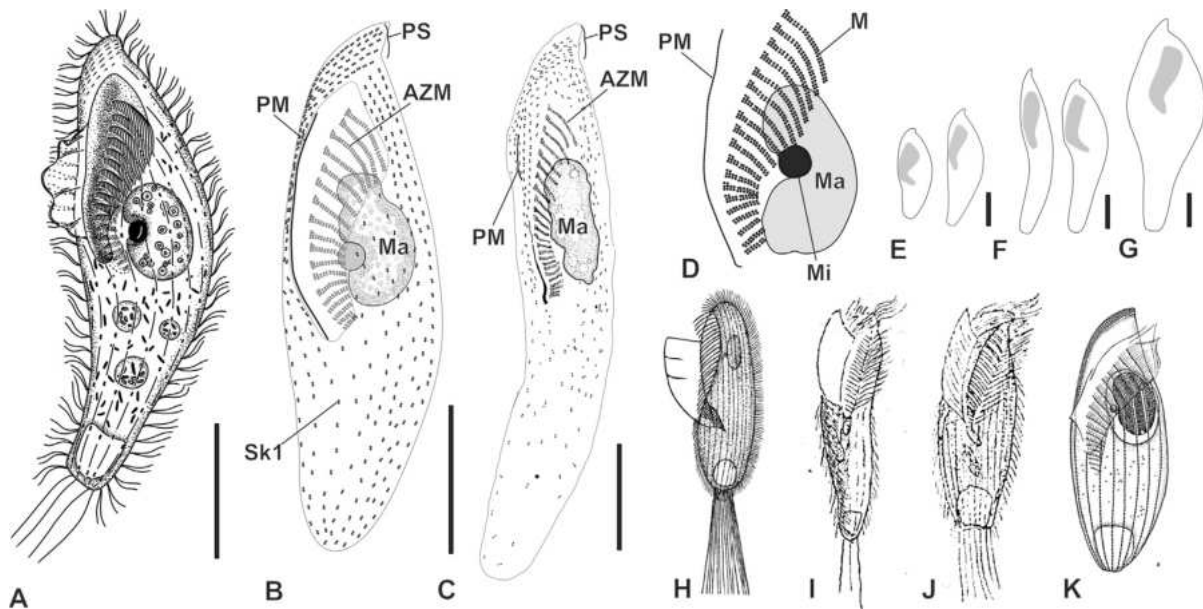


Figure 1. (A–K) *Bothrostoma undulans* from life (A, E–J), after mercuric chloride fixation (K), and after protargol impregnation (B, C, D). (A) Ventral view of a typical trophont. (B, C) Ventral view showing the infraciliature of a typical trophont (B) and a slender trophont (C). (D) Adoral zone of membranelles and nuclear apparatus. (E–G) Different morphotypes, typical trophonts (E), slender trophonts (F), and giant trophont (G). (H) After Stokes (1887). (I, J) After Kahl (1927, 1932). (K) After Jankowski (1964b). Scale bars: 20 µm.

Fig. 14M) is shared as an excretory pore with the contractile vacuole. All species have a slightly flexible cortex with ordinarily-spaced ciliary rows separated by flattened cortical strips. Colorless spherical cortical granules, probably mucocysts, about 0.5 µm in diameter, form distinct, oblique rows on the undersurface of the peristome, interkinetal discontinuous rows, and also small clusters within ciliary rows (Figs 9D, 13C). All strains, except *B. bimicronucleatum* sp. nov., were studied under UV fluorescence and showed autofluorescent rod-shaped prokaryotic symbionts in the cytoplasm (most likely methanogenic Archaea) (Figs 2F, 4M, 5K, 12I, 14K). Although *B. bimicronucleatum* sp. nov. was not examined by fluorescence microscopy we observed rod-shaped prokaryotes scattered in the cytoplasm, probably also methanogens (Fig. 8D).

Ciliary rows are longitudinal, ordinarily spaced, and composed of dikinetids, often with only the posterior basal body ciliated. The somatic ciliature is characterized by the presence of postoral kineties and a postoral suture along which right-sided and left-sided (i.e., postoral) somatic kineties converge. All species have variable numbers of elongated lank (i.e., never stiff or “setose”) caudal cilia arising from

the posteriormost somatic dikinetids. The perizonal ciliary stripe is invariably five-rowed with two basic arrangements; “even”, with all rows closely and regularly spaced, and “4 + 1” in which row 5 is separated from regularly-spaced rows 1–4 by a larger gap (Fig. 9E, F). Perizonal stripe dikinetids do not form “false-kineties”. Perizonal stripe row 1 typically terminates at, or anterior to the level of the cytostome. Three or more of the remaining rows continue posteriorly as “ordinary” somatic kineties (Fig. 5D).

The peristomial area is characteristically wide and shallow, extending obliquely from the buccal cavity to the anterior cell end. It is restricted to the ventral surface (i.e., not extending onto the dorsal side anteriorly), and occupies approximately one half of the cell length (Figs 1E–G, 3A, 5C, 7B, 9F, 11B, 13D, 17F). The distinctive, adoral membranelles are long, curved, very slender, and increase in length progressively from posterior to anterior (Figs 3D, 9C, 11C). All species inhabit hypoxic or anoxic freshwater sediments and feed on bacteria, but most species have a broader diet including unicellular algae and colorless flagellates. All species most likely form cysts but these were rarely observed. Conjugation is homopolar and isogamic for species in which it was observed (Fig. 2I).



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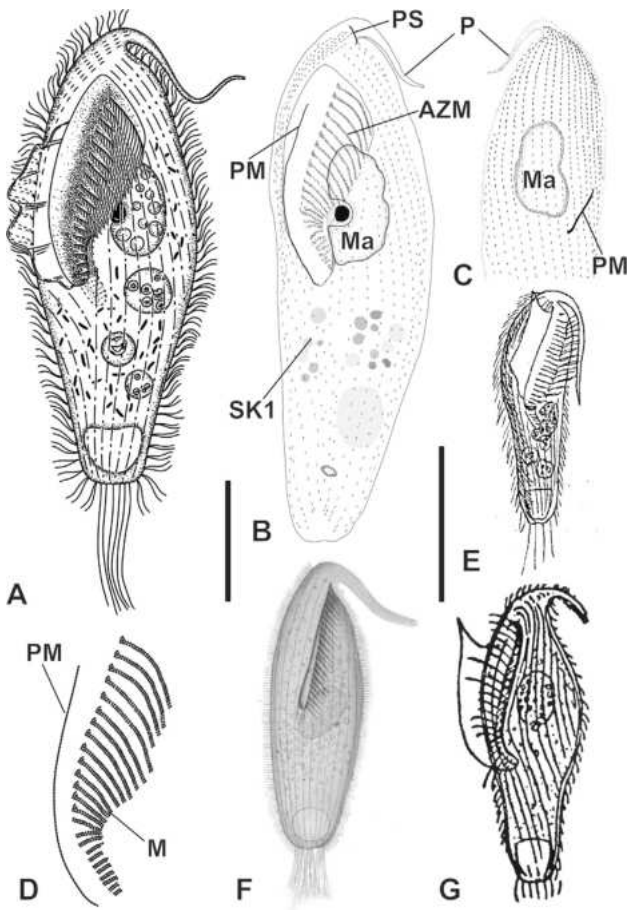


Figure 3. (A–G) *Bothrostoma nasutum* from life (A, E–G), and after protargol impregnation (B–D), (A) Ventral view of a typical specimen. (B) Ventral view showing the infraciliature. (C) Dorsal view showing the infraciliature on the anterior part and the distal part of the paroral membrane, as well as macronucleus. (D) Buccal ciliature. (E) After Kahl (1927). (F) After Cunha (1916). (G) After Penard (1922). AZM, adoral zone of membranelles; M, membranella; Ma, macronucleus; P, proboscis; PM, paroral membrane; PS, perizonal ciliary stripe; SK1, somatic kinety no. 1. Scale bars: 30 µm.

2.7 in vivo. Cell shape elongated, narrowly ellipsoidal, widest at or anterior to equator, ends anteriorly in conspicuous acute snout (about 5 µm long), posterior end truncated (Figs 1A, B, E, H; 2A, B). Cortical granules in five or six discontinuous oblique interkinetal rows and small clusters within kineties (Fig. 2E). Somatic, perizonal stripe, and membranella cilia about 10 µm long, caudal cilia about 40 µm long, in vivo. Somatic cilia arranged in an average of 18 longitudinal kineties (range 16–20) includ-

ing about five postoral kineties (Figs 1B; 2J, K). Perizonal stripe with even-type arrangement (Figs 1B; 2J, L). Adoral zone comprises 17 membranelles on average (range 15–19), occupies about 40% of body length. Paroral membrane extends > 70% length of adoral zone (Figs 1B, D; 2B, J, L, M–P).

Notes on LERMA1 strain: Phenotypically variable. In slender trophonts (Figs 1C, F, I; 2C, I, N, Q), cell shape elongated, flexible. Size 87–126 µm × 22–36 µm in vivo, average length:width ratio 3.9 in vivo, cells colorless. Swimming pace fast. In giant trophonts (Figs 1H, J; 2H), cell shape elongated, anterior half broad. Size 151–180 µm × 60–77 µm, average length:width ratio 2.5 in vivo, cells dark brownish due to many food vacuoles. Swimming pace very slow, usually resting.

Notes on BOPAT11 strain (Fig. 17K): Size 89–141 µm × 33–45 µm, average length:width ratio 3.0 in vivo. Cortical granules in 6 or 7 discontinuous oblique interkinetal rows and clusters.

***Bothrostoma nasutum* (Cunha, 1915) Jankowski, 1964** (Figs 3; 4; Table 3; Supplementary Material S1; Supplementary Material Video S2).

Description based on LERMA7 strain. Size 87–123 µm × 22–43 µm in vivo and 83–111 µm × 25–35 µm in protargol impregnated specimens, average length:width ratio 3.2 in vivo. Cell shape elongated ellipsoidal, widest at or anterior to equator, anterior end terminates in a long proboscis, posterior end truncated (Figs 3A, E–G; 4A–D, G, H). Proboscis glabrous, flexible, 18–40 µm long, digs and probes among debris. Cortical granules arranged in 5 or 6 discontinuous oblique interkinetal rows and small clusters within kineties (Fig. 4E). Somatic cilia about 10 µm long, perizonal stripe cilia about 15 µm long, caudal cilia about 40 µm long, in vivo. Somatic kineties comprise 29 (range 25–33) longitudinal rows, including about eight postoral kineties (Figs 3B, C; 4G, I–K). Perizonal stripe rows, in even-type arrangement. Adoral zone comprises 24 (range 21–26) membranelles, occupying approximately 50% of body length. Paroral membrane extends > 80% length of adoral zone (Figs 3B, D; 4D, J, L).

Notes on other strains: KUCR3 strain, size 88–123 µm × 31–44 µm, average length:width ratio 3.3 in vivo. Proboscis 18–40 µm in vivo (Table 3). Cultures could not be established for strains CEPIMAL1 and VLCIR, which presented the characteristic long proboscis. Only general morphologic observations were made of two additional populations, one from

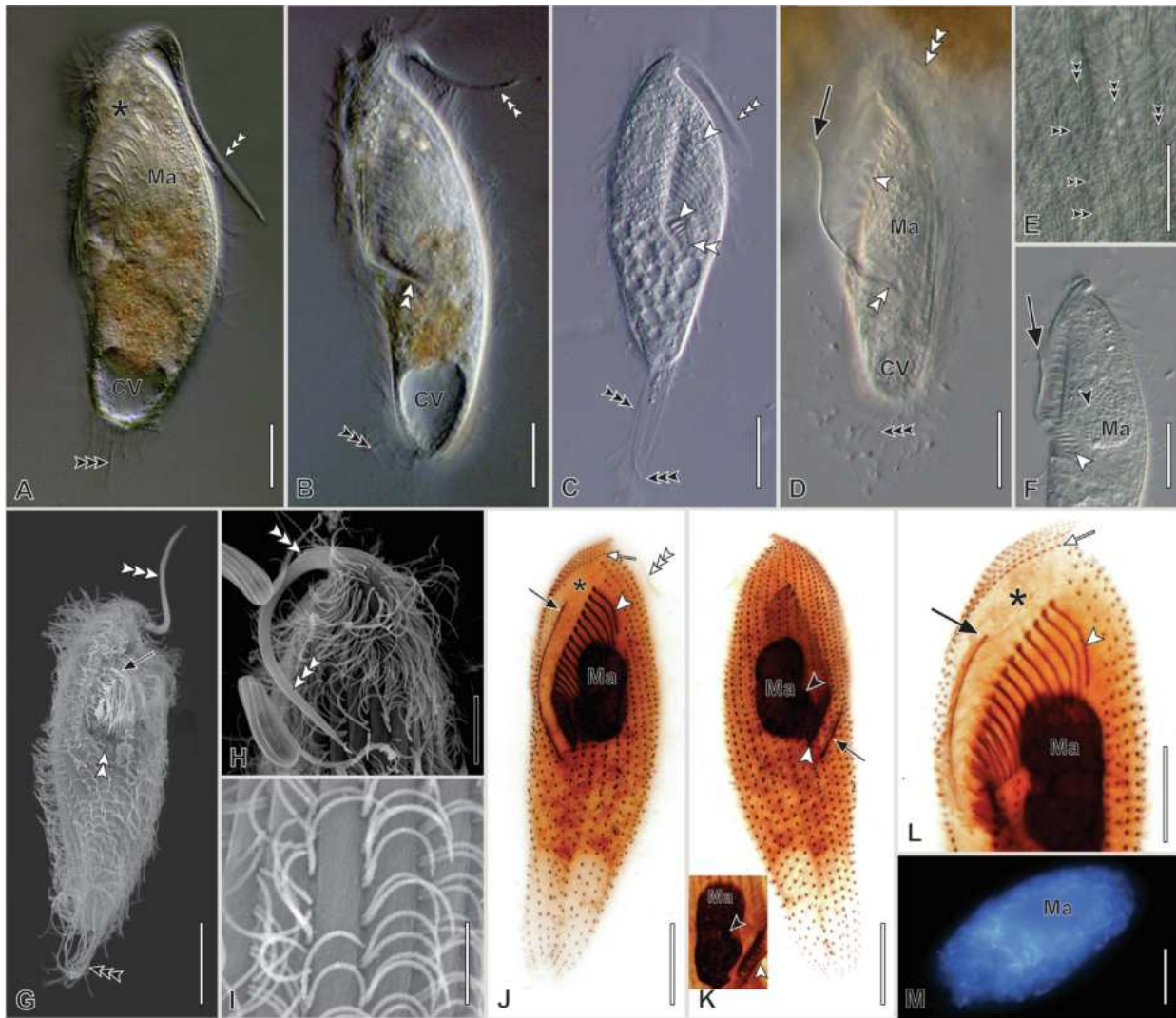


Figure 4. (A–M) *Bothrostoma nasutum* from life (A–F), in UV fluorescence microscopy (M), after protargol impregnation (J–L), and in SEM (G–I). (A–C, G) Ventral view of different individuals. (D) Left lateral view showing the paroral membrane. (E) Distribution of cortical granules. (F) Anterior region showing the paroral membrane, membranelles, and nuclear apparatus. (H) Anterior region showing the proboscis. (I) Disposition of the somatic kineties in furrows. (J, K) Ventral (J) and dorsal (K) view showing the infraciliature and macronucleus (inset in K). (L) Buccal ciliature and perizonal ciliary stripe. (M) Methanogen prokaryotic endosymbionts. Caudal cilia (black triple arrowheads); cortical granules (black double arrowheads); CV, contractile vacuole; distal portion of the buccal cavity (white double arrowheads); Ma, macronucleus; membranelles (white arrowheads); micronucleus (black arrowheads); paroral membrane (black arrows); perizonal row 1 (white arrows); proboscis (white triple arrowheads); undersurface of peristome (asterisks). Scale bars: 20 μm .

Idaho (Fig. 4A, B) and another from Petén Basin, Guatemala (not shown).

***Bothrostoma ovale* (Kahl, 1932) comb. nov.** (Figs 5; 6; Table 4; Supplementary Material S1).

Description based on EPOPOLO strain. Size 71–86 $\mu\text{m} \times 23$ –34 μm in vivo, 47–78 $\mu\text{m} \times 18$ –26 μm in protargol preparations, average length:width

ratio 2.9 in vivo. Cell shape broadly to narrowly ellipsoidal, widest in anterior half of cell, anterior snout conspicuous and rounded (about 5 μm long). Posterior end truncated (Fig. 5A–C, F–I). Small aggregate of argyrophilic granules in snout (Figs 5N; 6I, J). Cortical granules arranged in 3 or 4 discontinuous oblique interkinetal rows and scattered within ciliary

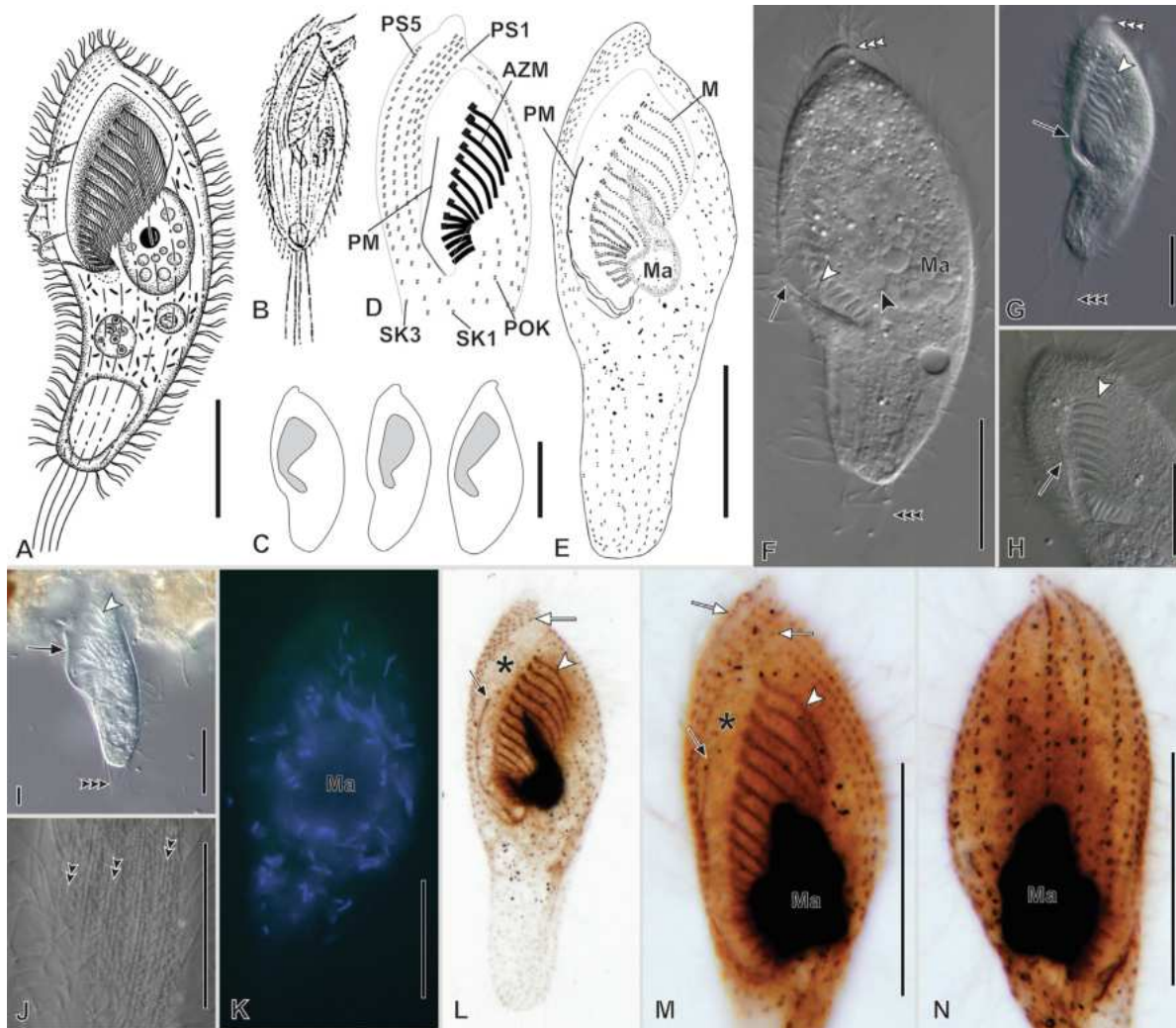


Figure 5. (A–N) *Bothrostoma ovale* (Kahl, 1932) comb. nov. from life (A, B, C, F–J), in UV fluorescence microscopy (K), after protargol impregnation (L–N). (A, F, G) Ventral view of different individuals. (B) After Kahl (1927). (C) Different cell shapes. (H) Anterior region showing the oral ciliature. (I) Left lateral view showing the paroral membrane. (J) Distribution of cortical granules. (K) Distribution of prokaryotic methanogens. (E, L) Ventral view showing the infraciliature. (D, M) Anterior region in ventral view showing the buccal ciliature and the 4 + 1 arrangement of the perizonal stripe. (N) Dorsal view showing the somatic kineties. AZM, adoral zone of membranelles; caudal cilia (black triple arrowheads); cortical granules (black double arrowheads); Ma, macronucleus; M, membranelles (white arrowheads); micronucleus (black arrowhead); PM, paroral membrane (black arrows); POK, postoral kineties; PS1, perizonal row 1 (white arrow in L); PS5, perizonal row 5 (white arrows); SK1, SK3, somatic kineties 1 and 3; snout (white triple arrowheads); undersurface of peristome (asterisks). Scale bars: 20 µm.

rows (Fig. 5J). Somatic cilia about 10 µm long, perizonal stripe cilia about 15 µm long, caudal cilia about 25 µm long, in vivo. Somatic kineties comprise 13 or 14 longitudinal rows, including 5 or 6 postoral kineties (Fig. 5E, L–N). Perizonal stripe of the 4 + 1-type or even-type arrangement (Fig. 5L, M). Adoral zone comprises 18 (range 16–18) membranelles,

occupying about 40% of body length. Paroral membrane extends > 60% length of adoral zone (Fig. 5E, G, I, L, M).

Notes on SMALLID strain (Fig. 6): Cells of SMALLID population were similarly shaped but slightly smaller, 62 × 29 µm on average in vivo, than those of EPOPOLO strain, with slightly more

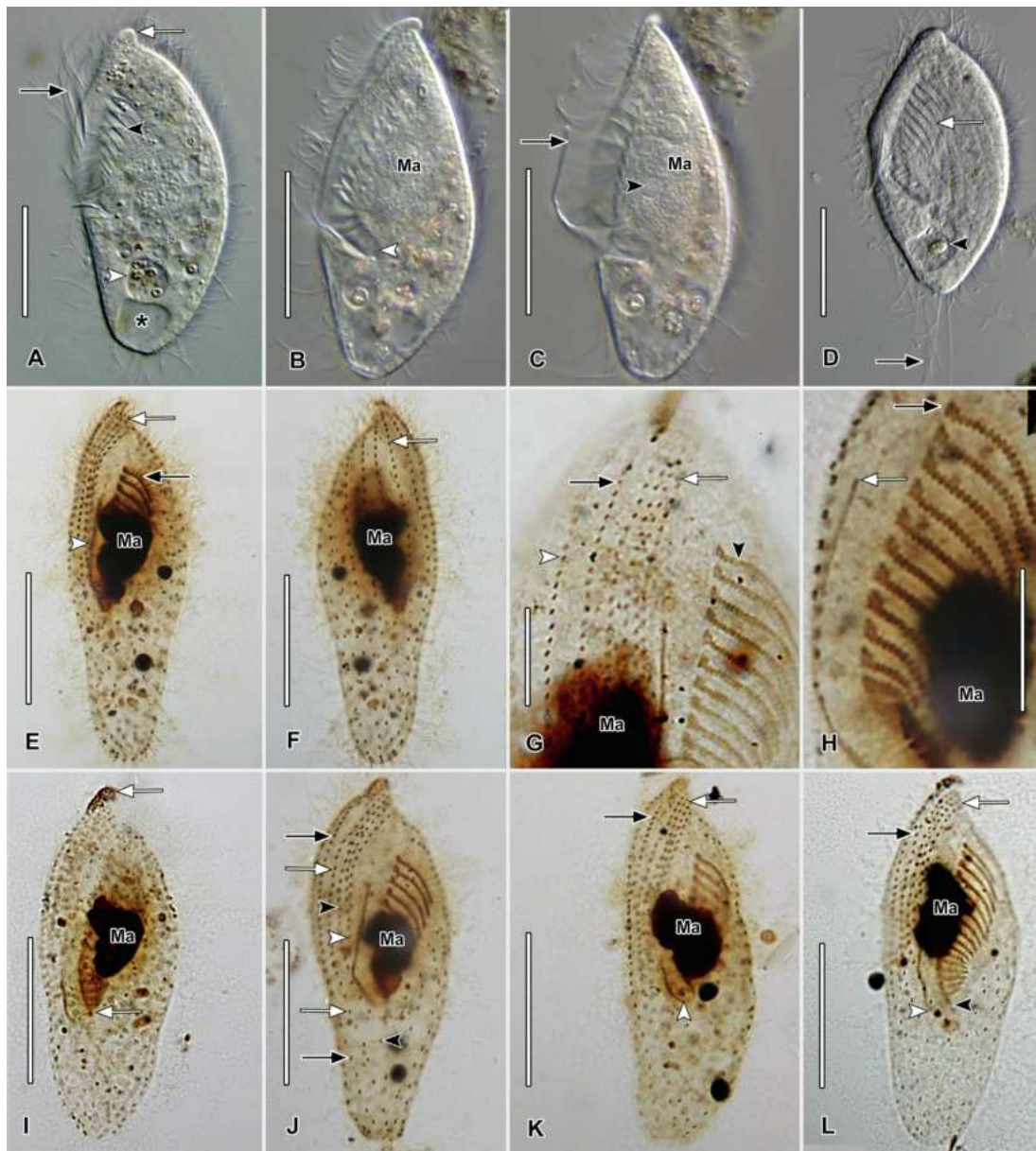


Figure 6. (A–L) *Bothrostoma ovale* (Kahl, 1932) comb. nov. SMALLID strain from life (A–D) and after protargol impregnation (E–L). (A, B, D) Ventral view showing the paroral membrane, macronucleus, and membranelles. (C) Left lateral view showing the paroral membrane. (E, F, I, K, L) Ventral (E, I, K, L) and dorsal (F) views showing the infraciliature. (G) Anterior region showing the 4 + 1 arrangement of the perizonal stripe, white arrow points the perizonal kinety 1, black arrow points the perizonal kinety 5, white arrowhead points the somatic kinety. (H) Buccal ciliature, white arrow shows the paroral membrane, black arrow shows the membranelles. (J) Arrangement of the somatic kineties and perizonal stripe kineties, black arrows point the perizonal stripe 5 and the third somatic kinety, white arrows point the perizonal stripe 4 and the second somatic kinety, black arrowhead point the perizonal kinety 3 and the first somatic kinety, white arrowhead points the perizonal kinety 1. Ma, macronucleus. Scale bars: 30 μ m.

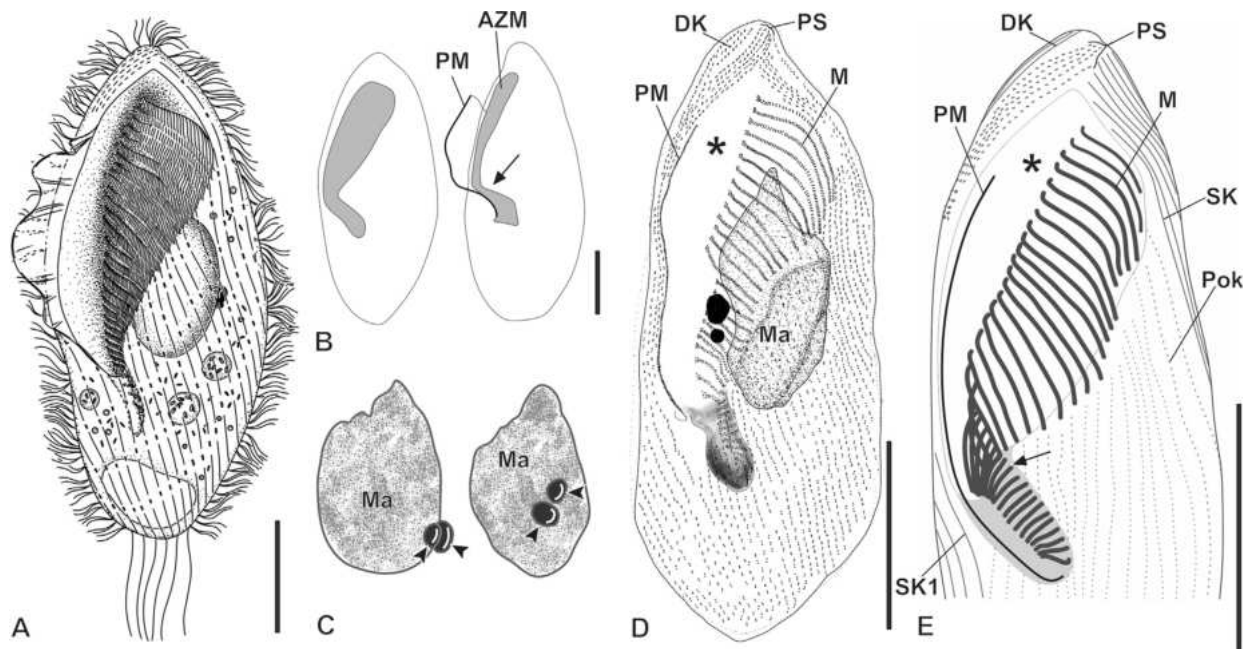


Figure 7. (A–E) *Bothrostoma bimicronucleatum* sp. nov. from life (A, B), and after protargol impregnation (C–E). (A) Ventral view of a typical specimen. (B) Ventral view (on the left) and left lateral view (on the right) showing the disposition of the adoral zone and paroral membrane. (C) Different shapes of macronucleus and disposition of micronuclei (black arrowheads). (D) Ventral view showing the infraciliature and nuclear apparatus. (E) Anterior ventral view showing the disposition of the adoral zone, the paroral membrane, and the infraciliature. AZM, adoral zone of membranelles; DK, dome kineties; M, membranelles; Ma, macronucleus; PM, paroral membrane; PS, perizonal ciliary stripe; SK, somatic kineties; Pok, postoral kineties; undersurface of peristome (asterisks). Scale bars: 30 μ m.

somatic kineties. Morphometric characteristics of the two populations in protargol preparations match very closely (Table 4).

***Bothrostoma bimicronucleatum* sp. nov.** (Figs 7; 8; Table 5).

Description based on KUNRA strain. Size 109–189 μ m $\times$ 44–83 μ m in vivo, 95–176 μ m $\times$ 34–70 μ m in protargol preparations, average length:width ratio 2.3 in vivo. Cell shape broadly ellipsoidal to ovoidal, widest equatorially, anteriorly rounded, anterior end terminates as an inconspicuous flattened snout, posterior end truncated (Figs 7A, B; 8A–C, G). Invariably two unequal-sized micronuclei adjacent to macronucleus (Figs 7A, C, D; 8A, B, E, G, K, L, N). Cortical granules arranged in 3 or 4 discontinuous oblique interkinetal rows and small clusters within kineties (Fig. 8F). Somatic cilia about 10 μ m long, perizonal stripe cilia about 15 μ m long, caudal cilia about 40 μ m long, in vivo. Somatic cilia arranged in an average of 55 longitudinal kineties (range 44–68), including an average of 20 postoral kineties (Figs 7D, E; 8K, L). Perizonal stripe rows in even-type arrangement (Figs 7D, E; 8K, M).

Adoral zone comprises 40 membranelles (range 35–45), occupying 45–75% of body length, distal portion inside of infundibulum (about 15 membranelles) and twisting about 180° clockwise and deviating toward the dorsal surface (Figs 7A, B, D, E; 8B–E, K, L). Paroral membrane extends > 70% length of adoral zone (Figs 7A, B, D, E; 8A, H, K, M). Early stages of cyst development sometimes observed, no mature cysts were found (Fig. 8I, J).

***Bothrostoma boreale* sp. nov.** (Figs 9; 10; Table 6).

Description based on strain MEDNIK3. Size 56–82 μ m $\times$ 21–33 μ m in vivo, 51–72 μ m $\times$ 14–23 μ m in protargol preparations, average length:width ratio 2.7 in vivo. Cell shape ellipsoidal, widest equatorially, ends anteriorly in short, rounded snout (about 4 μ m long), posteriorly end truncated (Figs 9G–I–III; 10D–H). Cortical granules arranged in 4 or 5 discontinuous oblique interkinetal rows and scattered within kineties (Figs 9D; 10K). Somatic and perizonal stripe cilia about 10 μ m long, caudal cilia about 20 μ m, in vivo. Somatic cilia arranged in average of 17 longitudinal kineties (range 15–19),



Figure 8. (A–O) *Bothrostoma bimicronucleatum* sp. nov. from life (A–J), and after protargol impregnation (K–O). (A) Left lateral view showing the paroral membrane. (B, C, H) Ventral view showing the adoral zone of membranelles. (D) Ventral view showing the aperture of the infundibulum (white double arrowhead) and some possible prokaryotic methanogens (black double arrowheads). (E) Left lateral view showing the infundibulum and the membranelles inside until the cytostome (white double arrowhead), as well as the nuclear apparatus. (F) Distribution of the cortical granules (black double arrowheads) and caudal cilia (black triple arrowheads). (G, N) Macronucleus with two micronuclei. (I, J) Cyst formation. (K, L) Ventral (K) and dorsal (L) views showing the infraciliature and nuclear apparatus in the holotype specimen. (M) Anterior ventral view showing the disposition of the perizonal stripe, the membranelles, and the paroral membrane. (O) Posterior region showing the distal portion of the adoral zone, and some ingested unicellular eukaryotes (white double arrowheads). CV, contractile vacuole; Ma, macronucleus; membranelles (white arrowheads); micronucleus (black arrowheads); paroral membrane (black arrows); peristomial roof (asterisks); perizonal kineties (white arrows). Scale bars: 20 µm.

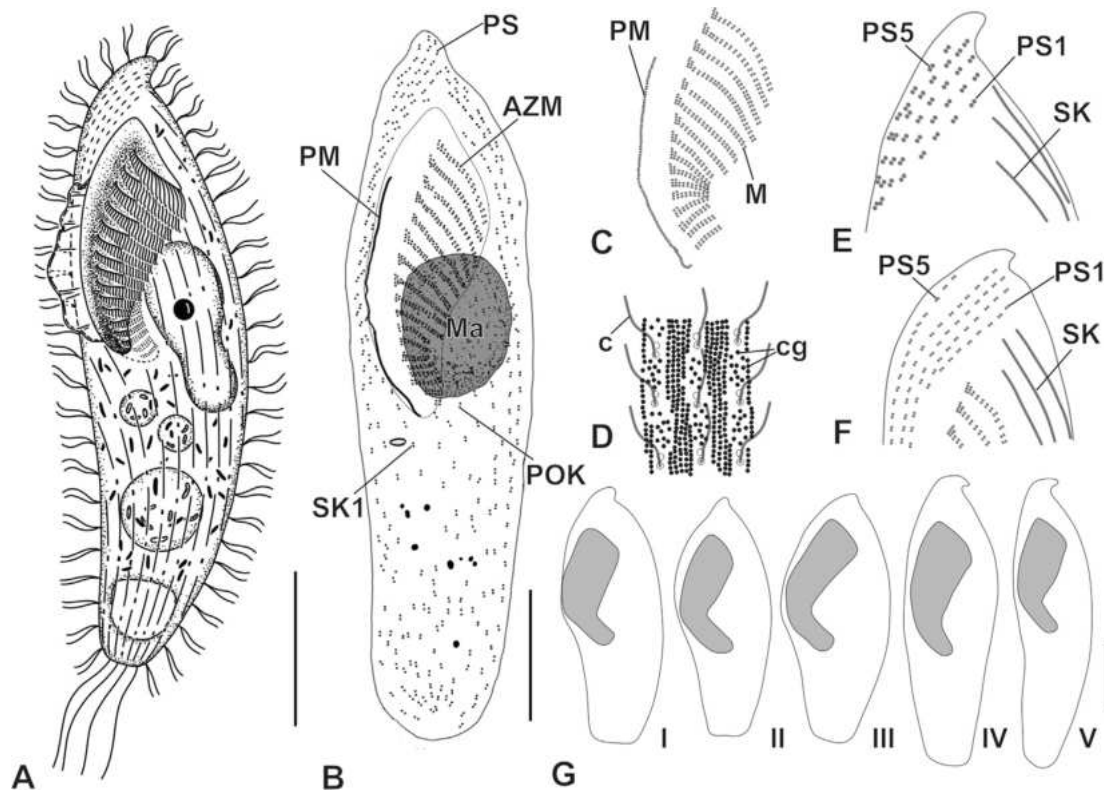


Figure 9. (A–G) *Bothrostoma boreale* sp. nov. from life (A, D, G), and after protargol impregnation (B, C, E, F). (A) Ventral view of a typical specimen. (B) Ventral view showing the infraciliature. (C) Adoral zone of membranelles and paroral membrane. (D) Disposition of cortical granules. (E, F) Arrangement of the perizonal stripe, even type (E) and 4 + 1-type (F). (G) Different cell shapes, I–III MEDNIK3 strain, IV–V GRANLX strain. AZM, adoral zone of membranelles; c, cilium; cg, cortical granules; M, membranelles; Ma, macronucleus; PM, paroral membrane; PS, perizonal ciliary stripe; PS1, perizonal stripe 1; PS5, perizonal stripe 5; SK, somatic kineties; POK, postoral kineties. Scale bars: 20 μ m.

including about 7 postoral kineties (Fig. 10O, P). Perizonal stripe in 4 + 1 arrangement (Figs 9F; 10R). Adoral zone comprises, on average, 17 membranelles (range 16–19), occupies about 40% of body length. Paroral membrane extends > 70% length of adoral zone (Fig. 10O).

Notes on GRNLX strain (Figs 9A–C, E, G–IV, G–V; 10A–C, L–N, Q): Size 86–92 μ m $\times$ 20–30 μ m in vivo and 55–79 μ m $\times$ 13–21 μ m in protargol stained specimens, average length:width ratio 3.8 in vivo. The shape is slightly different to MEDNIK3 strain, slender, or cylindrical, and narrower anteriorly, ending in a conspicuous acute snout (Figs 9A, G–IV, G–V; 10A–C). The arrangement of the perizonal stripe is usually even-type (Fig. 9E), but some cells were observed with 4 + 1-type arrangement.

***Bothrostoma robustum* sp. nov.** (Figs 11; 12; Table 7; Supplementary Material Video S3).

Description based on LIBL strain: Size 94–120 μ m $\times$ 32–46 μ m in vivo, 76–97 μ m $\times$ 24–34 μ m in

protargol preparations, average length:width ratio 2.5 in vivo. Cell shape stout, broadly ellipsoidal, elongate ellipsoidal, widest in anterior half of cell, anterior end terminates in rounded snout (about 4 μ m long), posterior end broadly truncated (Figs 11A; 12A–C). Cortical granules arranged in 5 or 6 discontinuous oblique interkinetal rows and small intrakinetal clusters (Fig. 12D). Somatic and perizonal stripe cilia about 13 μ m long, caudal cilia about 30 μ m long, in vivo. Somatic cilia arranged in 25 longitudinal kineties (range 24–27), including 8–10 postoral kineties (Figs 11D, E; 12J, K, P). Perizonal stripe rows in even-type arrangement (Figs 11D, E; 12J, L). Adoral zone comprises 25 (range 23–26) membranelles, occupies about 50% of body length. Distal portion inside of infundibulum (about 9 membranelles), twisting about 180° clockwise and deviating toward the dorsal surface. Paroral membrane extends > 80% length of adoral zone (Figs 11C–E; 12J, P).

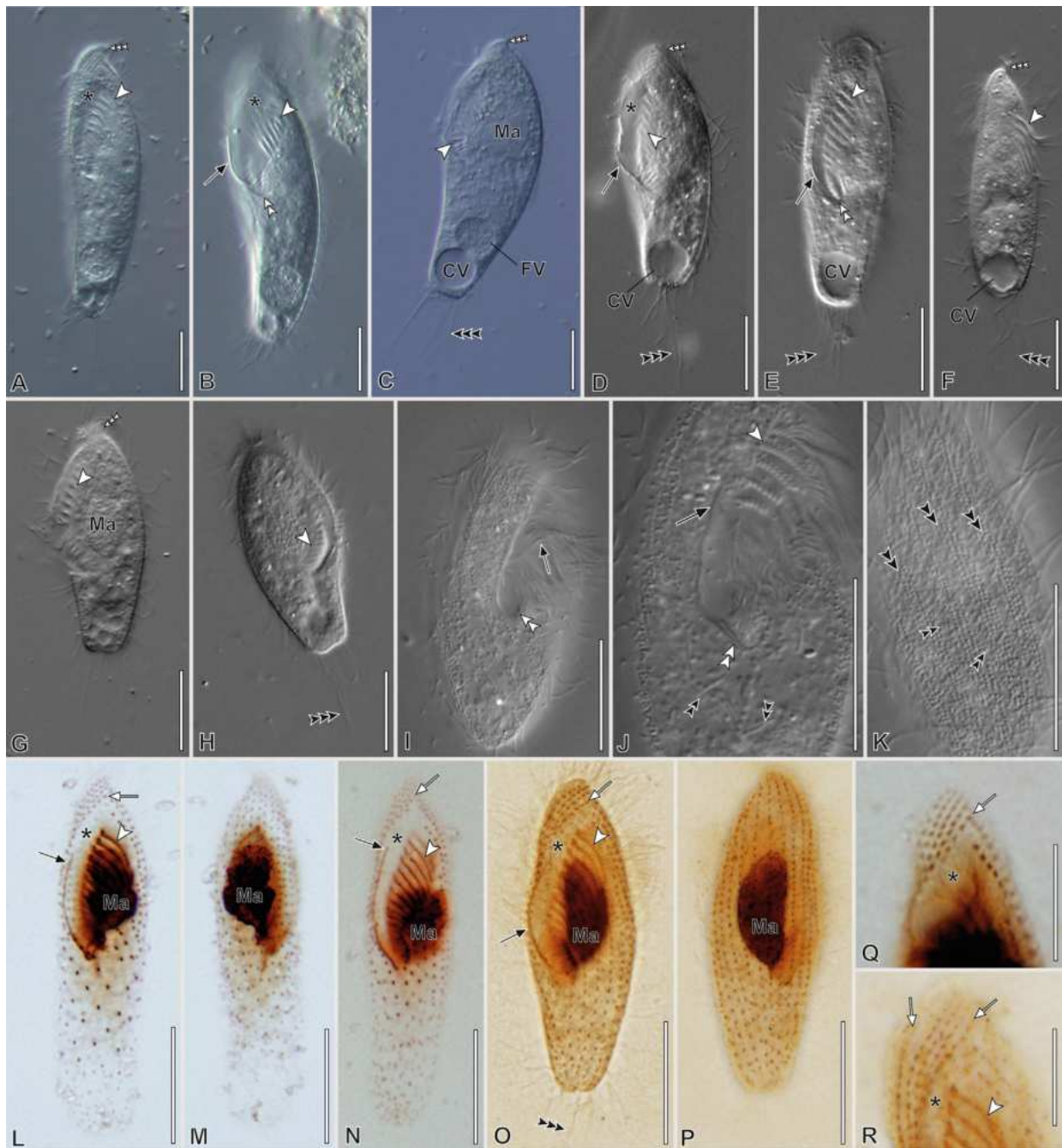


Figure 10. (A–R) *Bothrostoma boreale* sp. nov. from life (A–K), and after protargol impregnation (L–R). GRNLX strain (A–C, L–N, Q); MEDNIK3 strain (D–K; O, P, R). **(A–D, G)** Ventral view of different individuals. **(F)** Right lateral view showing the membranelles and the snout. **(H)** Dorsal view showing the distal part of the adoral zone. **(I, J)** Right ventrolateral view showing the aperture of the infundibulum, and the membranelles from the peristome and some prokaryotic symbionts (black double arrowheads in J). **(K)** Distribution of cortical granules (black double arrowheads). **(L–P)** Ventral (L, N, O) and dorsal view (M, P) showing the infraciliature, O and P belong to the holotype specimen. **(Q, R)** Arrangement of the perizonal stripe, even type (Q) and 4 + 1-type (R). Caudal cilia (black triple arrowheads); CV, contractile vacuole; FV, food vacuole; aperture of the infundibulum (white double arrowheads); Ma, macronucleus; membranelles (white arrowheads); paroral membrane (black arrows); perizonal row kineties (white arrows); snout (white triple arrowheads); undersurface of peristome (asterisks). Scale bars: 20 μ m.

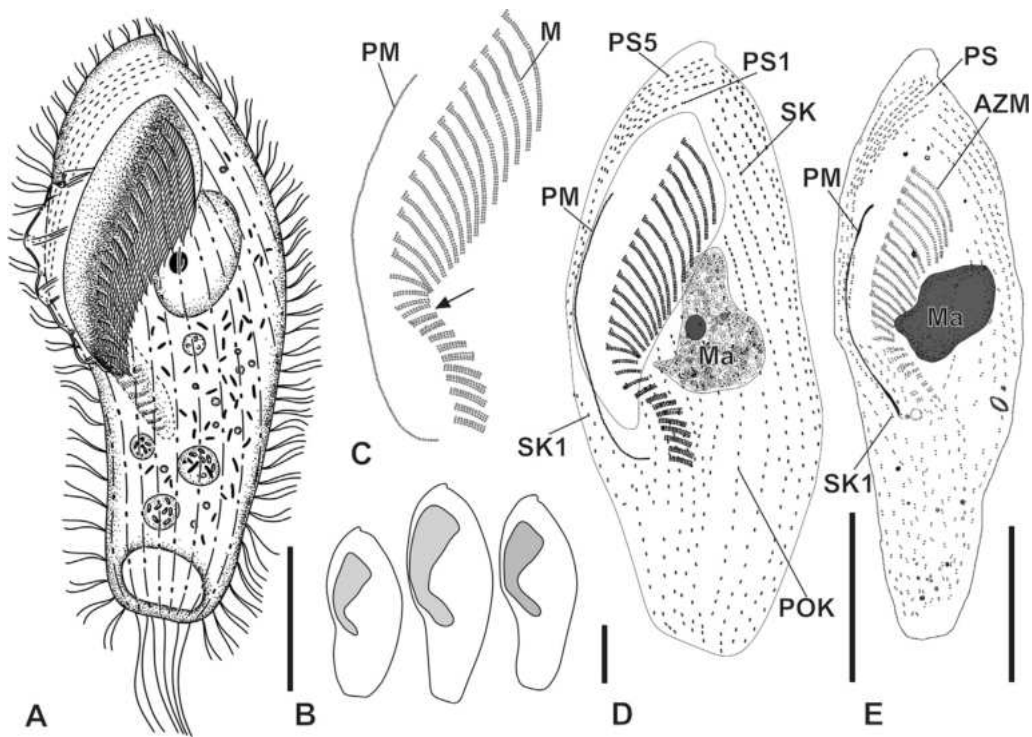


Figure 11. (A–E) *Bothrostoma robustum* sp. nov. from life (A, B), and after protargol impregnation (C–E). (A) Ventral view of a typical specimen. (B) Several cell shapes. (C) Adoral zone of membranelles and paroral membrane, arrow shows the torsion of the adoral zone. (D, E) Ventral view showing the infraciliature. AZM, adoral zone of membranelles; M, membranelles; Ma, macronucleus; PM, paroral membrane; PS, perizonal ciliary stripe; PS1, perizonal stripe 1; PS5, perizonal stripe 5; SK, somatic kineties; POK, postoral kineties. Scale bars: 20 µm.

Notes on PRASKX and CEPIMAL1 strains:

Cells of PRASKX strain, size 74–90 µm × 27–35 µm in vivo, with 19–24 membranelles, 26–33 somatic kineties, including 7–9 postoral kineties. CEPIMAL1 strain (Fig. 12E–H, M–O), size 113–155 µm × 38–60 µm in vivo, 28–32 membranelles, and 29–34 somatic kineties, including 12–16 postoral kineties.

***Bothrostoma kovalyovi* sp. nov.** (Figs 13; 14; Table 8; Supplementary Material Video S4).

Description based on PRAKBOT strain: Size 66–102 µm × 24–39 µm in vivo, 55–97 µm × 14–29 µm in protargol preparations, average length: width ratio 2.8 in vivo. Cell shape elongate ellipsoidal, widest equatorially, anterior end rounded, without snout, posterior end rounded. (Figs 13A; 14A, B, I). Cortical granules arranged in 5 discontinuous oblique interkinetal rows and small clusters within kineties (Figs 13C; 14C, L). Somatic and perizonal stripe cilia about 10 µm long, caudal cilia about 18 µm long, in vivo. Somatic cilia arranged in an average of 20 longitudinal kineties (range

18–22), including about 7 or 8 postoral kineties (Figs 13F; 14E, F, H, I). Perizonal stripe rows in even-type arrangement (Figs 13B, F; 14E, G, J). Adoral zone comprises 17 membranelles (range 16–18), occupying 37% of body length, angulation of posterior adoral zone less conspicuous than in other species. Paroral membrane extends > 70% length of adoral zone (Figs 13A, E, F; 14E, H, I, J).

Undescribed *Bothrostoma* spp. and *Planometopus contractus*

The strains STAN4, SUSBARB9 13, BORNEO, and UNDULID were characterized only by their 18S rRNA gene sequences. Based on our phylogenetic analysis, they likely represent three distinct *Bothrostoma* species (Fig. 15). The UNDULID population was previously misidentified by Bourland et al. (2017b) as *B. undulans* with which it is morphologically similar but genetically distant (Fig. 15; Supplementary Material Table S1).

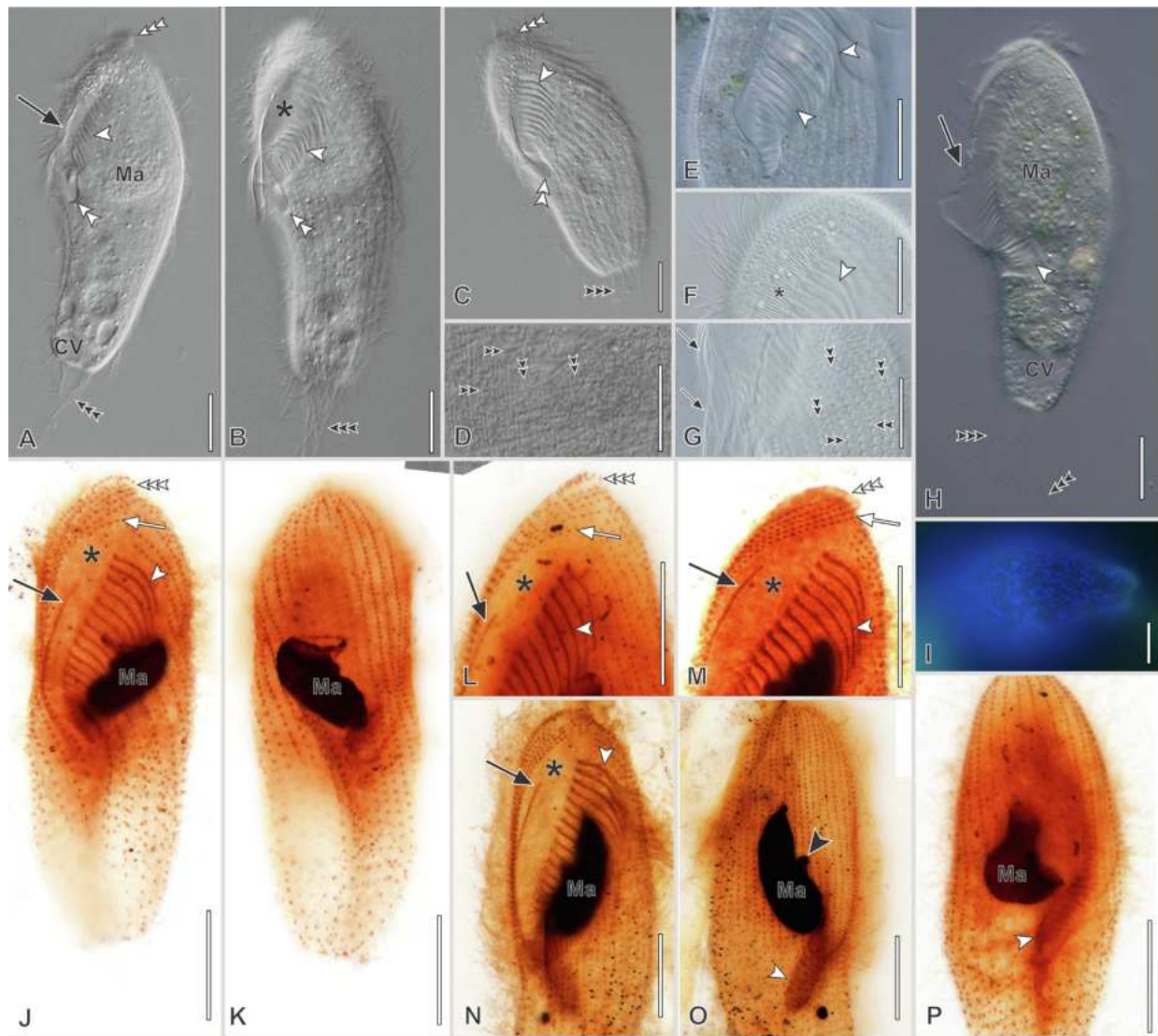


Figure 12. (A–P) *Bothrostoma robustum* sp. nov. from life (A–H), in UV fluorescence microscopy (I), and after protargol impregnation (J–P). LIBL strain (A–D, J–L, P); CEPIMAL1 strain (E–H, M–O). (A–C, H) Ventral view of different individuals. (D, G) Distribution of cortical granules (black double arrowheads), and cilia of the paroral membrane (in G). (E) Ventral view showing the aperture of the infundibulum, and membranelles. (F) Peristomial roof with cortical granules. (J, K, N, O) Ventral (J, N) and dorsal (K, O) views showing the infraciliature, J and K belong to the holotype specimen. (L, M) Anterior ventral view showing the disposition of the perizonal stripe kineties, the membranelles, and the paroral membrane. (P) Dorsal view showing the distal part of the adoral zone and the somatic kineties. CV, contractile vacuole; aperture of the infundibulum (white double arrowheads); Ma, macronucleus; membranelles (white arrowheads); micronucleus (black arrowhead); paroral membrane (black arrows); perizonal row kineties (white arrows); snout (white triple arrowheads); caudal cilia (black triple arrowheads); underside of peristome (asterisks). Scale bars: 20 µm.

A Czech population of the species *Planometopus contractus* (Penard, 1922) Rotterová, Bourland, and Čepička, 2018, LIB2P strain, was identified based on morphology (Fig. 16) and its 18S rRNA gene

sequence (GenBank accession number MZ054208), which is identical to that of the Idaho population (Bourland et al. 2014; Rotterová et al. 2018a, b).

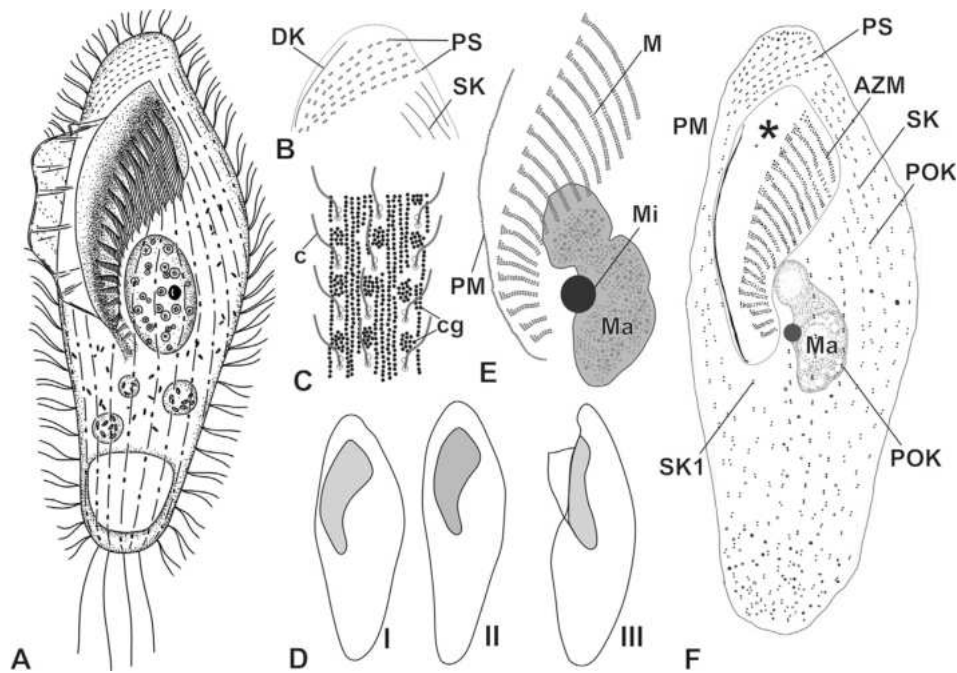


Figure 13. (A–F) *Bothrostoma kovalyovi* sp. nov. from life (A, C, D), and after protargol impregnation (B, E, F). (A) Ventral view of a typical specimen. (B) Distribution of the perizonal stripe. (C) Disposition of cortical granules. (D) Several cell shapes in ventral (I, II) and left lateral (III) views. (E) Adoral zone of membranelles, paroral membrane, and nuclear apparatus. (F) Ventral view showing the infraciliature. AZM, adoral zone of membranelles; c, cilium; cg, cortical granule; DK, dome kineties; M, membranelles; Ma, macronucleus; Mi, micronucleus; PM, paroral membrane; PS, perizonal ciliary stripe; SK, somatic kineties; POK, postoral kineties; undersurface of peristome (asterisks). Scale bars: 20 μ m.

Phylogenetic Analysis

The overall topology of the 18S rRNA gene tree is consistent with previous analyses (e.g., Bourland et al. 2020; Rotterová et al. 2018a). *Bothrostoma* forms a robust clade (bootstrap support, BS, 100; Bayesian posterior probability, BPP, 1). *Planometopus contractus* is sister to *Bothrostoma* with moderate support (BS 78; BPP 1); the clade of *Bothrostoma* + *Planometopus* is here referred as BoPI. The BoPI clade is a member of the HAC clade and seems to be sister to the PiCa clade (Fig. 15), though the support for the relationship was insufficient (BS < 50; BPP < 0.9).

Internal relationships of the genus *Bothrostoma* remained largely unresolved, but the genus splits into two clades. One consists of *B. kovalyovi* sp. nov. and the environmental sequence AY821942 and is strongly supported (BS 99; BPP 1). The second clade, comprising all the remaining *Bothrostoma*

species, has moderate support in ML and full support in BI (BS 73; BPP 1). The relationships between *B. ovale* comb. nov., *B. undulans*, *B. boreale* sp. nov., *B. nasutum*, and *Bothrostoma* sp. 1 STAN4 are unresolved. On the other hand, *B. bimicronucleatum* sp. nov., *B. robustum* sp. nov., *Bothrostoma* sp. 2 (SUSBARB9 13 and BORNEO), and *Bothrostoma* sp. 3 UNDULID form a robust clade (BS 100; BPP 1).

The intraspecific genetic distances (uncorrected p distances; Table S1) within *Bothrostoma* species were ≤ 0.005 , except for *B. nasutum* (0.005–0.036). The lowest interspecific genetic distance, 0.009, was found between *Bothrostoma* sp. 2 and *B. robustum* sp. nov., whereas the highest, 0.055, was found between *Bothrostoma* sp. 2 and *B. nasutum*. The interspecific genetic distances among *B. undulans*, *B. ovale* comb. nov., *B. boreale* sp. nov., and *B. kovalyovi* sp. nov., which are closely related and morphologically similar, ranged between 0.012 and 0.018.

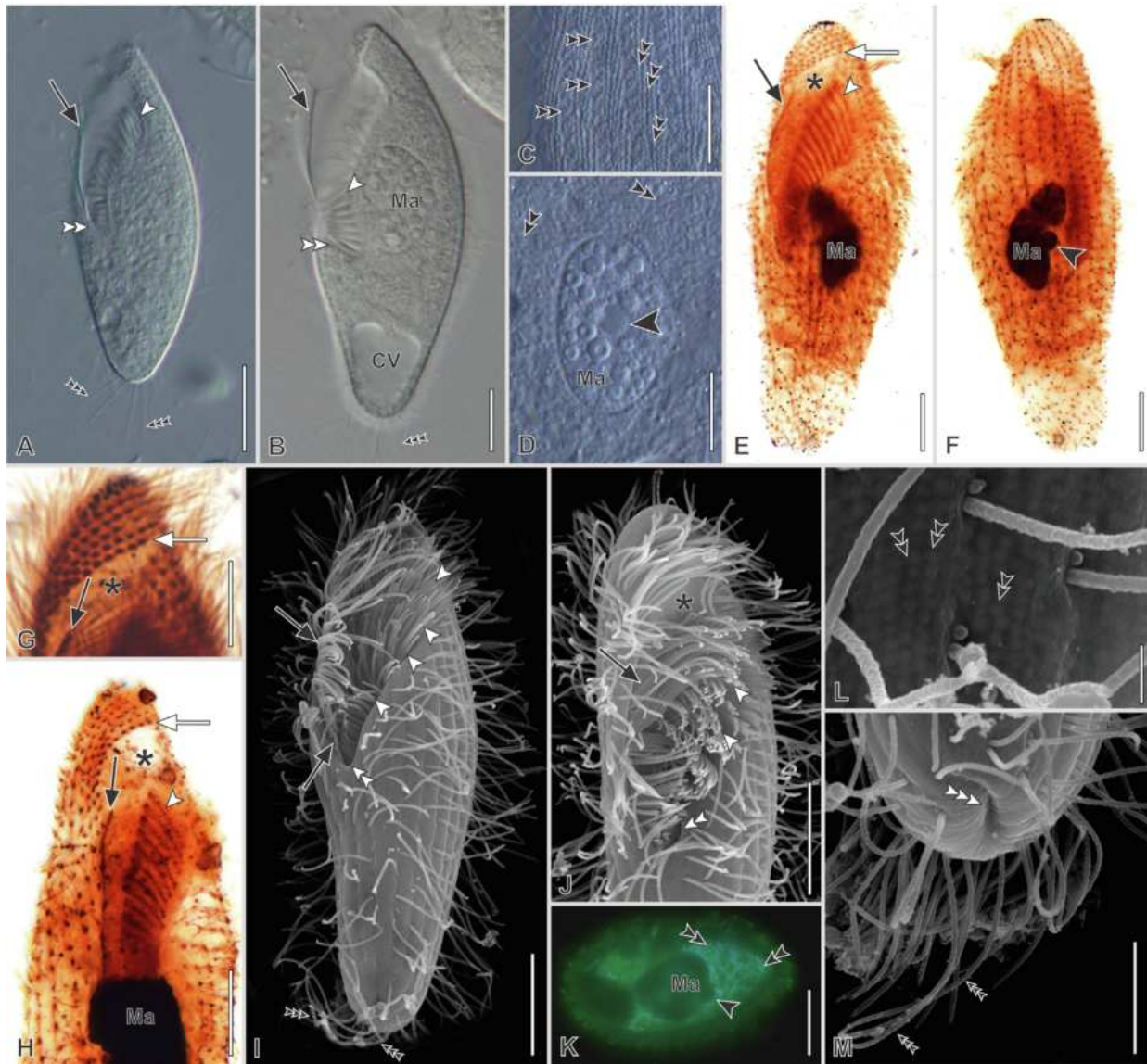


Figure 14. (A–M) *Bothrostoma kovalyovi* sp. nov. from life (A–D), in UV fluorescence microscopy (K), after protargol impregnation (E–H), and after SEM (I, J, L, M). (A) Ventral view of a typical specimen. (B) Left lateral view showing the paroral membrane. (C, L) Distribution of cortical granules (black double arrowheads). (D) Macronucleus and micronucleus. (E, F, I) Ventral (E, I) and dorsal (F) views showing the infraciliature; E and F belong to the holotype specimen. (G) Anterior region showing the perizonal stripe kineties. (H, J) Anterior region showing the buccal ciliature. (K) Prokaryotic methanogenic symbionts. (M) Posterior region showing the cytoproct (white triple arrowhead) and caudal cilia. CV, contractile vacuole; aperture of the infundibulum (white double arrowheads); Ma, macronucleus; membranelles (white arrowheads); micronucleus (black arrowheads); paroral membrane (black arrows); perizonal row kineties (white arrows); caudal cilia (black triple arrowheads); undersurface of peristome (asterisks). Scale bars: 20 µm.

Table 1. Origin of the studied strains of *Bothrostoma* and *Planometopus* used in this study.

Species	Strain	Locality	Sample type ¹	Coordinates
<i>B. undulans</i>	LERMA	Atarasquillo, Lerma, Mexico	Marsh, sediment	19°20'37" N, 99°30'09" W
	BOPAT11	Smíchovský rybník, Karlovice, Czech Republic	Pond, sediment	50°33'13.1" N, 15°10'59.8" E
<i>B. nasutum</i>	LERMA7	Atarasquillo, Lerma, Mexico	Marsh, sediment	19°20'37" N, 99°30'09" W
	CEPIMAL2	Žinkovy, Czech Republic	Pond, sediment	49°28'09.3" N, 13°29'09.7" E
	VLCIR	Vlčí rokle, Krhanice, Czech Republic	Pond, sediment	49°52'15.5" N, 14°31'46.8" E
	KUCR3	Červená Řečice, Czech Republic	Pond, sediment	49°33'22" N, 15°06'49" E
	IZR	Galilei, Israel	Pond, sediment	32° 36.80' N, 35° 33.046' E
<i>B. ovale</i>	SMALLID	Boise, Idaho, USA	Mesocosm sediment	43°33'04.57" N, 116°17'07.95" W
<i>B. bimicronucleatum</i>	EPOPOLO	Bayanga, Central African Republic	Pond, sediment	2°50'32" N, 16°27'56" E
	KUNRA	Kunratický les, Prague, Czech Republic	Pond, sediment	50°01'15" N, 14°28'46" E
<i>B. boreale</i>	GRNLX2	Tasersuaq Lake, Greenland, Denmark	Lake, sediment	66°59' N, 51°51' W
<i>B. robustum</i>	MEDNIK3	Medník Hill, Czech Republic	Pond, sediment	49°52'06.2" N, 14°26'34.9" E
	CEPIMAL1	Žinkovy, Czech Republic	Pond, sediment	49°28'09.3" N, 13°29'09.7" E
	LIBL	Libický luh, Czech Republic	Floodplain puddle, sediment	50°06'15.2" N, 15°10'08.4" E
<i>B. kovalyovi</i>	PRASKX	Prachovské skály, Czech Republic	Puddle, sediment	50°28'05.7" N, 15°17'06.4" E
	PRAKBOT	Prague, Czech Republic	Pond, sediment	50°04'20" N, 14°25'24" E
<i>B. sp. 1</i>	STAN4	Beaver lake, Vancouver, Canada	Pond, sediment	54°45'24.9" N, 111°55'11.1" W
<i>B. sp. 2</i>	BORNEO	Bako, Sarawak, Borneo, Malaysia	Pond, dried mud	1°43'07.2" N, 110°26'47.3" E
<i>B. sp. 3</i>	SUSBARB9 13	Bako, Sarawak, Borneo, Malaysia	Pond, Sediment	1°43'00.0" N, 110°28'00.0" E
	UNDULID	Boise, Idaho, USA	Stream, sediment	43°38'31.3" N, 116°11'12.31" W
<i>P. contractus</i>	LIB2P	Libický luh, Czech Republic	Pond, sediment	50°06'47.6" N, 15°10'33.9" E

¹ The area of lakes and ponds can be deduced from maps. Puddles were small (usually less than 20 m²).

Table 2. Morphometric data for *Bothrostoma undulans* Stokes, 1887. LERMA2 strain (L2 typical trophont), LERMA1 strain (L1 slender trophont), and BOPAT11 strain (B11).

Characteristic ^a	Met ^b	Strain	Mean	M	SD	CV	Min	Max	n
Body, length	IV	L2	81.5	80.5	8.24	10.1	67.0	95.0	14
	P	L2	60.5	60.0	8.64	14.3	45.0	75.0	26
	IV	L1	105.4	106.0	10.49	9.9	87.0	126.0	37
	P	L1	94.0	99.0	11.70	12.4	71.0	110.0	27
	IV	B11	106.8	100.5	20.13	18.8	89.0	141.0	6
	P	B11	102.5	102.0	11.77	11.5	80.0	120.0	11
Body, width	IV	L2	30.2	30.0	2.52	8.4	26.0	34.0	14
	P	L2	19.2	19.0	2.19	11.4	15.0	23.0	26
	IV	L1	27.1	27.0	3.57	13.2	22.0	36.0	37
	P	L1	18.5	19.0	2.50	13.5	14.0	24.0	28
	IV	B11	36.0	33.5	4.82	13.4	33.0	45.0	6
	P	B11	27.9	27.0	4.53	16.2	20.0	35.0	11
Body, length-width, ratio	IV	L2	2.7	2.7	0.39	14.2	2.1	3.5	14
	P	L2	3.2	3.1	0.37	11.8	2.5	3.9	26
	IV	L1	3.9	3.7	0.53	13.3	3.1	5.1	37
	P	L1	5.2	5.1	0.96	18.5	3.9	7.3	25
	IV	B11	3.0	3.0	0.21	7.0	2.7	3.2	6
	P	B11	3.7	3.7	0.41	11.1	3.1	4.6	11
Anterior cell end to posterior end of adoral zone	P	L2	32.5	32.0	2.93	9.0	26.0	38.0	26
	P	L1	43.9	43.0	2.36	5.4	40.0	48.0	29
	P	B11	48.0	48.0	3.68	7.7	42.0	56.0	10
Distance anterior cell end to posterior end of adoral zone-body length, ratio in %	P	L2	54.2	54.3	4.88	9.0	47.1	64.0	26
	P	L1	46.9	45.5	5.61	12.0	40.6	57.7	26
	P	B11	47.6	47.0	3.57	7.5	42.4	54.3	10
Anterior cell end to anterior end of adoral zone	P	L2	8.8	9.0	1.62	18.5	5.0	12.0	24
	P	L1	13.3	13.0	1.60	12.1	11.0	18.0	29
	P	B11	16.0	17.0	2.40	15.0	12.0	20.0	10
Distance anterior cell end to anterior end of adoral zone-body length, ratio in %	P	L2	14.3	14.4	2.37	16.6	10.0	19.2	24
	P	L1	14.2	14.0	1.56	11.0	11.3	16.9	26
	P	B11	15.7	15.7	1.10	7.0	14.2	17.0	10
Anterior cell end to posterior end of perizonal stripe row 1	P	L2	3.8	4.0	0.66	17.4	3.0	5.0	24
	P	L1	5.8	6.0	1.39	24.2	4.0	8.0	16
	P	B11	6.2	6.0	0.37	5.9	6.0	7.0	8
Distance anterior cell end to posterior end of perizonal stripe row 1-body length, ratio in %	P	L2	6.2	5.9	0.87	14.0	5.0	8.2	24
	P	L1	6.1	5.9	1.45	23.7	4.0	8.4	16
	P	B11	6.1	6.0	0.60	10.0	5.0	6.8	8
Anterior cell end to posterior margin of macronucleus	P	L2	34.3	34.0	4.21	12.3	26.0	42.0	26
	P	L1	44.2	43.0	3.68	8.3	37.0	54.0	29
	P	B11	51.5	50.0	3.69	7.2	48.0	57.0	9
Distance anterior cell end to posterior margin of macronucleus-body length, ratio in %	P	L2	57.0	55.6	4.74	8.3	50.0	68.0	26
	P	L1	47.3	45.9	5.66	12.0	38.6	57.8	26
	P	B11	49.8	48.0	4.05	8.1	45.8	58.9	9
Macronucleus, length	P	L2	13.3	14.0	2.04	15.3	10.0	17.0	26
	P	L1	20.1	20.0	2.64	13.1	14.0	25.0	31
	P	B11	25.0	25.0	3.00	12.0	21.0	30.0	11

(continued on next page)

Table 2 (continued)

Characteristic ^a	Met ^b	Strain	Mean	M	SD	CV	Min	Max	n
Macronucleus, width	P	L2	8.4	8.0	1.33	15.9	6.0	11.0	26
	P	L1	9.7	10.0	1.62	16.7	7.0	12.0	31
	P	B11	10.9	11.0	2.43	22.2	8.0	16.0	11
Micronucleus, diameter	P	L2	2.3	2.0	0.50	22.2	2.0	3.0	4
	P	L1	4.3	4.0	0.89	20.5	3.0	6.0	12
	P	B11	4.7	4.8	0.63	13.5	4.0	6.0	10
Adoral zone of membranelles, length	P	L2	24.4	24.0	1.81	7.4	21.0	29.0	26
	P	L1	30.7	30.0	2.06	6.7	27.0	34.0	29
	P	B11	32.0	31.0	3.06	9.5	30.0	39.0	10
Adoral zone length-body length, ratio in %	P	L2	41.0	38.7	6.27	15.3	34.3	57.8	26
	P	L1	32.7	31.7	4.84	14.8	26.7	42.3	26
	P	B11	31.9	31.6	4.19	13.2	25.4	39.1	10
Adoral membranelles, number	P	L2	17	17	1.02	6.0	16	20	26
	P	L1	18	18	1.32	7.2	16	22	30
	P	B11	19	19	2.14	11.1	17	22	7
Width of mid-adoral zone	P	L2	8.6	8.0	1.00	11.7	7.0	11.0	25
	P	L1	8.3	8.0	1.09	13.1	7.0	11.0	30
	P	B11	12.2	12.0	1.20	9.8	11.0	14.0	9
Paroral membrane, length ^c	P	L2	21.0	21.0	2.12	10.1	16.0	25.0	24
	P	L1	27.7	28.0	2.00	7.2	24.0	32.0	27
	P	B11	30.2	30.0	1.94	6.4	28.0	33.5	9
Length of paroral-cell length, ratio in %	P	L2	35.0	34.7	4.42	12.6	27.1	46.9	24
	P	L1	29.7	28.2	4.77	16.0	24.1	38.7	25
	P	B11	29.2	29.5	2.78	9.5	24.6	32.8	9
Length of paroral-adoral zone length, ratio in %	P	L2	85.9	87.2	8.23	9.6	69.0	96.2	24
	P	L1	90.7	90.0	3.79	4.2	81.8	97.0	27
	P	B11	94.1	96.7	7.91	8.4	83.3	108.1	9
Somatic kineties, number	P	L2	18.0	18.0	1.26	7.0	16	20	23
	P	L1	17.0	17.0	1.29	7.6	15	20	25
	P	B11	18.2	18.0	1.33	7.3	16	20	6
Postoral kineties, number	P	L2	5.2	5.0	0.58	11.1	4	6	14
	P	L1	5.5	5.5	0.52	9.5	5	6	12
	P	B11	5.5	5.0	1.00	18.2	5	7	4
Perizonal ciliary stripe rows, number	P	L2	5.0	5.0	0.00	0.0	5	5	17
	P	L1	5.0	5.0	0.00	0.0	5	5	27
	P	B11	5.0	5.0	0.00	0.0	5	5	9

CV, coefficient of variation (%); M, median; Max, maximum value; Mean, arithmetic mean; Met, method of study; Min, minimum value; n, number of cells studied; SD, standard deviation of the arithmetic mean.

^a All distances in μm .

^b Measurements and counts in vivo (IV) and from formalin-fixed protargol-impregnated permanent preparations (P) made from digital images using calibrated software.

^c Measured as the chord.

Discussion

Bothrostoma as a Distinct Genus within
Metopida

Despite Müller's (1786) recognition of *Metopus es* (original name *Trichoda s*) (Müller, 1786) Lauterborn, 1916, the type species of genus *Metopus*,

nearly a century and a half earlier, Kahl (1927, 1932) described most of the known diversity of *Metopus*, which he divided into six morphology-based species groups. Notably, group I included only species currently assigned to the genus *Bothrostoma*. Jankowski (1964a), recognizing the morphological differences between this group and the other metopids, resurrected *Bothrostoma*.

Table 3. Morphometric data for *Bothrostoma nasutum* (Cunha, 1915) Jankowski, 1964. LERMA7 strain (L7) and KUCR3 strain (K).

Characteristic ^a	Met ^b	Strain	Mean	M	SD	CV	Min	Max	n
Body, length	IV	L7	103.8	102.5	10.07	9.7	87.0	123.0	24
	P	L7	98.3	98.0	6.73	6.8	83.0	111.0	31
	IV	K	123.0	128.0	—	—	88.0	146.0	5
	P	K	92.2	92.0	8.02	8.7	73.0	104.0	17
Body, width	IV	L7	33.1	33.5	5.46	16.5	22.0	43.0	24
	P	L7	29.5	29.0	2.85	9.7	25.0	35.0	33
	IV	K	37.4	38.0	—	—	31.0	44.0	5
	P	K	27.4	28.0	3.46	12.7	21.0	34.0	17
Body, length-width, ratio	IV	L7	3.2	3.0	0.74	22.8	2.2	4.8	24
	P	L7	3.4	3.3	0.24	7.2	2.9	3.8	31
	IV	K	3.3	3.3	—	—	2.8	3.6	5
	P	K	3.4	3.3	0.39	11.4	2.9	4.0	17
Anterior cell end to posterior end of adoral zone	P	L7	52.5	52.0	3.38	6.4	45.0	58.0	33
	P	K	45.6	45.5	2.53	5.6	40.0	50.0	16
Distance anterior cell end to posterior end of adoral zone-body length, ratio in %	P	L7	53.3	54.3	3.86	7.2	43.7	59.0	31
	P	K	50.0	49.5	4.33	8.7	41.6	58.3	16
Anterior cell end to anterior end of adoral zone	P	L7	12.4	13.0	1.58	12.7	9.0	15.0	33
	P	K	11.6	12.0	1.77	15.3	8.0	15.0	17
Distance anterior cell end to anterior end of adoral zone-body length, ratio in %	P	L7	12.4	12.6	1.82	14.7	9.2	15.7	28
	P	K	12.6	13.0	1.82	14.4	9.3	15.3	17
Anterior cell end to posterior end of perizonal stripe row 1	P	L7	5.6	5.5	0.91	16.2	4.0	7.0	28
	P	K	—	—	—	—	—	—	—
Distance anterior cell end to posterior end of perizonal stripe row 1-body length, ratio in %	P	L7	5.8	5.7	0.93	16.1	3.9	7.4	28
	P	K	—	—	—	—	—	—	—
Anterior cell end to posterior margin of macronucleus	P	L7	48.2	48.0	4.50	9.3	41.0	56.0	33
	P	K	43.9	44.0	4.07	9.3	36.0	52.0	17
Distance anterior cell end to posterior margin of macronucleus-body length, ratio in %	P	L7	48.8	48.5	3.01	6.2	43.7	55.4	31
	P	K	47.8	48.0	3.93	8.2	41.9	55.3	17
Macronucleus, length	P	L7	24.4	24.0	2.42	9.9	20.0	29.0	33
	P	K	20.3	20.0	2.73	13.5	16.0	27.0	17
Macronucleus, width	P	L7	12.8	13.0	2.00	15.6	9.0	19.0	33
	P	K	10.1	10.0	1.56	15.5	8.0	15.0	17
Micronucleus, diameter	P	L7	3.2	3.0	0.42	13.1	3.0	4.0	23
	P	K	3.5	3.5	0.37	10.6	3.0	4.5	12
Adoral zone of membranelles, length	P	L7	40.0	40.0	2.65	6.6	33.0	45.0	33
	P	K	34.1	34.5	3.38	9.9	27.0	39.0	16
Adoral zone length-body length, ratio in %	P	L7	40.8	41.67	3.61	8.9	32.4	47.0	31
	P	K	37.4	37.8	4.69	12.6	26.7	46.4	16
Adoral membranelles, number	P	L7	24.6	25.0	1.03	4.2	21	26	33
	P	K	23.8	24.0	1.05	4.4	22	25	14
Width of mid-adoral zone	P	L7	13.0	13.0	1.30	10.0	9.0	15.0	33
	P	K	13.3	13.0	1.74	13.1	11.0	17.0	16
Paroral membrane, length ^c	P	L7	36.1	36.0	2.71	7.5	31.0	41.0	33
	P	K	32.8	32.0	2.46	7.5	29.0	37.0	15
Length of paroral-cell length, ratio in %	P	L7	36.7	36.5	3.30	9.0	30.4	44.8	31
	P	K	36.1	36.0	2.74	7.6	30.5	40.5	15
Length of paroral-adoral zone length, ratio in %	P	L7	90.2	90.7	4.27	4.7	80.0	97.5	33
	P	K	96.6	94.1	10.46	10.8	82.9	118.5	15

(continued on next page)

Table 3 (continued)

Characteristic ^a	Met ^b	Strain	Mean	M	SD	CV	Min	Max	n
Somatic kineties, number	P	L7	29.1	30.0	1.81	6.2	25	33	32
	P	K	31.8	31.5	0.96	3.0	31	33	4
Postoral kineties, number	P	L7	8.2	8.0	0.64	7.8	7	9	17
Perizonal ciliary stripe rows, number	P	L7	5.0	5.0	0.00	0.0	5	5	29
Proboscis, length	IV	L7	25.6	24.0	6.16	24.1	18.0	40.0	23
	IV	K	31.0	36.0	—	—	21.0	38.0	5
	P	L7	21.3	20.5	3.21	15.1	16.0	27.0	16
	P	K	31.1	33.0	4.66	15.0	21.0	37.0	13

CV, coefficient of variation (%); M, median; Max, maximum value; Mean, arithmetic mean; Met, method of study; Min, minimum value; n, number of cells studied; SD, standard deviation of the arithmetic mean.

^a All distances in μm .

^b Measurements and counts in vivo (IV) and from formalin-fixed protargol-impregnated permanent preparations (P) made from digital images using calibrated software.

^c Measured as the chord.

Bothrostoma differs morphologically from other metopid genera in the following features: 1) lack of the axial torsion of the anterior body part, 2) a more or less flattened rather than convex or vaulted preoral dome (*Planometopus contractus* being a notable exception, Fig. 16), 3) the anterior end of the cell terminates in a snout or proboscis (with the exception of *B. kovalyovi* sp. nov.), absent in other metopids, 4) a broad, shallow peristome confined to the ventral surface vs. the deeper, spiraling adoral zone in species of other metopid genera, 5) long, very slender, curved adoral membranelles, increasing in length from posterior to anterior vs. the absence of this combination of features in species of other genera, 6) a sail-like paroral membrane that extends > 60% of adoral zone length (except for *B. extantum*) with cilia increasing in length from posterior to anterior vs. a less conspicuous paroral membrane with shorter cilia in species of other metopid genera, and 7) distinct, oblique, discontinuous rows of cortical granules in the cortical strips and small clusters within ciliary rows vs. disorderly interkinetal arrangement of granules in species of other metopid genera (Bourland and Wendell 2014; Bourland et al. 2017a, b, 2018b, 2020; Foissner 2016a, b; Jankowski 1964b; Kahl 1926, 1932; Li et al. 2021; Omar et al. 2017; Rotterová et al. 2018a, b; Vd'ačný and Foissner 2017a, b, 2019; Fig. 17).

Small and Lynn (1985) and Lynn and Small (2002) mistakenly considered *Bothrostoma* species to have only four, not five, perizonal ciliary rows. Although Jankowski (1964b) depicted only three or four perizonal stripe rows (see Figures 15 and 16 therein), he mentioned the presence of five rows, which are confirmed by our results (Figs 1–14).

The discrepancies between some published illustrations of metopid species and their accompanying descriptions have been previously discussed (Bourland et al. 2017b).

Although both *Bothrostoma* and *Planometopus* Rotterová, Bourland, and Čepička, 2018 lack axial torsion of the anterior body part, *Bothrostoma* species are easily distinguished from *Planometopus contractus* by: 1) shape (elongate ellipsoidal vs. oblong to wedge-shaped or almost rectangular), 2) ventral curvature of the anterior margin of the preoral dome (absent vs. present), 3) dorsoventral flattening of the body (absent vs. conspicuous), 4) cortical surface (interkinetal cortical strips vs. distinct, sharp cortical ridges), 5) arrangement of somatic kineties (ordinarily spaced between cortical strips vs. widely spaced on the peaks of cortical ridges), 6) a prominent sail-like paroral membrane (present vs. absent), and 7) perizonal ciliary stripe “false-kinety” arrangement (absent vs. present) (Figs 1–14, 16, 17; Kahl 1932; Penard 1922; Rotterová et al. 2018a, b).

Phylogenetic Position of *Bothrostoma* within Metopida

The order Metopida includes two monophyletic families, Apometopidae and Tropidoatractidae, and the non-monophyletic Metopidae. A little more than a decade ago, the family Metopidae included seven genera (Lynn 2008). Today, the family has doubled in size and includes *Atopospira* Jankowski, 1964, *Bothrostoma*, *Brachonella* Jankowski, 1964, *Castula* Bourland, Rotterová, and Čepička, 2020, *Eometopus* Small and Lynn, 1985, *Heterometopus*

Table 4. Morphometric data for *Bothrostoma ovale* (Kahl, 1932) comb. nov. EPOPOLO strain (E), and SMALLID strain (S).

Characteristic ^a	Met ^b	Strain	Mean	M	SD	CV	Min	Max	n
Body, length	IV	E	78.3	78.5	4.98	6.4	71.0	86.0	16
	P	E	61.2	61.0	7.62	12.4	47.0	78.0	16
	IV	S	62.8	–	–	–	51.7	74.5	5
	P	S	61.2	61.0	7.71	12.6	47.0	77.0	43
Body, width	IV	E	27.3	26.5	3.16	11.6	23.0	34.0	16
	P	E	21.3	21.5	2.65	12.5	18.0	26.0	16
	IV	S	29.3	–	–	–	25.4	34.2	5
	P	S	22.0	22.0	3.07	13.9	17.0	31.0	43
Body, length-width, ratio	IV	E	2.9	3.0	0.31	10.6	2.4	3.3	16
	P	E	2.9	3.0	0.36	12.3	2.0	3.4	16
	IV	S	2.2	–	–	–	1.8	2.5	5
	P	S	2.8	2.8	0.35	12.6	1.9	3.4	43
Anterior cell end to posterior end of adoral zone	P	E	36.1	36.0	2.17	6.0	32.0	39.0	16
	P	S	37.1	37.0	3.45	9.3	29.0	46.0	36
Distance anterior cell end to posterior end of adoral zone-body length, ratio in %	P	E	59.1	59.0	5.02	8.5	50.0	68.1	15
	P	S	60.2	60.0	4.22	7.0	54.0	68.0	36
Anterior cell end to anterior end of adoral zone	P	E	9.8	10.0	1.20	12.3	7.0	12.0	17
	P	S	11.3	12.0	2.13	18.8	7.0	14.0	32
Distance anterior cell end to anterior end of adoral zone-body length, ratio in %	P	E	16.3	16.4	1.35	8.3	14.1	19.1	16
	P	S	18.3	18.0	2.86	15.7	12.0	26.0	32
Anterior cell end to posterior end of perizonal stripe row 1	P	E	5.1	5.0	0.86	16.8	3.0	6.0	14
Distance anterior cell end to posterior end of perizonal stripe row 1-body length, ratio in %	P	E	8.5	8.6	1.12	13.2	6.4	10.0	13
Anterior cell end to posterior margin of macronucleus	P	E	34.5	35.0	3.18	9.2	28.0	40.0	16
	P	S	32.0	31.2	4.10	12.8	24.0	44.0	38
Distance anterior cell end to posterior margin of macronucleus-body length, ratio in %	P	E	56.8	55.9	5.15	9.1	50.8	70.6	16
	P	S	52.4	52.0	3.85	7.3	43.0	66.0	37
Macronucleus, length	P	E	16.4	16.5	2.55	15.6	12.0	20.0	16
	P	S	13.8	13.5	1.94	14.1	11.0	20.0	37
Macronucleus, width	P	E	9.1	9.0	1.34	14.8	7.0	12.0	16
	P	S	9.3	9.0	1.26	13.6	7.0	12.0	37
Micronucleus, diameter	P	E	3.0	3.0	0.00	0.0	3.0	3.0	5
	P	S	2.4	2.5	0.44	18.3	2.0	3.0	17
Adoral zone of membranelles, length	P	E	26.3	26.0	1.84	7.0	22.0	29.0	15
Adoral zone length-body length, ratio in %	P	E	43.1	42.6	4.54	10.5	35.9	50.0	14
Adoral membranelles, number	P	E	18	18	0.67	3.9	16	18	12
	P	S	16	16	0.67	4.2	15	17	23
Width of mid-adoral zone	P	E	10.2	10.0	1.29	12.6	8.0	13.0	17
	P	S	9.8	10.0	0.95	9.7	8.0	11.0	29
Paroral membrane, length ^c	P	E	20.2	20.0	1.03	5.1	18.0	22.0	14
	P	S	21.9	22.0	1.82	8.3	17.0	26.0	24
Length of paroral-cell length, ratio in %	P	E	32.2	32.8	3.16	9.8	28.2	37.7	13
	P	S	35.6	34.0	4.66	13.1	29.0	48.0	24
Length of paroral-adoral zone length, ratio in %	P	E	75.3	76.9	5.83	7.7	64.3	84.0	13
	P	S	87.2	86.0	7.22	8.3	76.0	110.0	24
Somatic kineties, number	P	E	13.5	13.0	0.52	3.9	13	14	13
	P	S	16.3	16.0	0.65	4.0	15	17	22

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Table 4 (*continued*)

Postoral kineties, number	P	E	5.6	6.0	0.55	9.8	5	6	5
Perizonal ciliary stripe rows, number	P	E	4.7	5.0	0.48	10.3	4	5	10

CV, coefficient of variation (%); M, median; Max, maximum value; Mean, arithmetic mean; Met, method of study; Min, minimum value; n, number of cells studied; SD, standard deviation of the arithmetic mean.

^a All distances in μm .

^b Measurements and counts in vivo (IV) and from formalin-fixed protargol-impregnated permanent preparations (P) made from digital images using calibrated software.

^c Measured as the chord.

Table 5. Morphometric data for *Bothrostoma bimicronucleatum* sp. nov. KUNRA strain.

Characteristic ^a	Met ^b	Mean	M	SD	CV	Min	Max	n
Body, length	IV	151.0	145.0	22.03	14.6	109.0	189.0	20
	P	123.2	122.5	18.81	15.3	95.0	176.0	38
Body, width	IV	64.9	64.0	10.73	16.5	44.0	83.0	20
	P	47.8	44.5	10.21	21.4	34.0	70.0	38
Body, length-width, ratio	IV	2.3	2.3	0.23	9.7	2.1	2.8	20
	P	2.6	2.6	0.34	12.9	1.9	3.5	38
Anterior cell end to posterior end of adoral zone	P	69.6	71.0	6.06	8.7	56.0	79.0	31
Distance anterior cell end to posterior end of adoral zone-body length, ratio in %	P	58.3	60.2	7.87	13.5	40.9	71.4	31
Anterior cell end to anterior end of adoral zone	P	15.4	16.0	3.51	22.8	9.0	27.0	31
Distance anterior cell end to anterior end of adoral zone-body length, ratio in %	P	12.7	12.9	1.97	15.4	9.1	16.2	31
Anterior cell end to posterior end of perizonal stripe row 1	P	6.1	6.0	1.13	18.4	4.0	9.0	27
Distance anterior cell end to posterior end of perizonal stripe row 1-body length, ratio in %	P	5.3	5.3	1.38	25.9	2.8	8.8	27
Anterior cell end to posterior margin of macronucleus	P	64.2	65.0	8.02	12.5	53.0	88.0	31
Distance anterior cell end to posterior margin of macronucleus-body length, ratio in %	P	53.7	51.9	8.85	16.5	42.1	76.5	31
Macronucleus, length	P	32.4	32.0	6.74	20.8	22.0	50.0	33
Macronucleus, width	P	18.2	18.0	2.56	14.1	13.0	24.0	33
Micronucleus, number	P	2.0	2.0	0.00	0.0	2	2	18
Micronucleus, diameter	P	4.7	5.0	0.56	11.9	4.0	6.0	25
Micronucleus, diameter	P	2.9	3.0	0.66	22.4	2.0	4.0	17
Adoral zone of membranelles, length	P	54.6	55.0	6.15	11.3	45.0	75.0	31
Adoral zone length-body length, ratio in %	P	45.9	47.4	7.52	16.4	27.8	59.2	31
Adoral membranelles, number	P	40.6	41.0	2.36	5.8	35	45	24
Width of mid-adoral zone	P	16.8	17.0	1.39	8.3	14.0	20.0	29
Paroral membrane, length ^c	P	49.1	49.5	4.54	9.2	42.0	58.0	28
Length of paroral-cell length, ratio in %	P	40.9	41.3	6.51	15.9	26.7	52.3	28
Length of paroral-adoral zone length, ratio in %	P	88.9	91.4	7.50	8.4	70.7	98.2	27
Somatic kineties, number	P	55.7	55.0	5.35	9.6	44	68	36
Postoral kineties, number	P	20.3	20.0	1.74	8.6	17	22	11
Perizonal ciliary stripe rows, number	P	5.0	5.0	0.00	0.0	5	5	28

CV, coefficient of variation (%); M, median; Max, maximum value; Mean, arithmetic mean; Met, method of study; Min, minimum value; n, number of cells studied; SD, standard deviation of the arithmetic mean.

^a All distances in μm .

^b Measurements and counts in vivo (IV) and from formalin-fixed protargol-impregnated permanent preparations (P) made from digital images using calibrated software.

^c Measured as the chord.

Table 6. Morphometric data for *Bothrostoma boreale* sp. nov. GRNLX strain (G) and MEDNIK3 (M).

Characteristic ^a	Met ^b	Strain	Mean	M	SD	CV	Min	Max	n
Body, length	IV	G	89.8	90.5	—	—	86.0	92.0	4
	P	G	65.3	64.0	6.61	10.1	55.0	79.0	16
	IV	M	69.5	66.0	8.94	12.9	56.0	82.0	11
	P	M	60.1	59.0	6.92	11.5	51.0	72.0	15
Body, width	IV	G	24.0	23.0	—	—	20.0	30.0	4
	P	G	16.5	17.0	1.90	11.5	13.0	21.0	16
	IV	M	25.9	24.0	4.32	16.7	21.0	33.0	11
	P	M	18.1	17.0	3.03	16.7	14.0	23.0	15
Body, length-width, ratio	IV	G	3.8	3.9	—	—	2.9	4.6	4
	P	G	4.0	3.9	0.54	13.5	3.0	5.2	16
	IV	M	2.7	2.7	0.30	11.0	2.3	3.1	11
	P	M	3.4	3.4	0.42	12.5	2.7	4.0	15
Anterior cell end to posterior end of adoral zone	P	G	33.0	33.0	2.80	8.5	27.0	38.0	16
	P	M	34.2	33.7	2.70	7.9	30.0	39.0	15
Distance anterior cell end to posterior end of adoral zone-body length, ratio in %	P	G	50.9	49.3	6.09	12.0	39.2	61.8	16
	P	M	57.4	56.1	5.17	9.0	46.9	65.4	15
Anterior cell end to anterior end of adoral zone	P	G	9.3	9.0	1.62	17.4	7.0	12.0	16
	P	M	9.8	10.0	1.21	12.4	7.1	12.0	15
Distance anterior cell end to anterior end of adoral zone-body length, ratio in %	P	G	14.2	13.9	2.04	14.3	11.1	18.2	16
	P	M	16.4	16.2	2.21	13.5	12.5	20.8	15
Anterior cell end to posterior end of perizonal stripe row 1	P	G	4.2	4.0	0.58	13.7	3.0	5.0	14
	P	M	4.8	4.8	0.72	15.2	3.8	6.0	14
Distance anterior cell end to posterior end of perizonal stripe row 1-body length, ratio in %	P	G	6.4	6.3	0.96	15.0	4.5	8.1	14
	P	M	8.1	7.9	1.17	14.5	6.3	9.7	14
Anterior cell end to posterior margin of macronucleus	P	G	31.0	31.0	4.13	13.3	23.0	38.0	16
	P	M	32.5	33.0	3.01	9.3	25.7	37.0	15
Distance anterior cell end to posterior margin of macronucleus-body length, ratio in %	P	G	47.9	45.4	7.82	16.3	35.4	63.6	16
	P	M	54.5	55.0	4.79	8.8	45.1	62.5	15
Macronucleus, length	P	G	16.4	15.0	3.67	22.5	12.0	24.0	17
	P	M	16.7	17.1	2.52	15.1	11.0	20.0	15
Macronucleus, width	P	G	10.3	10.0	1.57	15.3	8.0	14.0	17
	P	M	8.1	8.0	1.29	15.9	6.5	10.0	15
Micronucleus, diameter	P	G	2.6	3.0	0.55	21.1	2.0	3.0	5
	P	M	2.9	2.9	0.46	16.1	2.5	4.0	10
Adoral zone of membranelles, length	P	G	23.7	24.0	2.33	9.8	20.0	27.0	16
	P	M	24.5	24.0	2.07	8.5	22.0	29.0	15
Adoral zone length-body length, ratio in %	P	G	36.7	36.7	5.63	15.3	25.3	49.1	16
	P	M	41.0	40.9	3.96	9.7	34.4	47.8	15
Adoral membranelles, number	P	G	17.0	17.0	0.94	5.5	16	18	19
	P	M	16.7	16.0	0.90	5.4	16	19	15
Width of mid-adoral zone	P	G	8.2	8.0	1.15	13.9	6.0	10.0	17
	P	M	8.1	8.1	0.97	11.9	6.4	10.0	15
Paroral membrane, length	P	G	19.4	19.0	2.42	12.5	17.0	24.0	16
	P	M	20.1	20.0	1.98	9.9	17.5	24.0	15
Length of paroral-cell length, ratio in %	P	G	30.4	30.5	5.38	17.7	21.5	43.6	15
	P	M	33.7	33.3	3.94	11.7	25.7	39.2	15
Length of paroral-adoral zone length, ratio in %	P	G	82.2	81.0	5.83	7.1	72.0	91.7	15
	P	M	82.1	82.8	5.30	6.5	69.2	91.3	15
Somatic kineties, number	P	G	16.6	16.5	0.74	4.5	16	18	14
	P	M	17.1	17.0	1.04	6.1	15	18	13

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Table 6 (*continued*)

Postoral kineties, number	P	G	5.1	5.0	0.74	14.5	4	6	10
	P	M	5.9	6.0	0.79	13.4	5	7	12
Perizonal ciliary stripe rows, number	P	G	4.4	4.0	0.52	11.8	4	5	8
	P	M	4.1	4.0	0.28	6.8	4	5	13

CV, coefficient of variation (%); M, median; Max, maximum value; Mean, arithmetic mean; Met, method of study; Min, minimum value; n, number of cells studied; SD, standard deviation of the arithmetic mean.

^cMeasured as the chord.

^a All distances in μm .

^b Measurements and counts in vivo (IV) and from formalin-fixed protargol-impregnated permanent preparations (P) made from digital images using calibrated software.

Table 7. Morphometric data for *Bothrostoma robustum* sp. nov. LIBL strain (LI), PRASK strain (PR), and CEPIMAL1 strain (C1).

Characteristic ^a	Met ^b	Strain	Mean	M	SD	CV	Min	Max	n
Body, length	IV	LI	102.8	100.0	8.13	7.9	94.0	120.0	10
	P	LI	84.6	82.5	7.07	8.4	76.0	97.0	18
	IV	PR	82.3	—	—	—	74.0	90.0	6
	P	PR	77.1	75.0	9.04	11.7	60.0	95.0	15
	IV	C1	126.8	125.0	12.72	10.0	113.0	155.0	14
	P	C1	108.5	105.0	11.76	10.8	95.0	137.0	19
Body, width	IV	LI	41.2	42.0	3.85	9.4	32.0	46.0	10
	P	LI	28.9	28.5	3.15	10.9	24.0	34.0	18
	IV	PR	30.3	—	—	—	27.0	35.0	6
	P	PR	31.8	30.0	5.82	18.3	22.0	40.0	15
	IV	C1	46.9	45.0	7.74	16.5	38.0	60.0	14
	P	C1	36.5	37.0	3.45	9.5	31.0	43.0	19
Body, length-width, ratio	IV	LI	2.5	2.5	0.27	10.6	2.2	3.1	10
	P	LI	3.0	2.9	0.44	14.7	2.4	3.8	18
	IV	PR	2.7	—	—	—	2.6	2.8	6
	P	PR	2.5	2.4	0.30	12.3	2.1	3.2	15
	IV	C1	2.7	2.8	0.31	11.4	2.1	3.1	14
	P	C1	3.0	3.0	0.39	13.1	2.3	3.7	19
Anterior cell end to posterior end of adoral zone	P	LI	53.3	52.5	3.51	6.6	47.0	60.0	18
	P	PR	46.7	45.5	4.53	9.7	40.0	55.0	16
	P	C1	66.2	66.5	5.23	7.9	58.0	77.0	20
Distance anterior cell end to posterior end of adoral zone-body length, ratio in %	P	LI	63.3	63.1	5.00	7.9	51.1	73.4	18
	P	PR	61.2	61.0	6.67	10.9	47.0	72.0	15
	P	C1	61.0	60.0	4.03	6.6	56.2	69.8	19
Anterior cell end to anterior end of adoral zone	P	LI	12.3	12.0	2.43	19.7	7.0	16.0	18
	P	PR	11.6	12.0	1.59	13.8	8.0	13.0	16
	P	C1	12.3	12.0	1.53	12.4	10.0	15.0	20
Distance anterior cell end to anterior end of adoral zone-body length, ratio in %	P	LI	14.6	14.8	2.70	18.5	7.6	19.2	18
	P	PR	15.1	15.0	1.71	11.3	12.0	18.0	15
	P	C1	11.3	11.0	1.30	11.4	8.9	14.3	19
Anterior cell end to posterior end of perizonal stripe row 1	P	LI	7.1	7.5	1.83	25.7	4.0	10.0	14
	P	PR	6.1	6.0	1.22	0.2	4.0	8.0	11
	P	C1	5.3	5.0	1.06	19.9	4.0	7.0	19

(continued on next page)

Table 7 (continued)

Characteristic ^a	Met ^b	Strain	Mean	M	SD	CV	Min	Max	n
Distance anterior cell end to posterior end of perizonal stripe row 1-body length, ratio in %	P	LI	8.4	8.9	2.23	26.6	4.3	12.0	14
	P	PR	8.2	8.2	1.79	0.2	5.7	12.1	11
	P	C1	5.1	5.0	1.12	22.2	3.4	7.1	18
Anterior cell end to posterior margin of macronucleus	P	LI	43.1	43.0	3.31	7.7	40.0	53.0	18
	P	PR	42.2	42.5	5.38	12.8	32.0	49.0	16
	P	C1	52.4	53.0	5.14	9.8	38.0	60.0	20
Distance anterior cell end to posterior margin of macronucleus-body length, ratio in %	P	LI	51.1	50.6	3.81	7.5	43.5	58.2	18
	P	PR	54.9	56.0	5.35	9.7	44.0	65.0	15
	P	C1	48.5	48.5	5.34	11.0	35.8	60.2	19
Macronucleus, length	P	LI	18.5	18.5	3.65	19.7	13.0	27.0	18
	P	PR	22.5	22.5	3.49	15.5	16.0	31.0	16
	P	C1	26.9	27.0	3.93	14.6	18.0	32.0	20
Macronucleus, width	P	LI	11.3	11.0	1.57	13.9	9.0	14.0	18
	P	PR	10.2	10.0	1.53	14.9	8.0	13.0	16
	P	C1	12.7	12.0	2.00	15.8	10.0	17.0	20
Micronucleus, diameter	P	LI	3.7	4.0	0.90	24.3	3.0	6.0	11
	P	PR	3.4	3.5	0.41	12.3	2.5	4.0	10
	P	C1	3.3	3.0	0.59	18.2	3.0	5.0	15
Adoral zone of membranelles, length	P	LI	41.0	40.5	2.85	6.9	37.0	48.0	18
	P	PR	35.8	35.0	4.07	11.4	31.0	43.0	16
	P	C1	53.9	54.0	4.55	8.4	47.0	63.0	20
Adoral zone length-body length, ratio in %	P	LI	48.7	48.7	4.49	9.2	43.0	58.2	18
	P	PR	49.5	50.4	6.53	0.1	38.3	59.1	14
	P	C1	49.6	48.5	3.81	7.7	45.0	57.3	18
Adoral membranelles, number	P	LI	24.9	25.0	1.02	4.1	23	26	18
	P	PR	22.0	23.0	1.71	7.6	19	24	13
	P	C1	29.9	30.0	1.11	3.7	28	32	18
Width of mid-adoral zone	P	LI	13.1	13.0	1.29	9.8	11.0	16.0	14
	P	PR	12.8	13.0	1.42	11.1	10.0	16.0	14
	P	C1	14.6	15.0	2.17	14.9	11.0	20.0	19
Paroral membrane, length ^c	P	LI	35.2	34.0	2.71	7.7	32.0	42.0	13
	P	PR	31.0	30.0	3.52	11.4	26.0	37.0	16
	P	C1	46.3	47.0	5.64	12.2	39.0	58.0	17
Length of paroral-cell length, ratio in %	P	LI	40.9	39.0	4.21	10.3	35.5	47.7	13
	P	PR	40.9	41.0	4.14	10.1	30.0	48.0	15
	P	C1	42.6	42.6	4.24	10.0	35.6	51.0	16
Length of paroral-adoral zone length, ratio in %	P	LI	85.3	85.0	3.67	4.3	80.0	92.3	13
	P	PR	87.3	86.0	7.58	8.7	68.0	100.0	15
	P	C1	85.0	86.5	5.13	6.0	76.5	93.5	17
Somatic kineties, number	P	LI	25.2	25.0	1.07	4.2	24	27	13
	P	PR	29.0	28.0	2.29	7.8	26	33	14
	P	C1	31.8	32.0	1.83	5.8	29	34	18
Postoral kineties, number	P	LI	8.7	9.0	0.67	7.8	8	10	10
	P	PR	8.0	8.0	0.59	7.3	7	9	13
	P	C1	13.9	14.0	1.35	9.7	12	16	7

(continued on next page)

Table 7 (continued)

Characteristic ^a	Met ^b	Strain	Mean	M	SD	CV	Min	Max	n
Perizonal ciliary stripe rows, number	P	LI	5.0	5.0	0.00	0.0	5	5	14
	P	PR	5.0	5.0	0.00	0.0	5	5	14
	P	C1	5.0	5.0	0.00	0.0	5	5	20

CV, coefficient of variation (%); M, median; Max, maximum value; Mean, arithmetic mean; Met, method of study; Min, minimum value; n, number of cells studied; SD, standard deviation of the arithmetic mean.

^a All distances in μm .

^b Measurements and counts in vivo (IV) and from formalin-fixed protargol-impregnated permanent preparations (P) made from digital images using calibrated software.

^c Measured as the chord.

Foissner, 2016, *Idiometopus* Bourland, Rotterová, Luo, and Čepička, 2018, *Lepidometopus* Vd'ačný and Foissner, 2017, *Longitaenia* Bourland, Rotterová, and Čepička, 2020, *Metopus*, *Parametopidium* Aescht, 2001, *Pileometopus* Bourland, Rotterová, and Čepička, 2020, *Planometopus*, and *Tesnospira* Jankowski, 1964 (Adl et al. 2019; Bourland and Wendell 2014; Bourland et al. 2014, 2017a, 2018a, b, 2020; Silva-Neto et al. 2016; Esteban et al. 1995; Foissner 2016a, b; Jankowski 1964a; Kahl 1927, 1932; Li et al. 2021; Lynn 2008; Rotterová et al. 2018a, b; Small and Lynn 1985; Vd'ačný and Foissner, 2017a, b). This reflects a recent resurgence of interest in ciliate anaerobiosis in general, and Metopida in particular.

The family Metopidae is polyphyletic, and the “true” members of Metopidae probably reside in the MeBr clade. Therefore, the HAC clade (PiCa clade + BoPI clade + *Heterometopus* + LAC clade (see Bourland et al. 2018b, 2020; Rotterová et al. 2018a) needs clarification through a multigene approach since the internal relationships are not well-resolved in 18S rRNA gene phylogenetic analyses (Fig. 15).

Phylogenetically, the genus *Bothrostoma* appears to be closely related to the monotypic genus *Planometopus*. Together they form the moderately supported clade BoPI, which is only distantly related to *Metopus* es, the type species of the type genus of the family Metopidae (see Bourland et al. 2017a; Rotterová et al. 2018a). In previous phylogenetic trees, *Planometopus contractus* and the environmental sequence AY821942 (which clusters with *Bothrostoma*) always branched within the HAC clade, where it was either sister to remaining HAC lineages (see figure 13 in Bourland et al. 2020) or a close relative of the PiCa clade (see figure 20 in Rotterová et al. 2018a; Fig. 15). The BS

value of the monophyly support for the BoPI clade has increased from 63% to 78% in the current tree, probably reflecting more extensive taxon sampling. The exact position of the BoPI clade remains uncertain pending more robust multigene analyses. Morphologically, the BoPI clade is also supported since both *Bothrostoma* and *Planometopus* lack the anterior axial torsion of the cell (see above) and have a flattened preoral dome.

The BoPI clade is phylogenetically distant from the type species of *Metopus*, and the members of BoPI lack one of the main diagnostic features of other Metopidae, i.e., “anterior part of body uniquely twisted to left” (Lynn 2008). It is probable that BoPI will be removed from Metopidae and possibly reclassified to its own family. However, at the current state of knowledge, we decided to keep these two genera inside Metopidae. Metopidae remains in sore need of a comprehensive revision. The anterior axial torsion is likely a plesiomorphic feature of Metopidae, having been lost only in the BoPI clade (Bourland et al. 2020; Silva-Neto et al. 2016; Omar et al. 2017; Vd'ačný and Foissner 2019).

The evolutionary origin of *Bothrostoma* was considered by Jankowski (1964a, b). He proposed that *Bothrostoma*, particularly *B. extantum*, had originated either from 1) a *Blepharisma*-like species, due to the high resemblance of both genera; or 2) from a *Metopus*-like species. In the second assumption, he considered that *Metopus* was derived from *Blepharisma* where, as a primary character, the anterior end of the cell twisted, therefore the ciliary rows on the twisted margin became more specialized as the perizonal stripe. Thus, he considered *Metopus* as the most primitive member of metopids and proposed that *Bothrostoma* had its origin in a *Metopus*-like species that secondarily lost the anterior torsion. Nevertheless, it is well known that *Ble-*

Table 8. Morphometric data for *Bothrostoma kovalyovi* sp. nov.

Characteristic ^a	Met ^b	Mean	M	SD	CV	Min	Max	n
Body, length	IV	80.4	79.0	10.02	12.5	66.0	102.0	25
	P	75.9	76.0	9.92	13.1	55.0	97.0	35
Body, width	IV	28.8	28.0	4.45	15.4	24.0	39.0	25
	P	21.3	21.0	3.70	17.4	14.0	29.0	35
Body, length–width, ratio	IV	2.8	2.7	0.29	10.3	2.4	3.4	25
	P	3.6	3.7	0.42	11.6	2.8	4.3	35
Anterior cell end to posterior end of adoral zone	P	40.0	40.0	3.48	8.7	30.0	47.0	31
Distance anterior cell end to posterior end of adoral zone-body length, ratio in %	P	52.8	53.2	3.79	7.2	44.3	62.5	31
Anterior cell end to anterior end of adoral zone	P	11.8	12.0	1.50	12.7	7.0	15.0	33
Distance anterior cell end to anterior end of adoral zone-body length, ratio in %	P	15.5	15.4	1.37	8.9	12.7	18.8	33
Anterior cell end to posterior end of perizonal stripe row 1	P	4.8	5.0	0.95	19.7	3.0	7.0	30
Distance anterior cell end to posterior end of perizonal stripe row 1-body length, ratio in %	P	6.4	6.5	1.25	19.5	4.1	9.1	30
Anterior cell end to posterior margin of macronucleus	P	45.4	44.0	6.03	13.3	36.0	57.0	33
Distance anterior cell end to posterior margin of macronucleus-body length, ratio in %	P	60.2	60.2	6.41	10.6	42.3	72.2	33
Macronucleus, length	P	19.0	19.5	2.97	15.7	13.0	25.0	34
Macronucleus, width	P	9.4	9.0	1.76	18.7	7.0	14.0	34
Micronucleus, diameter	P	3.3	3.0	0.47	14.2	3.0	4.0	23
Adoral zone of membranelles, length	P	28.3	29.0	2.44	8.6	23.0	34.0	31
Adoral zone length-body length, ratio in %	P	37.4	37.7	3.26	8.7	30.9	43.8	31
Adoral membranelles, number	P	16.9	17.0	0.85	5.0	16	18	20
Width of mid-adoral zone	P	8.4	8.0	1.29	15.4	7.0	14.0	32
Paroral membrane, length ^c	P	24.8	25.0	2.08	8.4	21.0	28.0	28
Length of paroral-cell length, ratio in %	P	33.0	32.9	3.70	11.2	24.5	40.6	28
Length of paroral-adoral zone length, ratio in %	P	88.3	89.8	6.69	7.6	70.0	96.6	28
Somatic kineties, number	P	19.8	20.0	1.04	5.3	18	22	21
Postoral kineties, number	P	7.4	7.0	0.50	6.8	7	8	14
Perizonal ciliary stripe rows, number	P	5.0	5.0	0.00	0.0	5	5	26

CV, coefficient of variation (%); M, median; Max, maximum value; Mean, arithmetic mean; Met, method of study; Min, minimum value; n, number of cells studied; SD, standard deviation of the arithmetic mean.

^a All distances in μm .

^b Measurements and counts in vivo (IV) and from formalin-fixed protargol-impregnated permanent preparations (P) made from digital images using calibrated software.

^c Measured as the chord.

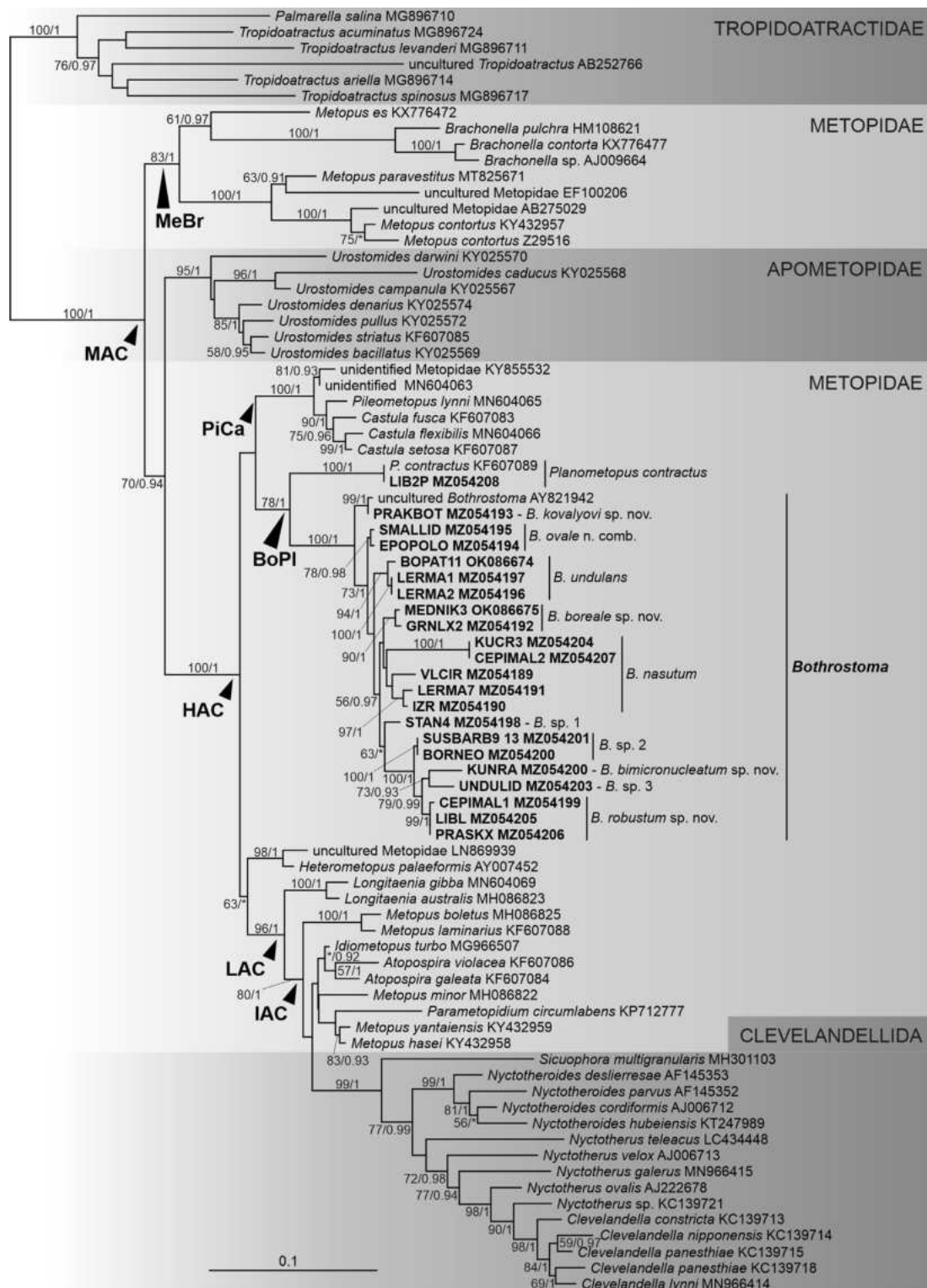


Figure 15. Phylogenetic tree based on the 18S rRNA gene sequences showing the position of *Bothrostoma* within Metopida and the new BoPI clade. The scale bar represents 10 substitutions per 100 positions.

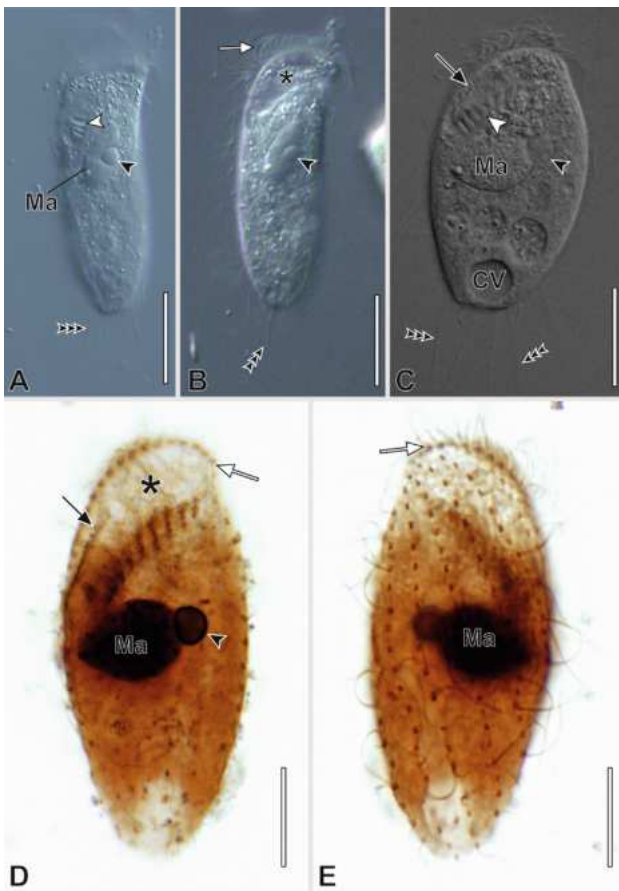


Figure 16. (A–E) *Planometopus contractus* (LIB2P strain) from life (A–C), and after protargol impregnation (D, E). (A–C) Ventral view of different specimens, and a compressed cell (C). (D, E) Ventral (D) and dorsal (E) views showing the infraciliature. Caudal cilia (black triple arrowheads); CV, contractile vacuole; Ma, macronucleus; membranelles (white arrowheads); micronucleus (black arrowheads); paroral membrane (black arrows); perizonal row kineties (white arrows); undersurface of peristome (asterisks). Scale bars: 20 μ m.

pharisma branches far from *Metopus* and *Bothrostoma*, inside the class Heterotrichea (Chi et al. 2021). Furthermore, our phylogenetic analyses support Jankowski's (1964b) scenario, in which the ancestor of *Bothrostoma* was a metopid species.

Species Diversity within *Bothrostoma*

Esteban et al. (1995) argued that Metopida, and particularly the genus *Metopus*, is less diverse than previously supposed due to several factors, e.g., the existence of polymorphic species and the lack of

detailed descriptions based on modern methods for most species. In their revision, they categorized metopids into five morphology-based groups that slightly differed from the six groups that Kahl (1927, 1932) established based on observations of living cells. The implementation of modern techniques, e.g., protargol impregnation, SEM, etc., has shed light on the diversity and the phylogenetic relationships of several metopid species (Bourland et al. 2014, 2017a, b, 2018a, b, 2020; Silva-Neto et al. 2016; Foissner 2016b; Li et al. 2021; Omar et al. 2017; Rotterová et al. 2018a; Vd'ačný and Foissner 2017a, b; Vd'ačný et al. 2019; Zhuang et al. 2021). Unfortunately, molecular data are still lacking for several recently described species, and their phylogenetic position remain unknown (Foissner 2016a; Vd'ačný and Foissner 2017a, b).

For the genus *Bothrostoma*, four described species were erected based only on the morphology of living cells. Our results clearly show that the genus is more diverse than previously thought, comprising at least nine species (including the new combination *B. ovale*) of which four, described in this report, are new. Furthermore, the actual diversity of *Bothrostoma* is certainly higher since four strains, that we characterized solely by their 18S rRNA gene sequences, possibly represent three distinct species. It is possible that *B. mirabilis* and *B. extantum*, which were not characterized in this study, are represented by some of these sequences.

Bothrostoma undulans is the most studied species of the genus (Babko et al. 2020; Foissner 1980; Foissner et al. 1992; Jankowski 1964b; Kahl 1927, 1932; Mermoud 1914; Şenler and Yildiz 2004; Stokes 1887; Wagener et al. 1986). The cells of this species present substantial phenotypic variability, particularly in size (Figs 1; 2). Kahl synonymized *Bothrostoma* and *Metopus* and, at different times (1927, 1932), divided *M. undulans* (Stokes, 1887) into three separate varieties, *M. undulans* var. *longior*, var. *major*, and var. *ovalis*. In his last revision (1932) he considered all but *M. undulans* var. *ovalis* as junior synonyms of *M. undulans* (Stokes, 1887). We sequenced the 18S rRNA gene of typical trophonts of *B. undulans* (Figs 1A, E; 2A, B). After a month, the culture became stable and contained mostly elongated slender trophonts (Figs 1C, F; 2C, N) whose 18S rRNA gene sequence was identical to that of the typical (i.e., less slender) trophonts. After some time, we mixed the culture of *B. undulans* (slender trophonts) with a thriving culture of *Tropidoatractus* for purposes

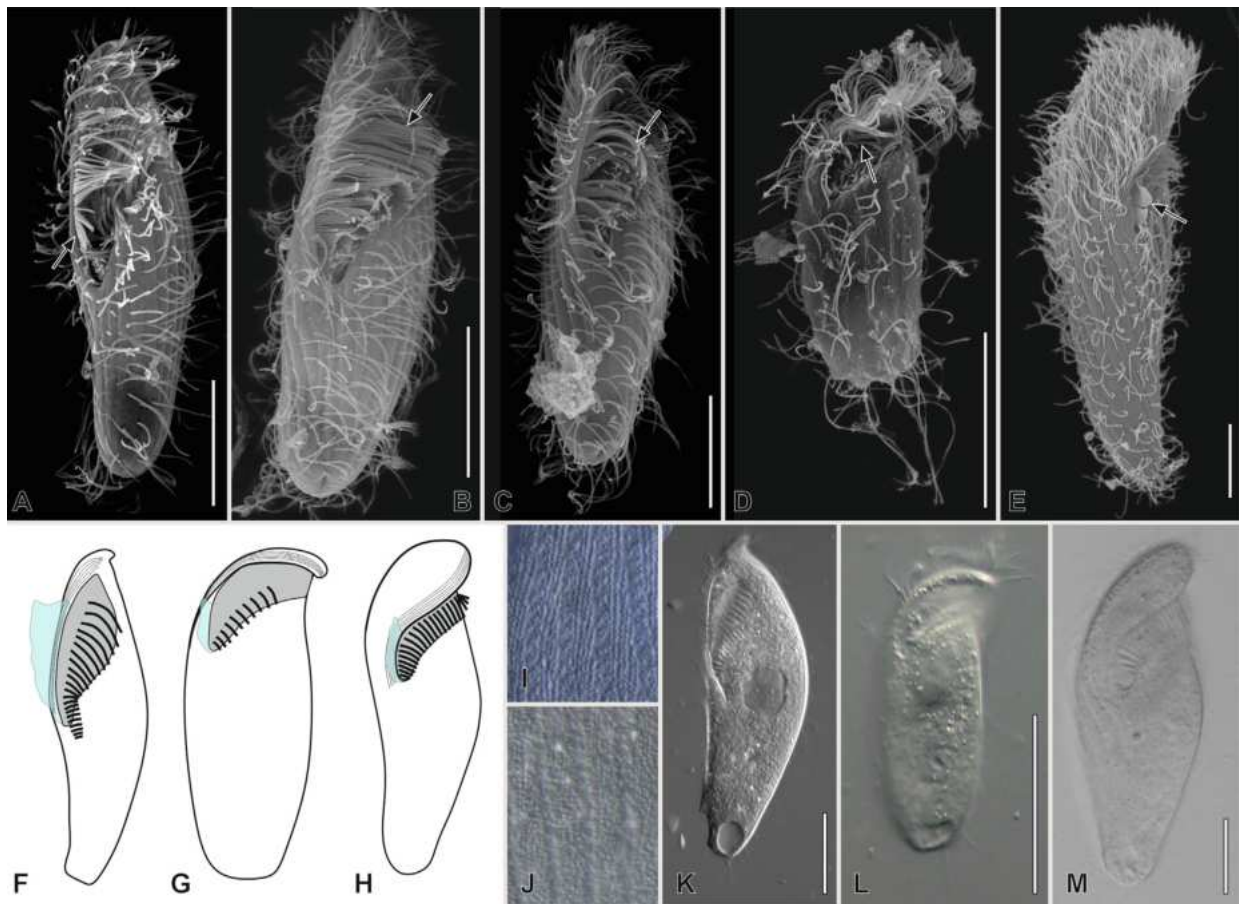


Figure 17. Morphological comparison of *Bothrostoma*, *Planometopus*, and *Metopus*, in SEM preparations (A–E), and living specimens (F–M). (A–C, F, I, K) *Bothrostoma* spp. (D, G, L) *Planometopus contractus*. (E, H, J, M) *Metopus* es. D (SMTUB strain; see Rotterová et al., 2018a) and E (MEBLUFF strain; see Bourland et al., 2017a). Scale bars: 20 µm.

of another project, and the cells of *Bothrostoma undulans* became plump and brownish due to numerous food vacuoles and giant trophonts appeared, some reaching 180 µm in length (Figs 1G, 2H). Perhaps the composition of the other culture contributed to an environment-dependent change in *B. undulans*. We suspect these two morphotypes (slender and giant) possibly represent the varieties *B. undulans* var. *major* and var. *longior* established by Kahl (1932). Interestingly, the number of membranelles and somatic kineties was similar among the different morphotypes (Table 2). Therefore, phenotypic variability in *Bothrostoma* might be driven by environmental conditions, especially nutritional factors. Esteban et al. (1995) also found dramatic phenotypic variability in cultured *Heterometopus palaeformis* (Kahl, 1927) Foissner, 2016 and diverse morphotypes are also known in

species of Tropidoatractidae (Rotterová et al. 2018a).

The third variety of *M. undulans* established by Kahl (1932), var. *ovalis*, represents a separate species, *B. ovale* (Kahl, 1932) comb. nov. Although Kahl (1932) did not provide many details for the population studied by himself, we consider the size and cell shape as strong characters to assign the populations EPOPOLO and SMALLID as conspecific with Kahl's population. The genetic distances between *B. undulans* and *B. ovale* were 0.015, further supporting the species separation (see Bourland et al. 2020).

Bothrostoma nasutum is another species that had been assigned to *Metopus*; however, morphologically and phylogenetically it belongs to *Bothrostoma* (Fig. 15), supporting Jankowski's (1964b) assignment. This species is widespread,

being found in the Nearctic, Neotropical, Palearctic, and Oriental biogeographical regions, as well as the Saharo-Arabian and Mexican transition zones proposed by [Morrone \(2015\)](#). The five 18S rRNA gene sequences obtained from different populations show a high degree of variability. The strains LERMA7 and KUCR3, which were studied in detail, were morphologically similar ([Table 3](#)), however, the genetic distance between both populations was 0.036, probably because they were isolated over 12,000 km from each other. Curiously, the 18S rRNA gene sequences of two populations isolated in different localities in the Czech Republic (KUCR3 and CEPIMAL2) were identical, while conversely, a third population from the Czech Republic (VLCIR) has a genetic distance of 0.034 from the other Czech populations. Interestingly, when the intraspecific genetic distances of the remaining *Bothrostoma* species are compared, including distant populations, the value is below 0.005. [Bourland et al. \(2020\)](#) suggested that genetic distances lower than 0.005 likely indicate conspecificity in the case of metopids. We observed substantially larger genetic distances in our isolates of *B. nasutum* and cannot exclude the possibility that it is a “species complex” reflecting cryptic diversity. However, we hesitate to embrace the description of ciliate species based solely on molecular sequences. Thus, we consider all our morphologically indistinguishable isolates of *B. nasutum* as conspecific. The long, flexible proboscis of *B. nasutum*, might qualify it as a “flagship” species (i.e., one that is unlikely to escape notice due to highly distinctive features). Nevertheless, it would not follow the role of a flagship species if it results to be a species complex due to its high genetic variability in the 18S rRNA gene (see [Bourland 2017](#)).

[Kahl \(1926\)](#) described *Metopus extentus* Kahl, 1926, and one year later established a new variety, *M. extentus* var. *steini*, based on a population of *Metopus* es studied by Stein as *M. sigmoides* ([Stein 1867](#), see Taf. XVI. figure 13 therein) ([Kahl 1927](#)). Based on morphology, [Jankowski \(1964a\)](#) transferred *Metopus extentus* to *Bothrostoma*. Since the genus name *Bothrostoma* is neuter, the species epithet must agree in gender, thus the correct names are *Bothrostoma extentum* (Kahl, 1926) Jankowski, 1964 and *Bothrostoma extentum steini* (Kahl, 1927) Jankowski, 1964. The paroral membrane of *B. extentum* bears the shortest cilia and has the shortest length among *Bothrostoma* spe-

cies. [Jankowski \(1964b\)](#) proposed this species as the earliest-diverging taxon of *Bothrostoma*. Molecular characterization of this species is needed to test Jankowski’s hypothesis. *Bothrostoma mirabilis*, another species described by [Kahl \(1927, 1932\)](#), and later redescribed by [Jankowski \(1964b\)](#), possesses a well-developed peristome that reaches almost the posterior end of the cell, has a short “thorn-like” proboscis, and, at least intermittently, inhabits a mucus case – “Gehäuse” in German. None of the populations herein studied by morphology could be identified as either *B. extentum* or *B. mirabilis*.

Interestingly, *B. robustum* sp. nov. showed considerable morphometric variability among the studied populations ([Table 7](#)). However, the genetic distance among the three strains was only 0.002, supporting their conspecificity. Other ciliate species also show wide morphological variability despite very low variability in the 18S rRNA gene ([Fan et al. 2021](#); [Fernandes et al. 2014](#); [Zhu et al. 2021](#)). We did not observe intermediate forms in *B. robustum*. Despite morphometric differences, there are features shared by all *B. robustum* populations that allow us to define them as a single species, i.e., body shape, torsion of the posterior part of the adoral zone, and a rounded snout.

Four strains that were not morphologically characterized, STAN4, SUSBARB9 13, BORNEO, and UNDULID, cannot be assigned to any of the seven *Bothrostoma* species proposed here; due to their phylogenetic position, as well as their genetic distances from the other species, and we categorized them as three separated unnamed species ([Fig. 15](#)).

Morphological Comparison Between *Bothrostoma* Species

The four new species, *Bothrostoma bimicronucleatum* sp. nov., *B. boreale* sp. nov., *B. robustum* sp. nov., and *B. kovalyovi* sp. nov., are erected due to morphological features and phylogenetic analysis of 18S rRNA gene sequences. Certain features, such as the number of adoral membranelles and somatic kineties are often stable morphologic characters for species delimitation in many ciliate groups ([Dragesco and Dragesco-Kernéis 1986](#); [Fernandes et al. 2014](#); [Foissner et al. 1992](#)). However, they are not ideal traits to discriminate between *Bothrostoma* species, namely *B. undulans*, *B. ovale* comb. nov., *B. boreale* sp. nov., and *B. kovalyovi* sp. nov., since these show an overlap for states of these charac-

ters. Nonetheless, the combination of other features such as the cell shape, the disposition of the perizonal stripe, the arrangement of cortical granules, the shape and prominence of the snout, and their phylogenetic position based on the 18S rRNA gene allow discrimination of these four species.

Bothrostoma extentum can be distinguished from its congeners by features of the paroral membrane (i.e., length less than 50% of the adoral zone length and bearing short cilia) (Kahl 1926, 1927, 1932). In all other species the paroral membrane occupies more than 60% of adoral zone length and bears long cilia, giving it a sail-like appearance. The anterior protuberance (snout or proboscis) and the shape of the adoral zone are also features that allow the distinction of *Bothrostoma* species. *Bothrostoma kovalyovi* sp. nov. differs from all other species by lacking the anterior snout (i.e., the cell tapers to a blunt anterior end) and having an arched adoral zone with an inconspicuous posterior angle as opposed to the distinctly geniculate shape of the zone in other species. *Bothrostoma bimicronucleatum* sp. nov. has an inconspicuous snout, however, it differs from all other *Bothrostoma* species by having two micronuclei. Also, it is the largest species and has the highest number of adoral membranelles and somatic kineties (35–45 and 44–68 vs. fewer than 32 and 24, respectively). *Bothrostoma robustum* sp. nov. can be distinguished from *B. bimicronucleatum* sp. nov. by the cell shape (i.e., more or less clavate vs. broadly ellipsoidal), fewer adoral membranelles (19–32 vs. 35–45), and fewer somatic kineties (24–34 vs. 44–68). *Bothrostoma robustum* sp. nov. differs from *B. mirabilis* by the shape of the snout (rounded, about 4 μ m vs. a short thorn-like proboscis), the relative length of the adoral zone (about 60% vs. 75% of body length), and occupation of a mucus case (absent vs. present, at least occasionally) (Kahl 1927, 1932; Jankowski 1964b). *Bothrostoma nasutum* differs from all other species by the elongated snout that forms a long flexible proboscis (up to 50 μ m) used to dig in and anchor to debris (Cunha 1915; Kahl 1927, 1932; Table 9).

The cell size and the number of adoral membranelles and kineties overlap among *B. undulans*, *B. ovale* comb. nov., *B. boreale* sp. nov., and *B. kovalyovi* sp. nov. *Bothrostoma undulans* has 5–7 interkinetal granule rows and small clusters within kineties, differing from *B. boreale* sp. nov. and *B. ovale* comb. nov. (both with 3–5 interkinetal rows and scattered cortical granules within kineties).

Bothrostoma ovale comb. nov. is distinguished from *B. boreale* sp. nov. mainly by the cell shape (broadly ovoidal vs. elongated ellipsoidal; Figs 5, 6, 9, 10; Table 9). Moreover, the minimum genetic distances (>0.012) support them as distinct species (see Bourland et al. 2020; Supplementary Material Table S1).

Occurrence and Ecology of *Bothrostoma* Species

To date, all species currently assigned to *Bothrostoma* have been collected from hypoxic/anoxic freshwater sediments or, in one instance (*Bothrostoma* sp. 2 BORNEO), dried mud (Table 1). In addition, all the species examined with UV excitation harbor autofluorescent cytoplasmic prokaryotic symbionts (probably methanogenic Archaea) (Wagener et al. 1986). All species feed on bacteria but some, if not most, are likely more omnivorous, feeding on heterotrophic flagellates and green algae (Fig. 8O). *Bothrostoma nasutum* has been found in the Palearctic, Nearctic, Neotropical, and Oriental biogeographical regions, as well as in the Saharo-Arabian transition zone (Cunha 1915, 1916; Kahl 1927, 1932; Kreutz and Foissner 2006; Mahajan and Nair 1971; Penard 1922). *Bothrostoma undulans* has been found in the Palearctic and Nearctic biogeographical regions (Babko et al. 2020; Foissner 1980; Kahl 1927, 1932; Mermod 1914; Stokes 1887; Şenler and Yildiz 2004; Wagener et al. 1986). *Bothrostoma ovale* has been found in the Nearctic, Palearctic, and Ethiopian biogeographical regions (Kahl 1927, 1932). *Bothrostoma boreale* sp. nov. was recorded from the Nearctic and Palearctic regions. *Bothrostoma robustum* sp. nov., *B. kovalyovi* sp. nov., and *B. bimicronucleatum* sp. nov. were found in the Palearctic region. Probably, *Bothrostoma* species are cosmopolitan, but the assessment of biogeography is hampered, as for many protist taxa, by global undersampling.

Taxonomic Summary

ZooBank registration of the work: urn:lsid:zoo bank.org:pub:5E6415B2-A1ED-4F65-B04F-A0EC8 A5A8DB3.

Taxonomic assignment: Phylum Ciliophora Doflein, 1901; Subphylum Intramacronucleata Lynn, 1996; Class Armophorea Lynn, 2004; Order Metopida Jankowski, 1980; Family Metopidae Kahl, 1927.

Table 9. Morphological comparison of *Bothrostoma* species.

Characteristic ^a	<i>B. undulans</i>	<i>B. nasutum</i>	<i>B. ovale</i>	<i>B. bimicro-nucleatum</i>	<i>B. boreale</i>	<i>B. robustum</i>	<i>B. kovalyovi</i>	<i>B. mirabilis</i>	<i>B. extantum</i>
Snout shape	Acute	Proboscis	Rounded	Flatten	Acute or rounded	Rounded	Without snout	Short proboscis	Rounded
Body, size*	60–180 × 22–45	80–150 × 22–44	51–86 × 23–34	109–189 × 44–83	56–92 × 20–33	74–155 × 27–60	66–102 × 24–39	91–102 × 25–36	140–200
Body, length-width, ratio	2.1–5.1	2.2–4.8	1.8–3.3	2.1–2.8	2.9–4.6	2.1–3.1	2.4–3.4	?	?
Micronucleus, number	1	1	1	2	1	1	1	?	?
AZM, length	21–39	27–45	22–29	45–75	20–29	31–63	23–34	?	?
AZM length-body length, ratio in %	26–57	26–47	36–50	28–59	25–49	38–59	31–44	75 ^b	?
Adoral membranelles, number	16–22	21–26	15–18	35–45	16–19	19–32	16–18	22	?
PM, length	16–33	29–41	17–22	42–58	17–24	32–58	21–28	?	?
Length of PM-cell length, ratio in %	24–47	30–45	28–48	27–52	21–44	30–51	24–40	?	?
Length of PM-AZM, ratio in %	69–108	80–118	64–110	71–98	69–91	68–100	70–97	75 ^b	30 ^b
SK, number	15–20	25–33	13–17	44–68	15–18	24–34	18–22	18–22	?
POK, number	4–7	7–9	5–6	17–22	5–7	7–16	7–8	?	?
PSK, number	5	5	4 + 1	5	4 + 1	5	5	?	?
Cortical granules disposition	5–7, clusters	6–7, clusters	3 or 4, scattered	3–4, clusters	4–5, scattered	6, clusters	5, clusters	?	?
Reference	a, b, c, d, this study	b, e, f, this study	b, this study	This study	This study	This study	This study	b, c	b

^aAll distances in µm; ^bestimation from drawings; * Measurements in vivo; AZM, adoral zone of membranelles; PM, paroral membrane; POK, postoral kineties; PSK, perizone stripe kineties, SK, somatic kineties. a–Stokes (1887), b–Kahl (1926, 1927, 1932); c–Jankowski (1964b); d–Foissner (1980); e–Cunha (1915); f–Penard (1922).

Genus *Bothrostoma* Stokes, 1887

Diagnosis: Small to medium-sized free-living freshwater Metopida. Shape elongate ellipsoidal to ovoidal, usually with anterior snout (proboscis). Leftward axial torsion of the anterior body part absent. Peristomial area wide, confined to ventral surface. Adoral zone usually posteriorly angled (geniculate) near entrance to buccal cavity. Adoral membranelles long, slender, curved, increase in length from posterior to anterior. Paroral membrane prominent, sail-like due to long cilia, usually extending > 60% of length of adoral zone.

Type species: *Bothrostoma undulans* Stokes, 1887, by monotypy.

Included species: *B. bimicronucleatum* sp. nov.; *B. boreale* sp. nov.; *B. extantum* (Kahl, 1926) Jankowski, 1964; *B. kovalyovi* sp. nov.; *B. mirabilis* (Kahl, 1927) Jankowski, 1964; *B. nasutum* (Cunha, 1915) Jankowski, 1964; *B. ovale* (Kahl, 1932) comb. nov.; *B. robustum* sp. nov.; and *B. undulans* Stokes, 1887.

Remarks: Genus *Bothrostoma* was erected by Stokes (1887), then it was synonymized with *Blepharisma* by Mermod (1914), and subsequently with *Metopus* by Kahl (1932). Mermod's synonymization of *Bothrostoma* and *Blepharisma* has not been accepted by others. Jankowski (1964a) resurrected the genus *Bothrostoma*, since then it has been recognized as a valid genus (Aesch 2001; Esteban et al. 1995; Lynn 2008).

Bothrostoma undulans Stokes, 1887

Diagnosis: Shape elongate slender to ellipsoidal, widest at, or anterior to, equator; size 60–180 × 22–45 µm in vivo; colorless to brownish. Conspicuous acute snout. 15–20 somatic kineties, including 4–7 postoral kineties; 15–22 adoral membranelles. Cortical granules, 5–7 discontinuous interkinetal rows and intrakinetal clusters.

Voucher material: Three slides, one from LERMA1, one from LERMA2, and one from BOPAT11 strains, each containing protargol-impregnated specimens, are deposited as vouchers in the collection of the National Museum in Prague, Czech Republic, inventory numbers: P6E 5181, P6E 5182, P6E 5183, respectively.

Gene sequence: Partial 18S rRNA gene sequences from LERMA1, LERMA2, and BOPAT11 strains of *B. undulans* are deposited in GenBank (accession numbers: MZ054196, MZ054197, OK086674, respectively).

Remarks: Kahl (1932) considered *Blepharisma bothrostoma* Mermod, 1914, *Metopus bothrostoma* (Mermod, 1914) Kahl, 1926 and *Metopus bothrostoma* var. *longior* Kahl, 1927 as junior synonyms of *Bothrostoma undulans* Stokes, 1887. *Bothrostoma undulans* Stokes, 1887 was combined with *Metopus* by Kahl (1932). After Jankowski (1964a) resurrected the genus *Bothrostoma* Stokes, 1887, *Metopus undulans* (Stokes, 1887) Kahl, 1932 became a junior synonym of *Bothrostoma undulans* Stokes, 1887.

Bothrostoma nasutum (Cunha, 1915) Jankowski, 1964

Diagnosis: Shape elongate ellipsoidal; size 80–150 × 22–44 µm in vivo. Long flexible proboscis (18–50 µm long in vivo). 25–33 somatic kineties, including 7–9 postoral kineties, 21–26 adoral membranelles.

Voucher material: Two slides, one from strain LERMA7 and one from strain KUCR3, each with numerous protargol-impregnated specimens, are deposited as vouchers in the collection of the National Museum in Prague, Czech Republic, inventory numbers: P6E 5184, P6E 5185, respectively.

Gene sequence: Five partial 18S rRNA gene sequences from LERMA7, KUCR3, IZR, CEPI-MAL2, and VELCIR strains of *B. nasutum* are deposited in GenBank (accession numbers: MZ054189, MZ054190, MZ054191, MZ054204, MZ054207, respectively).

Remarks: Previously, *Bothrostoma nasutum* (formerly *Metopus nasutus*) was transferred to the genus *Copemetopus* (Esteban et al. 1995). This combination has been rejected since both genera are morphologically distinct (Al-Rasheid 2001; Campello-Nunes et al. 2022). A recent phylogenetic analysis (Campello-Nunes et al. 2022) places *Copemetopus* as neither heterotrichean nor armophorean but, instead, as sister to *Protocruzia* with which it comprises a distinct early-branching lineage in Intramacronucleata.

Bothrostoma ovale (Kahl, 1932) comb. nov.

Diagnosis: Shape ellipsoidal to broadly ovoidal; size 51–86 × 23–34 µm in vivo. Conspicuous rounded snout. Posteriorly truncate or rounded. 13–17 somatic kineties including 5–6 postoral kin-

eties, 15–18 adoral membranelles. Cortical granules, 3 or 4 discontinuous rows and scattered intrakinetally. Perizonal stripe arrangement 4 + 1-type or even-type.

Voucher material: Two slides, one from EPOPOLO strain and one from SMALLID strain, with protargol-impregnated specimens, are deposited in the collection of the National Museum in Prague, Czech Republic, inventory numbers: P6E 5186, P6E 5187, respectively.

Gene sequence: Two partial 18S rRNA gene sequences from EPOPOLO, and SMALLID strains of *B. ovale* are deposited in GenBank (accession numbers: MZ054194, MZ054195, respectively).

Remarks: Kahl (1932) considered *Metopus bothrostoma* Kahl, 1926 as a junior synonym of *Metopus undulans* (Stokes, 1887) and erected a new variety, *M. undulans* var. *ovalis* therein. We elevate this variety (subspecies prior to 1961, ICZN Art 45.6.4) to species rank and transfer the species to *Bothrostoma* as the new combination *Bothrostoma ovale* (Kahl, 1932).

***Bothrostoma bimicronucleatum* sp. nov.**

Zoobank registration: urn:lsid:zoobank.org:act:E14F4AE3-8197-4922-B331-77F871095549.

Diagnosis: Shape broadly ellipsoidal to ovoidal; size 109–189 × 44–83 µm in vivo. Anteriorly rounded, inconspicuous flattened snout. Invariably two micronuclei.

Etymology: The Latin neuter adjective “*bimicronucleatum*” refers to the presence of two micronuclei.

Type locality: Eutrophic freshwater Pond in Kunratice forest, 50°01'15" N, 14°28'46" E, Prague, Czech Republic.

Type material: One slide containing protargol-impregnated specimens (the holotype and several paratypes) is deposited in the collection of the National Museum in Prague, Czech Republic, inventory number: P6E 5188. The holotype is marked with a black ink circle.

Gene sequence: An 18S rRNA gene sequence from *B. bimicronucleatum* is deposited in GenBank (accession number: MZ054202).

***Bothrostoma boreale* sp. nov.**

Zoobank registration: urn:lsid:zoobank.org:act:6A29BB79-8DB5-4934-ACA4-70C62CBF9D8B.

Diagnosis: Shape elongate to ellipsoidal, widest at or anterior to equator; size 56–92 × 20–33 µm

in vivo. Snout conspicuous, acute or rounded. 15–18 somatic kineties, including 5–7 postoral kineties; 16–19 adoral membranelles. Cortical granules in 4–5 discontinuous rows, also scattered within ciliary rows.

Etymology: From the Latin neuter adjective “*boreale*”, referring to the original discovery of this species in samples from the northern island of Greenland.

Type locality: Freshwater Pond in Medník hill, 49°52'06.2"N 14°26'34.9"E, Czech Republic.

Type material: One slide with the protargol-impregnated holotype specimen marked with a black ink circle, and one paratype slide of protargol-impregnated specimens of the GRNLX strain are deposited in the collection of the National Museum in Prague, Czech Republic, inventory numbers: P6E 5189, P6E 5190, respectively.

Gene sequence: Two partial 18S rRNA gene sequences, one from GRNLX and one from MEDNIK3 strains of *B. boreale* are deposited in GenBank (accession numbers: MZ054192 and OK086675, respectively).

***Bothrostoma kovalyovi* sp. nov.**

Zoobank registration: urn:lsid:zoobank.org:act:975B5924-B4AE-4575-A334-1027937FEF54.

Diagnosis: Shape cell elongated to ellipsoidal, widest equatorially, anteriorly rounded, snout absent; size 66–102 × 24–39 µm in vivo. 18–22 somatic kineties, including 7–8 postoral kineties, 16–18 adoral membranelles. Adoral zone in arch-like shape, not geniculate. Cortical granules, 5 discontinuous rows and clusters.

Etymology: The specific name, “*kovalyovi*”, is a noun in the genitive case, refers to Major Kovalyov, the protagonist of Gogol’s short story “The Nose”, who one day wakes up without his nose.

Type locality: Freshwater pond in the Botanical Garden, Faculty of Science, Charles University, 50°04'20" N, 14°25'24" E, Prague, Czech Republic.

Type material: One slide containing protargol-impregnated specimens (the holotype and several paratypes) is deposited in the collection of the National Museum in Prague, Czech Republic, inventory number: P6E 5191. The holotype is marked with a black ink circle.

Gene sequence: An 18S rRNA gene sequence from *B. kovalyovi* is deposited in GenBank (accession number: MZ054193).

***Bothrostoma robustum* sp. nov.**

Zoobank registration: urn:lsid:zoobank.org:act:3CD07571-9687-467D-A71A-28F87DE355AF.

Diagnosis: Shape stout, broadly ellipsoidal, posterior end broadly truncate; size 74–155 × 27–60 µm; inconspicuous rounded snout; 24–34 somatic kineties including 7–16 postoral kineties, 19–32 adoral membranelles. Posterior part of adoral zone inside infundibulum, rotating 180° clockwise and deviating toward dorsal surface. Cortical granules arranged in 5 or 6 discontinuous rows and clusters.

Etymology: From the Latin neuter adjective “*robustum*”, referring to the powerful or robust appearance of the species in vivo.

Type locality: Freshwater puddle in Libický luh, 50°06'15.2" N, 15°10'08.4" E, Czech Republic.

Type material: One slide from LIBL strain containing the protargol-impregnated holotype specimen, marked with a black ink circle, and two paratype slides, one each from CEPIMAL1 and PRASKX strains, with protargol-impregnated specimens are deposited in the collection of the National Museum in Prague, Czech Republic, inventory numbers: P6E 5300, P6E 5301, P6E 5302 respectively.

Gene sequence: Three partial 18S rRNA gene sequences from CEPIMAL1, LIBL, and PRASKX strains of *B. robustum* are deposited in GenBank (accession numbers: MZ054199, MZ054205, and MZ054206, respectively).

Methods

Sampling, culturing, and morphological characterization: Mud and sediment from freshwater ponds were sampled using Falcon tubes and plastic jars. Samples were collected from the littoral zone at the water–sediment interface at depths up to 30 cm. All samples were transported to the Department of Zoology, Faculty of Science, Charles University, Prague, CR, and inoculated into 15 ml Falcon® tubes containing 9 ml of Sonneborn's *Paramecium* medium (ATCC medium 802), maintained in polyxenic culture at room temperature, and transferred into fresh media at two-week intervals as described by Bourland et al. (2017a).

Observations of the strains were made on fresh material in all cases except *Bothrostoma kovalyovi* sp. nov. and *B. boreale* sp. nov. strain GRNLX, which had been kept as established cultures for two years as mentioned above. Strain IZR was kept for about 4 years. Strains LERMA1 (*B. undulans*) and PRAKBOT (*B. kovalyovi* sp. nov.) have been maintained in mixed cultures (for two years), usually with other ciliates, psalteriomonadid amoeboflagellates (Heterolobosea), and other anaerobic protists.

Living cells were studied at magnifications of 40–1000 × with brightfield and differential contrast illumination and protargol preparations were examined and imaged with brightfield illumination using Olympus BX51 (Olympus Corp, Tokyo, Japan) and Zeiss Axioskop 2Plus microscopes (Carl Zeiss Microscopy, LLC) equipped with an Olympus DP70 camera and Flex digital camera (Diagnostic Instruments), respectively. Video imaging was done using a Canon 6D

digital camera (Canon Inc.). Protargol (Polysciences Inc, no longer commercially available) was used for silver impregnation, mainly according to Foissner (2014) but with modifications according to Pan et al. (2013). In vivo measurements were made from photomicrographs of uncompressed, freely swimming cells. Counts and measurements from protargol preparations were made at 1000 × magnification. For SEM, cells were fixed for at least 30 minutes in a 1:1 solution of 5% glutaraldehyde and 2% osmium tetroxide and then processed according to Foissner (2014). Specimens were examined at an accelerating voltage of 10 kV in either a Hitachi SU3500 (Hitachi High Technologies America, Inc., USA) or JSM-6380 scanning electron microscope (JEOL LV, USA).

Terminology is according to Bourland et al. (2018a), Foissner and Agatha (1999), Foissner and Xu (2007), Jankowski (1964b), and Lynn (2008) except as noted; systematics mainly follows Jankowski (2007), Lynn (2008), and Adl et al. (2019).

DNA extraction, amplification, and sequencing: For all strains, except as noted below, five to ten cells were picked under an inverted microscope using micropipettes, washed several times using filtered medium (pore size 0.22 µm), and placed in Eppendorf tubes with 30 µl of DNA/RNA Shield (Zymo Research, Irvine, CA, USA). DNA was extracted with the DNeasy BloodTissue kit (Qiagen, Hilden, Germany) following the manufacturer's directions. For PCR amplification, primers Euk A (5'-AACCTGGTTGATCCTGCCAGT-3') and Euk B (5'-TGATCCATCTGCAGGTTACCT-3') or Euk A and Arm4R (5'-GWGGTTWTCCACACAGTC-3'), were used to amplify the 18S rRNA gene (Bourland et al. 2017a; Medlin et al. 1988). A DNA polymerase (PPP Master Mix, Top-Bio) was used with the following thermocycler parameters: 94 °C for 1 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 60 s at 72 °C, and final extension cycle at 72 °C for 10 min. Amplified DNA fragments were purified using the Gel/PCR DNA Fragments Extraction Kit (Qiagen). Sanger sequencing was performed using three internal primers, 577F (5'-GCCAGCACCGCGGT-3'), 577R (5'-ACCGCGGGTGCTGGC-3'), and 1055R (5'-GGTGGTGCATGGCCG-3') (Elwood et al. 1985). Sequencing was done at the Laboratory of DNA Sequencing (Charles University) on an ABI PRISM 3100 sequencer (Applied Biosystems).

For SMALLID and UNDULID strains, total DNA was extracted from washed single cells with 5% Chelex, the 18S rRNA gene was amplified by PCR using primers EukA and EukB. Sequencing was done by GeneWiz (South Plainfield, NJ, USA) as previously described (Bourland et al. 2017a).

For the strains KUCR3, IZR, BORNEO, SUSBARB9 13, and STAN4, 1.5 ml of thriving cultures were centrifuged for 8 min at 500 × g, 1.4 ml of the supernatant was removed, then the pellet and remaining 100 µl was resuspended and used for DNA isolation. DNA was isolated from the cultures using a DNA isolation kit (either Genomic DNA Minikit, Geneaid or DNeasy Blood & Tissue Kit, Qiagen, or ZR Genomic DNA Kit II TM, Zymo Research) according to the manufacturer's instructions. The 18S rRNA gene was amplified with primers designed for Armophorida, ArmF1 (5'-GCGAYATRT CATTCAAGT-3') and ArmR4 (5'-GWGGTTWTCCACACAGTC-3') and an annealing temperature of 60 °C (Bourland et al. 2017a).

In the case of the SUSBARB9 13 strain, it was necessary to clone the PCR product to obtain the target sequence. Cloning was done using a cloning kit (pGEM-T Easy Vector System I, Promega) and JM109 competent cells of *Escherichia coli* (High-Efficiency Competent Cells, Promega). Three clones were sequenced.

Phylogenetic analyses: A data set consisting of 18S rRNA gene sequences of Metopida and Clevelandellida was created with the new sequences obtained in this study and 58 additional sequences downloaded from the NCBI GenBank database. The sequences were aligned using the MAFFT method (Katoh et al. 2002) using the MAFFT 7 server <https://mafft.cbrc.jp/alignment/server/with> G-INS-i algorithm and default settings. The alignment was manually edited

using BioEdit 7.0.9.0 (Hall 1999) in order to trim primer sequences and remove hypervariable positions. The length of the final data set was 1665 positions. Phylogenetic trees were constructed by maximum likelihood (ML) and Bayesian inference (BI) methods. ML analysis was performed in RAxML 8.0.0 (Stamatakis 2014) under the GTRGAMMAI model. Node support was assessed by analysis of 1000 bootstrap replicates. Bayesian analysis was performed using MrBayes 3.2.7 on the Cipres Portal <https://www.phylo.org/portal2> (Miller et al. 2010) using the GTR + I + Γ model. Four MCMCs were run for 2,000,000 generations, with a sampling frequency of 1000 generations. The first 25% of sampled trees were removed as burn-in. Convergence was examined using RWTY (Warren et al. 2017; <https://CRAN.R-project.org/package=rwtty>).

Declaration of Interest

The authors declare no conflicts of interest.

CRedit Authorship Contribution Statement

Daniel Méndez-Sánchez: Methodology, Formal analysis, Investigation, Visualization, Writing – original draft. **Ondřej Pomahač:** Methodology, Formal analysis, Investigation. **Johana Rotterová:** Conceptualization, Methodology, Investigation. **William Bourland:** Conceptualization, Methodology, Formal analysis, Investigation, Supervision, Writing – review & editing. **Ivan Čepička:** Conceptualization, Methodology, Investigation, Formal analysis, Funding acquisition, Resources, Supervision, Writing – review & editing.

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Appendix A. Supplementary Material

Supplementary Material S1. Supplementary Material Table S1. Supplementary Material Video S1. *Bothrostoma undulans* <https://youtu.be/u3L8voJ0Wh4>. Supplementary Material Video S2. *Bothrostoma nasutum* <https://youtu.be/nB8Cqv7d7bQ>. Supplementary Material Video S3. *Bothrostoma robustum* <https://youtu.be/ZOIU19A72yY>. Supplementary Material Video S4. *Bothrostoma kovalyovi* <https://youtu.be/ufOJlzFRY3I>. Supplementary data to this article can be found online at <https://doi.org/10.1016/j.protis.2022.125887>.

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ORIGINAL ARTICLE



Morphology and phylogenetic position of three anaerobic ciliates from the classes Odontostomatea and Muranotrichea (Ciliophora)

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Abstract

The diversity of the classes Odontostomatea and Muranotrichea, which contain solely obligate anaerobes, is poorly understood. We studied two populations of *Mylestoma* sp., one of *Saprodinium dentatum* (Odontostomatea), two of *Muranotrix felix* sp. nov., and one of *Muranotrix* sp. (Muranotrichea) employing live observation, protargol impregnation, scanning electron microscopy, and 18S rRNA gene sequencing. Conspecificity of *Mylestoma* sp., described here, with a previously described species of this genus cannot be excluded since no species have been studied with modern methods. Phylogenetically, the genus *Mylestoma* is closely related to the odontostomatid *Discomorphella pedroeneasi*, although the phylogenetic position of class Odontostomatea itself remains unresolved. The newly described muranotrichean species, *Muranotrix felix* sp. nov., is morphologically similar to *M. gubernata* but can be distinguished by its fewer macronuclear nodules and fewer adoral membranelles; moreover, it is clearly distinguished from *M. gubernata* by its 18S rRNA gene sequence. Another population, designated here as *Muranotrix* sp., most likely represents a separate species.

KEY WORDS

18S rRNA gene, anoxia, marine, *Muranotrix*, *Mylestoma*, *Parablepharisma*, phylogeny, *Saprodinium*

INTRODUCTION

ANAEROBIC ciliates have received increased attention during the last decade. Most of the published studies deal with their taxonomy, diversity, and phylogenetic position using standard methods, for example, protargol impregnation, scanning electron microscopy, and single-gene phylogenies (Bourland et al., 2014, 2017a, 2017b, 2020, 2022; Bourland, Rotterová, & Čepička, 2018; Bourland, Rotterová, Luo, & Čepička, 2018; Bourland & Wendell, 2014; Campello-Nunes et al., 2020, 2022; da Silva-Neto et al., 2016; Fernandes et al., 2018; Foissner, 2016a, 2016b; Li et al., 2017; Li, Zhuang, Pérez-Uz, et al., 2021; Li, Zhuang, Wang, et al., 2021;

Méndez-Sánchez et al., 2022; Nitla et al., 2019; Orsi et al., 2012; Paiva et al., 2017; Poláková et al., 2021; Rotterová et al., 2018; Vďačný et al., 2019; Vďačný & Foissner, 2017a, 2017b, 2019; Zhuang et al., 2021, 2022). Other studies have provided insights into features of their anaerobic lifestyle, for example, evolution, metabolism, and symbioses with various prokaryotes (Beinart et al., 2018; Lewis et al., 2018; Muñoz-Gómez et al., 2021; Newbold et al., 2015; Omar et al., 2017; Rotterová et al., 2022). The more recent use of genomic/transcriptomic methods has more robustly clarified some phylogenetic relationships and further defined modifications of their mitochondrial metabolism (Rotterová et al., 2020).

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Recently, three new classes of anaerobic ciliates, Odontostomatea, Muranotrichea, and Parablepharisma, have been erected based on molecular and morphological data (Fernandes et al., 2018; Rotterová et al., 2020). According to 18S rRNA gene analyses, these three classes are closely related to Armophorea (Metopida + Clevelandellida) and, possibly, Cariacotrichea, forming a clade of exclusively obligate anaerobes (Rotterová et al., 2020).

Odontostomatids (order Odontostomatida Sawaya, 1940) have been well known for over a century and have been variously classified as members of the class Spirotrichea, Heterotrichea, Armophorea, or Plagiopylea (Corliss, 1979; Jankowski, 1964a, 2007; Lynn, 2008; Stoeck et al., 2007). Fernandes et al. (2018) elevated the order to class-level based on single- and two-gene phylogenies. According to morphological taxonomy, three families have been proposed: Mylestomatidae, Epalxellidae, and Discomorphellidae. All of their representatives are organisms with a rigid armor-like cortex, equipped with spines, and having a strongly reduced somatic cilia-ture arising from cortical pits or “sockets” (Fernandes et al., 2018; Jankowski, 1964a, 1964b; Kahl, 1932a; Lynn, 2008; Paiva et al., 2017). The family Mylestomatidae Kahl in Doflein and Reichenow, 1929 includes only two genera, *Mylestoma* and *Atopodinium*, both of which lack molecular characterization. The Epalxellidae include three genera, *Pelodinium*, *Saprodinium*, and *Epalxella*, and only the latter two genera have associated 18S rRNA gene sequences. *Epalxella* quite unexpectedly clustered inside the class Plagiopylea (Stoeck et al., 2007), and *Saprodinium*, along with *Discomorphella*, formed a deep lineage sister to Armophorea (Fernandes et al., 2018). The family Epalxellidae requires further investigation, particularly *Epalxella antiquorum* which most probably belongs to Odontostomatea.

Except for *Saprodinium dentatum* and *Discomorphella pedroeneasi*, all other odontostomatid species have an uncertain phylogenetic position and are awaiting redescription using modern methodologies, since most of the descriptions are based only on living or simply fixed specimens (Fernandes et al., 2018; Jankowski, 1964b; Kahl, 1932a, 1932b; Paiva et al., 2017; Schrenk & Bardele, 1991).

The recently erected class Muranotrichea currently comprises two monotypic genera of anaerobic ciliates (respective type species *Muranothrix gubernata* and *Thigmothrix strigosa*), inhabiting anoxic marine or brackish environments, characterized by slender cells with a rightward-spiraling anterior neck region, and invariably coated with prokaryotic ectosymbionts (Rotterová et al., 2020).

This study provides morphological and molecular characterization of five populations of anaerobic ciliates from these two poorly studied classes, broadening the diversity of the genus *Muranothrix*, describing a Czech

population of *Saprodinium dentatum*, and elucidating the phylogenetic position of the genus *Mylestoma*.

MATERIALS AND METHODS

Sampling

Anoxic sediments from six localities (see below) were sampled using Falcon tubes and plastic jars. The samples were examined with a compound microscope (Olympus BX51, Evident Europe, Hamburg). FARO samples were inoculated into 10 ml of ATCC Seawater Cereal Grass media (ATCC #1525). FRESHO samples were inoculated into a 1:1 mixture of medium #1525 and ATCC medium #802. Both were transferred into their respective fresh media every 2 weeks and maintained with undetermined prokaryotes in stable cultures.

Study localities

FRESHO. A brackish pond in Fresh River, Falmouth, Massachusetts, USA (41°32'30.804" N, 70°37'19.92" W).

FARO. A tidal brackish wetland sediment in Olhão, Algarve, Portugal (37°01'49.4" N, 7°48'32.0" W).

NAPAM. A small brackish pond in Napatree point, Rhode Island, United States (41°18'35.1" N, 71°52'42.5" W) in February 2022.

STRUJA. A brackish wetland near the sea, Strujan, Slovenia (45°31'33.1" N, 13°36'15.2" E), in September 2021.

VLKOV1. A small permanent freshwater pond in the village of Vlkov, Czech Republic (49°09'04.0" N 14°43'22.4" E) in October 2021.

SIPEK1A. A brackish lagoon in Agios Spiridonisos, Corfu, Greece (39°48'56" N, 19°51'35" E).

Morphological characterization

Living cells were studied at magnifications of 400–1000× with brightfield and differential interference contrast illumination, using an Olympus BX51 microscope equipped with an Olympus DP70 camera. Cells were fixed in 10% formalin (final concentration) and impregnated with protargol to reveal the infraciliature and nuclear apparatus (Dieckmann, 1995; Foissner, 2014; Pan et al., 2013). In vivo measurements were made from photomicrographs of uncompressed, freely swimming cells. Counts and measurements from protargol preparations were made at 1000× magnification using the software QuickPhotoCamera. In the case of odontostomatids, cells were examined by UV epifluorescence microscopy with a Zeiss Axioskop 2plus (Zeiss filter set 01) to search for methanogens according to Doddema and Vogels (1978). For scanning electron microscopy (SEM),

cells were fixed for at least 30 min in a 1:1 solution of 5% glutaraldehyde and 2% osmium tetroxide (v/v) and then processed according to Foissner (2014). Specimens were examined at an accelerating voltage of 10 kV in a JSM-6380 scanning electron microscope (JEOL LV, USA). Morphological terminology is based on Lynn (2008), Schrenk and Bardele (1991), and Paiva et al. (2017) except as noted.

DNA extraction, amplification, and sequencing

One to five cells from STRUJA, NAPAM, and VLKOV1 populations were hand-picked with glass micropipettes, washed five times with distilled water or sterile culture medium, and added into 30 µl of DNA/RNA shield (Zymo Research). Genomic DNA was isolated using the MasterPure™ Complete DNA and purification Kit following the manufacturer's instructions. For FESHO and SIPEK1A, 1.5 ml of the respective culture/sample were centrifuged at 4000g for 8 min, the supernatant was discarded, and the pellet was used for DNA isolation using the Genomic DNA Mini Kit Blood/Cultured cell (Geneaid) following manufacturer's instructions.

The 18S rRNA gene of VLKOV1, STRUJA, NAPAM, and SIPEK1A was amplified using the primers EukA (5'-AACCTGGTTGATCCTGCCAGT-3'), and EukB (5'-TGATCCATCTGCAGGTTACCT-3'; Medlin et al., 1988) with the following thermocycler parameters: 94°C for 5 min, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C, and final extension cycle at 72°C for 5 min. In the case of FRESHO, general eukaryotic primers EK42F (5'-CTCAARGAYTAAGCCATGCA-3') and EK1498R (5'-CACCTACGGAAACCTTGTTA-3') were used with the following thermocycler parameters: 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2.5 min at 72°C, and final extension cycle at 72°C for 10 min. PCR products were verified by 1% agarose gel electrophoresis and purified using the Gel/PCR DNA Fragments Extraction Kit (Qiagen). In the case of VLKOV1 and NAPAM samples, the purification was done using EXOSAP (Applied Biosystems).

For STRUJA and NAPAM, purified PCR products were directly sequenced using four internal primers, 577F (5'-GCCAGCACCCGCGGT-3'), 577R (5'-ACCGCGGGTGCTGGC-3'), 1055R (5'-GGTGGTGCATGGCCG-3'), and 1055F (5'-CGGCCATGCACCACC-3'; Elwood et al., 1985). The purified PCR products from FRESHO, VLKOV1, and SIPEK1A were cloned using the pGEM-T Easy Vector System I kit and JM109 competent cells of *Escherichia coli* (Promega). A total of three clones were obtained from SIPEK1A, five clones from FRESHO, and one clone from VLKOV1, all of which were sequenced using the primers T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-ATTTA GGTGACACTATAG-3'). Sequencing was done at the

Laboratory of DNA Sequencing (Charles University) on an ABI PRISM 3100 sequencer (Applied Biosystems).

Phylogenetic analyses

We assembled an 18S rRNA gene sequence data set comprising five newly determined sequences and 87 sequences obtained from GenBank and representing all ciliate classes. The sequences were aligned using MAFFT (Kato et al., 2002) under the G-INS-i algorithm. The alignment was manually edited using AliView (Larsson, 2014) to trim primer sequences and remove positions with single-nucleotide insertions within otherwise completely conserved positions. The final dataset included 1677 positions. Phylogenetic trees were constructed by maximum likelihood (ML) and Bayesian inference (BI) methods under the GTR+I+Γ as the best-fit model by ModelGenerator (Keane et al., 2006) according to the Akaike Information Criterion. ML analysis was performed in RAXML HPC 8.2.4 (Stamatakis, 2014). Node support was assessed by 1000 bootstrap pseudoreplicates. Convergence was assessed according to weighted Robinson-Foulds distance. Bayesian analysis was performed using MrBayes 3.2.7 (Ronquist et al., 2012). Four Markov chains Monte Carlo were run for 10,000,000 generations with a sampling frequency of 1000 generations. The first 25% of sampled trees were removed as burn-in; convergence was assessed using Tracer v1.7.2 (Rambaut et al., 2018), and RYTW (Warren, Geneva, & Lanfear, 2017).

RESULTS

Mylestoma sp.

Description based on FRESHO (Table 1; Table S1; Figures 1 and 2; Video S1)

Cells 18–25 × 13–21 µm in vivo and 15–25 × 13–21 µm in protargol-impregnated specimens. Shape semi-circular in outline, strongly laterally compressed, dorsal margin convex, ventral margin more or less straight (Figures 1A and 2A). Cortex rigid with four “spine-like” protrusions. Spine 1 or “frontal spine” appears as short spine in lateral views but as curved hood-like structure in ventral view (Figures 1A–C and 2A,C,F,L,M,O). Spine 2, largest, situated anteriorly in the first-third of the right side on ventral margin, prominent, strongly recurved, sharply pointed (Figures 1A–C and 2B,D,E,M,P). Spine 3, inconspicuous, situated on right side at posterior end of ventral margin, directed dorsally (Figures 1B and 2B,E,M). Spine 4 or “oral spine”, located just anteriorly to adoral zone, appears as inconspicuous short spine in lateral view but

TABLE 1 Summary morphometric data of *Mylostoma* sp. FRESHO and FARO populations.

Character ^a	Population	Mean	<i>M</i>	SD	CV	Min	Max	<i>n</i>
Body length in vivo	FRESHO	21.7	21.3	2.0	9.2	18.9	25.0	17
	FARO	20.7	21.3	1.4	6.8	18.3	22.5	12
Body width in vivo	FRESHO	18.0	18.0	2.3	13.0	13.6	21.2	17
	FARO	16.8	16.7	1.7	10.0	14.3	19.8	12
Body length:width ratio in vivo	FRESHO	0.8	0.8	0.1	6.1	0.7	0.9	17
	FARO	0.8	0.8	0.0	4.5	0.7	0.9	12
Membranelles, number	FRESHO	7.9	8.0	0.3	4.3	7.0	8.0	16
	FARO	7.5	7.0	0.5	7.0	7.0	8.0	11
Kineties in epistomial fringe, number	FRESHO	4.0	4.0	0.0	0.0	4.0	4.0	10
	FARO	4.0	4.0	0.0	0.0	4.0	4.0	9
Dikinetids in preoral kinety no. 1, number	FRESHO	6.0	6.0	0.0	0.0	6.0	6.0	5
	FARO	5.5	5.5	0.6	10.5	5.0	6.0	4
Dikinetids in preoral kinety no. 2, number	FRESHO	3.0	3.0	0.0	0.0	3.0	3.0	6
	FARO	3.0	3.0	0.0	0.0	3.0	3.0	2
Dikinetids in ventral kinety, number	FRESHO	3.9	4.0	0.4	9.8	3.0	4.0	7
	FARO	4.0	4.0	0.0	0.0	4.0	4.0	6
Caudal cirri, number	FRESHO	2.0	2.0	0.0	0.0	2.0	2.0	17
	FARO	2.0	2.0	0.0	0.0	2.0	2.0	11

Abbreviations: CV, coefficient of variation (%); *M*, median; Max, maximum value; Mean, arithmetic mean; Min, minimum value; *n*, number of cells studied; SD, standard deviation of the arithmetic mean.

^aMeasurements in μm and based on protargol-impregnated specimens, except as noted.

represents a cortical fold (Figures 1A,C and 2A,O). Cortex bears curved longitudinal rows of parasomal sac pores (possibly including subsurface basal bodies), two on right side, one on dorsal margin, three on left side (Figures 1B,C and 2J,M–O). Cytoplasm colorless with scattered spherical inclusions (Figures 1A and 2A–F), contains numerous rod-shaped prokaryotes showing UV autofluorescence (Figures 1A and 2A,B,E,G). Contractile vacuole not observed. Single ellipsoidal macronucleus in the posterior dorsal, with several nucleoli in impregnated cells, and single spherical micronucleus adjacent to macronucleus (Figures 1A,D and 2A,H,K). Swimming motion rapid, cells rotate clumsily on longitudinal axis.

Infraciliature reduced to frontal ciliary band, two preoral kineties, one ventral kinety, adoral zone of membranelles, and two caudal cirri (Figures 1D and 2H–K). Frontal ciliary band located ventrally and oriented to left side, composed of four longitudinal ciliary rows separated by notched interkinetal ridges or auricles (Figures 1D and 2H,I,K,P); two preoral kineties located between adoral zone and frontal ciliary band, composed of dikinetids, preoral kinety 1 composed of about six dikinetids, preoral kinety 2 composed of about three dikinetids (Figures 1D and 2H,I,K); ventral kinety, longitudinally oriented, situated at level of adoral zone, composed of four spaced dikinetids (Figures 1D and 2I–K); two caudal cirri near left posterior margin, each composed of single

obliquely oriented row of about six basal bodies separated by cortical ridge, cilia about $24\mu\text{m}$ long in vivo (Figures 1A,D and 2H,K–O).

Oral cavity located in left posterior quadrant of cell (Figures 1A,C and 2A,L); adoral zone composed of eight membranelles (Figures 1A,D and 2A,C,D,F,H,K,N,O), mostly covered by prominent cortical lip (Figures 1C and 2N,O); cytopharynx argyrophilic, situated above anteriormost adoral membranelle, crescentic in protargol preparations (Figures 1D and 2K).

Notes on FARO (Table 1; Table S1; Figure 2)

We were unsuccessful in sequencing the 18S rRNA gene of this population, however, we consider it to be conspecific with FRESHO since the two populations are morphologically indistinguishable. Cells $18\text{--}25\times 13\text{--}21\mu\text{m}$ in vivo and $15\text{--}25\times 13\text{--}21\mu\text{m}$ in protargol-impregnated specimens.

Voucher material

Two protargol slides, one from FRESHO and one from FARO populations, containing several protargol-stained specimens are deposited in the National Museum of Prague, Czech Republic (accession numbers P6E 5306 and P6E 5307, respectively).

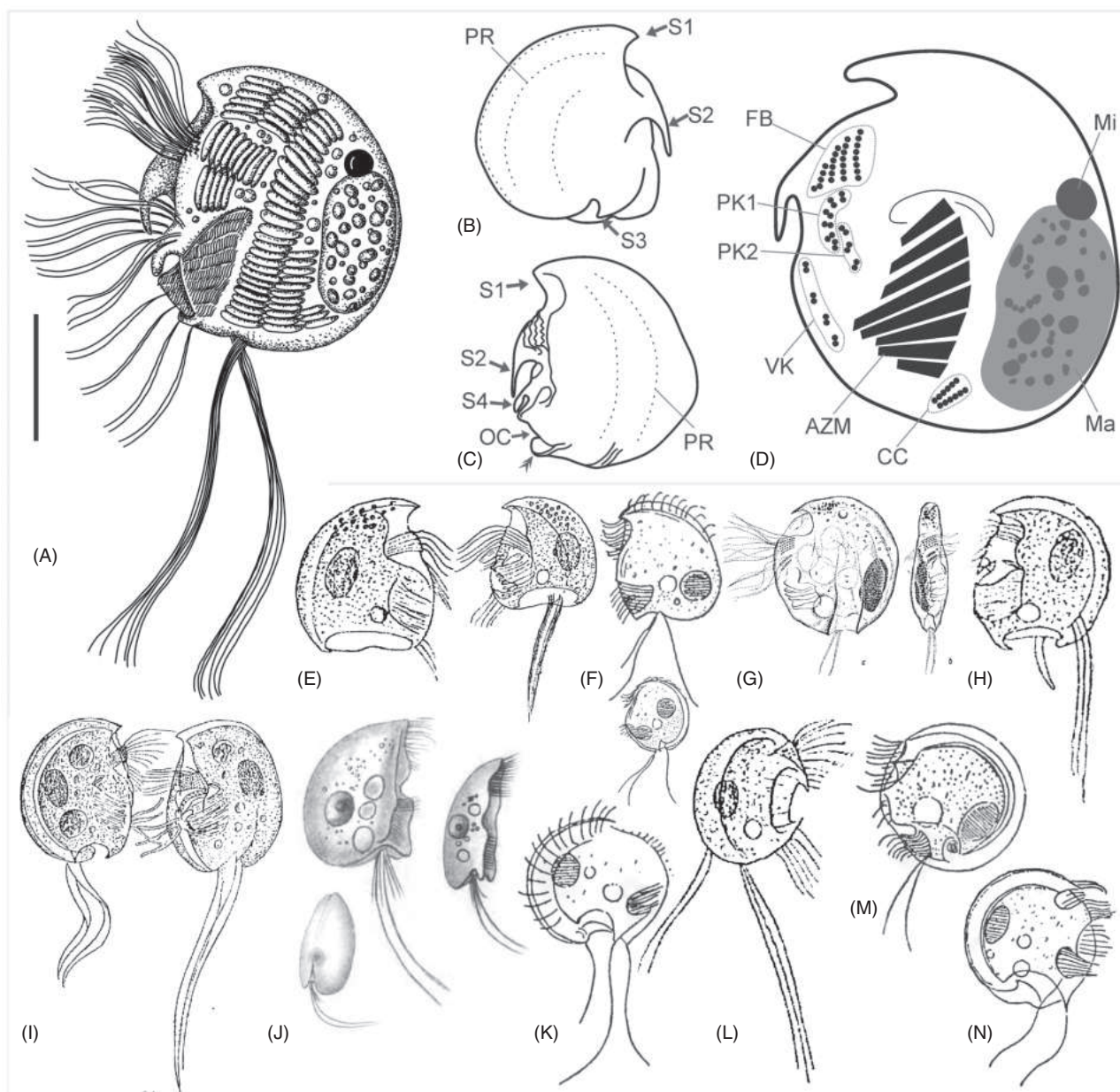


FIGURE 1 *Mylestoma* sp. FRESHO and other *Mylestoma* species. (A) A typical specimen of *Mylestoma* sp. Outline of the right (B) and left (C) side showing the spines and cortex pattern of *Mylestoma* sp., double arrowhead shows the cortical lip. (D) Left side showing the infraciliature and nuclear apparatus after protargol impregnation of *Mylestoma* sp. *M. anatinum* after Kahl (1932b) (E), after Penard (1922) (F), and after Jankowski (1964b) (G). (H) *M. spinigerum* after Kahl (1932b). *M. bipartitum* after Kahl (1932b) (I), and after Gourret and Roeser (1886) (J). (K) *M. flagellatum* after Kahl (1932b). (L) *M. pusillum* after Kahl (1932b). (M) *M. discoideum* after Kahl (1932b). (N) *M. uncinatum* after Kahl (1932b). AZM, adoral zone of membranelles; CC, caudal cirri; FB, frontal ciliary band; Ma, macronucleus; Mi, micronucleus; OC, oral cavity; PK1-2, preoral kinety 1-2; PR, parasomal sacs row; S1-4, spine 1-4; VK, ventral kinety. Scale bar, 10 μ m.

GenBank sequence

Five partial cloned 18S rRNA gene sequences of *Mylestoma* sp. have been deposited in GenBank, accession numbers: OP034695 (1631 bp, 37.89% G+C content), OP034696 (1667 bp, 38.03% G+C content), OP034697 (1674 bp, 38.11% G+C content), OP034698 (1675 bp, 38.15% G+C content), and OP034699 (1664 bp, 38.04% G+C content).

Saprodinium dentatum (Lauterborn, 1901) Lauterborn, 1908

Brief description of the VLKOV1 (Table 2; Table S2; Figure 3; Video S3)

Cells 65–78 $\times$ 56–75 μ m in vivo and 56–72 $\times$ 51–65 μ m in protargol-impregnated specimens. Shape almost circular in outline, strongly laterally compressed,

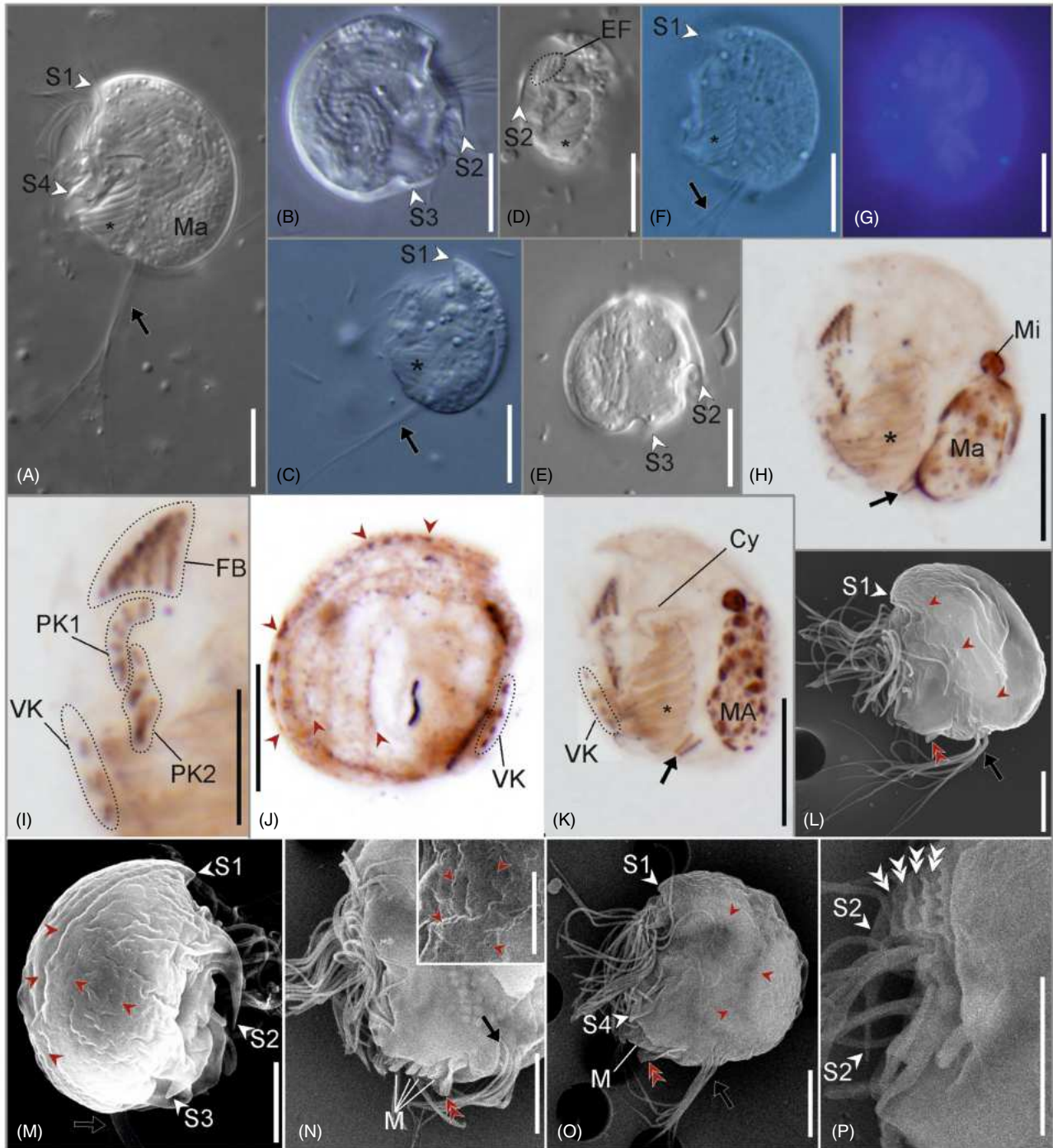


FIGURE 2 *Mylestoma* sp. FRESHO (A–L, N, O) and FARO (M, P) in vivo (A–F), under fluorescence microscopy (G), after protargol impregnation (H–K) and scanning electron microscopy (L–Q). Left (A, C, F) and right (B, E) lateral views of different individuals showing the spines, the membranelles (asterisks), and the caudal cirri (arrows). (D) Ventro-lateral view showing the frontal ciliary band, the spine 2 (double arrowhead) and the membranelles (asterisk). (G) Cytoplasm showing the prokaryotic methanogens. (H, I, K) Left lateral view showing the infraciliature. (J) Right lateral view showing parasomal sac pores (black arrowheads) and the ventral kinety. Right (M) and left (L, M, O) lateral view showing the spines, membranelles, cortical lip (red double arrowheads), the posterior cilia (arrows), and the parasomal sac pores (black arrowheads). Inset in N shows the parasomal sac pores (red arrowheads) on the surface. (P) Left lateral view showing the four auricles (white double arrowheads) of the frontal ciliary band followed by the spine 2 on the right side. FB, frontal ciliary band; Ma, macronucleus; Mi, micronucleus; PK1–2, preoral kinety 1, 2; S1–4, spine 1–4; VK, ventral kinety. Scale bars, A–H, J, K 10 µm, I, L–Q 5 µm.

dorsal keel terminates anteriorly in distinct frontal spine; posterior cell margin with concavities between slender sharp spines, four spines on right posterior

margin, three on left posterior margin, one at posterior end of dorsal keel (Figures 3A,E). Cortex rigid. Cytoplasm colorless, contains rod-shaped prokaryotes

TABLE 2 Summary morphometric data of *Saprodinium dentatum* VLKOV1 population.

Character ^a	Mean	M	SD	CV	Min	Max	n
Body length in vivo	73.9	75.3	4.5	6.1	65.4	78.4	10
Body width in vivo	67.3	67.1	6.1	9.0	56.6	75.2	10
Body length:width ratio in vivo	1.1	1.1	0.0	4.3	1.0	1.2	10
Macronuclear nodules, number	1.6	1.5	0.7	42.2	1.0	3.0	12
Micronucleus, diameter	4.2	4.0	0.4	9.3	4.0	5.0	12
Kineties, right epistomial fringe, number	21.2	21.0	1.9	9.2	17.0	24.0	11
Kineties, left epistomial fringe, number	10.5	10.0	1.7	16.1	8.0	14.0	11
Kinetids, right dorsal kinety, number	96.8	94.5	8.9	9.2	88.0	110.0	6
Kinetids, left dorsal kinety, number	77.5	77.5	3.0	3.9	73.0	82.0	6
Adoral membranelles, number	8.0	8.0	0.0	0.0	8.0	8.0	11
Right kineties, number	5.0	5.0	0.0	0.0	5.0	5.0	11
Left kineties, number	1.0	1.0	0.0	0.0	1.0	1.0	11

Abbreviations: CV, coefficient of variation (%); M, median; Max, maximum value; Mean, arithmetic mean; Min, minimum value; n, number of cells studied; SD, standard deviation of the arithmetic mean.

^aMeasurements in μm and based on protargol-impregnated specimens, except as noted.

(methanogens), autofluorescent under UV light (Figure 3F).

One unciliated kinety along right side and one ciliated kinety along left side of dorsal keel margin, both composed of dikinetids (Figure 3M); six longitudinal kineties with unciliated basal bodies and associated parasomal sacs, five on right side, and one on left side (Figure 3G,J,L). Frontal ciliary band composed of 21 ciliary rows on the right side, and about 10 on the left side (Figure 3D,G,H,L,N). Two preoral kineties located between adoral zone and frontal ciliary band, composed of dikinetids (Figure 3H,N). Adoral zone encompasses about eight membranelles (Figure 3N). Oral spine just above the aperture of the buccal cavity (Figure 3B,C,E). Ventroposterior spine bearing the distal portion of the two ventral kineties (Figure 3L,M). Four posterior spines, two on the right side and two on the left side, bearing somatic kineties composed of dikinetids (Figure 3J–M). One to three spherical macronuclei, and a single spherical micronucleus (Figure 3M). Swimming speed moderate, rotating on longitudinal axis.

Muranothrix felix sp. nov.

Description based on NAPAM (Table 3; Table S3; Figures 4 and 5; Video S2)

Cells $80\text{--}140 \times 14\text{--}21 \mu\text{m}$ in vivo. Cells elongated, narrowly the postoral body part broadly ellipsoidal, “pin-shape” (Figures 4A,B and 5A,B). Slender anterior neck region spiralized rightward approximately 360° (Figures 4A,B and 5E–I). Cortex flexible, with

longitudinal furrows (Figures 4A,B and 5B,E,F,K,M). Surface of the cortex coated with rod-shaped bacteria (ca. $2 \mu\text{m}$ long) oriented transversely between ciliary rows (Figures 4C and 5C,E). Cytoplasm with sparse globular inclusions up to $5 \mu\text{m}$ in diameter, food vacuoles contain prokaryotes, diatom frustules (Figures 4A and 5B). Contractile vacuole terminal (Figures 4A and 5A,B,D). Macronuclei comprise 6–14 spherical to ellipsoidal nodules (about $10 \mu\text{m}$), each with several nucleoli, clustered in mid-body region, one single spherical micronucleus (ca. $2.7 \mu\text{m}$ in diameter) among macronuclear nodules (Figures 4A,F and 5J,M–O). Swims rapidly, intermittently reverses course.

Somatic ciliature composed of ordinarily spaced dikinetids, only anterior basal body ciliated, cilia about $10 \mu\text{m}$ long, arranged in 10–12 narrowly spaced somatic kineties, lying on more or less straight longitudinal furrows (Figures 4A,E and 5B,J,K,M). Bristle-like somatic cilia (ca. $10 \mu\text{m}$ long) interspersed at regular intervals along kineties and perpendicularly oriented to body margin (Figures 4A,C and 5B,C,F). Perizonal stripe mostly comprises five rows of dikinetids arranged in about 12 false kineties, although this feature is not very stable (Figures 4D and 5L,M). Caudal cilia intermittently converge in an inverted cone arrangement surrounding stiffened much longer ($34\text{--}65 \mu\text{m}$) coherent caudal cilia (Figures 4A and 5A,B,D).

Peristome elongated, inverted keyhole shaped, spirals around anterior neck region (Figures 4B,E and 5F,P,N). Buccal cavity more or less equatorial (Figures 4E and 5E,P). Buccal ciliature comprises long paroral membrane parallel to adoral zone of membranelles, both spiralize rightward about 360° extending

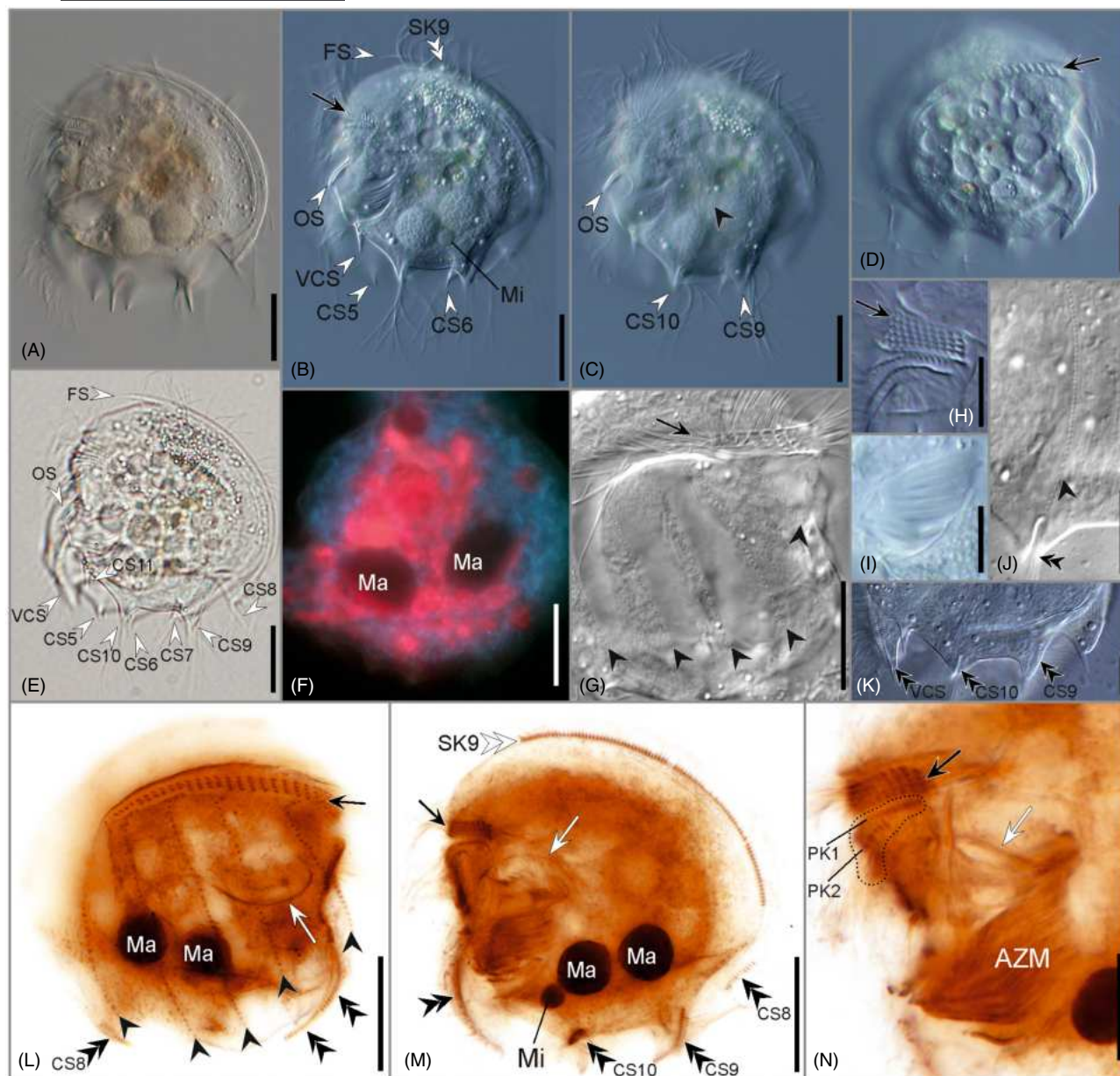


FIGURE 3 *Saprodinium dentatum* VLKOV1 in vivo (A–E, G–K), under fluorescence microscopy (F), and after protargol impregnation (L–N). Left (A–C, E) and right (D) lateral sides showing the spines and general shape. (F) methanogenic archaea symbionts (in blue). (G, J) Right (G) and left lateral views showing the kineties with unciliated basal bodies and associated parasomal sacs (black arrowheads) and the frontal ciliary band (black arrow). (H) Left side of the frontal band (black arrow) and the somatic kineties 1 and 2. (I) Adoral zone of membranelles. (K) Posterior cell end showing the spines bearing somatic kineties. Right (L) and left (M) lateral views showing the infraciliature, cytopharynx (white arrows) and nuclear apparatus. (N) Left lateral view showing the frontal band, the somatic kineties 1 and 2, the adoral zone, and the cytopharynx (white arrow). AZM, adoral zone of membranelles; CS, caudal spine; FS, frontal spine; Mi, micronucleus; OS, oral spine; SK, somatic kineties; VCS, ventro-caudal spine. Scale bars, 20 μ m (A–G, L, M), 10 μ m (H–K, N).

almost to anterior end of cell (**Figures 4A,B,D,E and 5B,E–J,M,N**). Adoral zone extends from buccal cavity to terminate subapically, comprises 26–33 membranelles, all of similar size (ca. 5 μm long), composed of two rows of about eight basal bodies (**Figures 4D and 5L,N,P**). Paroral membrane long reverse J-shape, originates on left posterior wall of buccal cavity, borders its posterior margin, and extends anteriorly in

peristome, occupies about 90% of adoral zone length, bears long cilia (about 15 μm) resembling a “velum” or “sail,” seemingly comprises a single continuous file of basal bodies, precise organization not determined (Figures 4A,B,D,E and 5A,B,G,I,N,P).

Postdividers elongated ellipsoidal; with about six macronuclear nodules and several membranelles; adoral zone terminates apically (Figure 5Q,R).

TABLE 3 Summary morphometric data of *Muranothrix felix* sp. nov. NAPAM and STRUJA populations.

Character ^a	Population	Mean	<i>M</i>	SD	CV	Min	Max	<i>n</i>
Body, length in vivo	NAPAM	110.2	113.6	17.0	15.4	80.9	141.3	13
	STRUJA	119.4	123.2	11.4	9.6	98.9	133.9	10
Body, width in vivo	NAPAM	18.2	18.5	2.1	11.8	14.6	21.4	13
	STRUJA	22.3	23.5	2.7	11.9	17.0	25.0	10
Body length:width, ratio in vivo	NAPAM	6.1	5.8	0.7	11.0	5.2	7.0	13
	STRUJA	5.4	5.4	0.4	6.7	4.8	5.9	10
Adoral zone of membranelles, length	NAPAM	44.0	44.3	4.9	11.1	29.4	53.9	32
	STRUJA	30.1	30.0	6.7	22.4	17.0	39.0	11
Adoral zone length-body length, ratio in %	NAPAM	48.2	48.2	5.5	11.3	40.2	58.6	32
	STRUJA	42.6	43.1	8.9	21.0	26.3	54.2	11
Paroral membrane, length	NAPAM	41.2	41.7	5.0	12.1	28.1	49.4	23
Paroral mem length: adoral zone length, % ratio	NAPAM	91.8	91.7	8.6	9.4	71.6	107.1	23
Micronucleus, number	NAPAM	1.0	1.0	0.0	0.0	1.0	1.0	32
	STRUJA	1.0	1.0	0.0	0.0	1.0	1.0	12
Macronuclear nodules, number	NAPAM	8.6	8.0	1.9	21.8	6.0	14.0	33
	STRUJA	8.9	9.0	0.7	7.8	8.0	10.0	7
Adoral membranelles, number	NAPAM	28.6	28.0	2.0	7.0	26.0	33.0	27
Somatic kineties, number	NAPAM	11.0	11.0	0.8	7.2	10.0	12.0	31
	STRUJA	11.0	11.0	0.8	7.0	10.0	12.0	11
False kineties, number	NAPAM	11.7	12.0	0.9	7.7	10.0	13.0	15
Caudal cilia, length in vivo	NAPAM	50.3	50.3	22.1	43.9	34.7	65.9	2
	STRUJA	49.6	42.0	21.7	43.8	26.0	98.0	10

Abbreviations: CV, coefficient of variation (%); *M*, median; Max, maximum value; Mean, arithmetic mean; Min, minimum value; *n*, number of cells studied; SD, standard deviation of the arithmetic mean.

^aMeasurements in μm and based on protargol-impregnated specimens, except as noted.

Notes on STRUJA (Table 3; Table S3; Figure 5)

This population closely resembles the NAPAM population. Cell size $98\text{--}134 \times 17\text{--}25\mu\text{m}$ in vivo. Caudal cilia $26\text{--}98\mu\text{m}$ long (1/5 to 3/4 of body length). Protargol preparations were of suboptimal quality and did not reveal several important features, such as the number of adoral membranelles and paroral membrane length. However, cell size, shape, the number of somatic kineties, and macronuclear nodule number are consistent with NAPAM population. The 18S rRNA gene sequences of STRUJA and NAPAM populations were identical.

Muranothrix sp. SIPEK1A

The observations of this population include only few specimens (Figure 4I). Cells slender, size about $135 \times 20\mu\text{m}$, peristome extends about 60% of body length. About seven macronuclear nodules arranged in longitudinal row. Somatic ciliature arranged as longitudinal kinetids with bristle-like cilia (ca. $15\mu\text{m}$ long) interspersed with somatic cilia. Caudal cilia intermittently converge

in an inverted cone arrangement surrounding stiffened much longer caudal cilia.

GenBank sequence

Three partial cloned 18S rRNA gene sequences of *Muranothrix* sp. have been deposited in GenBank, accession numbers: OP034691 (1705 bp, 44.46% G+C content), OP034692 (1705 bp, 44.40% G+C content), and OP034693 (1700 bp, 44.59% G+C content).

18S rRNA gene sequences and phylogenetic analysis

Eleven 18S rRNA gene sequences were determined, two from *Muranothrix felix* (NAPAM and STRUJA, respectively), three from *Muranothrix* sp. (SIPEK1A), five from *Mylestoma* sp. (FRESHO), and one from *Saprodinium dentatum* (VLKOV1) (GenBank accession numbers OP034689–OP034699). The new sequence of *S. dentatum* differs at 29 nucleotide positions (1.7%)

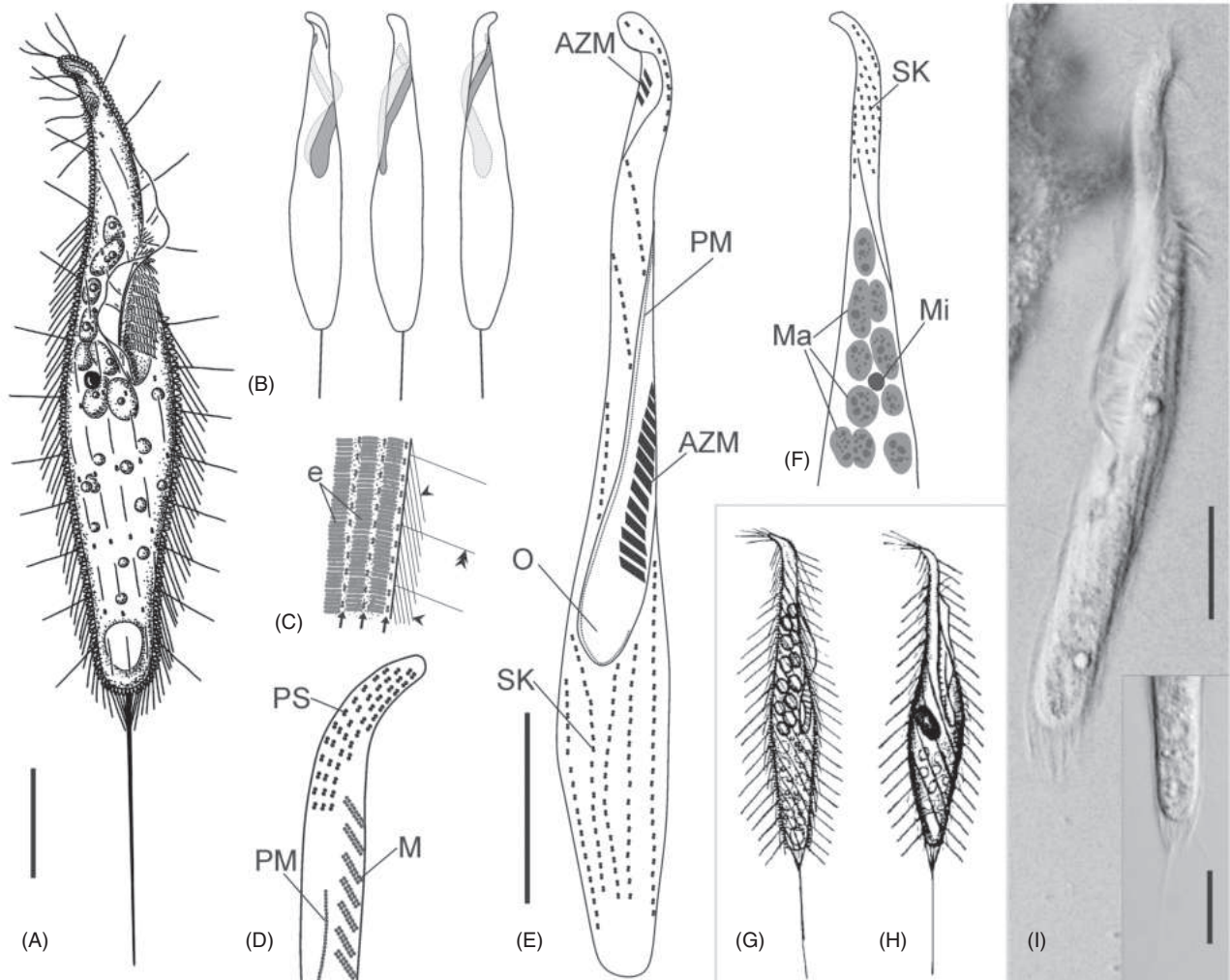


FIGURE 4 *Muranothrix felix* sp. nov. (A–F), *Parablepharisma kahli* (G, H), and *Muranothrix* sp. SIPEK1A (I) in vivo (A–C, G–I), and after protargol impregnation (D–F). (A) General shape of a typical specimen in ventral view. (B) Ventral, left lateral, and dorsal views of an individual. (C) Cell cortex showing the disposition of ectosymbionts between ciliary rows (arrows). (D) Anterior portion of the cell showing the perizonal stripe, membranelles and distal part of paroral membrane. (E) Ventral view showing the infraciliature. (F) Nuclear apparatus. (G, H) *Parablepharisma kahli*, and its uninucleate form (H) after Tucolesco (1962). (I) Differential interference contrast of *Muranothrix* sp. SIPEK1A showing the general shape, the inset shows the arrangement of caudal cilia. AZM, adoral zone of membranelles; C, bristles-like cilia; e, ectosymbionts; M, membranelle; Ma, macronucleus; Mi, micronucleus; O, oral opening; PM, paroral membrane; PS, perizonal stripe; SK, somatic kineties. Scale bars, 20µm.

from the published sequence of the Brazilian population (Fernandes et al., 2018).

The uncorrected p-distances among the five cloned sequences of *Mylestoma* sp. range from 0.001 to 0.004, whereas for those of *Muranothrix* sp. SIPEK1A ranged from 0.002 to 0.004. In both cases, only the sequence with the lower number of changes (OP034698 for *Mylestoma* sp. and OP034693 for *Muranothrix* sp.) was used in the phylogenetic trees.

The identical sequences of *Muranothrix felix* sp. nov. (NAPAM and STRUJA) differ from the sequence of *M. gubernata* (MT177189) and *Muranothrix* sp. SIPEK1A (OP034693) at 52 (3.0%) and 38 (2.2%) nucleotide positions, respectively.

The ML and BI phylogenetic trees were mostly congruent, and only the ML is shown with branch supports

from both methods (Figure 6). *Mylestoma* sp. clusters with *Discomorphella pedroeneasi* and the *Saprodinium dentatum* sequence from VLKOV1 groups with the *S. dentatum* sequence from GenBank, both clades are fully supported. *Discomorphella*, *Mylestoma*, and *Saprodinium* form the strongly supported clade of Odontostomatea. This clade has a sister relationship to Litostomatea (77 BS/0.97 BPP).

The four muranotrichean species for which sequence data are available (*Thigmothrix strigosa*, *Muranothrix gubernata*, *M. felix* sp. nov., and *Muranothrix* sp. SIPEK1A) form a clade (BS 92/BPP 1.0). The two identical sequences of *Muranothrix felix* sp. nov. strongly cluster with *Muranothrix* sp. SIPEK1A and *M. gubernata* (BS 100/BPP 1.0). *Muranothrix* sp. SIPEK1A clusters with *M. felix* sp. nov. (BS 65/BPP 0.70).

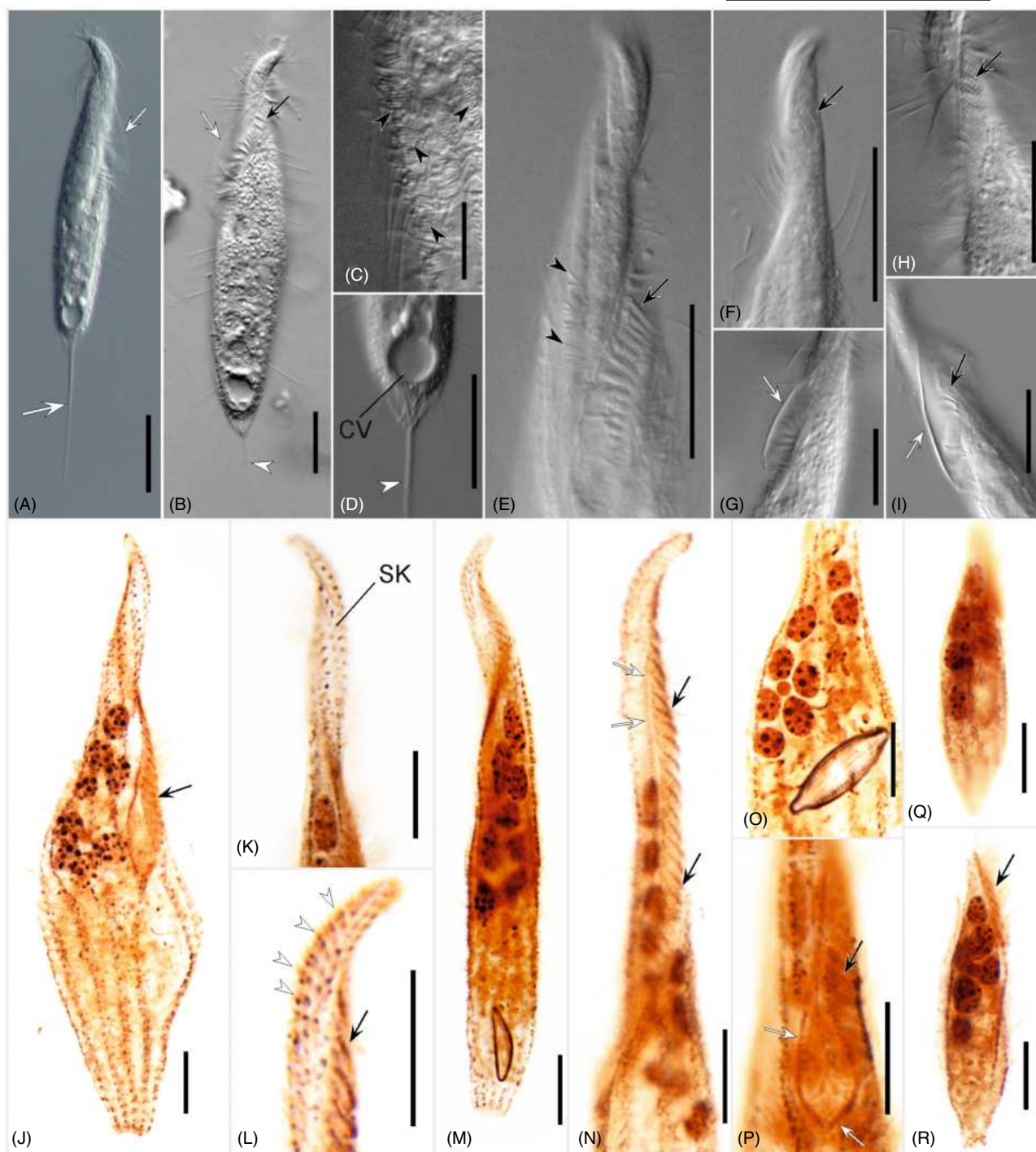


FIGURE 5 *Muranothrix felix* sp. nov. NAPAM in vivo (A–I), and after protargol impregnation (J–R). (A, B) Different specimens showing the general shape. (C) Cortex showing the organization of ectosymbionts (black arrowheads) and the cilia. (D) Posterior cell end showing the contractile vacuole, inverted cone of cilia, and caudal cilia (white arrowhead). Ventral (E), left lateral (G, H, I), and dorsal (F) views of the anterior cell half showing the spinalization, the adoral zone (black arrows), and the paroral membrane (white arrows). (J, M) General view showing the infraciliature and nuclear apparatus. (K) Dorsal view of the anterior cell end showing the somatic kineties. (L) Anterior cell end showing the perizonal stripe (white arrowheads) and the distal part of adoral zone (black arrow). (N) Anterior cell showing the torsion, the adoral zone, (black arrows), and the paroral membrane (white arrows). (O) Nuclear apparatus. (P) Ventral view showing the buccal cavity, the paroral membrane (white arrows), and the adoral zone (black arrow). (Q, R) Postdivisor showing the nuclear apparatus and the adoral zone. CV, contractile vacuole; SK, somatic kineties. Scale bars, 20 μ m.

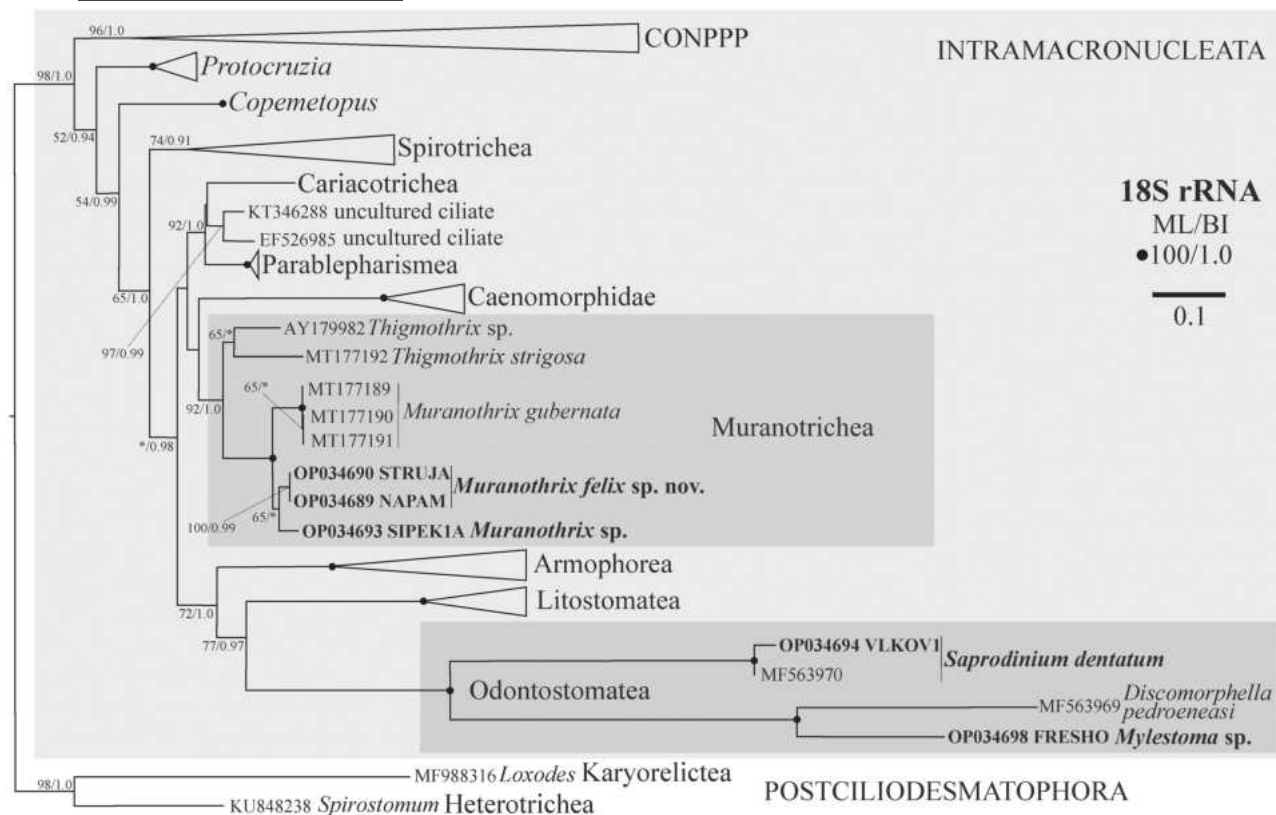


FIGURE 6 Maximum likelihood tree showing the phylogenetic position of *Mylestoma* sp., *Muranothrix felix* sp. nov., and *Muranothrix* sp. SIPEK1A (in bold). Node values represent bootstrap support of ML and Bayesian posterior probabilities of BI, values below 50 and 0.90, respectively, are not shown or are represented by an (*). Scale bar, 10 changes per 100 nucleotides.

In our phylogenetic analyses, Caenomorphidae appear as a sister group of Muranotrichea (BS 25/BPP 0.81).

DISCUSSION

Genus assignment of the new isolates

The class Odontostomatea encompasses ciliates with a reduced somatic ciliature, frontal ciliary band kineties (frontal band), and a rigid or “armor-like” cortex with spine-like protrusions. The frontal ciliary band in Mylestomatidae is sparse, extending from the ventral margin slightly onto the left side, unlike in Epalxellidae and Discomorphellidae in which the frontal band extends from the right side, around the ventral margin, and onto the left side (Foissner & Berger, 1996; Kahl, 1932b). The Mylestomatidae includes genus *Mylestoma* and the monotypic genus *Atopodinium*. Posterior marginal spines are either absent or inconspicuous in *Mylestoma* spp. *Atopodinium fibulatum* possesses a very prominent frontal spine and conspicuous right and left-sided posterior marginal spines. Morphologically, FRESHO and FARO populations can be assigned to genus *Mylestoma* (Kahl, 1932b).

Saprodinium includes at least seven species of which only *S. dentatum* has been well-studied by modern methods (Fernandes et al., 2018; Schrenk & Bardele, 1991). Species of this genus are characterized by prominent, sometimes thorn-like posterior spines upon which ciliated portions of the somatic kineties terminate. *Saprodinium* species have an unusually complex somatic ciliature consisting of somatic kineties with ciliated parts and unciliated parts comprising subsurface basal bodies, both accompanied by prominent parasomal sacs (Foissner et al., 1992; Kahl, 1932b). The VLKOV1 population morphologically corresponds to previous descriptions of *Saprodinium dentatum* (Jankowski, 1964b; Schrenk & Bardele, 1991).

The only family within the class Muranotrichea, Muranotrichidae, comprises two genera, *Thigmothrix* and *Muranothrix*. Morphologically, *Muranothrix* can be easily distinguished from *Thigmothrix* by the posterior somatic ciliature (inverted cone-shape arrangement of shorter cilia converging on bundle of very long, stiff caudal cilia vs. absence of elongated caudal cilia) and by bristle-like cilia interspersed among ordinary somatic cilia (present vs. absent; Rotterová et al., 2020). The caudal ciliary arrangement and bristle-like so typical of genus *Muranothrix* were present in STRUJA, NAPAM, and SIPEK1A populations.

Saprodinium dentatum, a well-known odontostomatid species

Lauterborn (1901) first described this species as *Discomorpha dentata* (genus replacement name *Discomorphella* Corliss, 1960) and later erected the genus *Saprodinium* (Lauterborn, 1908) with *S. dentatum* as its type and only species. Since its description, this large and easily recognized species has been well-characterized from a morphologic and molecular standpoint (Dragesco, 1960; Fernandes et al., 2018; Foissner et al., 1992; Jankowski, 1964b; Schrenk & Bardele, 1991; Sola et al., 1992; Wetzel, 1928). At least six additional species have been erected within, or transferred to *Saprodinium* (i.e. *S. integrum*, *S. halophilum*, *S. mimeticum*, *S. putrinum*, *S. spinigerum*, and *S. triangulum*; Kahl, 1932b).

The morphological characterization of the VLKOV1 population matches previous descriptions and depictions of *Saprodinium dentatum* (Dragesco, 1960; Fernandes et al., 2018; Foissner et al., 1992; Jankowski, 1964b; Kahl, 1932b; Kreutz & Foissner, 2006; Lauterborn, 1901, 1908; Schrenk & Bardele, 1991; Sola et al., 1992; Wetzel, 1928). Although the newly determined 18S rRNA gene sequence differs in 29 nucleotide positions (i.e. 1.7%) from the only sequence available in GenBank (Fernandes et al., 2018), both populations are indistinguishable. The intraspecific 18S rRNA gene sequence variability of odontostomatids, and particularly *S. dentatum*, cannot yet be assessed due to lack of data. The possibility that VLKOV1 represents a separate, possibly cryptic species cannot be excluded.

Mylestoma sp. as an unassigned species and comparison with similar species

The introduction of methodologies such as protargol impregnation, scanning electron microscopy, and molecular sequencing have revolutionized the taxonomy of ciliates and better characterized many poorly described taxa and their affinities (Li et al., 2017; Nitla et al., 2019; Rotterová et al., 2018; Warren, Patterson, et al., 2017). Even so, most of the available descriptions of odontostomatid species are based solely on observations of living and simply fixed, unstained specimens (Jankowski, 1964b; Kahl, 1932b; Penard, 1922) and have not been studied using modern techniques (Warren, Patterson, et al., 2017). Thus, nearly all species in this group are in urgent need of redescription and molecular sequencing.

Although Klein (1930) and Tuffrau (1992) applied silver nitrate methods to various odontostomatids and to *Discomorphella pectinata*, respectively, these methods can only provide partial morphological information (e.g. the nuclear apparatus is not demonstrated). So far, only

two odontostomatid species, *Saprodinium dentatum* and *Discomorphella pedroeneasi*, have been well characterized both morphologically and molecularly (Fernandes et al., 2018; Paiva et al., 2017; Schrenk & Bardele, 1991). The rigid armor-like cortex and its various spine-like protrusions have been considered important taxonomic characters at the family, genus, and species level (Jankowski, 1964a, 1964b; Kahl, 1932b; Lynn, 2008; Paiva et al., 2017).

The genus *Mylestoma* currently comprises seven described species (Figure 1E–N; Kahl, 1932b). *Mylestoma pusillum*, *M. uncinatum*, *M. flagellatum*, and *M. discoideum* have a discoidal or circular body shape in contrast with the semicircular to rounded triangular shape of *Mylestoma* sp. studied by us. *M. spinigerum* possesses a prominent right posterior spine, which is absent in our populations. *Mylestoma bipartitum*, the type species, originally described by Gourret and Roeser (1886) as *Aspidisca bipartita*, is about 50 µm long, has two curved ventral spines on the right side of the cortex, and, according to Kahl (1928), there is a “wind-mill-like” group of cilia near the oral apparatus that beat in a “grinding” a fashion (“...heftiger mahlender Bewegung”). It was with this feature in mind that Kahl erected the genus with the name *Mylestoma* (*myle-* Gr. mill). Ironically, after describing additional species, Kahl (1932b) concluded that this distinctive feature was present only in *M. bipartitum* and absent in all other *Mylestoma* species. Interestingly, we observed a similar structure in FRESHO and FARO populations, and we refer it to as the ventral kinety (Figure 1D) consisting of four dikinetids. Thus, FRESHO and FARO populations can be distinguished from *M. bipartitum* by the number of dikinetids in the ventral kinety (four vs. three), smaller size (19–25 µm vs. 35–50 µm long), and ventral spines (absent vs. present).

Mylestoma anatinum (original combination *Epalxis anatina*), which has been reported in freshwater (Foissner et al., 1992; Kahl, 1932b; Kreutz & Foissner, 2006; Penard, 1922), resembles *Mylestoma* sp. from FRESHO and FARO populations. Penard (1922) depicted this species with a row of cilia along the dorsal margin or keel as in *Saprodinium* or *Epalxella*. Kahl (1932b), transferred *Epalxis anatina* to *Mylestoma*, and considered the dorsal ciliary row depicted by Penard (1922) as a misinterpretation. He considered the Swiss population as conspecific with the German population he later studied. The *Mylestoma* sp. we describe lacks the curious posterior marginal “heart-shaped indentation” depicted by Kahl (1932b, Fig. S. 528, 9, 10 therein). Jankowski (1964b) described a population of *M. anatinum* that differs from the previous descriptions by a more circular shape, absence of posterior margin spines, and a broad left sided cortical groove, raising doubts about its identification.

Previously, some photomicrographs of living cells of *M. anatinum* (see Figure 20 and 21 in Foissner et al., 1992,

p. 445) and *M. discoideum* (see figure 3 in Kreutz & Foissner, 2006, p. 139) were provided, both slightly resemble our *Mylestoma* sp., however, the absence of a detailed characterization has limited us to compare their morphology with our populations. *Mylestoma anatinum* is in urgent need of redescription and redefinition.

At the present state of knowledge, we refrain from erecting a new species for the FRESHO and FARO populations and prefer to wait for further information on additional *Mylestoma* populations to avoid species inflation in this group.

Comparison of *Muranothrix felix* sp. nov. with similar species

The class Muranotrichea and family Muranotrichidae were recently established by Rotterová et al. (2020). Muranotrichidae comprise two monotypic genera. *Muranothrix* (type species *M. gubernata*) and *Thigmothrix* (type species *T. strigosa*). Rotterová et al. (2020) compared their populations with a similar species and a “form” assigned to *Parablepharisma* because of their resemblance to *Muranothrix*; namely *P. kahli* Tucolesco, 1962 and *P. kahli* f. *uninucleata* (the latter is considered infrasubspecific according to ICZN 1999 Art. 45.6.4.).

According to the diagnosis of Muranotrichea, the paroral membrane is heteromorphic, either tripartite in *Muranothrix* or bipartite in *Thigmothrix*. In both, the distal part of the paroral membrane consists of a single file of basal bodies (Rotterová et al., 2020). The distal part of the paroral membrane of *Muranothrix felix* sp. nov. comprises a single row of basal bodies, however, the morphology of the proximal portion could not be determined.

Muranothrix gubernata and *Parablepharisma kahli* are morphologically similar to each other and to *M. felix* sp. nov. *Muranothrix felix* sp. nov. can be easily distinguished from *M. gubernata* by having fewer adoral membranelles (26–33 vs. 40–56) and macronuclear nodules (6–14 vs. 15–33; Rotterová et al., 2020, Table S1 therein). Although *P. kahli*, and its uninucleate “form,” was described solely from live specimens and have not been reported since, they are distinguishable from *M. felix* sp. nov., which is smaller (80–141 µm vs. 175–180 µm) and has fewer somatic kineties (12 vs. about 18), and obviously differs from the uninucleate “form” in having multiple macronuclear nodules (Tucolesco, 1962).

Muranothrix sp. SIPEK1A most likely represents a different species since its 18S rRNA gene sequence forms a separated branch within the *Muranothrix* clade (Figure 6), pending morphological studies are needed to prove otherwise.

Tucolesco (1962) erected *Parablepharisma kahli* (Figures 4G,H) based on living specimens and provided only a brief description and a single drawing. It is characterized by being coated with prokaryotic

ecotsymbionts, a bundle of very long stiff caudal cilia and a convergent cone of shorter cilia, somatic ciliature of ordinary cilia with distinctive bristle-like interspersed cilia, and a slender twisted anterior neck region with a long peristome and a conspicuous paroral membrane. *Parablepharisma kahli* differs from other members within *Parablepharisma* (e.g. *P. pellitum*, *P. bacteriophora*, *P. brasiliensis*, or *P. granulata*) and has a much closer morphological affinity to the recently erected genus *Muranothrix* due to the presence of the aforementioned features (Campello-Nunes et al., 2020; Rotterová et al., 2020). Although there is no available molecular data for *P. kahli* and its description is brief, at least on morphologic grounds, this species resides elsewhere, likely the Muranotrichidae.

Besides *P. kahli* there are also other putative parablepharismids with clear morphological affinities to Muranotrichea (e.g. *P. collare*, *Kahlum chlamydophorum*; Kahl 1932, Campello-Nunes et al., 2020). It should also be noted that Jankowski's (2007) proposal of epibiotic symbionts as a generic synapomorphy of *Parablepharisma* (Campello-Nunes et al., 2020) is not supported since this is a feature shared with Muranotrichidae (Rotterová et al., 2020).

Muranotrichean diversity in brackish and marine anoxic habitats is likely much greater than current reports would suggest since we have encountered several other populations likely to be muranotricheans based on morphology but from which we have not yet been able to obtain molecular sequences.

Phylogenetic position of the studied populations

The overall phylogenetic tree topology differs slightly from that of previous analyses (Bourland et al., 2022; Campello-Nunes et al., 2022; Zhuang et al., 2022). Interestingly, the clade of Caenomorphidae resulted sister of Muranotrichea in our phylogenetic analyses, throughout low support, rather than to Litostomatea as in previous analyses (Li et al., 2017; Rotterová et al., 2020). The family Caenomorphidae is a clade of anaerobic ciliates with an uncertain position within Ciliophora (Rajter & Dunthorn, 2021).

In previous 18S rRNA analyses, the class Odontostomatea was sister to Armophorea (Metopida + Clevelandellida), branching within the APM clade (Bourland et al., 2022; Fernandes et al., 2018; Rotterová et al., 2020; Zhuang et al., 2022). In the present phylogenetic trees, as well as in others (Campello-Nunes et al., 2022), the class Odontostomatea is sister to Litostomatea. As in case of Caenomorphidae and Cariacotrichea, the phylogenetic position of Odontostomatea remains unclear. Rotterová et al. (2020) inferred that Odontostomatea belong to the obligate anaerobic APM clade. According to this hypothesis, odontostomatids would have an anaerobic common ancestor with Armophorea (Metopida +

Clevelandellida), Parablepharisma, Muranotrichea, and, possibly, Cariacotrichea. The genomic/transcriptomic data, whose analyses will likely be necessary to clarify these deeper relationships, have not yet been published.

The genus *Mylestoma* finally finds its phylogenetic position within Odontostomatida, as sister to *Discomorphella*. Confirmation of the phylogenetic positions of genera *Atopodinium*, *Pelodinium*, and *Epalxella*, currently considered as belonging to Odontostomatea (Fernandes et al., 2018; Stoeck et al., 2007), awaits the results of further molecular sequencing efforts.

TAXONOMIC SUMMARY

Zoobank registration of the work: urn:lsid:zoobank.org:pub:EFBE5C91-F199-4D35-A89D-AA22DBE4D7AA.

Phylum Ciliophora Doflein, 1901 Subphylum Intramacronucleata Lynn, 1996 Class Odontostomatea Fernandes, Vizzoni, Borges, Soares, Silva-Neto, Paiva, 2018 Order Odontostomatida Sawaya, 1940 Family Mylestomatidae Kahl in Doflein and Reichenow, 1929

Genus *Mylestoma* Kahl, 1928

Improved diagnosis: Small odontostomatid; circular or semicircular body shape; rigid armor-like cortex with a conspicuous hood-like protrusion appearing as a frontal spine when viewed laterally; posteriorly rounded, truncate, or concave; posterior margin without or with only inconspicuous spines; oral spine near buccal apparatus; frontal ciliary band located ventrally and oriented to the left side, reduced to a few kineties; somatic ciliature limited to two preoral kineties between frontal ciliary band and buccal apparatus, one kinety on ventral margin at level of buccal apparatus, two posterior marginal cirri; freshwater, brackish or marine anoxic habitats.

Type species: *Mylestoma bipartitum* (Gourret & Roeser, 1886) Kahl, 1928 by monotypy (original combination *Aspidisca bipartita* Gourret & Roeser, 1886).

Included species: *Mylestoma anatinum* (Penard, 1922) Kahl, 1932, *M. bipartitum* (Gourret & Roeser, 1886) Kahl, 1928, *M. discoideum* (Penard, 1922) Kahl, 1932, *M. flagellatum* (Penard, 1922) Kahl, 1932, *M. pusillum* Kahl, 1932, *M. spinigerum* Kahl, 1932, and *M. uncinatum* (Penard, 1922) Kahl, 1932.

Remarks: The name of this genus has been incorrectly recorded as *Myelostoma* (see Warren et al., 2016).

Family Epalxellidae Corliss, 1960.

Saprodinium dentatum (Lauterborn, 1901) Lauterborn, 1908

Improved diagnosis: Large odontostomatid; circular body shape; rigid armor-like cortex with a conspicuous frontal spine followed by a dorsal keel; posteriorly concave with eight thorn-like spines, three on the left, three on the right, one dorsal, and one ventral; oral spine near buccal apparatus; frontal band encircles the forehead commencing on the left side margin and ending on the right side

almost at the dorsal edge; rows of parasomal sacs present, five on the right and one on the left side; two preoral kineties between frontal band and buccal apparatus; five of the caudal spines bearing ciliary rows; one somatic kinety on the left margin of dorsal keel; eight adoral membranelles; one micronucleus, one to three macronucleus; freshwater.

Original combination: *Discomorpha dentata* Lauterborn, 1901.

Type locality: A small freshwater sulfidic pond in Ludwigshafen, Germany.

Voucher material: One protargol slide containing several protargol-stained specimens is deposited in the National Museum of Prague, Czech Republic (accession number P6E 5309).

GenBank sequence: A partial 18S rRNA gene sequence of *Saprodinium dentatum* has been deposited in GenBank, accession number: OP034694 (1678 bp, 41.78 G+C% content).

Remarks: The genus *Saprodinium* was included in the family Epalxellidae (Adl et al., 2019; Jankowski, 1964a, 1964b, 2007; Kahl, 1932b; Lynn, 2008), however, Fernandes et al. (2018) considered Epalxellidae as incertae sedis within Ciliophora despite molecular data placing *Saprodinium dentatum* in class Odontostomatea. We consider *Saprodinium dentatum* as a member of Epalxellidae within Odontostomatea.

Phylum Ciliophora Doflein, 1901 Subphylum Intramacronucleata Lynn, 1996 Class Muranotrichea Rotterová, Salomaki, Pánek, Bourland, Žihala, Táborský, Edgcomb, Beinart, Kolísko & Čepička, 2020 Order Muranotrichida Rotterová et al., 2020 Family Muranotrichidae Rotterová et al., 2020 Genus *Muranotrix* Rotterová et al., 2020

Muranotrix felix sp. nov.

Zoobank registration: urn:lsid:zoobank.org:act:7D5A5DB5-9B9D-403E-BAB0-175F9FB504A1.

Diagnosis: Size 80–141 × 14–25 μm; anterior neck-like region spirals rightward about 360° around long axis; 6–14 macronuclear nodules grouped in mid-body (10 μm); peristome about 1/2 of body length; 26–33 adoral membranelles; perizonal stripe comprises about 12 false kineties; long caudal cilia present.

Etymology: The name *felix* (L. fem. adj.) acknowledges the luck in finding two populations of this species, allowing more detailed study of its morphology.

Type locality: A small brackish pond in Napatree point, Rhode Island, United States (41°18'35.1" N, 71°52'42.5" W).

Type material: One protargol slide containing the holotype specimen (marked with a black ink circle) and several paratype specimens is deposited in the National Museum of Prague, Czech Republic (accession number P6E 5308).

GenBank sequence: Two partial 18S rRNA gene sequences, one from NAPAM and other from STRUJA populations, of *Muranotrix felix* sp. nov. have been

deposited in GenBank, accession numbers: OP034689 (1709bp, 44.12% G+C content) and OP034690 (1709bp, 44.12% G+C content).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

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Article

Rediscovery of Remarkably Rare Anaerobic Tentaculiferous Ciliate Genera *Legendrea* and *Dactylochlamys* (Ciliophora: Litostomatea)

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Simple Summary: Rare organisms represent a challenge for researchers in all fields of study in biology. In the realm of ciliatology, the genera *Dactylochlamys* and *Legendrea* are considered to be such cases. Very little information has accrued in over a century since their first descriptions; only a few published reports, online images or videos of rarely encountered individual specimens exist. *Dactylochlamys* and *Legendrea* are also morphologically remarkable for their likely independently evolved tentacle-like structures. Recently, the first molecular data were published for the species *L. loyezae*. In our study, we present more robust phylogenetic analysis based on three molecular markers of *Dactylochlamys pisciformis* and all of the three known *Legendrea* species, showing that they likely represent a new anaerobic lineage of ciliates. We first provide a detailed morphological characterization of both genera using modern microscopy and staining methods. We identify and discuss the bacterial (Syntrophaceae) and archaeal endosymbionts harbored by both genera based on 16S rRNA gene sequences. We also discovered that *Legendrea* preys on gastrotrichs, which is supported by molecular data and a unique video material documenting the feeding behavior of one *Legendrea* species. This study brings essential information needed to better understand the phylogeny, life strategies, and rarity of these organisms and emphasizes the importance of citizen science.

Abstract: Free-living anaerobic ciliates are of considerable interest from an ecological and an evolutionary standpoint. Extraordinary tentacle-bearing predatory lineages have evolved independently several times within the phylum Ciliophora, including two rarely encountered anaerobic litostomatean genera, *Legendrea* and *Dactylochlamys*. In this study, we significantly extend the morphological and phylogenetic characterization of these two poorly known groups of predatory ciliates. We provide the first phylogenetic analysis of the monotypic genus *Dactylochlamys* and the three valid species of *Legendrea* based on the 18S rRNA gene and ITS-28S rRNA gene sequences. Prior to this study, neither group had been studied using silver impregnation methods. We provide the first protargol-stained material and also a unique video material including documentation, for the first time, of the hunting and feeding behavior of a *Legendrea* species. We briefly discuss the identity of methanogenic archaeal and bacterial endosymbionts of both genera based on 16S rRNA gene sequences, and the importance of citizen science for ciliatology from a historical and contemporary perspective.

Keywords: convergent evolution; infraciliature; methanogenic archaea; predatory ciliates; Spathidiidae; Syntrophaceae



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1. Introduction

The phylum Ciliophora comprises a bewildering diversity of ciliated unicellular eukaryotes inhabiting virtually every biotope on Earth as both free-living organisms or

symbionts. Their enormous morphologic variety is matched by the many indispensable ecologic roles they play in terrestrial, freshwater, and marine microbial food webs. Eukaryovorous ciliates, an important guild of microorganisms, use various morphologic features for prey capture, and tentacles represent one of the most intriguing structures of this kind. Tentacles, as a feature of the ciliate cortex, have evolved several times in very distantly related lineages; e.g., the family Mesodiniidae and classes Phyllopharyngea and Litostomatea [1]. Ciliates of the class Litostomatea are known mainly as free-living aerobic eukaryovorous predators (subclasses Rhynchostomatia and Haptoria) or anaerobic endosymbionts inhabiting the guts of invertebrate and vertebrate hosts (subclass Trichostomatia) [2,3]. In addition to these varied lifestyles, litostomateans also exhibit some spectacularly diverse morphologic features, e.g., the skeletal plates of the endosymbiotic entodiniomorphids, the triple proboscises of *Teuthophrys* Chatton and Beauchamp, 1923, and the tentacles and papillae of *Actinobolina* Strand, 1928, *Dactylochlamys* Lauterborn, 1901, and *Legendrea* Fauré-Fremiet, 1908 [1,4,5].

The rarely encountered tentaculiferous ciliate genera *Legendrea* and *Dactylochlamys* have been assigned to the litostomatean family Spathidiidae based solely on their morphology [1,6]. The most distinctive features of *Legendrea* are tassel-like, ciliated, and extrusome-bearing tentacles which, in two species (*L. bellerophon* Penard, 1914 and *L. loyezae* Fauré-Fremiet, 1908), are highly extensible and have been assumed, but not proven, to be involved in prey capture [7]. On the other hand, the tentacles of *Dactylochlamys pisciformis* Lauterborn, 1901, the type and only species of the genus, are morphologically quite different from those of *Legendrea* and are more similar in appearance to those of *Actinobolina* and *Belonophrya* Andre, 1914, yet their functions remain unknown [4,5]. *Legendrea* and *Dactylochlamys* are vanishingly rare genera that may elude even the most widely traveled ciliatologists. To date, there have been only a few published reports, website images, and videos of *Legendrea* species and *Dactylochlamys* [5,8]. Thus, little information has accrued in over a century since their first descriptions.

In the genus *Legendrea*, three currently accepted species were originally described based on shared morphological features: *L. loyezae*, the type species, *L. bellerophon*, and *L. pespelicani* Penard, 1922. Four other species previously included in *Legendrea* by Penard (original combinations *L. crassa*, *L. interrupta*, *L. porculus*, and *L. simplex*) have been transferred to other genera in the order Spathidiida [9–11].

We present a phylogenetic analysis based on the ribosomal RNA cistron of *D. pisciformis* and all three *Legendrea* species, and also provide more detailed morphological characterization of *Legendrea* and *Dactylochlamys*, using protargol-impregnated specimens and scanning electron microscopy. We also present unique video material, including documentation of the feeding behavior of one *Legendrea* species, and discuss the putative bacterial (Syntrophaceae) and archaeal (*Methanobacterium* sp., *Methanocorpusculum* sp., and *Methanosaeta* sp.) endosymbionts harbored by both genera based on 16S rRNA gene sequences. Our results provide unique data essential to a better understanding of the phylogeny, life strategies, and rarity of these remarkable organisms.

2. Materials and Methods

2.1. Sampling

Sulfidic sediment samples from four freshwater localities in Belgium, the Czech Republic, Germany, and the USA were collected in 50 ml Falcon tubes. All populations in the original raw samples were kept in closed tubes for two to three weeks. Attempts to establish enriched and clonal cultures were unsuccessful.

The *Dactylochlamys pisciformis* population VB2A was found in the Venusbassin in Tiergarten Park in Berlin, Germany (52°30'52.5" N 13°22'14.2" E), and the MRATIN population in a eutrophic pond, Mratín, Czech Republic (50°11'58.7" N 14°33'17.2" E). The Czech populations of *Dactylochlamys* sp. (MOKOTP1Q), *Legendrea loyezae* (MOKOTL), and *L. ornata* (MOKOTP1) were found in a small permanent pond adjacent to the Vltava river in Prague (49°59'24.9" N 14°24'04.1" E). The US population of *L. ornata* (SAGEGLEN) came

from a small permanent eutrophic pond near Boise, Idaho (43°40′57.20″ N 116°15′15.44″ W), the Belgian population of *L. ornata* came from a freshwater garden pond in Tontelange, Province de Luxembourg (49°43′36.7″ N 5°48′32.1″ E), and the GTUB population from a garden mesocosm (see [12]). The Czech population of *L. pespelicani* (VLKOV) was found in a small permanent concrete-lined freshwater reservoir in the village of Vlkov (49°09′04.0″ N 14°43′22.4″ E).

2.2. Morphological Characterization

Living cells were hand-picked with glass micropipettes and studied at magnifications of 100–1000× with brightfield and differential interference contrast illumination, using an Olympus BX51 microscope equipped with an Olympus DP70 camera (Olympus Corp., Tokyo, Japan). Cells of VB2A were fixed with 4% (*v/v*) formalin and impregnated with protargol (Polysciences, Warrington, PA, USA) according to a modified Dieckmann's protocol [13–15]. In vivo measurements were made from photomicrographs of uncompressed, freely swimming cells, using calibrated software. Counts and measurements from protargol preparations were made at 1000× magnification. For scanning electron microscopy (SEM), cells were fixed for at least 30 min in 2.5% glutaraldehyde or in a 1:1 (*v/v*) solution of 5% glutaraldehyde and 2% osmium tetroxide, processed according to [14], and examined JSM-IT200 scanning electron microscope (JEOL LV, Peabody, MA, USA). Except as noted, morphological terminology and size classification follows [1,2,9,14]. For clarity, we refer to the cortical pellicular projections of *Dactylochlamys pisciformis* and the three species of *Legendrea* as “tentacles” and the trichocyst-bearing oral bulge structures of *L. pespelicani* as “papillae”.

2.3. DNA Extraction, Amplification, and Sequencing

In total, 3 cells from the VB2A population and single cells from the VLKOV, MOKOTL, MOKOTP1, and MOKOTP1Q populations were hand-picked with glass microcapillary pipettes, washed 5 times with distilled water, and added into 30 µL of DNA/RNA shield (Zymo Research, Irvine, CA, USA). The total DNA was isolated using the MasterPure™ Complete DNA and Purification Kit following the manufacturer's instructions. Three individuals of SAGEGLEN were picked for DNA extraction with a modified Chelex method [16].

The 18S rRNA gene (complete or almost complete) of SAGEGLEN and VLKOV was amplified using the primers EukA 5′-AACCTGGTTGATCCTGCCAGT-3′ and EukB 5′-TGATCCATCTGCAGGTTACCT-3′ [17], VB2A and MOKOTL were amplified with primers 82F 5′-GAAACTGCGAATGGCTC-3′ [18] and EukB, and the annealing temperature was 55 °C. MOKOTP1 was amplified with the primers 82F and the newly designed LEGR3 5′-TTCTCCTTCCTCTAGGTGATAAGG-3′, and the annealing temperature was 55 °C. An approximately 1200 bp long fragment of the ITS region and 5′ end of the 28S rRNA gene was amplified using the primers ITS-F 5′-GTAGGTGAACCTGCGGAAGGATCATTA-3′ [19] and LO-R 5′-GCTATCCTGAGRGAACTTCG-3′ [20], and the annealing temperature was 54 °C. The 16S rRNA gene fragment (approx. 460 bp) of methanogenic Archaea was amplified using the primers Arc915F 5′-AGGAATTGGCGGGGAGCAC-3′ and ArcR1326 5′-TGTGTGCAAGGAGCAGGGAC-3′ [21,22], and the annealing temperature was 55 °C. The PCR products were purified using EXOSAP (Applied Biosystems, Waltham, MA, USA). Amplicons were directly sequenced on an ABI PRISM 3100 sequencer (Applied Biosystems). For taxonomic assignment of archaeal 16S rRNA gene fragments, the BLASTn algorithm was used (<https://blast.ncbi.nlm.nih.gov>, accessed on 29 March 2023).

The V4 variable region fragment of 16S rRNA gene (approx. 300 bp) from VLKOV and VB2A populations was amplified from extracted DNA (see above) for Illumina sequencing, using barcoded primers 515F 5′-GTGYCAGCMGCCGCGGTAA-3′ and 806R 5′-GGACTACNVGGGTWTCTAAT-3′ [23,24]. Paired-end (2 × 250 bp) Illumina amplicon sequencing (NovaSeq6000) and demultiplexing was performed at SEQme company (Dobříš, Czech Republic). Microbiome bioinformatic analyses were performed using the QIIME2

2022.8 platform with DADA2 plugin for denoising and chimera removal [25,26]. Amplicon sequence variants (ASVs) were taxonomically classified using the classify-sklearn naïve Bayes taxonomy classifier (via q2-feature-classifier plugin) [27] against the Silva 138 99% OTUs reference sequences of the 515F/806R region [28]. Dominant archaeal ASVs were identified as putative symbionts based on their relative frequency.

2.4. Phylogenetic Analyses

An alignment of 18S rRNA gene sequences included 167 taxa, comprising 135 litostomateans and 32 outgroup taxa representing Armophorea and CONThreeP [29]. Five litostomatean sequences are newly reported in this study and all other sequences were obtained from GenBank. The dataset of concatenated 18S-ITS-28S rRNA region sequences consisted of 168 taxa, including 6 newly determined sequences and 162 sequences (135 litostomateans and 32 outgroup taxa) obtained from GenBank. The sequences were aligned using the G-INS-i algorithm [30] on the MAFFT server (<https://mafft.cbrc.jp/alignment/software/>, accessed on 15 March 2023). The alignment was manually edited using AliView [31] to trim primer sequences. The length of the final 18S rRNA gene data set was 1825 positions and the concatenated data set comprised 3163 positions. Phylogenetic trees were constructed using the maximum likelihood (ML) and Bayesian inference (BI) methods. The best fit substitution model (GTR + I + Γ) was selected using Modeltest-NG [32,33]. ML analysis was performed in RAxML-NG [34] on the CIPRES portal (http://www.phylo.org/sub_sections/portal/, accessed on 15 March 2023) under the GTR + I + Γ substitution model. Statistical support was assessed using 1000 bootstrap pseudoreplicates and convergence assessed using weighted Robinson–Foulds distance. Bayesian analysis was performed using MrBayes 3.2.7 [35] on the CIPRES portal using the GTR + I + Γ substitution model. For the 18S rRNA gene alignment, Markov chain Monte Carlo (MCMC) analyses of four chains (three hot, one cold, temperature 0.2) and for the concatenated alignment six chains (five hot, one cold, temperature 0.1) were each run for 10 million generations, with a sampling frequency of 1000 generations. The first 25% of sampled trees were removed as burn-in. Convergence was assessed using RWTY [36]. Uncorrected p-distances were calculated using MEGA v10.2.6.

3. Results

3.1. Morphological Descriptions and Diagnoses

3.1.1. *Dactylochlamys pisciformis* Lauterborn, 1901

Amended diagnosis based on [10,37,38] and current study. Moderately small-to-medium-sized spathidiid. Shape fusiform to clavate. Cortex rugose. One equatorial, globular to ellipsoidal macronucleus with one adjacent ellipsoidal micronucleus. Contractile vacuole subterminal. Somatic kineties follow left-hand spiral course. Long fine somatic cilia interspersed with very slender, rigid, tubular, capitate, retractile tentacles. Long filiform extrusome occupies lumen of each tentacle, and extends into cytoplasm when tentacles retract. Oral bulge truncate conical; base surrounded by short oblique pectinelles at anterior ends of somatic kineties.

Description based on VB2A (Figures 1 and 2; Table S1; Video S1). Size 85–110 $\times$ 25–35 μ m in vivo ($n = 2$), about 60 $\times$ 18 μ m after protargol impregnation, length:width ratio from 2.5–3.7 in vivo ($n = 2$) and about 3.6 in protargol preparations. Shape fusiform to clavate. Colorless. Single equatorial globular to ellipsoidal macronucleus about 8 $\times$ 6 μ m with many small, scattered nucleoli in protargol preparations; single adjacent globular micronucleus about 2–3 μ m across (Figure 2C). Contractile vacuole subterminal; excretory pores not observed (Figure 1A,B,F,G). Long, fine filiform extrusomes occupy lumen of tentacles; proximal ends extend into cytoplasm. Cortical granules not observed. Cytoplasm filled with refractive globules and large food vacuoles.

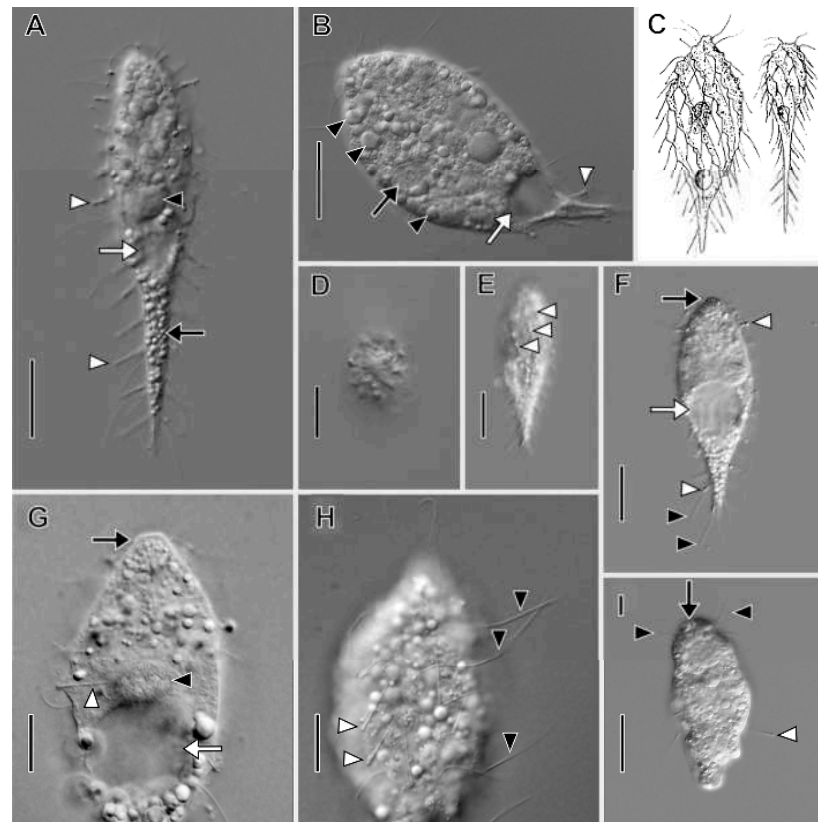


Figure 1. *Dactylochlamys pisciformis* from VB2A (A,B,E–H) and MRATIN (D) in vivo and a sucktorian swarmer (I) in vivo. (A) Lateral view of a slender individual showing the inconspicuously capitate tentacles in varying states of extension (white arrowheads), the macronucleus (black arrowhead), the subterminal contractile vacuole (white arrow), and the refractile cytoplasmic globules (black arrow). (B) Lateral view of a broadly ellipsoidal individual showing the cytoplasmic globules (black arrowheads), the contractile vacuole (white arrow), and a retracted tentacle (white arrowhead). (C) Two variations in body shape, modified from Kahl presenting two morphotypes of *D. pisciformis*. (D) Circular cell shape viewed in optical cross section. (E) Lateral view showing the spiral cortical ridges (white arrowheads). (F) Lateral view showing capitate tentacles (white arrowheads), somatic cilia (black arrowheads), the contractile vacuole in diastole (white arrow), and the bluntly conical oral bulge (black arrow). (G) Lateral view, optical section showing extrusomes of a retracted tentacle extending into the cytoplasm (white arrowhead), the contractile vacuole in diastole (white arrow), the macronucleus (black arrowhead), and the oral bulge (black arrow). (H) Surface view showing the long, fine somatic cilia (black arrowheads) and the tentacles (white arrowheads). (I) Lateral view of sucktorian swarmer form (similar to *Parapopodophrya solaris*) from the same sample as VB2A, showing an extended capitate tentacle (white arrowhead), the apical part of the cell superficially resembling the oral bulge of *Dactylochlamys* (black arrow), and the somatic cilia resembling the pectinellae of *Dactylochlamys* (black arrowheads); otherwise somatic cilia are absent unlike in *Dactylochlamys*. Scale bars: 20 μ m (A–F,I), 10 μ m (G,H).

About 10–12 ordinarily spaced somatic ciliary rows on slightly wavy left-hand spiraling ridges, short inclined ciliated pectinelle between anterior end of each somatic kinety and base of oral bulge, each pectinelle composed of three or four basal bodies. Kineties composed of monokinetids bearing long fine cilia (12–20 μ m long), interspersed with tentacles. Tentacles very slender, tubular, retractile, about 15 μ m long when extended, inconspicuously capitate when retracted, and each encloses an argyrophilic extrusome. Dorsal brush not observed.

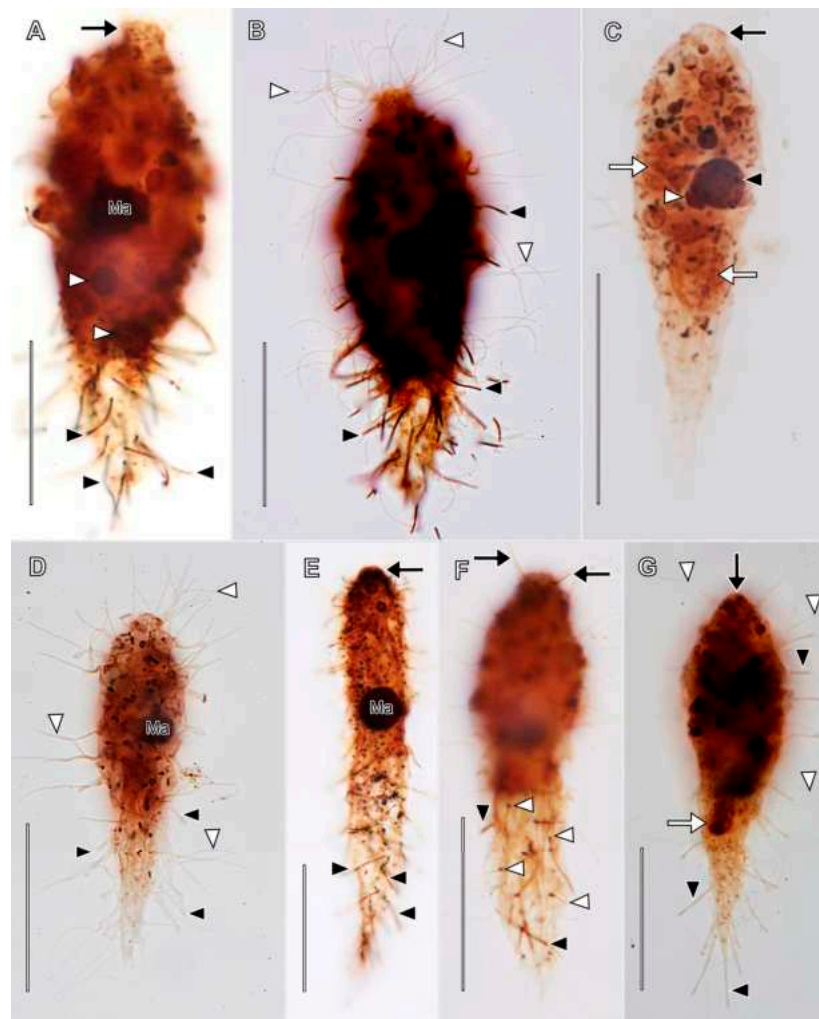


Figure 2. *Dactylochlamys pisciformis* from VB2A after protargol impregnation. (A) Optical section showing the oral bulge with left-hand-spiraling pectinellae comprising 3–4 basal bodies (black arrow), food vacuoles (white arrowheads), and the strongly impregnated (argyrophilic) tentacles (black arrowheads). (B) High-contrast view showing the long fine somatic cilia (white arrowheads) and the argyrophilic tentacles (black arrowheads). (C) Optical section showing the macronucleus with multiple small nucleoli (black arrowhead), the micronucleus (white arrowhead), the oral bulge (black arrow), and the food vacuoles (white arrow). (D) Cell showing long fine somatic cilia (white arrowheads) and inconspicuous unimpregnated tentacles (cf. A,B). (E) Slender individual showing the oral bulge (black arrow) and the tentacles (black arrowheads). (F) View showing the cilia of the oral bulge pectinellae (black arrows), the left-hand-spiraling somatic kineties (white arrowheads), and the tentacles (black arrowheads). (G) View showing the oral bulge (black arrow), a food vacuole with an ingested flagellate (white arrow), and the inconspicuous somatic cilia (white arrowheads) interspersed between tentacles (black arrowheads). Ma, macronucleus. Scale bars: 25 µm.

Oral bulge truncate conical, inconspicuous in vivo, about 2 µm high, 4 µm in diameter after protargol impregnation. Oral bulge extrusomes and cytopharyngeal basket not observed (Figure 2).

Based on our own observations, we accept Kahl's synonymization of *D. hystrix* Wetzel, 1928 with *D. pisciformis* [10]).

3.1.2. Legendrea Fauré-Fremiet, 1908

Amended diagnosis: Moderately small to large spathidiid. Tentacles, each with circumtentacular kinety and extrusome bundle, sometimes highly extensible, located on

dorsal and ventral margins or restricted to posterior one-half of cell. Sometimes with papillae in oral bulge. Inhabits anoxic or microoxic freshwater biotopes.

- *Legendrea loyezae* Fauré-Fremiet, 1908

Amended diagnosis (based on [7–10,39] and current study): With characters of genus. Size 70–132 × 62–85 µm in vivo. Shape broadly elliptical to almost spherical. Eccentric posterior contractile vacuole may appear as two vacuoles due to median cleft at posterior end when tentacles retracted. Up to 25 tentacles clustered posteriorly, never anterior to cell equator, trail limply behind swimming cells. Tentacles more or less tubular when retracted, distal ends mushroom-shaped when extended, often with an eccentric distal luminal vacuole, tentacular extrusome bundles 4.5–14 µm long, layer of smaller rod-shaped extrusomes beneath terminal cap of tentacles. Extended tentacles reach up to 6.5 times retracted length.

Description based on in vivo observations of MOKOTL ($n = 3$; Figure 3; Video S2): Size 85–102 × 55–102 in vivo, contractile. Shape broadly obovate to nearly globular, anterior end obliquely truncate; up to 25 flexible, trailing tentacles restricted to posterior one half of cell. Horseshoe-shaped macronucleus, micronuclei not observed. Contractile vacuole eccentrically located at posterior end, sometimes indented by median pellicular cleft when tentacles retracted, occupies entire posterior end in diastole (Figure 3C,D). Extrusomes in oral bulge, morphology not determined, tentacles with two types of extrusomes; one type curved, filiform, closely packed in central bundles at distal ends of tentacles (about 4.5 µm long); second type comprises layer beneath distal ends of tentacles, small (about 1.5 µm long), rod-shaped (Figure 3F,H,I). Ejected extrusomes not observed. Tentacles extensible, covered with granular layer near junction with cell body, distinct cortical ruffles (collar) not observed. Cytoplasm colorless but cells appear dark under low magnification. Cells packed with refractive globules, food vacuoles with ingested prey. Cells swim steadily at moderate pace and rotate rightward about long axis (Video S2).

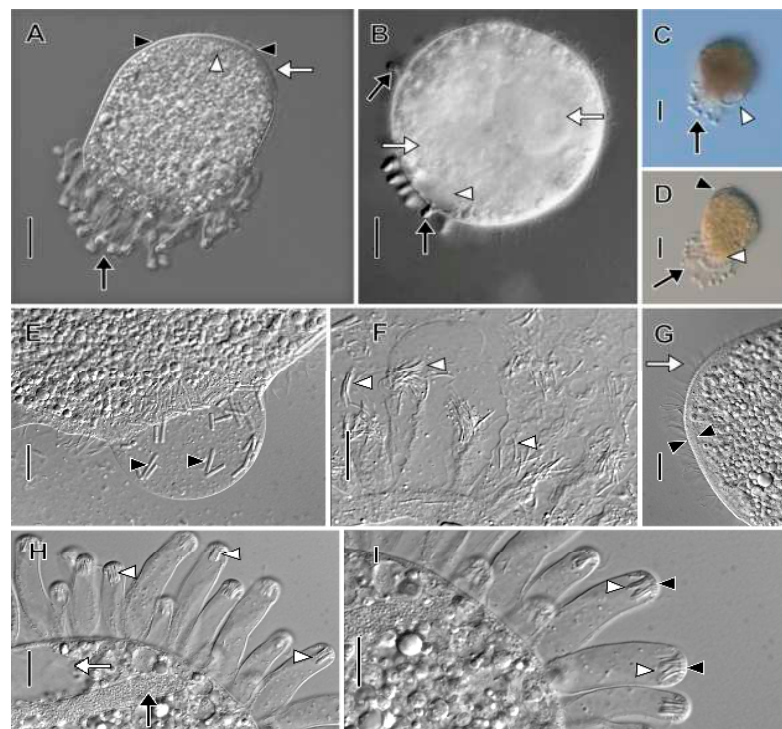


Figure 3. *Legendrea loyezae* from MOKOTL in vivo. (A) Lateral view of a swimming cell showing the oral bulge (between black arrowheads), the position of the dorsal brush (white arrow), the oral basket (white arrowhead), and trailing partially retracted tentacles (black arrow). (B) Lateral view of

a globular cell with retracted tentacles (black arrows) and ingested prey (white arrows) and showing the contractile vacuole (white arrowhead). (C) Lateral view showing the typical shape of a swimming cell and showing trailing retracted tentacles (black arrow) and the eccentric position of the contractile vacuole (white arrowhead). (D) Lateral view of the same specimen as A showing trailing tentacles (black arrow), the oral bulge (black arrowhead), and the contractile vacuole (white arrowhead), the same specimen as A. (E) Squashed cell showing prokaryotic endosymbionts (black arrowheads). (F) Squashed tentacles showing curved filiform tentacular extrusomes (white arrowheads). (G) The hyaline subcortical layer probably composed of mucocysts (between black arrowheads) and somatic cilia (white arrow). (H) Detail view of the posterior part of the cell showing tentacular extrusome bundles (white arrowheads), one end of the horseshoe-shaped macronucleus (black arrow), and the eccentrically located contractile vacuole (white arrow). (I) Detail of tentacles with approximately 5 μm long curved filiform extrusomes in bundles (white arrowhead) and a subcortical layer of much smaller (about 1.5 μm) rod-shaped extrusomes (black arrowheads). Scale bars: 20 μm (A–D), 10 μm (E–I).

Ciliature holotrichous, somatic cilia about 6 μm long in closely spaced longitudinal kineties; number of kineties not determined. Each tentacle with single circumferential subterminal kinety. Dorsal brush inconspicuous with approximately 3 μm long clavate cilia; number and morphology of brush rows not determined.

Oral bulge inconspicuous, less than 3 μm high, occupies two-thirds of anterior end, eccentric (i.e., displaced slightly ventrally). Oral basket inconspicuous.

Remark: We were unsuccessful in obtaining protargol preparations of *L. loyezae*.

- Legendrea pespelicani Penard, 1922

Amended diagnosis based on [9] and current study: With characters of genus. Size 180–210 $\times$ 60–90 μm in vivo. Broadly spatulate in lateral view, laterally compressed. Oral bulge oblique, invariably contains one trichocyst-bearing papilla at either end, usually one or two papillae in between; thus, oral bulge outline moniliform or dumbbell-shaped when viewed anteriorly. Macronucleus filiform, very long, tortuous strand. About six stout, mushroom-shaped, ciliated, extrusome-bearing tentacles commence at or anterior to equator, distributed at irregular intervals on dorsal and ventral margins of cell, always one posterior polar tentacle. Tentacular extrusomes longer (about 25 μm) than those in tentacles of *L. ornata* or *L. loyezae*. Distinctly spathidiid somatic ciliary pattern. Circumtentacular kineties composed of many more dikinetids than in *L. ornata*. Ciliary rows interrupted by tentacles while deviating around tentacles in *L. ornata*. Tentacle extension characteristic for *L. loyezae* and *L. ornata* not observed.

Description based on VLKOV (Figures 4–6; Table S2; Videos S3–S5): Size about 180 $\times$ 90 μm in vivo, about 165 $\times$ 120 after protargol impregnation, length:width ratio 2 in vivo and 1.4 in protargol preparations. Shape broadly spatulate in lateral view, laterally compressed in vivo, less so in protargol preparations. Cells appear dark in vivo under low magnification. Single very long (up to 331 μm), tortuous, filiform macronucleus; nucleoli prominent in vivo (Figures 4I and 5). Five to nine scattered micronuclei (2.5–4 μm across). Extrusomes of one type, restricted to closely packed bundles in distal ends of tentacles and in oral bulge papillae, curved, filiform (about 25 $\times$ 1 μm), do not impregnate with protargol. Contractile vacuole large, terminal, several subterminal excretory pores on right side (Figures 4F and 5C). Cortex flexible (Figure 4C). Cortical granules not observed. Cytoplasm filled with refractive globules (about 2–4 μm across) and large food vacuoles. Swims lazily (Videos S3–S5).

Somatic ciliation holotrichous, longitudinal ciliary rows interrupted by tentacles. Somatic cilia about 8 μm long, arranged in about 70 rows. Ciliary pattern distinctly spathidiid (i.e., right-side kineties curve dorsally to meet circumoral kinety, left kineties curve ventrally). Each tentacle with ciliated subcapital circumtentacular kinety composed of dikinetids (about 35 on average). Stout (about 8 μm high $\times$ 10 μm across) mushroom-shaped tentacles (six on average) at irregular intervals on dorsal, ventral margins, invariably one tentacle at posterior pole (Figure 6A). Active tentacle extension not observed. Total

of 3 dorsal brush rows composed of dikinetids bearing $3.5\ \mu\text{m}$ long clavate cilia, rows commence to left of dorsal oral bulge papilla; B1 and B2 extend posteriorly about 20% of cell length; B3 highly unusual, i.e., proximal part similar in morphology and length to B1 and B2 but posterior part composed of patchily distributed monokinetids and groups of dikinetids extending about 50% of cell length (Figure 6C,D,F).

Oral bulge oblique (about 40° to long axis of cell), convex anteriorly, long, narrow (about $80 \times 5\ \mu\text{m}$). Circumoral kinety about $80\ \mu\text{m}$ long, $5\ \mu\text{m}$ wide between papillae, invariably encloses one extrusome-bearing papilla at dorsal and ventral end, usually one or two additional papillae between (two of nine individuals had only two oral bulge papillae: one dorsal, one ventral). Oral bulge papillae about $3.5\ \mu\text{m}$ high $\times$ $10\ \mu\text{m}$ across. Outline of circumoral kinety moniliform or dumbbell-shaped when viewed anteriorly due to deviation around large papillae (Figure 5C,D,G). Large broadly conical oral basket extending nearly entire length of cell (Figure 5H).

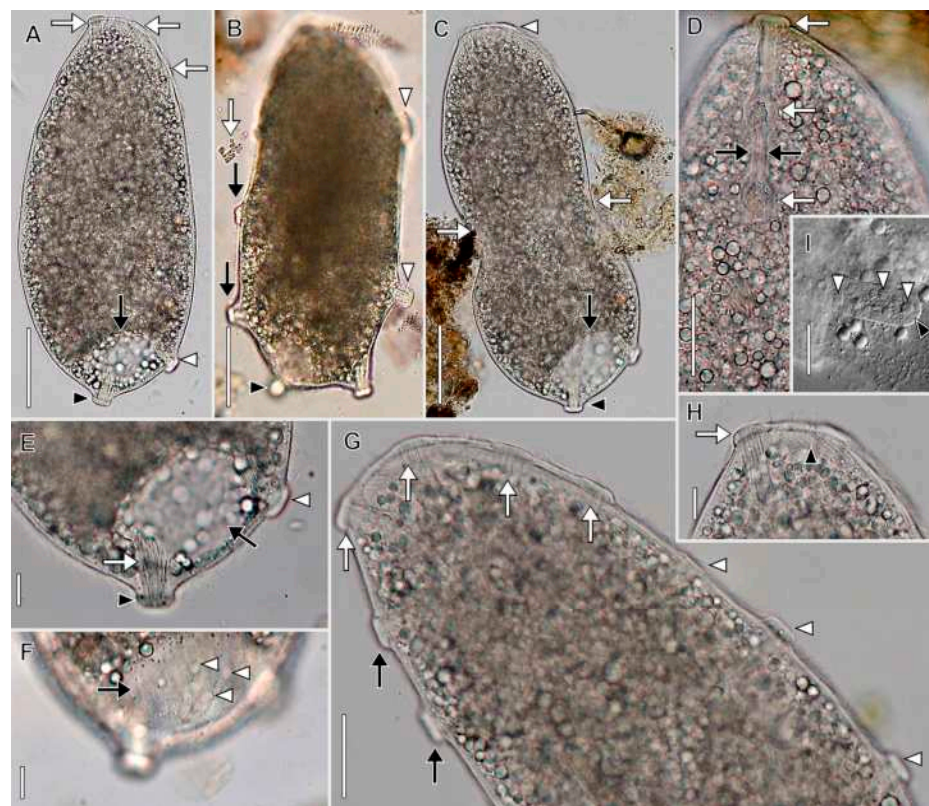


Figure 4. *Legendrea pespelicani* from VLKOV in vivo. (A) Right dorsolateral view showing three oral papillae (white arrows), the posterior terminal tentacle (black arrowhead), a ventral tentacle (white arrowhead), and the contractile vacuole (black arrow). (B) Lateral view showing dorsal tentacles (black arrows), ventral tentacles (white arrowheads), the terminal tentacle (black arrowhead), and a *Thiopedia rosea* platelet (white arrow). (C) Optical section showing the oral bulge (white arrowhead), flexible cortex (white arrows), the terminal tentacle (black arrowhead), and the contractile vacuole (black arrow). (D) Ventral view showing three oral papillae (white arrows) encompassed by the circumoral kinety (black arrows). (E) Detail of posterior end of the cell showing an extrusome bundle (white arrow) the terminal tentacle (black arrowhead), a ventral tentacle (white arrowhead), and the contractile vacuole (black arrow). (F) Surface view, the same cell as (E), showing excretory pores (white arrowheads) of the contractile vacuole (black arrow). (G) Optical section showing four oral papillae (white arrows), dorsal somatic tentacles (black arrows), and ventral tentacles (white arrowheads). (H) First oral papilla (white arrow) and somatic kinety furrows (black arrowhead). (I) The filiform macronucleus (black arrow) and nucleoli (white arrowheads). Scale bars: $50\ \mu\text{m}$ (A–C), $25\ \mu\text{m}$ (D,G), $10\ \mu\text{m}$ (E,F,H).

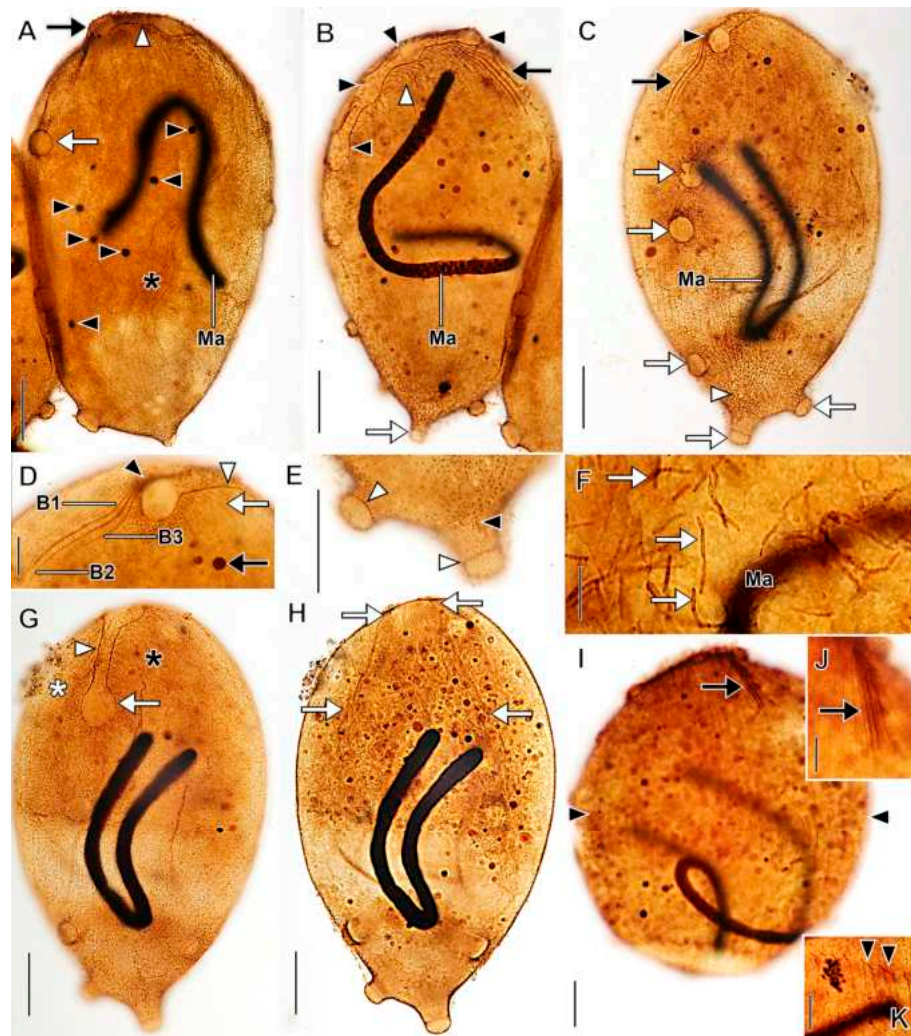


Figure 5. *Legendrea pespelicani* from VLKOV after protargol impregnation. (A) Right dorsolateral view showing the circumoral kinety (white arrowhead), an oral papilla at the dorsal end of the oral bulge (black arrow), the first dorsal tentacle (white arrow), multiple micronuclei (black arrowheads), and fibrils of the oral basket (asterisk). (B) Left ventrolateral view showing four oral papillae (black arrowheads), the anterior ends of the left somatic kineties curving ventrally (white arrowhead), dorsal brush rows extending to the left of the dorsal oral papilla (black arrow), and the posterior terminal tentacle (white arrow). (C) Right dorsolateral view showing a dorsal oral papilla (black arrowhead), the dorsal brush rows (black arrow), tentacles (white arrows), and excretory pores of the contractile vacuole (white arrowhead). (D) Detail of the same cell as (C) showing three dorsal brush rows extending to the left of the dorsal oral papilla (black arrowhead), the circumoral kinety (white arrowhead), a micronucleus (black arrow), and the right somatic kineties curving slightly dorsally (white arrow). (E) Detail of the posterior end of the cell showing the circumtentacular kinety (white arrowheads) and a somatic kinety extending onto the posterior terminal tentacle (black arrowhead). (F) Detail showing cytoplasmic endosymbionts (white arrows). (G) Left ventrolateral view showing right (white asterisk) and left (black asterisk) somatic kineties, the circumoral kinety (white arrowhead) encompassing the ventral oral papilla (white arrow). (H) Optical section showing the long, broadly conical oral basket (white arrows) extending from the oral bulge. (I) An early divider showing dorsal brush rows (black arrow) and the level of the future division furrow (black arrowheads). (J) Anterior detail, the same cell as (I), showing the dorsal brush (black arrow). (K) Detail of the same cell as (I), showing holotelokinetal morphogenesis with basal bodies proliferating from somatic kineties and curving leftward at the level of the future division furrow (black arrowheads). B1–3, dorsal brush rows 1, 2, and 3; Ma, macronucleus. Scale bars: 25 μ m (A–C,G–I), 10 μ m (D,F,J,K).

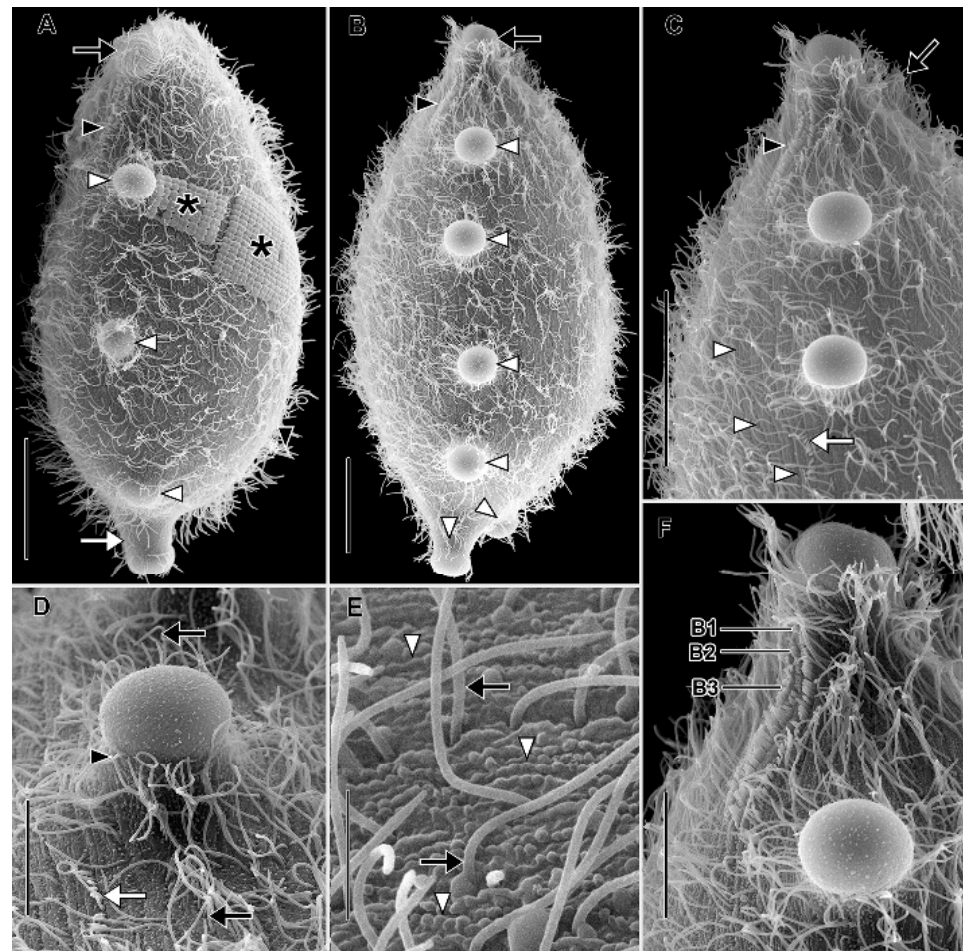


Figure 6. *Legendrea pespelicani* from VLKOV in the scanning electron microscope. (A) Dorsal view showing an oral bulge papilla (black arrow), the clavate cilia of the dorsal brush (black arrowhead), dorsal tentacles (white arrowheads), the terminal tentacle (white arrow), and *Thiopedia rosea* platelets adhering to the right side of the cell (asterisks). (B) Dorsal view showing an oral papilla (black arrow), dorsal brush rows (black arrowhead), and tentacles (white arrowheads). (C) Detail of the same cell as (B) showing the cilia of the circumoral kinety (black arrow), dorsal brush rows (black arrowhead), somatic kineties (white arrowheads), and isolated posterior groups of clavate cilia in brush row 3 (white arrow). (D) Detail showing a tentacle interrupting a somatic kinety (black arrows), an isolated group of clavate cilia posterior to dorsal brush row 3 (cf. (C)), and a ciliated circumtentacular kinety (black arrowhead). (E) Detail of the cortex showing rows of probable mucocysts (white arrowheads) and cilia of somatic monokinetids (black arrows). (F) Detail of the three inconspicuous dorsal brush rows extending to the left side of the dorsal oral papilla. B1–B3, dorsal brush rows 1–3. Scale bars: 25 µm (A–C,F), 10 µm (D), 5 µm (E).

Stomatogenesis holotelokinetal; proliferation of basal bodies first occurs in opisthe kineties in line with proter dorsal brush rows. Developing anterior ends of opisthe kineties curve ventrally (Figure 5K).

- *Legendrea ornata* (Stokes, 1887) Penard, 1914
- Original combination, *Holophrya ornata* Stokes, 1887

Amended diagnosis based on [7,10,40] and current study: With characters of genus. Size 70–180 × 60–90 µm. Shape oblong ellipsoidal when swimming, broadly ovoidal when at rest with tentacles extended. Rows of tentacles at alternating angles on dorsal and ventral margins, commence in anterior one-fourth of cell, continuous around posterior end. Extrusome bundles about 5–7 × 5 µm in vivo. Tentacles highly extensible, up to >25 times retracted length. Ciliary pattern distinctly enchelyodontid, unlike spathidiid pattern of

L. pespelicani, i.e., anterior ends of somatic kineties perpendicular to circumoral kinety. Ciliary rows not interrupted by tentacles as seen in *L. pespelicani*. Fewer (ten on average) circumtentacular kinety dikinetids than seen in *L. pespelicani* (35 on average). Three dorsal brush rows; B1 and B2 approximately equal in length, longer than B3.

Description based on BELG, GTUB, MOKOTP1, and SAGEGLEN (Figures 7–10; Table S3; Videos S6 and S7):

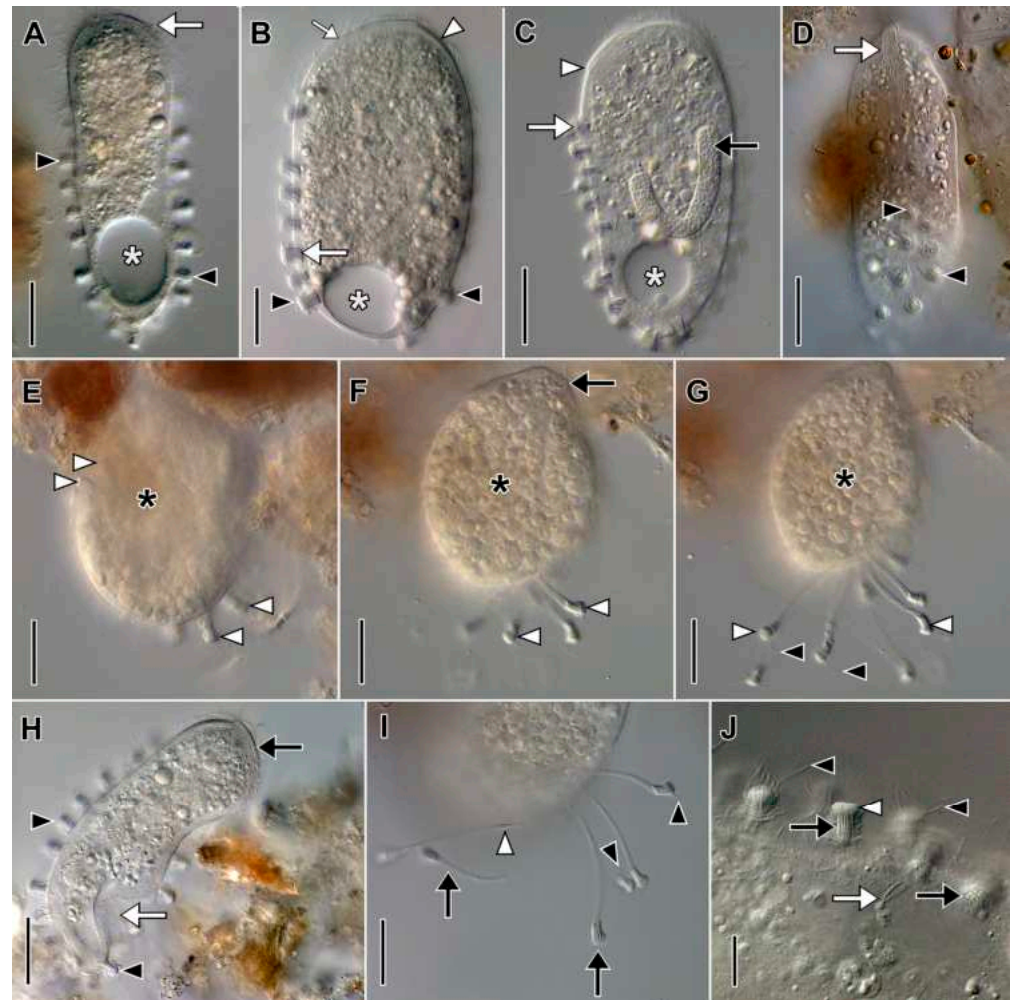


Figure 7. *Legendrea ornata* from SAGEGLEN in vivo. (A) Optical section showing the oral bulge (white arrow), retracted marginal tentacles (black arrowheads), and the contractile vacuole (asterisk). (B) Optical section showing the oral bulge (small white arrow), retracted marginal tentacles (black arrowheads), the location of the dorsal brush (white arrowhead), a tentacle extrusome bundle (white arrow), and the contractile vacuole (asterisk). (C) Optical section showing the ventral end of the oral bulge (white arrowhead), the anteriormost retracted ventral marginal tentacle (white arrow), the macronucleus (black arrow), and the contractile vacuole (asterisk). (D) Ventral view showing the oral bulge (white arrow) and retracted ventral marginal tentacles (black arrowheads). (E–G) The same cell (asterisk) during progressive extension of marginal tentacles (white arrowheads), each of which bears a ciliary wreath (black arrowheads, (G)) and a bundle of extrusomes (cf. (J)). (H) Cell showing the highly flexible cortex (white arrow), retracted marginal tentacles (black arrowheads), and the oral bulge (black arrow). (I) Detail of extended marginal tentacles (black arrows) the cilia of the circumtentacular kineties (black arrowheads), and extrusomes transiting a tentacle stalk (white arrowhead). (J) Detail of retracted marginal tentacles showing extrusomes (black arrows), ciliary wreaths of circumtentacular kineties (black arrowheads), and cytoplasmic extrusomes (white arrow). Scale bars: 25 μ m (A–I), 10 μ m (J).

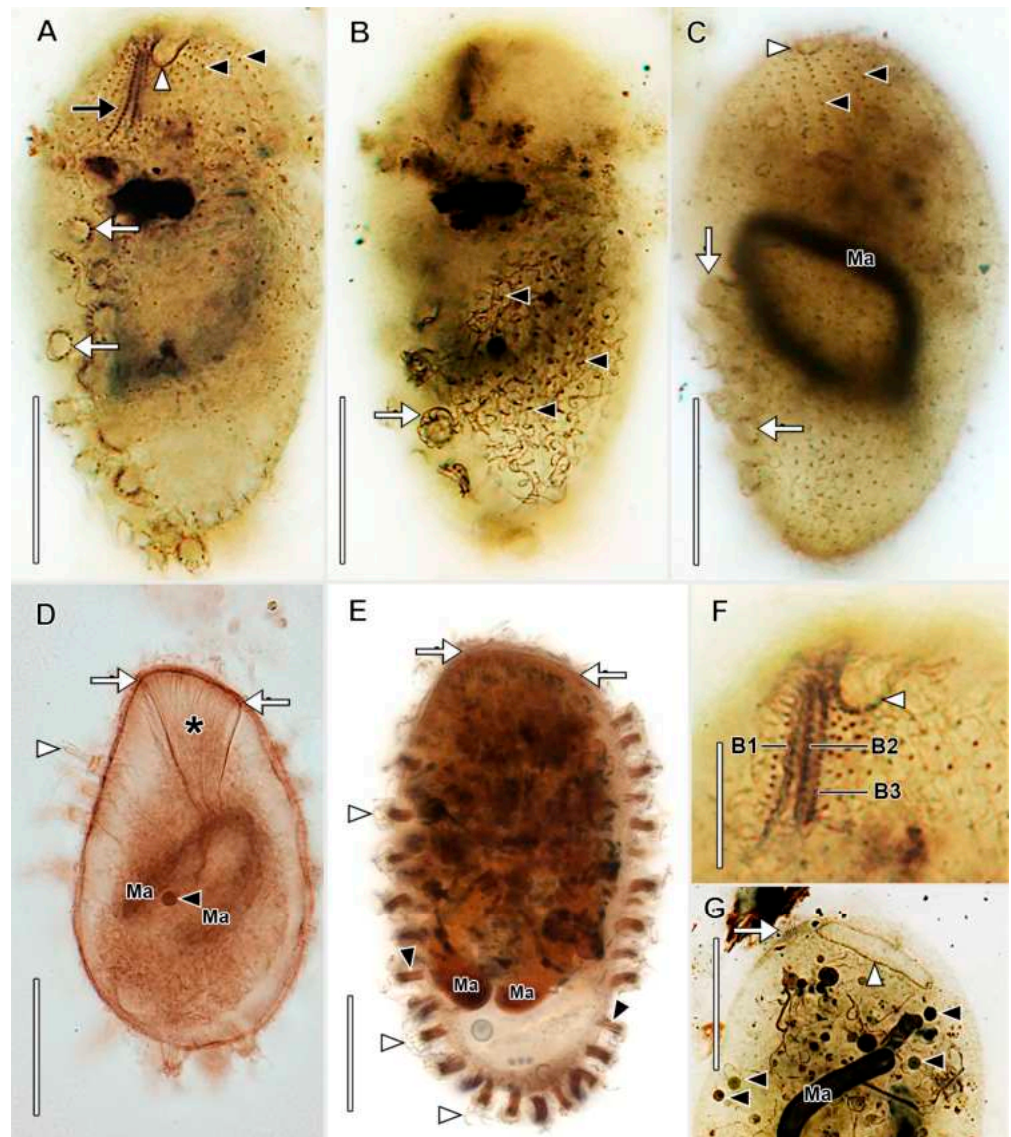


Figure 8. *Legendrea ornata* from GTUB after protargol impregnation. (A) Right dorsolateral view showing right somatic kineties (black arrowheads) perpendicularly abutting the circumoral kinety (white arrowhead), the dorsal brush (black arrow), and the dikinetids of the circumtentacular kineties (white arrows). (B) Surface view of the same cell as (A) showing circumtentacular cilia (white arrow) and longitudinal somatic kineties (black arrowheads). (C) Ventral view of the same cell as (A) showing retracted marginal tentacles (white arrows) and somatic kineties (black arrowheads) perpendicularly abutting the circumoral kinety (white arrowhead). (D) Optical section showing broad obconical oral basket (asterisk) originating from the oral bulge (between white arrows), ejected tentacular extrusomes (white arrowhead), and one of several micronuclei (black arrowhead). (E) Lateral view showing the oral bulge (between white arrows), some cilia of the marginal tentacles (white arrowheads), and tentacular extrusome bundles (black arrowheads). (F) Detail of the dorsal brush which extends to the left of the circumoral kinety (white arrowhead). (G) Dorsolateral view showing the dorsal brush (white arrow), the elliptical circumoral kinety (white arrowhead), and several micronuclei (black arrowheads). Scale bars: 25 μ m (A–E,G), 10 μ m (F).

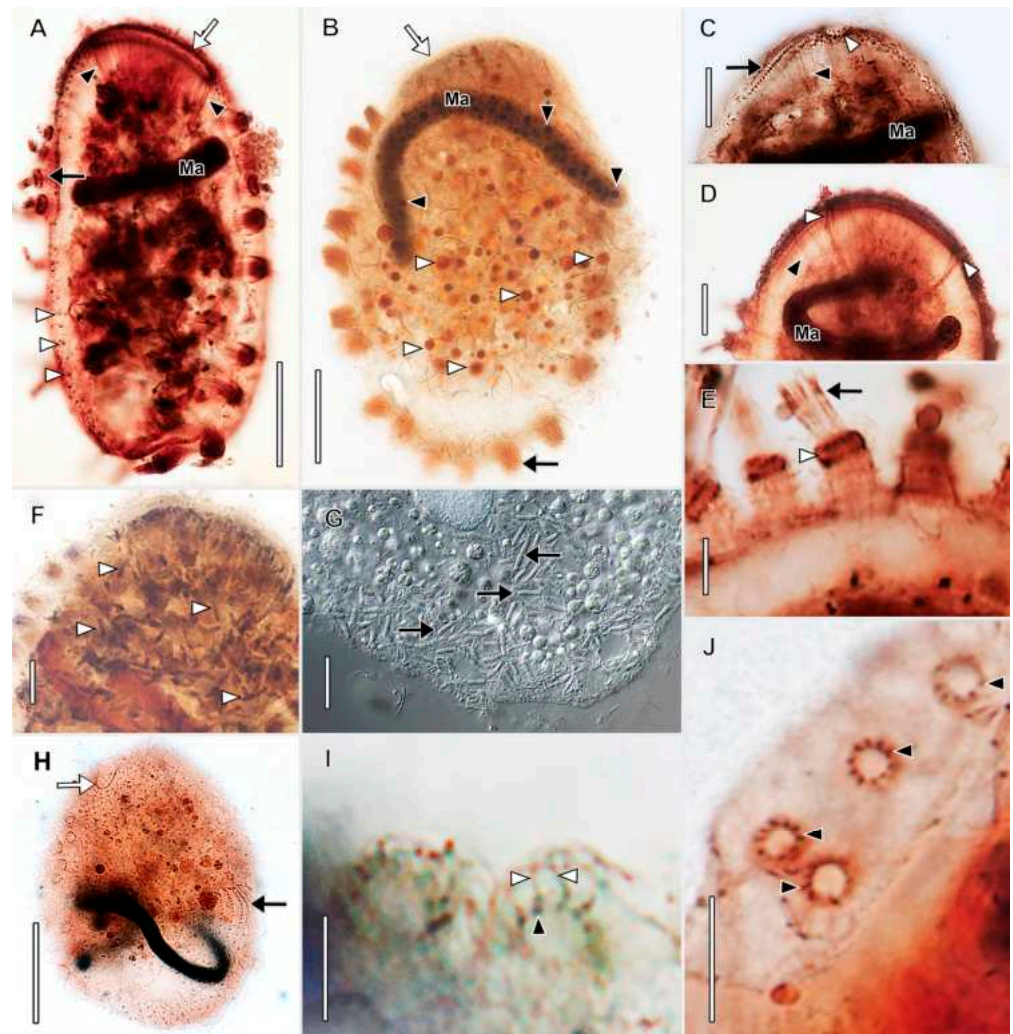


Figure 9. *Legendrea ornata* from SAGEGLEN (A,C–E,J), GTUB (H,I), and BELG (B,F) after protargol impregnation and from SAGEGLEN (G) in vivo. (A) Lateral view showing a retracted marginal tentacle with ejected extrusomes (black arrow), the obconical oral basket (between black arrowheads), the circumoral kinety (white arrow), and monokinetics of a somatic kinety (white arrowheads). (B) Optical section showing the oral bulge (white arrow), retracted marginal tentacles (black arrow), and numerous small nucleoli (black arrowheads). Numerous cytoplasmic globules (white arrowheads) make identification of micronuclei difficult. (C) Detail showing the circumoral kinety (white arrowhead). Dorsal brush row dikinetids (black arrow) give rise to oral basket nematodesmata (black arrowhead). (D) Optical section showing oral basket nematodesmata (black arrowhead) arising from dorsal brush row dikinetids to join those arising from the oral bulge (between white arrowheads). (E) Retracted marginal tentacles showing the cap (white arrowhead) and ejected extrusomes (black arrow). (F) Detail of argyrophilic cytoplasmic symbionts (white arrowheads). (G) Squashed cell showing large cytoplasmic prokaryotic endosymbionts (black arrows). (H) Dikinetids of circumtentacular kinety bear a single cilium (white arrowhead, cf. (I)). (I) Detail of a circumtentacular kinety with axially oriented dikinetids (black arrowheads) each bearing a single cilium (white arrowheads). (J) Apical view of circumtentacular cilia (black arrowheads). Ma, macronucleus. Scale bars: 25 μ m (A,B), 10 μ m (C,D,F,J), 5 μ m (E,G–I).

With characters of genus. Size 88–141 $\times$ 30–80 μ m in vivo. Shape narrowly to broadly oblong when swimming, broadly ovoidal to almost globular when at rest with tentacles extended. Anterior end obliquely truncate; posterior end broadly rounded. Macronucleus horseshoe-shaped or tortuous strand (49–80 $\times$ 3.5–5.5 μ m), numerous nucleoli approximately 1 μ m in diameter in protargol preparations, inconspicuous in vivo, multiple (2–7)

globular micronuclei (about $2.5\ \mu\text{m}$ across) in protargol preparations, not discernible in vivo (Figures 7, 8C and 9B). Contractile vacuole large, terminal, multiple excretory pores at right posterior side of cell. Two types of extrusomes: ordinary rod-shaped subcortical mucocysts (about $1 \times 0.5\ \mu\text{m}$); curved, filiform tentacular extrusomes (about $6 \times 1\ \mu\text{m}$), impregnate densely with protargol. Retracted mushroom-shaped tentacles in continuous row at alternating angles on dorsal, ventral, and posterior margin, commence in anterior one-fourth of cell, retracted tentacles with telescoping ruffled cortical collar (Figure 7A,J and Figure 10A,C); tentacles highly extensible (up to >25 times retracted length), used to immobilize prey (Video S7). Cortex flexible. Cytoplasm colorless, filled with refractive globules, prokaryotic symbionts (Figure 9F,G). Rod-shaped prokaryotic ectosymbionts in SEM preparations of some populations (Figure 10D). Swims at a moderate pace, rotating on the long axis (Video S6).

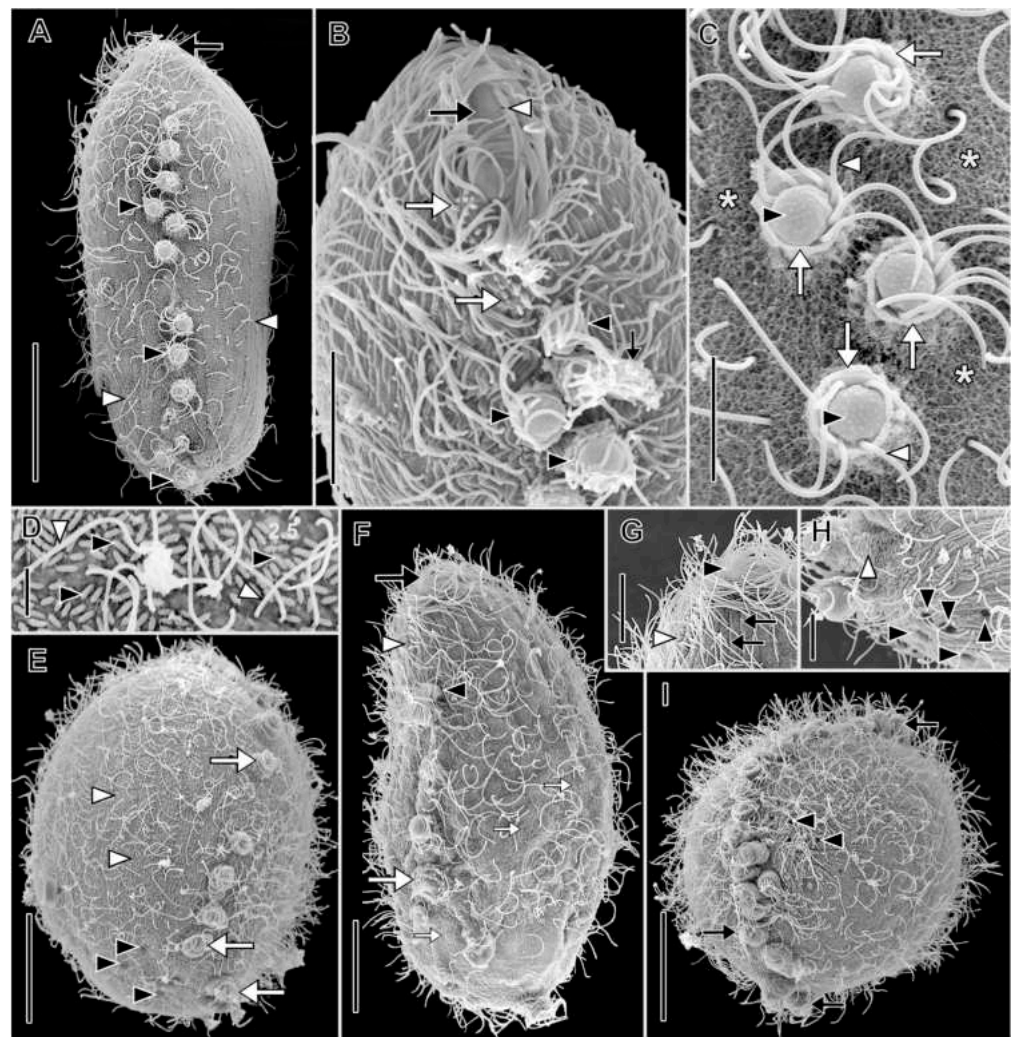


Figure 10. *Legendrea ornata* from GTUB (A,C) and BELG (B,D–I) in the scanning electron microscope. (A) Ventral view showing retracted marginal tentacles (black arrowheads), cilia of somatic monokinetids (white arrowheads), and the oral bulge (black arrow). (B) Dorsal view showing the oral bulge (black arrow), cilia of the circumoral kinety (white arrowhead), the clavate cilia of the dorsal brush (white arrows), retracted marginal tentacles (black arrowheads), and ejected tentacular extrusomes (small black arrow). (C) Detail showing docked extrusomes dotting the cap of retracted

marginal tentacles (black arrowheads), ciliary wreaths of the circumtentacular kineties (white arrowheads), the telescoped collars surrounding retracted tentacles (white arrows), and the dense reticular coating of the cortex, possibly a glycocalyx (asterisks). (D) Detail of cortical ectosymbionts (black arrowheads) and somatic cilia (white arrowheads). (E) Right ventrolateral view showing cortical symbionts (white arrowheads), retracted marginal tentacles (white arrows), and excretory pores of the contractile vacuole (black arrowheads). (F) Right dorsolateral view showing the oral bulge (black arrow), the inconspicuous dorsal brush (white arrowhead), the anteriormost dorsal marginal tentacle (black arrowhead), cortical granules—probably mucocysts (small white arrows), and the rosette-like collar of a retracted tentacle (white arrow). (G) Detail of the same cell as (F) showing the clavate cilia of the inconspicuous dorsal brush (white arrowhead), a cilium of the circumoral kinety (black arrowhead), and cortical furrows of somatic kineties (black arrows). (H) Detail of the posterior end of the cell showing cortical symbionts (white arrowhead) and the excretory pores of the contractile vacuole (black arrowheads). (I) Right posterolateral view showing retracted marginal tentacles (black arrow) and the excretory pores of the contractile vacuole (black arrowheads). Scale bars: 25 μm (A,E,F,I), 10 μm (B,G), 5 μm (C,H), 2.5 μm (D).

Somatic cilia about 12 μm long in about 30 longitudinal rows, pattern of infraciliature distinctly enchelyodontid, i.e., anterior ends of somatic kineties perpendicular to circumoral kinety. Ciliary rows not interrupted by tentacles. Dorsal brush inconspicuous; three rows of dikinetids with 3 μm long clavate cilia, brush rows commence to left of circumoral kinety; B1 and B2 of equal length (about 15 μm), B3 slightly shorter (Figure 8A,F).

Oral bulge inconspicuous, slightly oblique. Circumoral kinety narrow elliptical, composed of dikinetids. Oral bulge extrusomes not observed. Oral basket inconspicuous in vivo, obconical, extends about one-half length of cell in protargol preparations (Figures 8D and 9A,D).

Stomatogenesis holotelokinetal, basal body proliferation commences first in somatic kineties in line with dorsal brush rows (Figure 9H).

3.2. Molecular Data and Phylogenetic Analysis

Analyses of the concatenated data set revealed that the genera *Legendrea* and *Dactylochlamys* form a supported clade (bootstrap value 80, Bayesian posterior probability 1) together with *Arcuospathidium* sp. and *Apertospathula oktemae*; *Legendrea* sequences formed a fully supported clade (Figures 11 and S1).

As the 18S rRNA gene sequences of *L. loyezae* and *L. pespelicani* are identical and differ from that of *L. ornata* by only one nucleotide, the analysis based on the 18S rRNA gene did not resolve the relationships between *Legendrea* species (Figure S2), and neither did the analysis of the concatenated dataset resolve the relationships among the three *Legendrea* morphospecies. *L. ornata* MOKOTP1 and SAGEGLEN sequences cluster together. Sequences of *L. loyezae* OP352778 and *L. pespelicani* VLKOV are more closely related to each other than to *L. loyezae* MOKOTL (Figures 11 and S1). However, the published sequence of *L. loyezae* OP352778 represents only the V4 region of the 18S rRNA gene (1007 bp), while *L. pespelicani* VLKOV and *L. loyezae* MOKOTL are concatenated sequences (18S-ITS-28S). Genetic distances (uncorrected p-distances) between ITS-28S fragments (1183 bp) of *L. ornata* MOKOTP1, *L. loyezae* MOKOTL, and *L. pespelicani* VLKOV range between 0.008 and 0.009. Only amplification of the ITS-28S rRNA gene region of *Dactylochlamys* sp. MOKOTP1Q isolate was successful. Newly determined sequences are available in GenBank (accession numbers: OP985785–OP985794).

We also obtained two partial 18S rRNA gene sequences of prey gastrotrichs (family Chaetonotidae) from a cell of *L. loyezae* (MOKOTL; Figure 3B) and from the only cell of *L. ornata* from MOKOTP1 (GenBank accession numbers: OQ848030–OQ848031).

Using Sanger sequencing of 16S rRNA gene fragments, we identified two methanogenic archaeal endosymbionts as *Methanobacterium* sp. (*L. pespelicani* VLKOV) and *Methanosaeta* sp. (*L. loyezae* MOKOTL) (GenBank accession numbers: OQ843028–OQ843029). Using Illumina sequencing of the V4 region of 16S rRNA gene, we identified *Methanocorpusculum*

sp. as the dominant archaeal methanogenic symbiont of *D. pisciformis* VB2A, representing around 95% of all archaeal ASVs. *Methanobacterium* sp. was the only archaeal ASV present in *L. pespelicani* VLKOV. In *L. pespelicani* VLKOV, we also identified a bacterial symbiont belonging to the Syntrophaceae (Table S4).

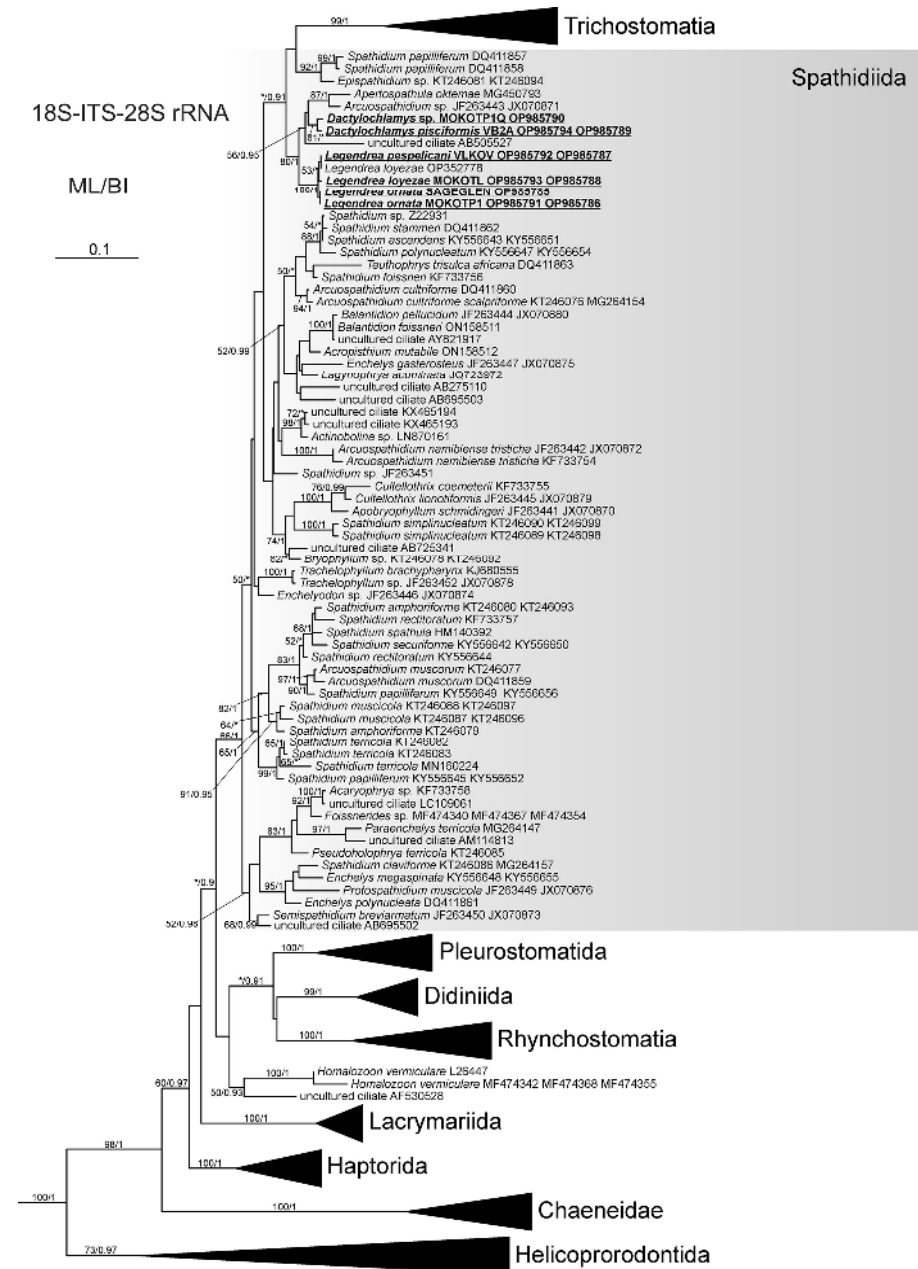


Figure 11. Maximum likelihood tree of the class Litostomatea based on 18S-ITS-28S rRNA region sequences. The tree is unrooted. The values at the branches represent statistical support in maximum likelihood bootstrap values/Bayesian posterior probabilities. Support values below 50/0.90 are not shown or depicted by an asterisk. Newly determined sequences are in bold. Accession numbers for 18S and ITS-28S GenBank sequences follow taxon names. Scale bar: 10 substitutions/100 nucleotide positions.

4. Discussion

4.1. Morphological Comparison of *Legendrea* Species and Similar Species

Legendrea pespelicani can be easily distinguished from *L. ornata* and *L. loyezae* by size (180–210 vs. <180 µm), cell shape (broadly spatulate vs. oblong, resp. broadly obovate), and by possession of oral papillae (present vs. absent). *L. pespelicani* also differs from

L. ornata by number of tentacles (4–8 vs. 10–43), number of circumoral kineties (30 vs. 10 in average), number of somatic kineties (66–70 vs. 28–36), and the disposition of the ciliary rows (interrupted by tentacles vs. deviating around the tentacles). *L. loyzae* can be distinguished from *L. ornata* by the position of tentacles (bundle at the posterior vs. margin of the cell on $\frac{3}{4}$ of the cell length), and from both other species by the eccentric posterior contractile vacuole which appears as two vacuoles due to median cleft at posterior. All *Legendrea* species are different to any other spathidiid in terms of tentacles (present vs. absent), except *Dactylochlamys pisciformis*, which are not similar to those of *Legendrea* (very slender, not ciliated vs. thick with circumtentacular kineties). *L. pespelicani* differs from *Spathidium papilliferum* in morphology of the oral papillae (circumtentacular kineties present vs. absent).

4.2. Remarks on the Rarity and Ecology of *Dactylochlamys* and *Legendrea*

Both *Dactylochlamys* and *Legendrea* spp. have been very rarely reported, although Lauterborn and Penard suggested that the populations could be quite abundant [9,37]. Our experience was similar: the VB2A sample was unusually rich in *Dactylochlamys* (approx. 4 cells/mL of water and sediment), but the abundance quickly declined in several days, and we were unable to maintain the ciliate in long-term culture. In other cases (MRATIN, MOKOTP1Q), only a few cells were found in the whole volume of a 50 mL sample. In the case of *Dactylochlamys*, Penard cautioned about possible misidentification, because some swimmers of suctorian ciliates (Phyllopharyngea: Suctoria) (e.g., *Enchelyomorpha vermicularis* Smith, 1899) appear similar in vivo (compare Figure 1F,I) [9]. Thus, mentions in the literature not accompanied by illustrations are questionable, e.g., [41,42]. In the case of *Legendrea*, we found two relatively rich localities in the Czech Republic and one in the USA; other known localities are, for example, in France, Germany, Belgium, and Poland. This might suggest that the rarity of *Dactylochlamys* and *Legendrea* is rather a matter of specific habitat conditions than endemism.

Interestingly, gastrotrich rRNA gene partial sequences (family Chaetonotidae) were recovered from cells of *L. loyzae* and *L. ornata*, indicating ingestion of gastrotrichs as prey. The process of *L. ornata* from sediments of a German freshwater pond catching and digesting a gastrotrich (family Chaetonotidae) is the first documentation of the role played by the tentacles in prey capture (Video S7). Chaetonotidae is a species-rich and widely distributed gastrotrich family that commonly inhabits hypoxic lacustrine sediments [43]. Although chaetonotid gastrotrich 18S rRNA gene sequences were the only eukaryotic contaminants from *Legendrea*, the importance of gastrotrichs in the diet of *Legendrea* species remains unknown.

4.3. Phylogenetic Analysis and the Relationship of Tentaculiferous Ciliates

A recent report (8) included a 994 bp V4–V9 rRNA region sequence from *L. loyzae*. Phylogenetic analysis could not resolve a position of *Legendrea* within Haptorida. The problems with attempting to resolve relationships within Litostomatea based on analysis of the rRNA cistron have been emphasized previously [44,45]. Consistent with the studies mentioned above, we did not succeed in resolving the internal relationships among *Legendrea* species in spite of addition of two more markers, i.e., using three times more positions in comparison to [8].

Despite pronounced morphological differences, all three identified *Legendrea* species have identical (*L. loyzae* and *L. pespelicani*) or almost identical (*L. ornata*) 18S rRNA gene sequences. However, the existence of the three *Legendrea* morphospecies is further supported by the analysis based on additional molecular markers; namely, part of the ITS region and part of the 28S rRNA gene. Clustering of *L. loyzae* OP352778 with *L. pespelicani* was interpreted as an artifact due to the short length of the former (1007 nucleotides) and ambiguous sites in both of the sequences of *L. loyzae*. This view is also supported by the p-distances of the ITS–28S fragments of each of the three *Legendrea* species, which range from 0.008 to 0.009. On the other hand, Jankowski even erected a monotypic genus for each

of the three *Legendrea* species solely on morphology, but we consider with respect to the morphological and molecular analysis presented above [46].

We also obtained a partial ITS-28S rRNA gene sequence of a second *Dactylochlamys* population (MOKOTP1Q), but the material was insufficient for morphometric analysis. Molecular data and in vivo observation suggest that it is possibly a new species (Video S9).

Our analysis showed that *Dactylochlamys* and *Legendrea* are not closely related to *Actinobolina*, contradicting the traditional notion of the family Actinobolinidae, comprising the genera *Actinobolina*, *Belonophrya*, *Legendrea*, and *Dactylochlamys*, based on the presence of tentacle-like structures which have evolved independently [1,10,47]. As noted previously, homoplasies are rife throughout the Spathidiidae and, together with many pleiomorphies, have so far obscured the evolutionary relationships in the group [44,48–50]. Interestingly, *Dactylochlamys* and *Legendrea* are members of the same well-supported clade despite their obvious morphological differences. The other members of the clade (i.e., *Apertospathula oktemae* and an undescribed *Arcuospathidium* sp.) belong to spathidiid genera which do not bear any tentacle-like structures [2]. *Spathidium papilliferum* Kahl, 1930, another only distantly related spathidiid, has oral bulge papillae superficially similar to those of *L. pespelicani* but lacking circumtentacular kineties [10,48]. Analysis also showed that a supported clade including *S. papilliferum* (DQ411857, DQ411858; morphologically uncharacterized population) and an *Epispathidium* species (KT246081, KT246094; undescribed) are not closely related to the other *S. papilliferum* (KY556645, KY556652), suggesting possible misidentification (Figure 11). Thus, the diversity of tentacle- or papillae-bearing spathidiids could be even higher than currently indicated. The genus *Belonophrya* is highly similar to *Actinobolina*, but molecular data and ultrastructural details of its tentacles are still unavailable [51]. Another tentacle-bearing ciliate, *Holophrya ornata* is now synonymized with *Legendrea bellerophon* based on its morphological characteristics (Figure 12) [40].

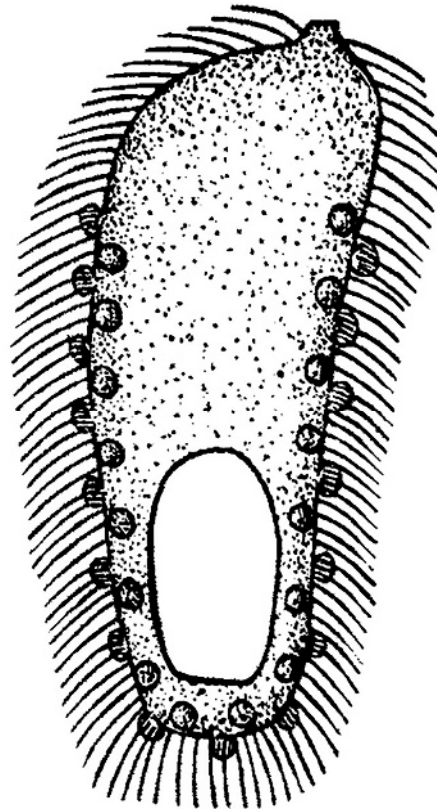


Figure 12. Illustration of *Holophrya ornata* from Stokes (1887). Cell length 100 μ m.

Given the complex relationships between tentaculiferous lineages in Spathidiidae or Haptoria, it is not surprising that tentacles of other litostomateans are also likely not

homologous. Tentacles in the family Mesodiniidae, possibly a sister group to Litostomatea, are placed anteriorly, are reinforced by a cylindrical structure of 14 microtubules, and bear extrusomes [1,52,53]. Another tentacle-bearing species with unclear phylogenetic position is *Enchelyomorpha vermicularis*, once included in Actinobolidae, tentacles of which are also reinforced by microtubules and do not bear extrusomes, which is now considered to be the swarmer of a globular suctorian on morphological grounds but has not yet been sequenced [1,54]. Tentacles of the members of the class Phyllopharyngea have a completely different morphology compared to both *Legendrea* and *Dactylochlamys*. Typical suctorian feeding tentacles are formed by two concentric cylinders of microtubules, the inner one being reinforced by microtubule fibrils, and the outer with various taxon-specific microtubular structures [1,55]. Although, as indicated by their name, suctorians have been assumed to “suck” cell contents from their prey, this mechanism has been cast into doubt by Rudzinska [56]. In the parasitic subclass Rhynchodia, only a single sucking tentacle-like structure, probably a transformed cytostome, is present [1].

4.4. Putative Prokaryotic Endosymbionts

Using Sanger sequencing of the partial 16S rRNA gene, we identified putative methanogenic endosymbionts of *L. pespellicani* as *Methanobacterium* sp. and *Methanosaeta* sp. in *L. loyzeae*. Using Illumina sequencing, we corroborated the identity of the archaeal symbiont of *L. pespellicani* as *Methanobacterium* sp. and identified the archaeal symbiont of *Dactylochlamys pisciformis* as *Methanocorpusculum* sp. Autofluorescence typical of archaeal methanogens was also observed in *Dactylochlamys* (Video S8) [57]. Interestingly, it seems that there is some symbiont diversity among the studied species, where genera belonging to the same clade and even each of the *Legendrea* species harbor different methanogenic symbionts. Methanogenic Archaea commonly form syntrophic symbioses with anaerobic ciliates using the hydrogen and acetate produced by the host mitochondria for their metabolism, e.g., [21,58–60]. This suggests that both *Dactylochlamys* and *Legendrea* are most likely facultative or obligate anaerobes, potentially having hydrogen-producing mitochondria. The role of these prokaryotes as endosymbionts requires confirmation by fluorescence in situ hybridization and/or transmission electron microscopy. Nevertheless, our results indicate that both *Dactylochlamys* and *Legendrea* represent a novel anaerobic lineage of ciliates. Interestingly, in addition to archaeal symbionts, we also identified a putative bacterial symbiont related to the family Syntrophaceae. The Syntrophaceae are strictly anaerobic and grow only in the presence of hydrogen-utilizing partners, such as archaeal methanogens [61]. Further investigations of symbiotic interactions between the host, archaeal, and bacterial endosymbionts in *Legendrea* are needed.

4.5. The Role of Citizen Science in Ciliatology

Taxonomic and biogeographic studies of a wide variety of groups, including protists, have always been plagued by the problem of undersampling. This fact, in part, underlies the uncertainty and contentiousness associated with such topics as protist endemism [62–64]. Since the number of academic professionals dedicated to and funded for mainly taxonomic work has rapidly declined, the importance of the much larger population of “expert amateurs” (interested individuals without formal academic credentials in the particular specialty) in species identification, specimen collection, ecology, and biogeographical data collection has come into clearer focus [65–70]. The role of the expert amateur was central to science world-wide prior to the professionalization of science in the early 20th century, after which contributions by non-academics were increasingly ignored or criticized as inferior. Alfred Kahl, one of the most notable ciliatologists of the last century, a high school teacher by training, was probably an unfortunate object of this developing attitude [38,71]. Although a formal definition of “citizen-science” infers that it occurs mainly as an activity in collaboration with, or under the direction of, academic professionals usually as part of structured projects [72], we consider the data generated by expert amateurs working independently to be of considerable value, especially with regard to rare taxa such as

Legendrea. Most of the information that has accrued in the century since the first description of the genus *Legendrea* has been gathered almost exclusively by expert amateurs and made publicly available in online forums and social media. The study of Weiss et al. and this study are perfect examples of the fruitfulness of such cooperation [8].

5. Conclusions

Although both genera, *Legendrea* and *Dactylochlamys*, presented in this study are considered to be remarkably rare, we were able to collect enough data to not only corroborate their phylogenetic position close to or within Spathidiidae but also revealed that both genera are closely related to each other despite apparent differences in their tentacle-like structures. We studied their morphology with modern methods and found out that these two ciliate genera are in fact anaerobes harboring prokaryotic endosymbionts. Our work demonstrates that for studying rare organisms, both modern single-cell methods and the contribution of citizen science are essential. Thus, we encourage the increasing collaboration of academic professionals with their expert amateur counterparts, inclusion of their data in peer-reviewed research publications, and acknowledgement of their contributions.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/biology12050707/s1>, Figure S1: Maximum likelihood tree of the class Litostomatea based on 18S-ITS-28S rRNA region sequences, Figure S2: Maximum likelihood tree of the class Litostomatea based on 18S rRNA gene sequences; Table S1: Morphometric table of *Dactylochlamys pisciformis*, Table S2: Morphometric table of *Legendrea pespelicani*, Table S3: Morphometric table of *Legendrea ornata*, Table S4: Illumina consensus nucleotide sequences of putative prokaryotic symbionts; List of Videos S1–S9.

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Original Article

Morphology matters: congruence of morphology and phylogeny in the integrative taxonomy of Clevelandellidae (Ciliophora: Armophorea) with description of six new species

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ABSTRACT

Armophorid ciliates of family Clevelandellidae represent ecologically interesting symbionts of ecologically interesting hosts: wood-eating cockroaches of subfamily Panesthiinae unrelated to the termite/*Cryptocercus* lineage. Moreover, these protists exhibit a peculiar morphology, with the posteriorization of oral structures being the most striking of their unique characters. Despite that, the family is relatively understudied, with only a handful of morphologically novel species being described since its erection more than 80 years ago. Recently, however, several clevelandellid species were described solely on the basis of molecular characters and it has been suggested that morphology should be abandoned in favour of purely molecular taxonomy. In our study we report on the diversity of Clevelandellidae from the widest host spectrum assessed so far, characterize a majority of previously described Clevelandellidae species, and describe six new *Clevelandella* species. By applying an integrative taxonomical approach, using molecular and modern morphological methods, we demonstrate the pitfalls of a purely molecular approach and show that morphology still has its place in the taxonomy of Clevelandellidae. Moreover, thanks to a combination of observations of *in vivo* cells, protargol preparations, and scanning electron microscopy (used for the first time in Clevelandellidae), we were able to clarify morphological uncertainties of previous works and discuss various morphological peculiarities of Clevelandellidae.

Keywords: biogeography; Clevelandellida; cockroach; host specificity; Panesthiinae; scanning electron microscopy; symbiont; 18S rRNA gene

INTRODUCTION

Blattodea (cockroaches and termites) are a morphologically and ecologically diverse lineage of insects with significant economic impact. Several globally distributed cockroach species are infamous synanthropic pests and sources of allergens (Cochran 1999). Termites, on the other hand, are the major structural and agricultural pests in tropical and subtropical areas and are also of great ecological importance (Govorushko 2019). Most termite species feed on wood and, since they are unable to completely digest wood particles by themselves, the ancestors of termites and wood-eating Cryptocercidae cockroaches entered into symbioses with various organisms more than 130 million years ago (Evangelista *et al.* 2019). These partners, either alone or as part

of a microbial consortia, are able to degrade cellulose and other compounds of wood, making themselves indispensable for their hosts (Brune 2014, Chouvenec *et al.* 2021). Cryptocercidae, and the so-called lower termites, host in their hindgut endosymbiotic flagellates belonging to Parabasalia and Preaxostyla. These protists are excellent and well-studied examples of coevolution with their hosts and are notable for having evolved into huge and morphologically bizarre forms (Noda *et al.* 2007, Čepička *et al.* 2010, Taerum *et al.* 2018). Additionally, an independent evolution of wood-eating occurred in the distantly related cockroach subfamily Panesthiinae (Blaberoidea: Blaberidae) (Bell *et al.* 2007, Djernæs *et al.* 2020). Similar to the lower termites and cryptocercids, panesthiines also host morphologically unusual

protists in their hindguts. However, these are not metamonad flagellates, but ciliates of the family Clevelandellidae (Armophorea: Clevelandellida). As in the case of termites, several symbiont species are usually found simultaneously in a single host organism (Kidder 1937). Whether Clevelandellidae play any role in the cellulose digestion of panesthiines is unknown. But even if they do, it seems unlikely to be of great benefit to the host, since it has been shown that at least one panesthiine, *Panesthia cribrata* Saussure, 1864, produces endogenous cellulase and can be maintained for at least 12 weeks in the absence of protozoan and most of the bacterial endosymbionts (Scrivener et al. 1989, Slaytor 1992). Moreover, unlike hypermastigotes, which visibly ingest large particles of wood into their cell, members of Clevelandellidae have not been reported to do so.

In contrast to their more or less ovoid nyctotherid relatives, Clevelandellidae are immediately recognizable by their bizarre morphology. The anterior end of the cell is tapered to a rounded point and the oral apparatus is situated at the posterior end of the cell, giving a false impression of the ciliate swimming backwards. In most species, the buccal cavity occupies a posterior extension: the peristomial projection. The distinctive appearance is further accentuated by the conspicuously asymmetric cell shape of most species.

The family Clevelandellidae gave the name to the whole order Clevelandellida, which consists entirely of ciliates living as symbionts of various animals. The endosymbiotic lifestyle has obviously originated in an exclusive common ancestor of Clevelandellida, because the order forms an internal branch of predominantly free-living ciliates of the order Metopida (Lynn and Wright 2013, Rotterová et al. 2018). While representatives of the family Clevelandellidae inhabit only Blattodea, members of the other four families of the order Clevelandellida (Nyctotheridae, Sicutophoridae, Neonyctotheridae, and Inferostomatidae) live as symbionts in a wide variety of both vertebrate and invertebrate animals. Clevelandellidae comprise five genera: *Anteclevelandella* Pecina and Vďačný, 2022, *Clevelandella* Kidder, 1938 (a replacement name for the preoccupied *Clevelandia* Kidder, 1937), *Paraclevelandia* Kidder, 1937, *Metaclevelandella* Uttangi and Desai, 1963, and *Rhynchoclevelandella* Pecina and Vďačný, 2022. Genera *Clevelandella* (with seven species) and *Paraclevelandia* (with two species) were described more than 80 years ago by Kidder (1937) from the cockroaches *Panesthia angustipennis* (Illiger, 1801) (known as *Panesthia javanica* Serville, 1831 at that time) from Philippines and *Panesthia angustipennis spadica* Shiraki, 1906 from Japan, respectively. Yamasaki (1939), apparently unaware of Kidder's work, described the genus *Emmaninius* Yamasaki, 1939, comprising three species, from an unidentified subspecies of *Panesthia angustipennis* (Illiger, 1801) from Japan. However, the genus name *Emmaninius* was unavailable, since Yamasaki (1939) did not designate the type species. Jankowski (2007) made the name available, designating *E. papilloris* as the type species. We consider *Emmaninius* Jankowski, 2007 to be a junior synonym of *Clevelandella* Kidder, 1938 and *E. papilloris*, *E. plantiformis*, and *E. longicollis* to be junior synonyms of *C. panesthia*, *C. constricta*, and *C. nipponensis*, respectively. Since 1939, only two morphologically described species of *Clevelandella* from host genus *Panesthia* Serville, 1831 have been added (Mandal and

Nair 1974, Pecina and Vďačný 2020a). Recently, Pecina and Vďačný (2022) split the genus *Clevelandella* into three genera: *Clevelandella*, *Anteclevelandella*, and *Rhynchoclevelandella*. They also described eight new species between the three genera; however, the descriptions are based solely on molecular methods and no morphological differences are mentioned. *Metaclevelandella termitis* Uttangi and Desai, 1963, the sole representative of its genus, was, surprisingly, described not from a Panesthiinae cockroach but from a termite, *Dicuspitermes incola* (Wasmann, 1893) (known as *Capritermes longicornis* Wasmann, 1902 at that time) from India (Uttangi and Desai 1963).

Molecular data for Clevelandellidae were unavailable until Lynn and Wright (2013) analyzed the 18S rRNA gene sequences of four species of *Clevelandella* from Australia, all of which they considered to be conspecific with ones previously described by Kidder (1937). It was shown that Clevelandellidae form an internal branch of the genus *Nyctotherus* Leidy, 1849 (Clevelandellida: Nyctotheridae), and that *Clevelandella* is specifically related to *Nyctotherus* lineages from cockroaches, suggesting that the morphologically divergent Clevelandellidae evolved within cockroaches from a ciliate ancestor with a much less derived morphology (Albaret 1975). More recent studies that dealt with additional species and added more molecular markers (Pecina and Vďačný 2020a, b, 2022) were congruous with the findings of Lynn and Wright (2013). Somewhat surprisingly they pointed to a peculiar inconsistency of morphology and genetics in Clevelandellida (Pecina and Vďačný 2020b) leading to a proposal to shift from morphology-based species descriptions in the group to a purely molecular approach (Pecina and Vďačný 2022).

The occurrence of morphologically similar organisms in geographically distant hosts from Philippines, Japan, and Australia suggested a rather cosmopolitan distribution for a number of Clevelandellidae species (Kidder 1937, Yamasaki 1939, Lynn and Wright 2013). More recent works (Pecina and Vďačný 2020b, 2022), on one hand, found similar organisms also in mainland Asia, but, on the other, showed that some of the species or lineages may exhibit some geographical specificity. In regard to host specificity, the diversity of the hosts assessed so far is too low to draw meaningful conclusions as, until now, only five species of the cockroach genus *Panesthia*, one species of *Salganea* Stål, 1877, and one species of *Macropanesthia* Saussure, 1895 were inspected for gut ciliates (Kidder 1937, Yamasaki 1939, Mandal and Nair 1974, Lynn and Wright 2013, Pecina and Vďačný 2020a, b, 2022). This represents only a small fraction of subfamily Panesthiinae (including Geoscapheinae, see: Beasley-Hall et al. 2021), which comprises at least 169 species in 11 genera distributed in Sino-Japanese, Oriental, and Australian regions (Beccaloni 2014).

In this study we report on the diversity of Clevelandellidae from 13 host populations, the widest host spectrum assessed to date. We dissected 104 cockroach specimens and examined the diversity of their Clevelandellidae symbionts based on the morphology and single-cell 18S, ITS, and partial 28S rRNA gene sequences. We report nine previously described species, describe six new species, and report one new, as yet, undescribed species. We combine molecular and morphological methods and show that morphology is important in the taxonomy of

Clevelandellidae, contrary to a previous suggestion to abandon morphology in favour of a purely molecular approach (Pecina and Vďačný 2022). We propose synonymy of eight previously described species. We also discuss numerous morphological peculiarities, the phylogeny, diversity, host specificity, and biogeography of Clevelandellidae.

MATERIALS AND METHODS

Sample collections

To obtain Clevelandellidae symbionts, 104 individuals from 13 Panesthiinae host populations were dissected. *Ancaudellia pygmaea* Roth, 1982 (AP), *Ancaudellia serratissima serratissima* (Brunner von Wattenwyl, 1865) (ASS), *Panesthia angustipennis* (PA), *Panesthia angustipennis angustipennis* (PAA), *Salganea raggei* Roth, 1979 (SR), *Salganea rugulata* Saussure, 1895 (SRU), *Salganea ternatensis hirsuta* Roth, 1979 (STH), and *Salganea taiwanensis ryukyuanus* Asahina, 1988 (STR) were collected from nature. *Ancaudellia hamifera* (Hanitsch, 1930) (AH), *Miopanesthia polita* (Krauss, 1902) (MP), *Panesthia angustipennis cognata* Bey-Bienko, 1969 (PAC), *Panesthia angustipennis angustipennis* 'gold wing' (PGW), and *Panesthia triangulifera* Hanitsch, 1927 (PT) were obtained from hobby cultures. The origin of host cockroaches is summarized in the [Supporting Information, Table S1](#). The cockroach colonies were housed separately in plastic containers filled with decaying wood with appropriate humidity and room temperature. Close attention was paid to avoid any contact of animals and substrate between colonies to prevent symbiont cross-contamination.

The cockroaches were euthanized by ethyl acetate vapors. The hindgut was extracted, freed from the fat body, homogenized in the liquid phase of Dobell and Laidlaw's medium (Dobell and Laidlaw 1926), and stored in 1.5 mL Eppendorf tubes, where the ciliates survived from a few hours to several days at room temperature. Since the high viscosity of the culture medium due to added egg white severely hinders work with micropipettes, aliquots of the gut were transferred to drops of Ringer's solution for manual picking of ciliate cells under the dissecting microscope. The ciliates usually died within 20–40 min after being placed on slides.

Light microscopy

The morphology of live and protargol-impregnated cells was examined with an Olympus BX51 or BX53 microscope equipped with either a DP71 (Olympus, Japan) or Canon EOS 80D camera, and an AE2000 inverted microscope equipped with a BTU10 camera (Motic, Xiamen, China). Protargol (Polysciences Inc., Warrington, PA, no longer commercially available) preparations were done according to Bourland and Wendell (2014). Images of protargol-impregnated specimens were taken under brightfield illumination. Images of live cells were taken using differential interference contrast illumination (DIC). Measurements were performed using calibrated QuickPHOTO CAMERA 2.3 (Promicra) or ImageJ (National Institutes of Health, <https://imagej.nih.gov/ij/index.html>) software. Due to variability in cell orientation, not all characters could be measured in all cells.

Scanning electron microscopy

Ciliates collected as described above were placed in a drop of Ringer's solution on a glass slide, selected with a glass

micropipette, and washed free of detritus by transfer to three successive drops of fresh Ringer's solution. The cells were then placed in 1 mL of 2.5% glutaraldehyde in 1.5-mL Eppendorf tubes for at least 30 min and up to several days. Glutaraldehyde-fixed cells were washed in distilled water under the dissecting microscope, placed in brass sample chambers (Foissner 2014), and dehydrated in the chambers through a graded ethanol series (50%, 70%, 90% for 5 min each, and three exchanges of 100% for 15 min each). Critical point drying was done in a BalTech CPD030 critical point dryer (Leica Microsystems, Vienna, Austria) and dried cells were scattered on adhesive carbon tabs on aluminium specimen stubs and sputter coated with gold (vacuum 0.1 mbar, current 40 mA for 90 s). Observations were done in a JSM-6380 scanning electron microscope (JEOL LV, USA) in secondary electron and back-scattered electron detection modes at an accelerating voltage of 15 kV.

Statistical analyses

All statistical analyses were performed in R 4.2.0 (R Core Team 2023). For the purpose of a detailed morphological investigation of ciliate species, the following features were determined in a total of 455 protargol-stained cells from 33 strains of 16 species ('brevis' and 'simplex' morphs of *Paraclevelandia brevis* were considered as separate species in this analysis): (i) body length, (ii) body width, (iii) the presence of peristomial projection, (iv) peristomial projection length, (v) peristomial opening width, (vi) adoral zone of membranelles length, (vii) adoral membranelles number, (viii) distance from the anterior of the cell to the posterior of macronucleus, (ix) macronucleus length, (x) macronucleus width, (xi) micronucleus length, and (xii) micronucleus width. From 7 to 73 cells (24 in average) were morphologically analyzed per each species. Missing data were imputed with a principal component analysis (PCA) using the 'missMDA' package (Josse and Husson 2016) applying the iterative PCA algorithm. An NMDS (non-parametric multi-dimensional scaling) analysis was carried out with the 'vegan' package (Oksanen et al. 2020), based on the imputed dataset and using a total of 1000 random starts in search of a stable solution. The morphological similarities of analyzed cells were visualized with a scatterplot of the first two axes (stress factor = 0.076).

In addition, we performed a linear discriminant analysis (LDA) using the 'MASS' package (Venables and Ripley 2022) and calculated a classification matrix measuring the performance of the model. This matrix reports the number of cases correctly and incorrectly assigned to each of the groups (species) based on the discriminant analysis of determined morphological traits.

To assess shrinkage of protargol preparations we have compared measurements of the cell size (length or width) for a given species between protargol preparations and *in vivo* measurements. We used a generalized linear model (GLM) model with gamma distribution and link function log to test the effect of species, cell condition (live cell/protargol preparation), and their interaction on the measured cell size. Reduced major axis (RMA) regression from the package 'lmodel2' was used to analyze the effect of species cell size (length or width) on shrinkage.

DNA isolation, amplification, and sequencing

Individual ciliates were handpicked from the gut content with micropipettes, washed three times in Ringer's solution, and

either processed immediately or stored frozen at -80°C in 30 μL of Ringer's solution (Supporting Information, Table S2; Figs S1–S3). Genomic DNA was isolated with the MasterPure™ Complete DNA & RNA Purification Kit (Lucigen, Middleton, WI, USA).

The 18S rRNA gene was amplified using primers ArmF1 (Bourland *et al.* 2017a) and EukB (Medlin *et al.* 1988). PCR conditions were as follows: denaturation at 94°C for 4 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 4 min, and final extension at 72°C for 15 min. The ITS1–5.8S–ITS2 region, along with the first two domains of the 28S rRNA gene, were amplified using primers ITS-F (Miao *et al.* 2008) and LO-R (Pawlowski 2000). PCR conditions were as follows: denaturation at 95°C for 15 min, 30 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 1 min, and extension at 72°C for 150 s, and final extension at 72°C for 10 min. EconoTaq® PLUS GREEN 2X Master Mix (Lucigen, Middleton, WI, USA) was used for both protocols. Amplified DNA was checked by agarose gel electrophoresis and purified with ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA). The purified PCR products were sequenced either on ABI 3730 automatic sequencer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) at Macrogen, Amsterdam, the Netherlands or on an ABI PRISM 3100 (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) at Charles University, Prague, Czech Republic.

Phylogenetic methods

The 18S rRNA gene was sequenced from all 147 cells and the ITS1–5.8S–ITS2 region and the first two domains of the 28S rRNA were sequenced from 48 cells (Supporting Information, Table S2). All sequences relevant to Clevelandellida and outgroup sequences were downloaded from GenBank (Supporting Information, Table S3). For outgroups, we chose sequences of the metopid group IAC (defined by: Bourland *et al.* 2018), the closest known relatives of Clevelandellida. Two datasets were created: (i) a full dataset containing all newly determined sequences and all available 18S, ITS, and 28S sequences of Clevelandellidae and outgroups (Nyctotheridae and Metopida) and (ii) a reduced dataset, derived from the full dataset, containing only the strains with all three genes available. The sequences were aligned using the G-INS-I algorithm in MAFFT (Katoh *et al.* 2002, Katoh and Standley 2013) with otherwise default settings on the GENEIOUS PRIME 2023.1.1 platform. The unedited alignment comprised 3012 sites and was manually edited using BioEdit 7.0.9.0 (Hall 1999), retaining 2962 sites (98.3%). The GTR+I+G nucleotide substitution model was chosen based on analysis by ModelTest-ng (Darriba *et al.* 2020). Phylogenetic trees were constructed using maximum likelihood (ML) and Bayesian methods. ML analysis was performed with RAxML-ng (Kozlov *et al.* 2019) under the GTR+G model using 20 starting trees (10 random, 10 parsimony). Node support was assessed with 1000 bootstrap replicates. Bayesian analysis was performed using MrBayes 3.2.7a on the Cipres Portal (Miller *et al.* 2010) using the GTR+I+G model. Two parallel runs of four chains each were run for 5 000 000 generations, with a sampling frequency of 1000 generations. The first 25% of sampled trees were discarded as burn-in. The average

standard deviation of split frequencies was 0.007 after 5 million generations. Convergence was assessed by RWTY (Warren *et al.* 2017). Trees were visualized in FigTree v. 1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>).

RESULTS

Phylogenetic analyses

The phylogenetic tree of the Metopida/Clevelandellida lineage based on the full dataset inferred from the 18S rRNA, ITS, and 28S rRNA gene sequences is shown in the Supporting Information, Figure S4. The phylogenetic tree of the Clevelandellida lineage based on the reduced dataset is shown in the Supporting Information, Figure S5. Photomicrographs of the individual cells included in the tree, as well as genes sequenced from them, are summarized in the Supporting Information, Table S2 and Figures S1–S3. Topology and support values of both trees are highly consistent, with the only notable difference being higher support value for the monophyly of the genus *Rhynchoclevelandella* in the tree based on the reduced dataset (bootstrap support, BS, 99 vs. 78, Bayesian posterior probability, BPP, 1 vs. 0.99). The part of the full dataset tree showing only the Clevelandellidae lineage is shown in Figure 1. The overall topology of the tree is consistent with previous analyses (Lynn and Wright 2013, Bourland *et al.* 2017a, b, Li *et al.* 2018, Rotterová *et al.* 2018, Pecina and Vďačný 2020a, b, 2022). The order Clevelandellida is robustly monophyletic (BS 98, BPP 1). The family Clevelandellidae is monophyletic (BS 91, BPP 1) and forms an internal lineage of the family Nyctotheridae. *Anteclevelandella constricta* (Kidder, 1937) forms a branch sister to the remaining Clevelandellidae, which form a robust clade (BS 89, BPP 1). Genera *Rhynchoclevelandella* and *Paraclevelandia* form a well-supported clade (BS 89, BPP 1) sister to the genus *Clevelandella*. Genus *Rhynchoclevelandella* is moderately supported (BS 78, BPP 1). The isolates with *R. nipponensis* (Kidder, 1937) morphology are paraphyletic with respect to *R. hastula* (Kidder, 1937). This may be a case of cryptic diversity. The supports in the genus are, however, too weak for any meaningful assumptions. Genus *Paraclevelandia* is fully supported (BS 100, BPP 1). All lineages in the genus include isolates of both *Paraclevelandia brevis* Kidder, 1937 and *Paraclevelandia simplex* Kidder, 1937 morphology, with a majority of them including genetically identical isolates of both morphologies. Genus *Clevelandella* is moderately supported (BS 71, BPP 1) and splits into 12 strongly supported lineages considered here as separate species on the basis of morphology (see below). The relationships among individual species are largely unresolved.

Morphology

Terminology: We use 'anterior' and 'posterior' in the sense of Kidder (1937) and Albaret (1975). Following Kidder (1937) we designate the margin on which the lateral part of the peristomial opening and cytoproct lie as 'left' and the margin bearing the long suture between dorsal and ventral kineties suture as 'right'. We define the portion of the cell anterior to the peristomial projection as the 'body proper' (Kidder 1937, Mandal and Nair 1974). In agreement with Kidder's original terminology (1937) followed by Mandal and Nair (1974) and Pecina, Vďačný (2020a, b), we consider cell flattening to be dorsoventral. It should be

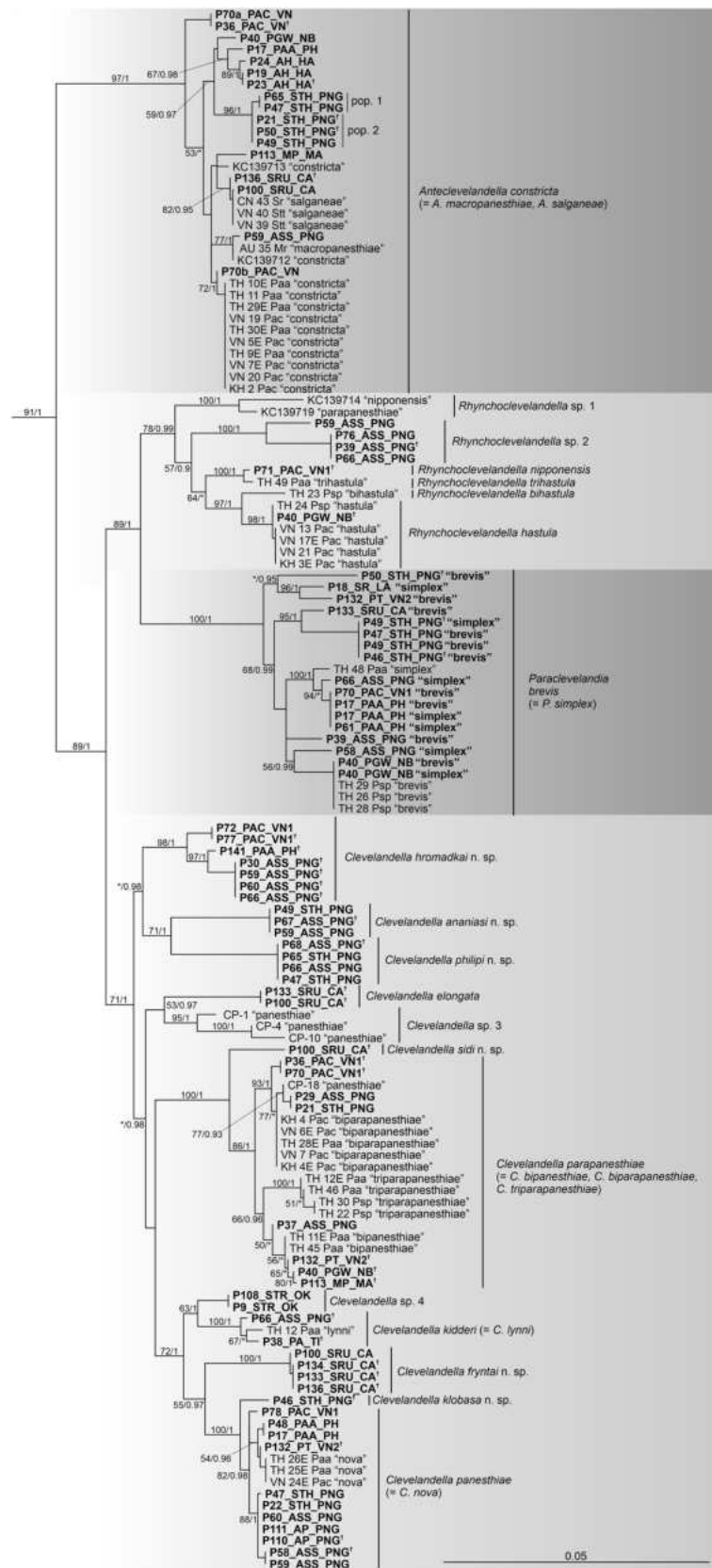


Figure 1. Part of the maximum likelihood phylogenetic tree based on the full dataset using 18S rRNA gene, ITS1-5.8S-ITS2 region, and 28S rRNA gene sequences showing the relationships among Clevelandellidae. Sequences of Nyctotheridae, Sicutophoridae, and Metopida were removed (for the complete tree figure see [Supporting Information, Fig. S4](#)). Metopida was used as an outgroup. The values at the branches represent statistical support in maximum likelihood bootstrap values/Bayesian posterior probabilities. Support values below 50/0.90 are not shown or are depicted by an asterisk. Newly determined sequences are in bold. Scale bar: 5 substitutions/100 nucleotide positions. * indicates

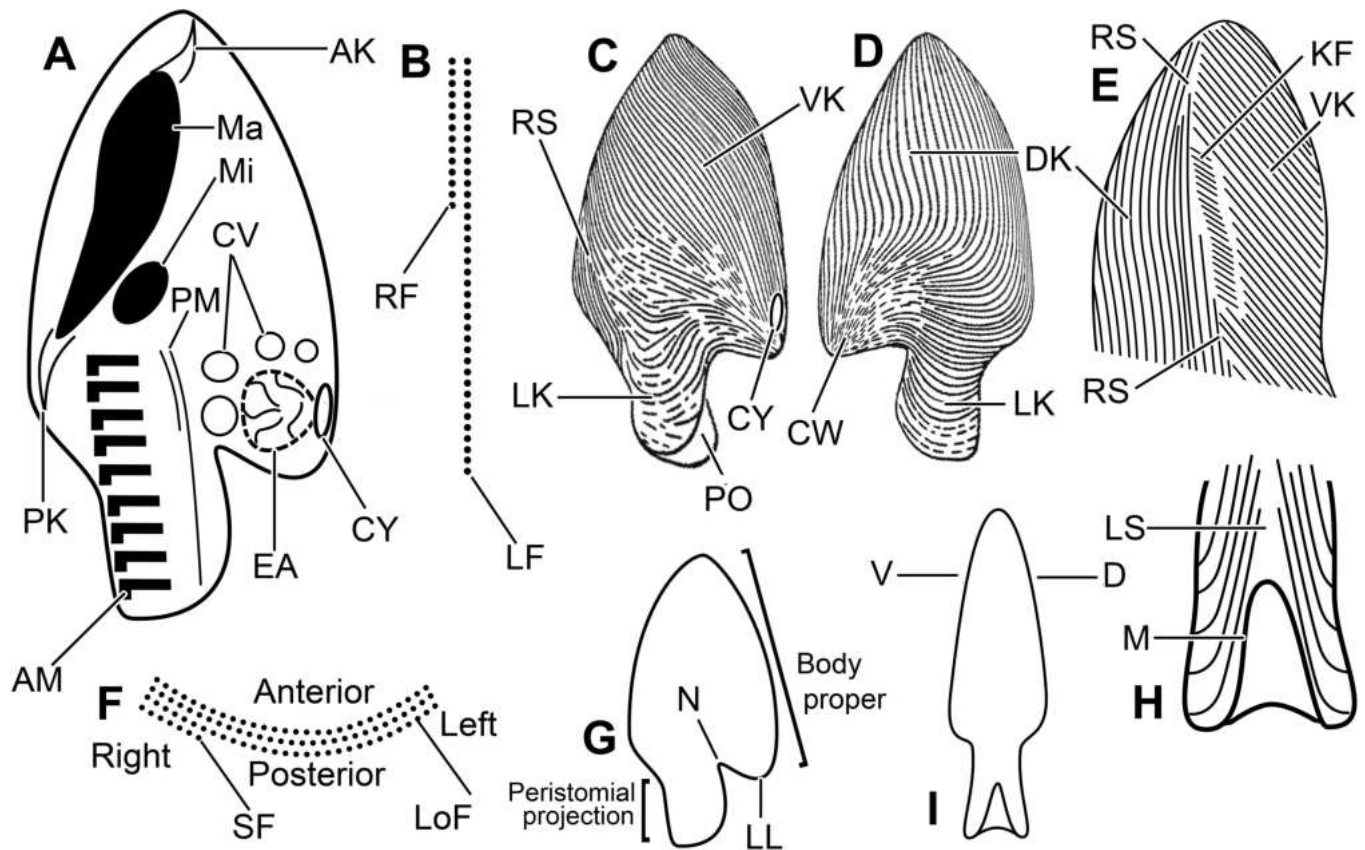


Figure 2. Schematic illustrations of various morphologic features of Clevelandellidae. A, ventral view (optical section) of a spade-shaped species. B, ventral view. C, dorsal view. D, right lateral view of *Anteclevelandella constricta*. E, composition and orientation of adoral membranelle. F, composition of diplostichomonad paroral membrane. G, left lateral view of peristomial projection. H, outline showing body parts of spade-shaped species. I, left lateral view showing dorsoventral flattening. AK, anterior karyophore; AM, adoral membranelle; CY, cytoproct; CV, collecting vesicles of contractile vacuole; CW, ciliary whorl; D, dorsal; DK, dorsal kineties; EA, ciliated excretory antrum; KF, kinetofragments; LF, left file of paroral membrane basal bodies; LL, left lobe; LoF, long file of adoral membranelle basal bodies; LS, short left suture; M, margin of the lateral extension of the peristomial opening; Ma, macronucleus; Mi, micronucleus; N, notch between left lobe and peristomial projection; PK, posterior karyophore; PM, diplostichomonad paroral membrane; PO, peristomial opening; RS, right suture; SF, short file of adoral membranelle basal bodies; V, ventral; VK, ventral kineties. See text for discussion of terminology regarding morphologic features and orientation.

noted, however, that Yamasaki (1939) and Albaret (1975) considered the same species as being laterally flattened. We refer to the distinctive incomplete somatic ciliation as ‘hemiciliation’, recognizing that the extent of partial ciliation varies somewhat between species. We define the cytoproct as the opening in the pellicle through which faecal material is discharged (Lynn 2008). We define the unique ‘cloaca-like’ ciliated intracellular antrum, through which faecal material and contractile vacuole contents pass, as the ‘excretory antrum’ (see Discussion). We use the terms ‘peristomial overtire’, ‘peristomial opening’, and ‘buccal overtire’ interchangeably. The peristomial overtire has two continuous portions, lateral (left) and terminal (posterior), except for *Paraclevelandia brevis* in which the posterior peristomial projection is absent and the buccal overtire is terminal, extending anteriorly only slightly. We consider the terms ‘spade-shaped’

and ‘spear-shaped’ as synonymous. Otherwise the terminology used mainly follows Lynn (2008) and Foissner and Xu (2007). A schematic illustration of an idealized Clevelandellidae cell showing morphologic features and orientation can be found in Figure 2.

Features common to all Clevelandellidae

Morphologic organization: Clevelandellidae show a wide diversity of body shapes and sizes but share the same general morphologic organization, i.e. ‘posteriorization’ of the oral structures in comparison with representatives of nyctotherid families of order Clevelandellida. In all Clevelandellidae, the anterior end of the cell is tapered to a rounded point oriented in the direction of swimming, while the posterior end of the cell bears the buccal

that at least two cells with identical sequences from the same host individual were characterized. The names in quotation marks indicate the species name originally assigned by previous authors and/or morphotype in case of *Paraclevelandia brevis*. For GenBank accession numbers of newly determined sequences, as well as images of individual cells, see Supporting Information, Table S2 and Figs S1–S3. For accession numbers of sequences downloaded from GenBank see Supporting Information Table S3.

or peristomial overture. All Clevelandellidae show some degree of dorsoventral flattening, most conspicuous in spade-shaped taxa and least conspicuous in *Anteclevelandella constricta* and *Paraclevelandia brevis*.

The peristome leads internally to a tubular infundibulum bearing the adoral zone and paroral membrane that both end anteriorly at the cytostome, typically to the right of the midline in the posterior half of the body proper. The peristomial opening occupies the posterior end of the cell and has an inverted U- or V-shaped lateral part extending anteriorly toward the junction of the peristomial projection and the body proper. We consider the lateral part of the overture an important landmark designating the left side of the cell. All species except *Anteclevelandella constricta*, *C. elongata* (Kidder 1937), *C. klobasa* sp. nov., and *Paraclevelandia brevis* have a distinct, more or less cylindrical posterior peristomial projection. In the other four species mentioned, the projection is less developed (the former three) or absent (*Paraclevelandia brevis*).

Cytoplasm and food vacuoles: All members of Clevelandellidae are colourless to slightly brownish depending on cytoplasmic contents. Food vacuoles are quite indistinct in all species, giving the cytoplasm a rather featureless, coarsely granular appearance. The cytoplasm occasionally contains colourless platelets, consistent with polysaccharide granules (Fig. 17C) possibly composed of amylopectin, also known as paraglycogen, the carbohydrate storage product found also in nyctotherids and many other free-living and parasitic protists. Although Albaret (1975) showed them to consist of polysaccharide by the Bauer reaction, no more specific identification of their composition is available (Kidder 1937, Hoyte 1961, Pecina and Vďačný 2020b, 2022, Ralton et al. 2021).

Cortex: The cortex is flexible without distinct cortical granules. However, all species studied *in vivo* by DIC have a distinct 1.5–2.5 µm-thick, hyaline subcortical layer as noted in brightfield illumination by Kidder (1937). As in nyctotherids, this probably comprises a layer of closely-packed mucocyst-type extrusomes (Albaret 1975).

Nuclear apparatus and karyophore: The nuclear apparatus of all species is located in the body proper, usually on the right in its midportion or anterior part. A notable exception is *Anteclevelandella constricta*, which has a transversely oriented macronucleus attached to the right and left cell margins by the karyophore. Although features of the karyophore, a system of macronuclear suspensory fibres attached to the somatic cortex in Clevelandellida, have been considered taxonomically valuable (Kidder 1937, Albaret 1975, Pecina and Vďačný 2020a, b), in our hands, demonstration of this feature is highly inconsistent in the majority of species, except *Anteclevelandella constricta* and *C. elongata* (Figs 19A, E, 25B, C, 26A, B). It is often undetectable in both live cells observed with DIC and in protargol preparations. Thus, presence or absence of the karyophore is a highly inconsistent morphologic character.

Contractile vacuole, ciliated excretory antrum, and cytoproct: The contractile vacuole is closely associated with the ciliated

excretory antrum on the left posterior margin of the body proper (Fig. 2A). The morphology is consistent with Patterson's (1980) type I contractile vacuole. The excretory antrum itself consists of a permanent, intracellular cavity, to the left of the cytostome and lined by motile cilia. The faecal material and contractile vacuole contents are propelled through this excretory antrum to empty via a slit-like cytoproct on the left margin of the body [*C. panesthiae* (Kidder, 1937): [Supplementary Information, Video S1](#)].

Somatic ciliature: In all Clevelandellidae, only the anterior portion of the cell bears cilia (i.e. 'hemiciliation'), a fact recognized by Albaret (1975), Mandal and Nair (1974), and Yamasaki (1939) but, curiously, overlooked by others (Pecina and Vďačný 2020a, b, 2022), including Kidder himself (1937). The remainder of the cell cortex bears barren dikinetids, except for the ciliated circumperistomial kineties. The somatic ciliation of the body proper extends furthest posteriorly along the right cell margin, most notably in *P. brevis* in which the somatic ciliation on the right cell margin can extend to the posterior end (Fig. 24A, B, E, F). The somatic ciliature follows a similar 'Clevelandellidae pattern' in all species, i.e. leftward-spiralling, narrowly (about 1.5–2.0 µm apart; e.g. *C. panesthiae*) to very narrowly spaced (≤ 1 µm apart; e.g. *C. elongata*) kineties consisting of densely spaced dikinetids radiating outward from the region of the cytoproct on to the ventral and dorsal surfaces of the body proper and with more widely spaced kineties looping posteriorly on to the peristomial projection. The dorsal kineties tend to be less spiralled. The ventral and dorsal kineties of the body proper meet at a long suture on the right lateral margin of the body proper and a short, inconspicuous left posterior suture near the junction of the peristomial projection with the body proper (with the exception of *Paraclevelandia brevis*, which lacks a distinct peristomial projection). The density of the ciliary pattern and the occurrence of shortened or incomplete kineties hinder precise determination of numbers of somatic kineties. Within the right suture, several species [*Anteclevelandella constricta*, *C. hromadkai* sp. nov., *C. parapanesthiae* (Kidder, 1937), *C. philipi* sp. nov., *C. kidderi* Mandal and Nair, 1974, *Paraclevelandia brevis*, and *R. hastula*] have short transverse to oblique kinetofragments consisting of about five basal bodies bearing clavate cilia (Fig. 2E), as also noted by Albaret (1975). This feature was absent in *C. sidi* sp. nov., *C. fryntai* sp. nov., *C. panesthiae*, and *C. elongata* and could not be reliably assessed in *C. ananiasi* sp. nov., *C. klobasa* sp. nov., *R. nipponensis*, and *Rhynchoclevelandella* sp. 2. All species have two or three ciliated circumperistomial kineties at the posterior margin of the buccal overture (e.g. Figs 4A, F, 5A, C). The somatic cilia of all examined species are distinctly shorter than those typical of free-living ciliates (about 4.5–5.5 µm vs. about 7–15 µm).

Oral structures: In all species, the adoral zone of membranelles (AZM), in contrast to nyctotherids, is located on the right wall of the infundibulum and terminates anteriorly at the cytostome. The adoral zone is usually straight or slightly oblique to the long axis of the cell, usually curving as it ends at the cytostome. The adoral membranelles consist of two or three long files of basal bodies and one short file. The polarity of the membranelles themselves is also reversed, i.e. in contrast to nyctotherids; the

short file of basal bodies is located on the posterior rather than anterior margin of each membranelle (Fig. 2A, F). The paroral membrane (POM) is opposite the AZM on the left wall of the infundibulum (Fig. 2SB) and about same length as the adoral zone. In all but one species (*C. sidi* sp. nov.) with adequate preparations, the paroral membrane is diplostichomonad (i.e. comprises two parallel files of basal bodies separated by a cortical ridge) and the right file of basal bodies is usually shortened posteriorly (de Puytorac and Grain 1976).

The above features are common to all species except as noted. The following species descriptions include only character states that differ between species.

Newly described species

Clevelandella ananiasi sp. nov.

(Figs 3–5; Supporting Information, Table S4)

Description based on population from ASS hosts from Papua New Guinea: Small *Clevelandella*, size *in vivo* 69–87 × 35–52 µm, usually about 77 × 41 µm; size in protargol preparations 44–67 × 25–34 µm, usually about 55 × 29 µm. Body proper dorsoventrally flattened; overall outline in ventral view asymmetrically spade-shaped, with prominent left lobe, anterior cell end bluntly pointed; peristomial projection joins body proper to right of midline. Left cell margin notched at base of peristomial projection forming a lobe that overhangs the peristomial projection for about one-fifth of its length. Macronucleus ellipsoidal (Figs 3A, 4A) to inverted teardrop-shape (Figs 3B, 4B–E, G), anterior end rounded, posterior end acutely tapered, chromatin coarsely granular. Karyophore attached to posterior margin of macronucleus (Figs 3A, 4A, B). Micronucleus ellipsoidal (4.4 × 3.4 µm), adjacent to anterior margin of macronucleus (Figs 3B, 4G). Swims slowly.

Somatic ciliature consists of about 60 kineties, only anterior two-thirds of body properly ciliated (Fig. 5A, B). Right sutural kinetofragments not confirmed, cannot be completely excluded due to suboptimal orientation of cells in protargol preparations.

Peristomial projection extends an average of 28% of total cell length. Lateral part of peristomial opening extends about three-quarters of length (Figs 4A, F, 5A) of peristomial projection. Adoral zone usually extends about 52% of cell length, composed of an average of 23 membranelles. Fine structures of POM unrecognizable due to poor staining but visibly stichomonad at the posterior (Fig. 5B–D).

Occurrence: *Clevelandella ananiasi* sp. nov. was found in the hindgut of two Panesthiinae species from Papua New Guinea: ASS and STH from the same locality (Wanang 3). It occurred in 56% of ASS individuals regardless of age and sex. In STH the ciliate was present only in one juvenile host individual among 10 dissected insects. When present, the ciliate is typically abundant. In ASS, infection with *C. ananiasi* sp. nov. frequently co-occurs (60%) with *C. sidi* sp. nov. requiring special care in species identification.

Clevelandella philipi sp. nov.

(Figs 6, 7; Supporting Information, Tables S5, S6)

Description based on populations from hosts ASS and STH from Papua New Guinea: Small- to medium-sized *Clevelandella*, size *in vivo* 74–99 × 40–54 µm, usually about 90 × 49 µm; size in

protargol preparations 59–88 × 35–47 µm, usually about 75 × 41 µm in STH population; size *in vivo* 73–95 × 43–59 µm, usually about 87 × 49 µm; size in protargol preparations 49–78 × 29–41 µm, usually about 63 × 34 µm in ASS population. Body proper dorsoventrally flattened; overall outline spade-shaped in ventral view, widest near junction of body proper with peristomial projection, cell outline asymmetric due to prominent left lobe, anterior end of cell tapers to blunt point; peristomial projection joins body proper near right margin; left cell margin conspicuously notched at base of peristomial projection, left lobe overhangs peristomial projection up to one-third of its length (Figs 6A–D, 7B–D, G, H). Macronucleus usually inverted teardrop-shape (Fig. 7B, E, F), right margin slightly convex, or ellipsoidal (Figs 6A, 7A, G, H), karyophore unobserved. Chromatin coarsely granular. Micronucleus ellipsoidal (5.5 × 2.5 µm), adjacent to right anterior margin of macronucleus (Figs 6B, 7G). Swims slowly.

Somatic ciliature arranged in about 55 kineties. Cilia present only on anterior two-thirds of cell (Fig. 7B). About nine widely-spaced, curved, free transverse ciliary rows on peristomial projection. Right sutural kinetofragments numerous, well developed (Fig. 7C, E). Peristomial projection averages about 24% of total body length in STH population, 28% in ASS population. Peristomial opening extends one-half (Fig. 7A) to three-quarters (Fig. 7B, H) of length of peristomial projection. Adoral zone extends anteriorly about 51% (STH) to 57% (ASS) of body length to end near posterior margin of macronucleus, composed of an average of 30 (ASS) to 32 (STH) membranelles, adoral zone widest in peristomial projection, narrows anteriorly, POM as described for the family.

Occurrence: *Clevelandella philipi* was found in the hindgut of two Panesthiinae species from Papua New Guinea: ASS and STH from the same locality (Wanang 3). Of STH individuals, all of which were juveniles, 30% harboured *C. philipi*. Since only two adults of the STH population (both males) were dissected, no conclusions can be reached about possible life-stage specificity. In the ASS population, *C. philipi* was present in 37% of cockroach individuals regardless of age and sex. When present, the ciliate is typically abundant. In ASS the infection with *C. philipi* sp. nov. frequently (90%) co-occurred with *C. ananiasi* sp. nov., requiring care in identification.

Clevelandella hromadkai sp. nov.

(Figs 8–11; Supporting Information, Tables S7–S10)

Description based on populations from hosts ASS from Papua New Guinea and PAC from Vietnam: Medium to very large *Clevelandella*, widely variable in both size and shape. In ASS population, size *in vivo* 103–262 × 39–81 µm, usually about 164 × 57 µm; size in protargol preparations 77–164 × 25–66 µm, usually about 117 × 40 µm; in PAC population, size *in vivo* 127–339 × 44–85 µm, usually about 190 × 58 µm; size in protargol preparations 110–276 × 31–83 µm, usually about 155 × 45 µm. Body shape usually broadly cultriform, ventral surface of the body proper convex, dorsal surface concave, anterior end of cell bluntly pointed and curved more (Figs 9E, 10C, E) or less (Figs 9C, H, 10B, 11A, D, E) in dorsal direction. Peristomial projection flares to merge gradually with body proper, prominence of

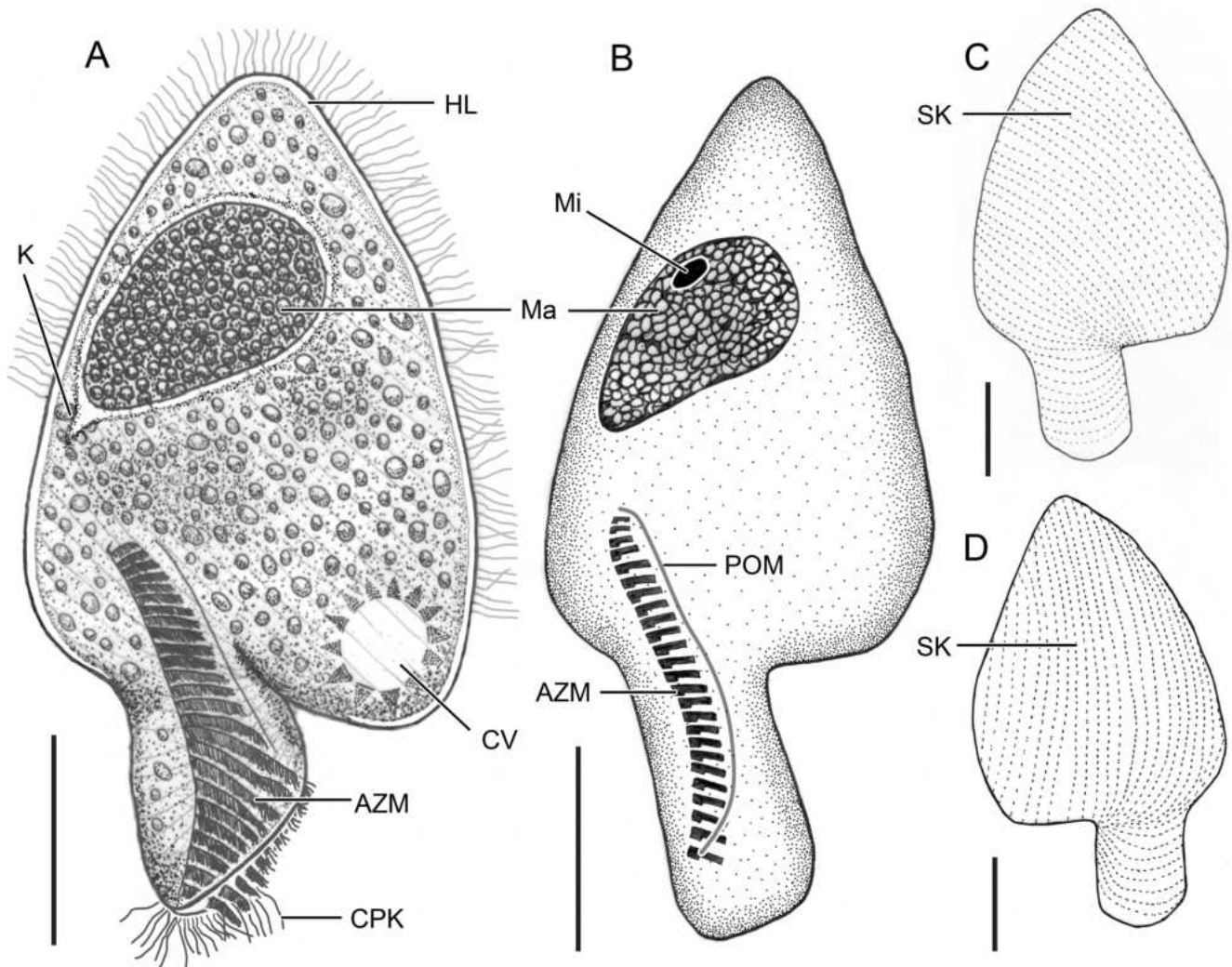


Figure 3. Schematic drawings of *Clevelandella ananiasi* from life (A) and protargol impregnation (B–D). A, ventral view of a typical individual. B, ventral view showing the oral apparatus and nuclear structures. C, ventral infraciliature. D, dorsal infraciliature. AZM, adoral zone of membranelles; CPK, circumperistomal cilia; CV, contractile vacuole; HL, hyaline subcortical layer; K, karyophore; Ma, macronucleus; Mi, micronucleus; POM, paroral membrane; SK, somatic kineties. Scale bars: 20 µm.

left lobe varies from less conspicuous (Figs 9A, B, 10A) to more prominent (Figs 8A, D, F, 9D, F–H, 10F, 11B, C, E) with a notch formed between the lobe and peristomial projection (Figs 8F, 9F), in contrast to spade-shaped species, the notch degree between peristomial projection and left lobe seems never to be under 90°; posterior margin of peristomial projection truncate. L/W ratio highly variable in individuals from both hosts, from shorter stouter forms (Figs 8D–G, 9A–I, 11A–E) to longer slender cells (Figs 8A, B, 10A–F). Macronucleus broadly ellipsoidal in short forms (Figs 8D, E, 9A–F), to narrowly ellipsoidal in long forms (Fig. 10A–C, E, F). Karyophore inconspicuous; when detectable, attached to posterior end of macronucleus and right body margin (Figs 8A, B, D, 10A, D). Micronucleus ellipsoidal, usually adjacent to right anterior margin of macronucleus, enclosed in separate membrane (Figs 8A, B, D, 9A, C, E, F, 10A, C–E). Swims moderately fast while rotating around long axis.

Somatic ciliature composed of about 60–85 somatic ciliary rows in Clevelandellidae pattern; cilia present only on approximately anterior half of cell; about 20–25 ciliary rows on the

peristomial projection in ASS population. Ciliary rows in PAC population not counted. Right suture has only two or three small free kinetofragments (not shown).

Peristomial projection occupies an average of 35% of total body length. Lateral part of peristomial opening extends about one-half (Figs 9B, 11B, D, E) length of peristomial projection in short cells, about one-third (Fig. 10A, B) length of peristomial projection in long cells. Adoral zone usually extends about 50% of body length, composed of an average of 54 (ASS) to 62 (PAC) membranelles, base of membranelles longest in midportion of peristomial projection. POM as described for the family (Fig. 9I).

Remarks on short and long morphotypes: *Clevelandella hromadkai* shows considerable intraspecific phenotypic variability. The ASS population, while genetically uniform, can be divided in two distinct phenotypes: short (Figs 8D–G, 9A–I, 11A–F) and long (Figs 8A, B, 10A–F). In a single host, only one phenotype seems to occur at a given time; from nine thoroughly inspected

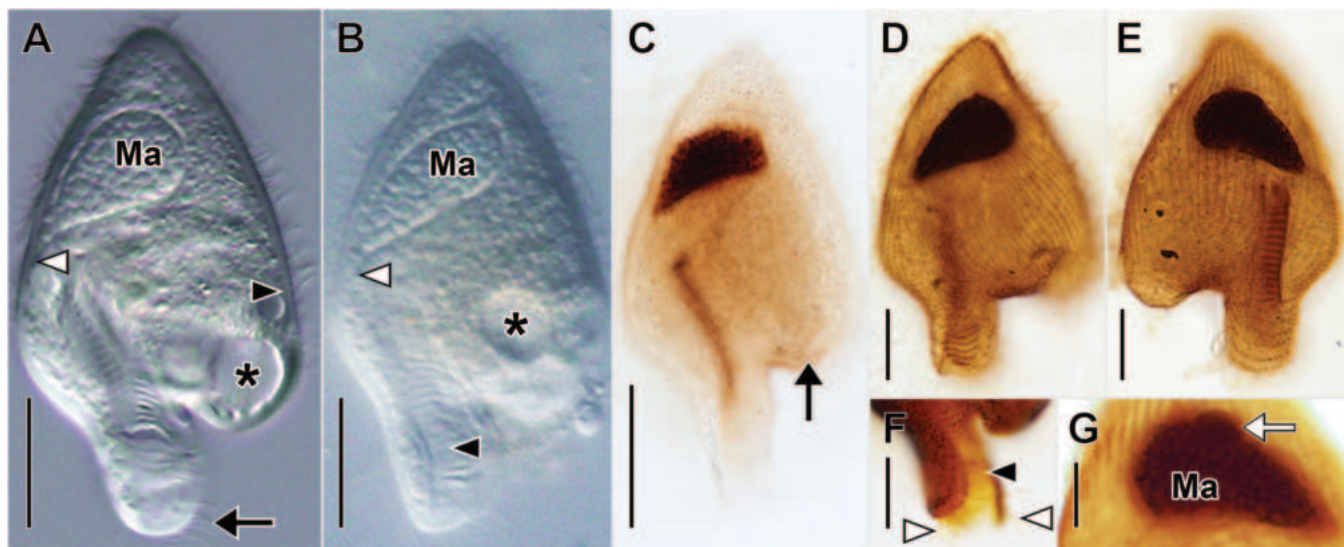


Figure 4. *Clevelandella ananiasi* sp. nov. from ASS. *In vivo* (A, B) and after protargol impregnation (C–G). A, ventral view showing karyophore (white arrowhead), contractile vacuole (asterisk), posterior extent of somatic cilia (black arrowhead), and cilia of circumperistomial kineties (black arrow). B, ventral view showing a more slender macronucleus, karyophore (white arrowhead), contractile vacuole (asterisk), and adoral membranelles (black arrowhead). C, ventral view showing excretory (black arrow). D, ventral infraciliature. E, dorsal infraciliature of same cell as (D). F, detail view of peristomial projection showing the peristomial overture (black arrowhead) and cilia of the circumperistomial kineties (white arrowheads). G, detail view showing position of micronucleus (white arrow). Ma, macronucleus. Scale bars: 20 μm (A–C), 10 μm (D–F), 5 μm (G).

hosts the short morph occurred in two females and two larvae, the long morph occurred in four males and one larva. Apart from the obvious length difference (mean *in vivo* 127 μm vs. 215 μm) and other morphological characteristics (e.g. number of adoral membranelles—see [Supporting Information, Tables S8, S9](#)), both morphs can be distinguished by their shapes: the short one is stouter (body L/W ratio 2.5) with a more rounded anterior end and the long one is more slender (body L/W ratio 3.7).

Occurrence: Three distinct populations of *C. hromadkai* were found in the hindguts of three Panesthiinae populations: ASS from Papua New Guinea, PAA from Philippines, and PAC from Vietnam. The ASS population occurred in 63% of host individuals regardless of age and sex. When present, *C. hromadkai* was less abundant (a few tens of individuals) compared to other ciliate species present in a host. The PAC population occurred in 28% of host individuals. Out of nine infected hosts, one was a large larva and eight were visibly old adult males. Most of the PAC hosts exhibited low occurrence of *C. hromadkai* (around 10 cells) with other ciliate species being more abundant. The exception was one male that harboured around 50 cells of *C. hromadkai*. When *C. hromadkai* was present in PAC hosts, the populations of other ciliates were visibly low compared to PAC host, in which *C. hromadkai* was absent. The PAA population occurred in only 17% of host individuals. Out of two infected hosts (both adult males), one exhibited a low occurrence of the *C. hromadkai* with the other ciliate species being more abundant. The other host exhibited high occurrence of *C. hromadkai* with other ciliates being scarce.

Clevelandella klobasa sp. nov.

([Figs 12, 13](#); [Supporting Information, Table S11](#))

Description based on population from STH hosts from Papua New Guinea: Large *Clevelandella*, size *in vivo* 146–206 $\times$ 61–98

μm , usually about 170 $\times$ 80 μm ; size in protargol preparations 123–163 $\times$ 61–80 μm , usually about 135 $\times$ 71 μm . Overall cell outline in ventral view more or less symmetrical, broadly lanceolate, widest at middle of cell, anterior cell end bluntly pointed, dorsoventrally flattened; peristomial projection inconspicuous, merges gradually with cell body proper at midline, thus predominant left lobe absent. Peristomial opening broad. Macronucleus long, slender sausage-shaped (mean 63 $\times$ 12 μm) ([Figs 12A, B, 13A, C, D](#)), anterior end rounded, posterior end rounded ([Fig. 13A, B](#)) or abruptly tapered to short point ([Fig. 13C](#)); extends along nearly entire right margin of body proper, convex toward right body margin ([Fig. 13A, C](#)). Chromatin coarsely granular. Karyophore not identified. Micronucleus (4.7 $\times$ 4.3 μm) globular, adjacent to anterior right margin of macronucleus ([Figs 12A, B, 13B, D](#)). Swims slowly.

Somatic cilia present on only anterior two-thirds of cell. Somatic ciliature composed of about 102 narrowly-spaced kineties, arranged in typical *Clevelandella* pattern. Right sutural kinetofragments not observed.

Peristomial projection indistinct, i.e. flares as it gradually merges with body proper. Lateral part of peristomial opening wide (23 μm) ([Fig. 13A–C](#)). Adoral zone extends about 56% of body length, composed of 59 membranelles ($N = 3$), markedly curved right at about three-quarters of length. Fine morphology of POM ([Fig. 13D](#)) not determined with certainty.

Occurrence: *Clevelandella klobasa* is extremely rare as it was found only in two out of 10 dissected individuals of STH: a male and a small juvenile, each of them from a different family group. The population density was very low in both cases, with no more than 15 cells of *C. klobasa* sp. nov. present in the entire hindgut. In both cases, *C. klobasa* co-occurred with cells of *Paraclevelandia brevis* (once also in 'simplex' form) and *Nyctotherus* sp..

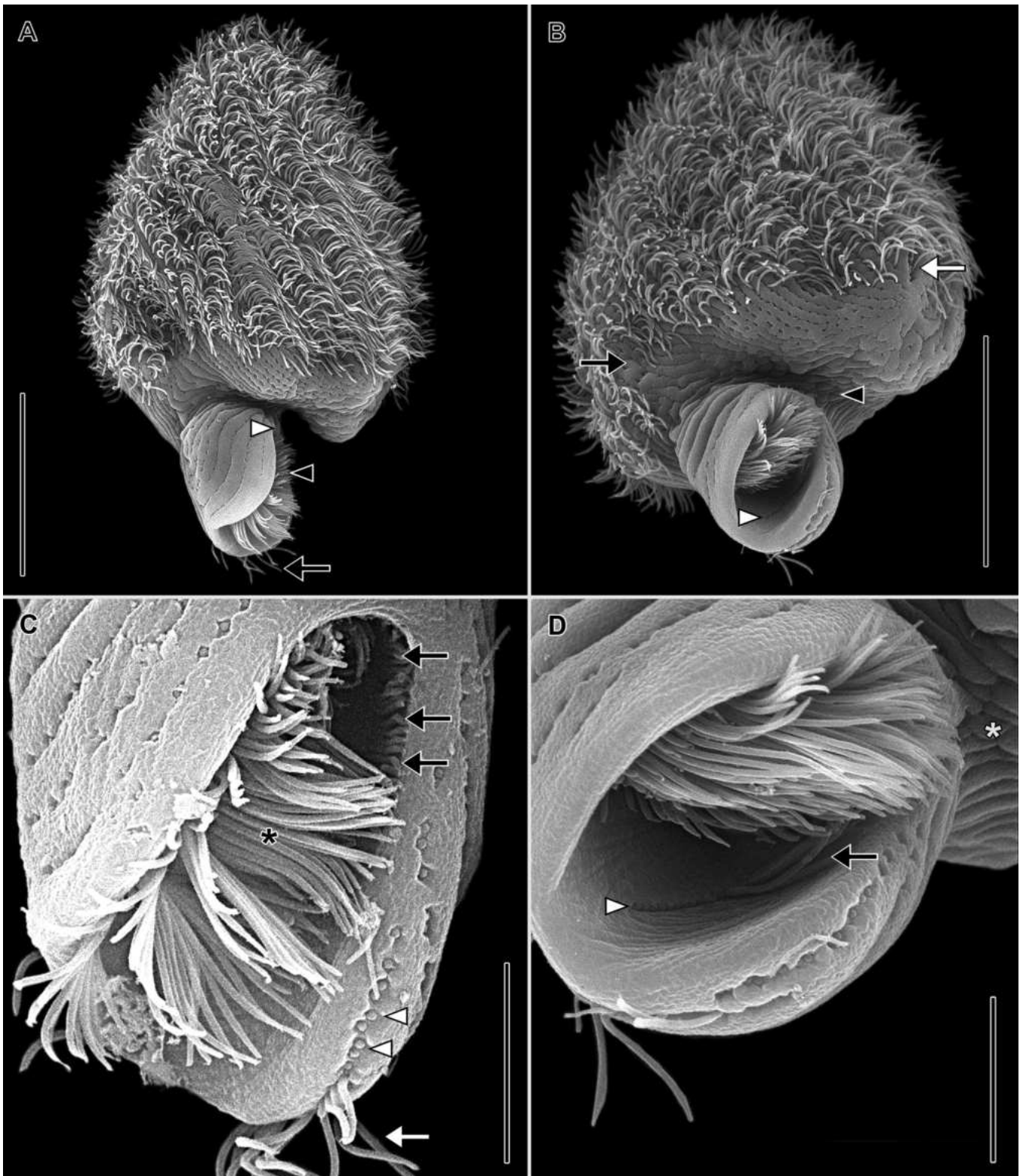


Figure 5. *Clevelandella ananiasi* sp. nov. (from ASS) in the scanning electron microscope. A, ventral view showing anterior margin of peristomial overtube (white arrowhead), cilia of adoral membranelles (black arrowhead), and cilia of circumperistomial kineties (black arrow). B, posteroventral view showing posterior end of the right suture (black arrow), the cytoproct with protruding cilia (white arrow), the inconspicuous left suture (black arrowhead), and the posterior end of the paroral membrane (white arrowhead). C, detail view of the peristomial overtube showing adoral membranelles (black asterisk), cilia of the posterior end of the paroral membrane (black arrows), dikinetids of the posteriormost circumperistomial kinety (white arrowheads), and cilia of the circumperistomial kineties (white arrow). D, detail [same cell as (B)] showing the single file of basal bodies comprising the posterior part of the paroral membrane (white arrowhead), cilia of the paroral (black arrow), and the left suture (asterisk). Scale bars: 20 μm (A, B), 10 μm (C, D).

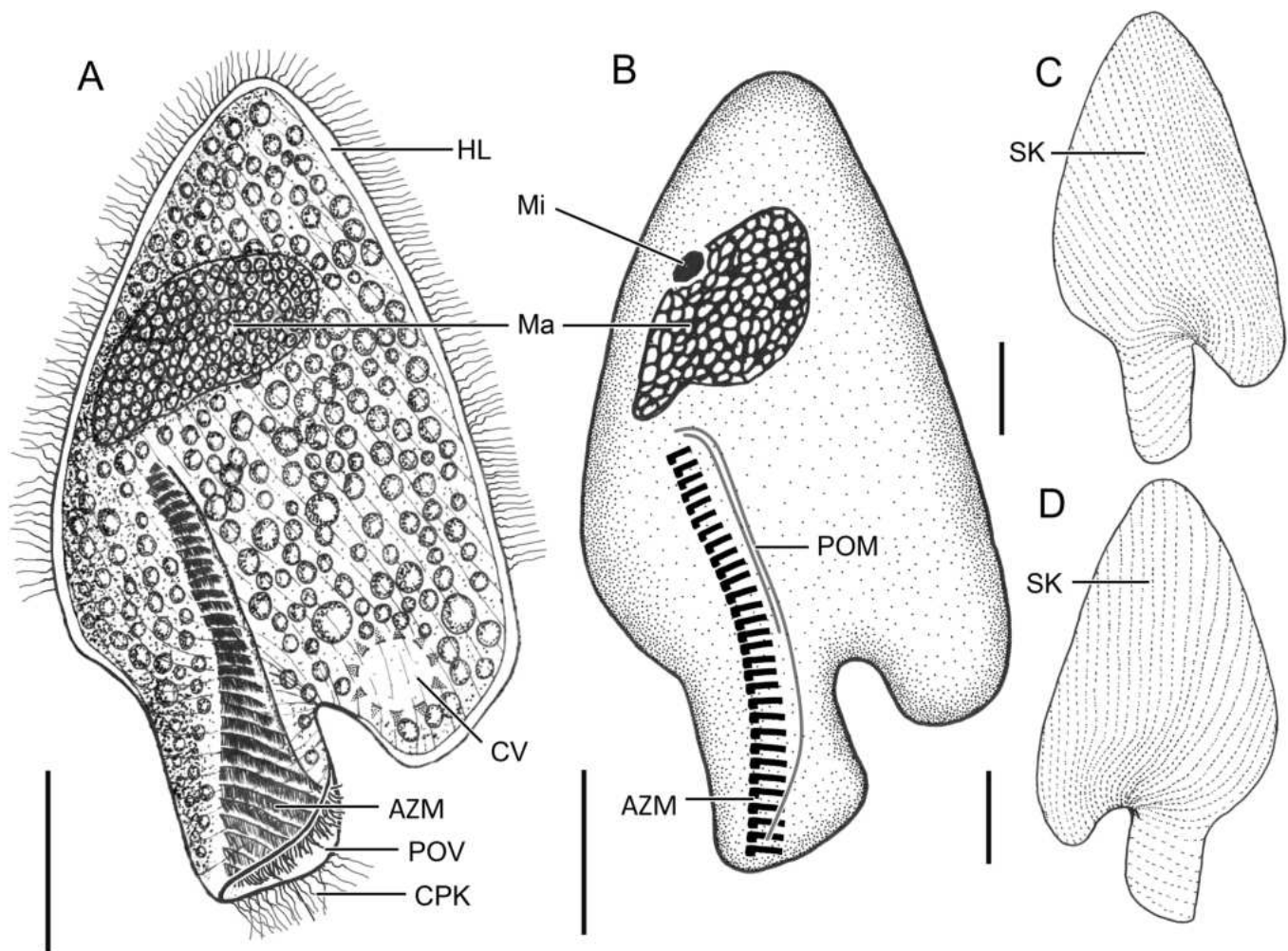


Figure 6. Schematic drawings of *Clevelandella philipi* sp. nov. from life (A) and after protargol impregnation (B–D). A, ventral view of a typical individual. B, ventral view showing the oral apparatus and nuclear structures. C, ventral infraciliature. D, dorsal infraciliature. AZM, adoral zone of membranelles; CPK, cilia of circumperistomial kineties; CV, contractile vacuole; HL, hyaline subcortical layer; Ma, macronucleus; Mi, micronucleus; POM, paroral membrane; POV, peristomial overtire; SK, somatic kineties. Scale bars: 20 µm.

Clevelandella sisi sp. nov.

(Figs 14, 15; Supporting Information, Table S12)

Description based on population from a single SRU host individual from Cambodia: Medium-sized, spade-shaped *Clevelandella*, size *in vivo* 126–154 × 50–64 µm, usually about 142 × 59 µm, in protargol preparations 95–142 × 37–59 µm, usually about 117 × 46; cell dorsoventrally flattened, prominent notch between left lobe and peristomial projection; peristomial projection about 28% of cell length, peristomial overtire extends to about three-quarters of peristomial projection (Fig. 15A, C). Macronucleus broad inverted teardrop-shaped (Figs 14A, B, 15A–C), in anterior half of cell, narrow end at right cell margin, micronucleus ellipsoidal (usually about 4.5 × 2.7 µm), adjacent to macronucleus (Fig. 15G); karyophore usually undetectable or inconspicuous in both live, protargol-impregnated cells, located at posterior end of macronucleus (visible in 3 of 15 protargol-impregnated cells) (Fig. 15F). Swims lazily. Somatic ciliature composed of about 80–90 somatic ciliary rows in Clevelandellidae pattern, about 20–25 ciliary rows extend on to peristomial projection; cilia restricted to approximately anterior

half of cell. Sutural kinetofragments absent. Adoral zone usually extends about one-half of body length, composed of an average of 52 membranelles, base of membranelles longest in posterior one-third of adoral zone. POM entirely diplostichomonad (Fig. 15H).

Occurrence: *Clevelandella sisi* was obtained only from one wild-born SRU subadult female. Later, another wild-born female from the same family group and four of her offspring were dissected, but none of them contained *C. sisi*.

Clevelandella fryntai sp. nov.

(Figs 16–18; Supporting Information, Tables S13, S14)

Description based on populations from SRU hosts from Cambodia: Spade-shaped *Clevelandella*, size *in vivo* quite variable 113–189 × 55–79 µm, usually about 143 × 63 µm; size in protargol preparations 110–167 × 51–82 µm, usually about 130 × 65 µm; dorsoventrally flattened, widest in posterior half of cell, anterior end broadly tapered, cell shape varies depending on host and possibly nutritional status, i.e. morphology less variable within

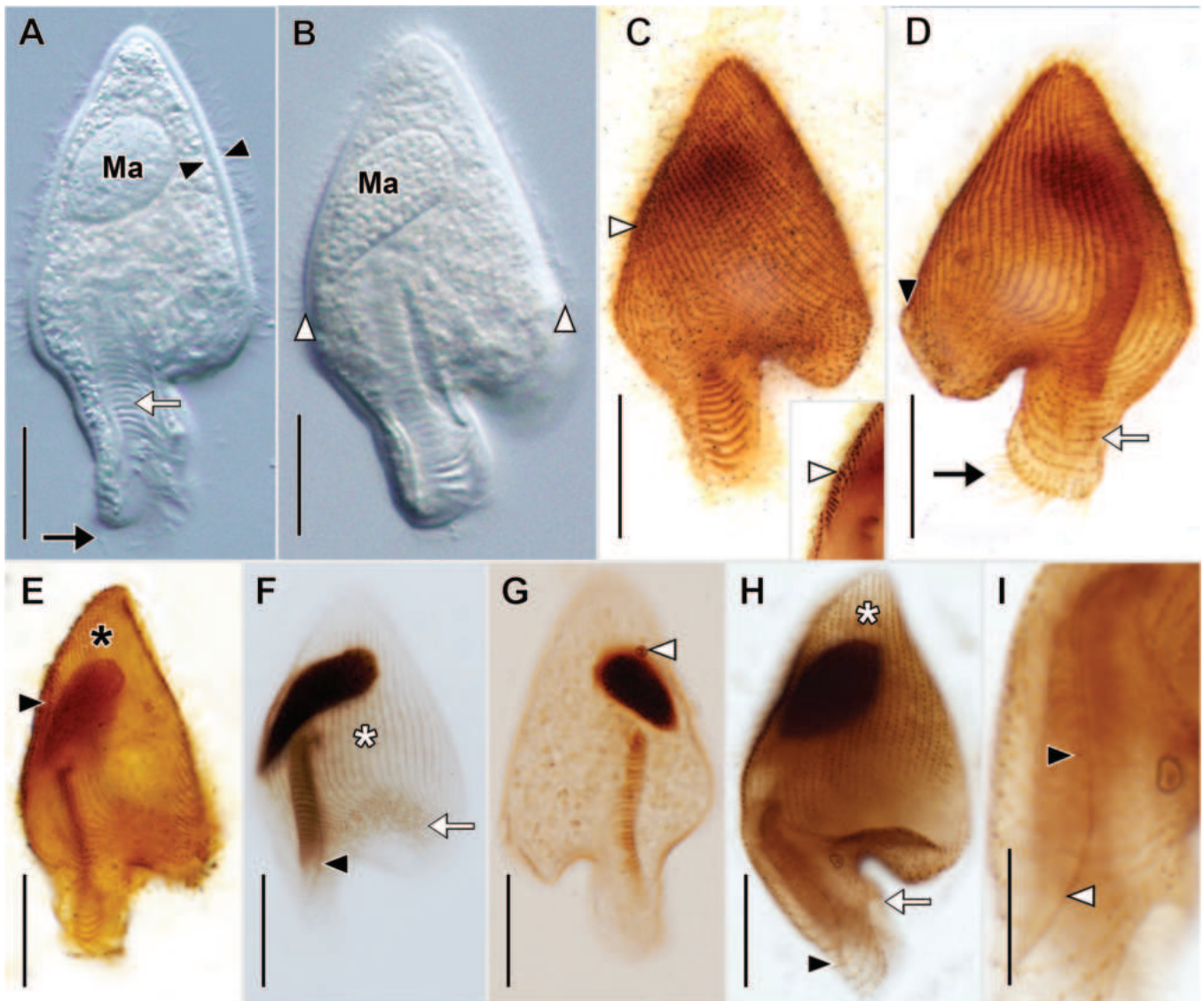


Figure 7. *Clevelandella philipi* sp. nov., *in vivo* (A, B) and after protargol impregnation (C–I). From STH (A–G) and ASS (H, I). A, ventral view showing thick, hyaline cortical layer (between black arrowheads), cilia of the circumperistomial kineties (black arrow) and adoral membranelles (white arrow). B, ventral view showing posterior extent of somatic ciliation (white arrowheads). Note variability of macronuclear shape (A, B). C, ventral infracapitulum with detail (inset) showing right sutural kinetofragments (white arrowheads). D, dorsal view showing infracapitulum, ciliated circumperistomial kineties (black arrow), looping kineties of peristomial projection (white arrow), and cytoproct (black arrowhead). E, ventral view showing left-hand spiralling ventral somatic kineties (black asterisk) and right sutural kinetofragments (black arrowhead). F, dorsal view showing straight somatic kineties (white asterisk), disordered basal bodies on left protuberance (white arrow), and paroral membrane (black arrowhead). G, dorsal view showing micronucleus (white arrowhead). H, dorsal view showing straight dorsal somatic kineties (asterisk), proximal margin of the peristomial overture (white arrow), and looping kineties of peristomial projection (black arrowhead). I, detail view showing diplostichomonad paroral membrane composed of shorter right file (black arrowhead) and longer left file (white arrowhead) of basal bodies. Ma, macronucleus. Scale bars: 20 μ m (A–H) 10 μ m (I).

than between hosts, well-fed cells plump (L/W ratio about 2.6), less well-fed cells narrower (L/W ratio about 2.1), cells of intermediate length–width ratio common (range 1.6–2.8). Peristomial projection length relative to cell length quite variable depending on nutritional status (average in all cells about 19%; about 25% in more slender, less well-fed cells; about 17% in plumper, well-fed cells), peristomial overture extends nearly entire length of peristomial projection regardless of nutritional state (Fig. 18A–D). Macronucleus in anterior half of cell, inverted teardrop-shaped (Fig. 17G) to lenticular (Fig. 17B, H),

chromatin granular; micronucleus distinctly ellipsoidal (Fig. 17H), adjacent to macronucleus, about $7 \times 4 \mu$ m. Presence of karyophore highly variable, not seen or quite inconspicuous *in vivo*, when present, appearance in protargol preparations (Fig. 17G) highly variable (seen at anterior and posterior ends of macronucleus in 11 of 39 cells, anterior end only in 7 of 39 cells, at posterior end only 4 of 39 cells, undetectable in 17 of 39 cells). Swims lazily. Somatic ciliature composed of about 80–100 somatic ciliary rows in Clevelandellidae pattern, about 20–25 ciliary rows extend on to peristomial projection, somatic

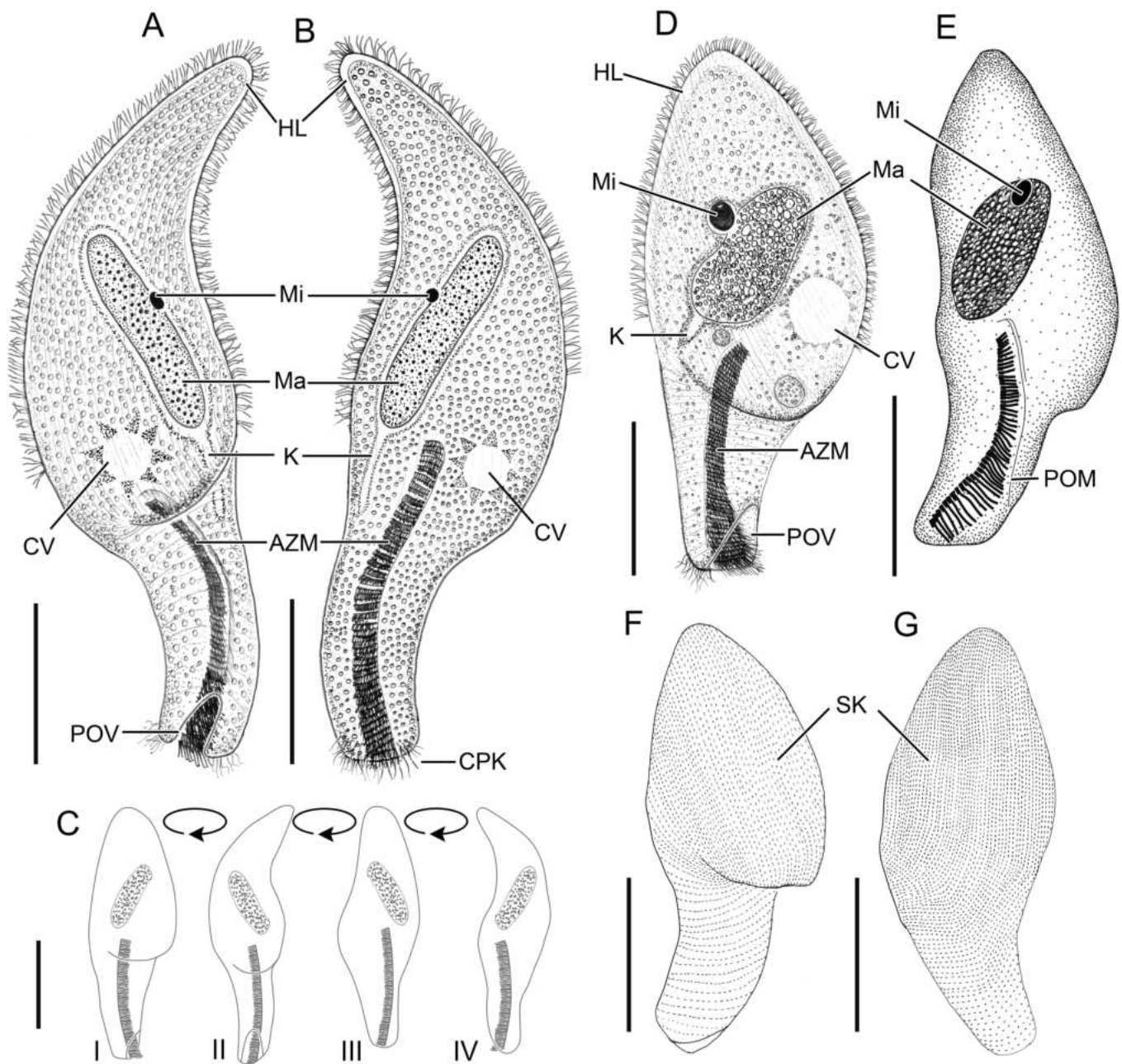


Figure 8. Schematic drawings of *Clevelandella hromadkai* sp. nov. from life (A–D) and after protargol impregnation (E–G); long morphotype (A–C), short morphotype (D–G). A, left lateral view. B, right lateral view. C, (I–IV) cell outline in clockwise rotation around long axis. D, ventral view. E, ventral view. F, ventral infraciliature. G, dorsal infraciliature. AZM, adoral zone of membranelles; CPK, cilia of circumperistomial kineties; CV, contractile vacuole; HL, hyaline subcortical layer; K, karyophore; Ma, macronucleus; Mi, micronucleus; POM, paroral membrane; POV, peristomial overtire; SK, somatic kineties. Scale bars: 50 µm.

cilia restricted to approximately anterior two-thirds of cell except for four or five ciliated circumperistomial kineties. Sutural kinetofragments absent. Adoral zone usually extends about 45% of body length, composed of an average of 60 membranelles, base of membranelles longest in posterior one-third of adoral zone. POM as described for the family.

Remarks on phenotypic variability: Two genetically different populations were detected in SRU host: (i) (Fig. 17A) and (ii) (Figs 17B–K, 18). Cells of population (i) were markedly larger than cells of population (ii)—cell size *in vivo*

180 × 65 µm vs. 141 × 64 µm (Supporting Information, Tables S13, S14).

Based on overall morphology, the cells can be divided into two more or less distinct morphotypes: slender (Figs 16A, 17A, B, G, 18A, B) and broad (Figs 16B–E, 17C–E, H–K, 18C–F). In population (i) only the slender morphotype was detected.

Occurrence: *Clevelandella fryntai* occurred in all six dissected SRU individuals (all from the same family group). Population (i) was detected in only one individual (subadult female), the very same in which *C. sidi* (see above) was found. The five remaining hosts

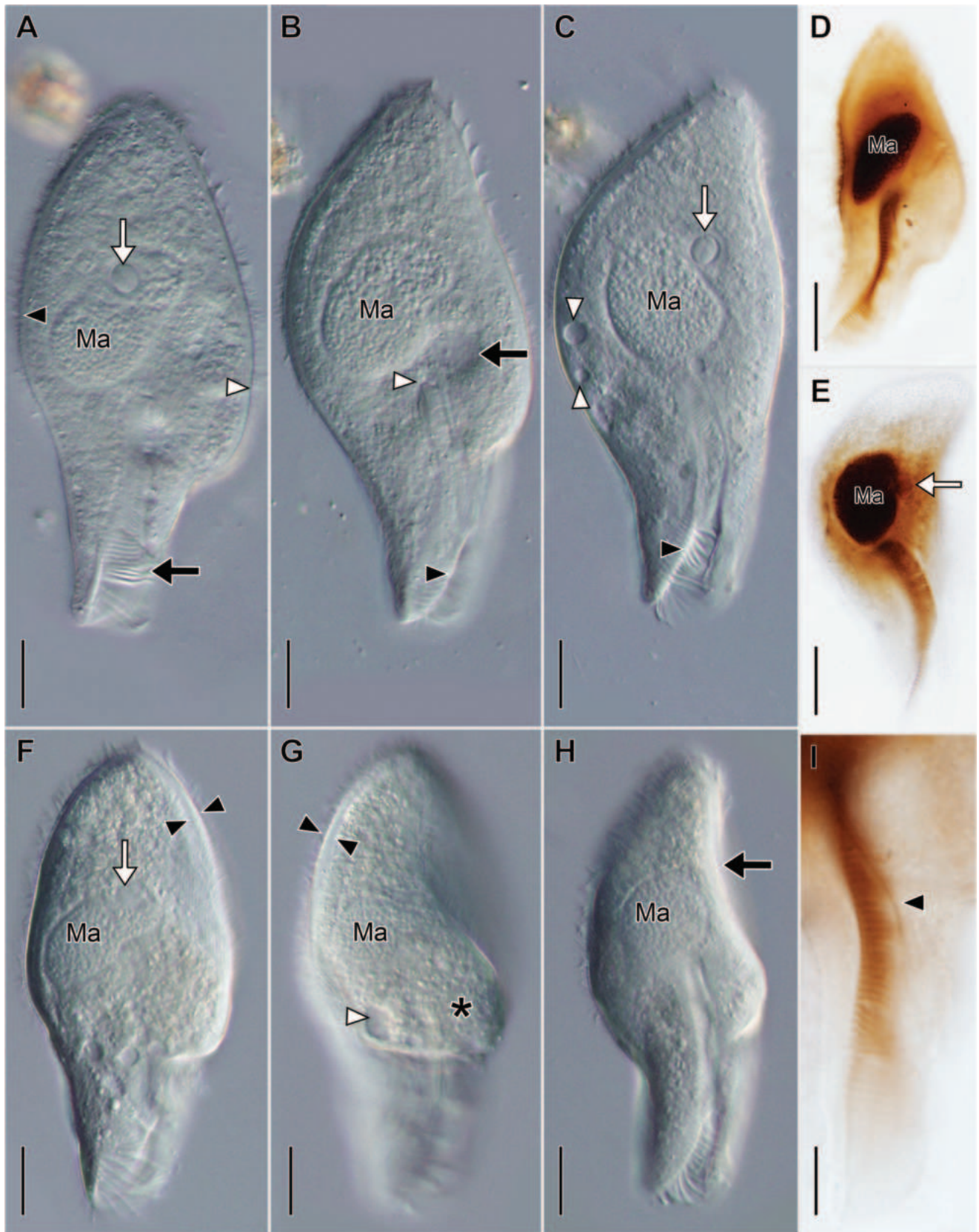


Figure 9. *Clevelandella hromadkai* sp. nov. short morph: from life (A–C, F–H) and after protargol impregnation (D, E, I). From ASS. A, left ventrolateral view showing micronucleus (white arrow), adoral membranelles (black arrow), and the posterior extent of ciliation on the right (black arrowhead) and left (white arrowhead) margins. B, ventral view, (optical section) showing the cytostome (white arrowhead), contractile vacuole (black arrow) and margin of the peristomial overtube (black arrowhead). C, left lateral view showing collecting vesicles of the contractile vacuole (white arrowheads), the micronucleus (white arrow) and the margin of the peristomial overtube (black arrowhead). D,

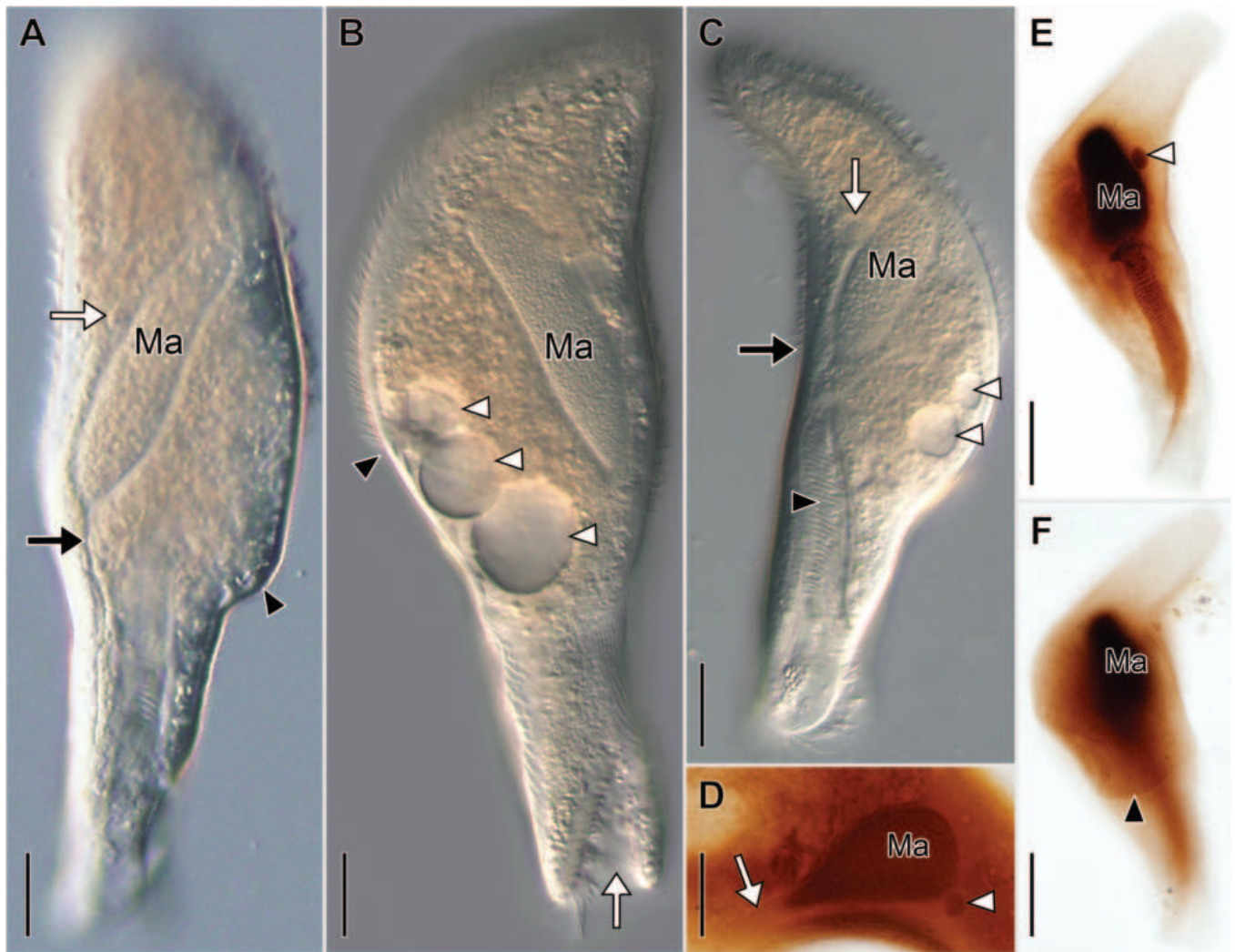


Figure 10. *Clevelandella hromadkai* sp. nov. long morph from life (A–C) and after protargol impregnation (D–F). From ASS (A, E, F), and PAC (B–D). A, ventral view showing micronucleus (white arrow), left protuberance (black arrowhead), and karyophore (black arrow). B, left ventrolateral view showing peristomial overtube (white arrow), collecting vesicles of contractile vacuole (white arrowheads), and the posterior extent of ciliation on the left ventrolateral margin (black arrowhead). C, right dorsolateral view showing contractile vacuole collecting vesicles (white arrowheads), the posterior extent of ciliation on the dorsolateral margin (black arrow), the micronucleus (white arrow), and the adoral membranelles (black arrowhead). D, detail showing micronucleus (white arrowhead) and karyophore (white arrow). E, left ventrolateral view showing micronucleus (white arrowhead). F, (same cell as E) showing posterior margin of left protuberance. Ma, macronucleus. Scale bars: 20 μ m (A–C, E, F), 10 μ m (D).

harboured population (ii): in four (male, two females, and a larva) of them the slender and broad morphotypes co-occurred and in one (larva) only the slender was present.

Redescriptions of known species of Clevelandellidae

In addition to the newly described species, a number of known species was observed, namely: *Anteclevelandella constricta*, *R. nipponensis*, *R. hastula*, *Paraclevelandia brevis*, *Paraclevelandia simplex*, *C. elongata*, *C. parapanesthia*, *C. kidderi* (we consider *C. lynni*

Pecina and Vďačný, 2020 as a junior synonym of *C. kidderi*, see discussion), and *C. panesthia*. Most, but not all available ciliate populations were morphologically characterized. For known species, except *C. elongata*, which has not been reported since its original discovery by Kidder (1937), descriptions are limited to those characters not previously reported, previously recorded inaccurately (e.g. somatic ciliature), or deviating significantly from previous descriptions. Apart from them, two species were detected but left undescribed, namely: *Rhynchoclevelandella* sp.

left ventrolateral view. E, left lateral view showing micronucleus (white arrow). F, left ventrolateral view showing thick hyaline subcortical layer (black arrowheads) and micronucleus (white arrow). G, ventral view showing left protuberance overlying left cell margin (asterisk), contractile vacuole (white arrowhead), and thick hyaline subcortical layer (black arrowheads). H, left lateral view showing concavity of dorsal surface (black arrow). I, detail of diplostichomonad paroral membrane showing the end of the shorter right file (black arrowhead). Ma, macronucleus. Scale bars: 20 μ m (A–H), 10 μ m (I).



Figure 11. *Clevelandella hromadkai* sp. nov. (from ASS) in the scanning electron microscope. A, right lateral view showing overall cell shape with concave dorsal margin (white arrowhead) and convex ventral margin (black arrowhead), ciliated anterior parts (black asterisk) and unciliated posterior parts (white asterisk) of somatic kineties, and prominent posterior peristomial projection (black arrow). B, posterodorsal view showing left posterior protuberance (black arrowhead) and peristomial overture (black arrow). C, left lateral view showing dorsoventral flattening, cilia protruding from the cytoproct (black arrowhead) on the left protuberance (white asterisk). D, posterodorsal view (down left) showing spiralling somatic kineties (white asterisk) and peristomial overture (black arrow) and right lateral view (black asterisk). E, posterodorsal view showing the cytoproct on the left protuberance (black arrow), the ciliated circumperistomial kineties (white arrowhead), and the adoral membranelles on the right wall of the infundibulum (black arrowhead). F, detail view of the left protuberance showing unciliated parts of kineties (white arrowheads) converging on the ciliated cytoproct (black arrowhead) and the fully ciliated dikinetids of the anterior body part (black arrow). Scale bars: 20 µm (A–E), 5 µm (F).

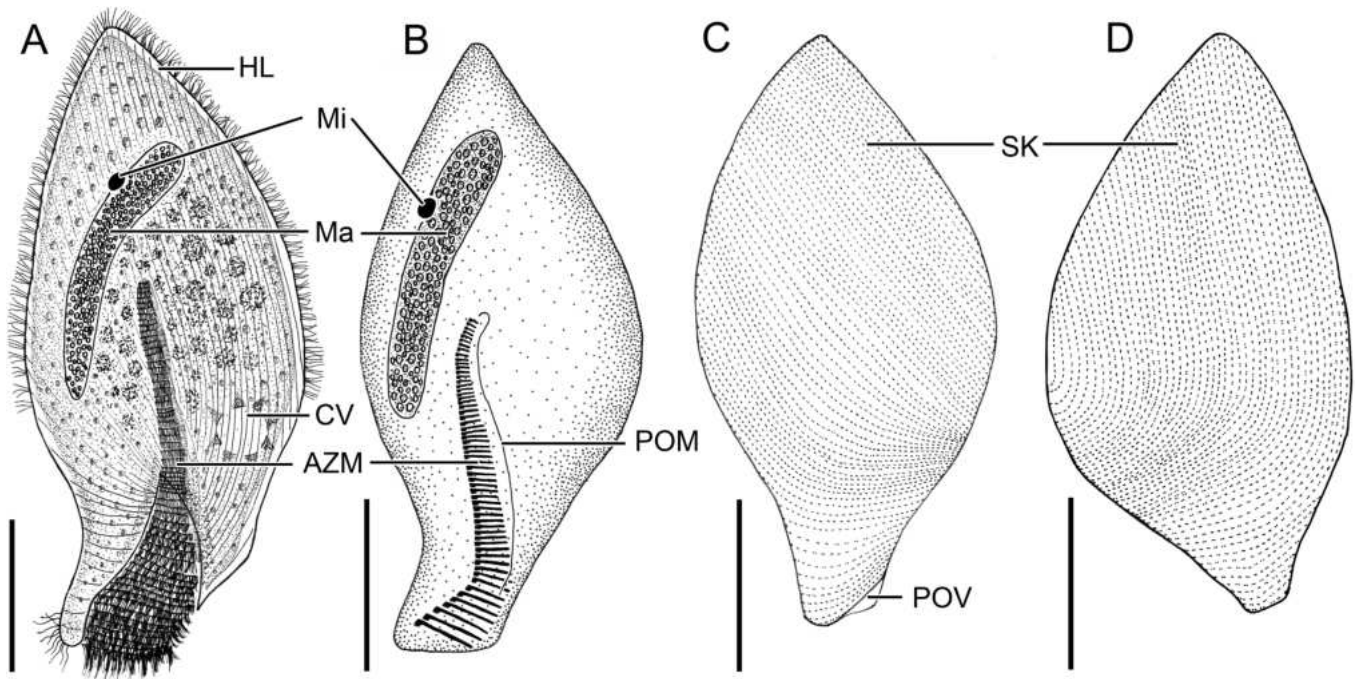


Figure 12. Schematic drawings of *Clevelandella klobasa* sp. nov. from life (A) and after protargol impregnation (B–D). A, ventral view of a typical individual. B, ventral view. C, ventral infraciliature. D, dorsal infraciliature. AZM, adoral zone of membranelles; CPK, cilia of circumperistomial kineties; CV, contractile vacuole; HL, hyaline subcortical layer; Ma, macronucleus; Mi, micronucleus; POM, paroral membrane; POV, peristomial overtire; SK, somatic kineties. Scale bars: 40 μ m.

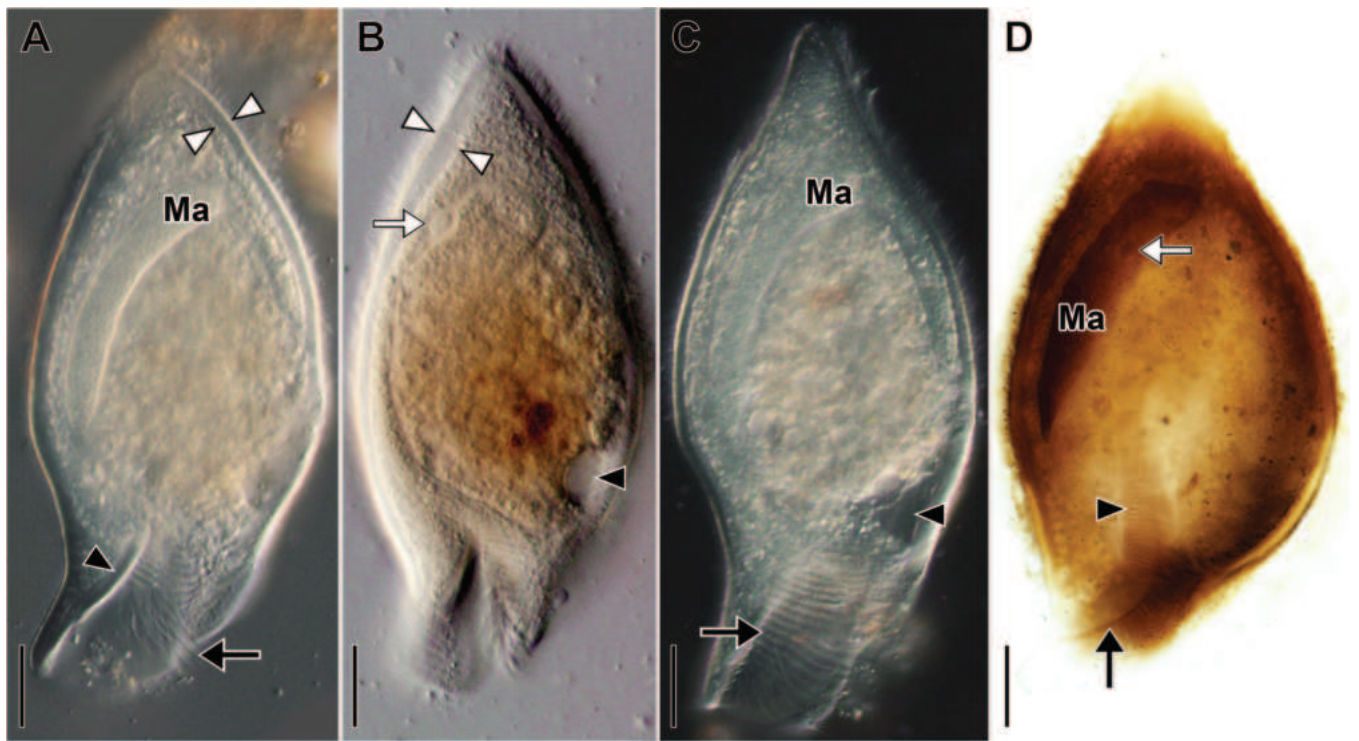


Figure 13. *Clevelandella klobasa* sp. nov. *in vivo* (A–C) and after protargol impregnation (D). From STH. A, left ventrolateral view showing thick hyaline subcortical layer (white arrowheads), cilia of adoral membranelles (black arrow), and margin of the peristomial overtire (black arrowhead). B, left lateral view showing thick hyaline subcortical layer (white arrowheads), contractile vacuole (black arrowhead), and micronucleus (white arrow). C, optical section showing adoral membranelles (black arrow) and contractile vacuole (black arrowhead). D, left ventrolateral view (optical section) showing micronucleus (white arrow), adoral membranelles (black arrowhead), and paroral membrane (black arrow). Ma, macronucleus. Scale bars: 20 μ m.

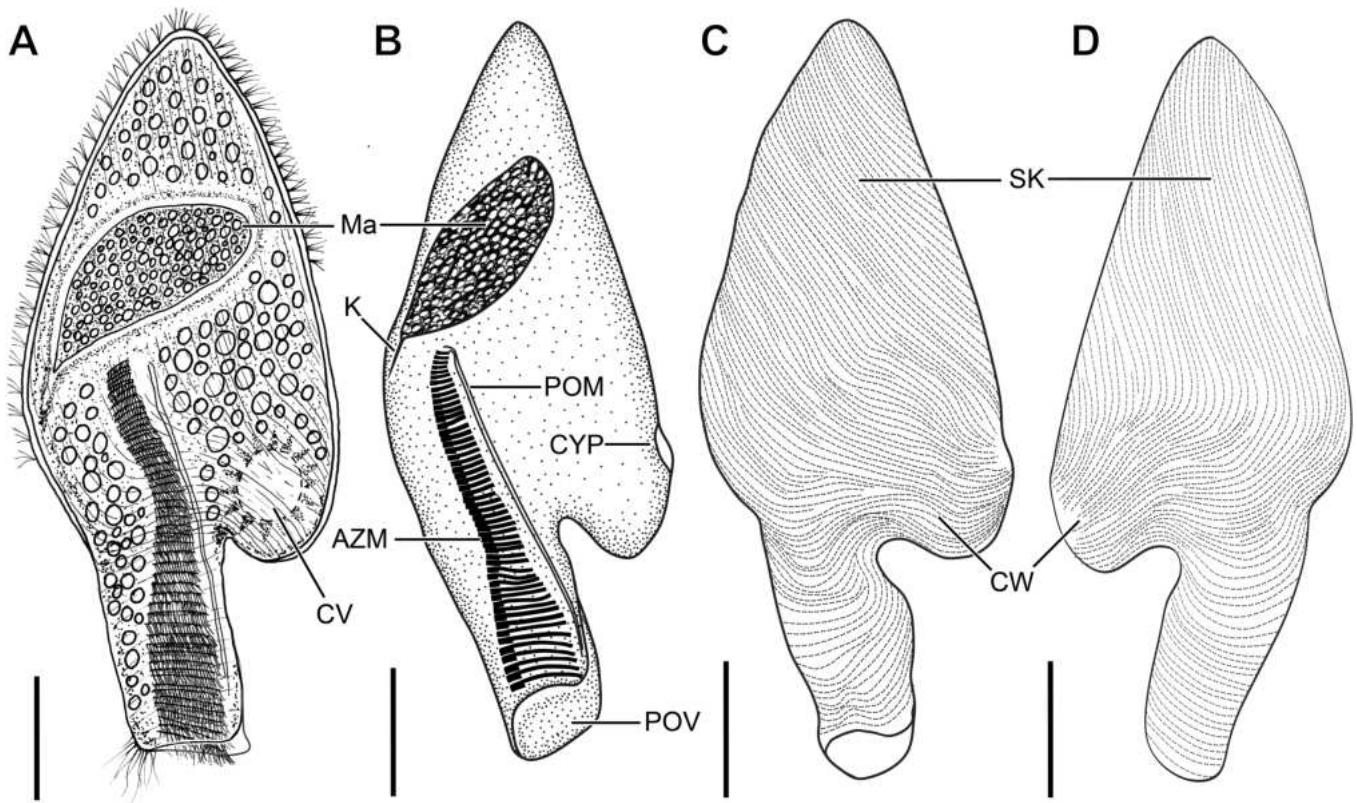


Figure 14. Schematic drawings of *Clevelandella sisi* sp. nov. from life (A) and after protargol impregnation (B–D). A, ventral view of a typical individual. B, ventral view. C, ventral infraciliature (holotype). D, dorsal infraciliature (holotype). AZM, adoral zone of membranelles; CV, contractile vacuole; CW, ciliary whorl of left protuberance; CYP, cytoproct; K, karyophore; Ma, macronucleus; POM, paroral membrane; POV, peristomial overtube; SK, somatic kineties. Scale bars: 20 μ m.

2 and *Clevelandella* sp. 4. The hosts infected and the occurrence of individual species are summarized in [Supporting Information, Table S48](#).

Anteclevelandella constricta (Kidder, 1937)

(Figs 19, 20; [Supporting Information, Tables S15–S21](#))

Six populations (ASS, PAA, PRG, SRU, STH #1, and STH #2) were morphologically characterized by both *in vivo* observations and protargol preparations, and one population (PAC) was characterized *in vivo* only. When present, the ciliate was usually abundant.

Description based on the above populations: Medium- to large-sized Clevelandellidae (on average $128 \times 38 \mu\text{m}$ *in vivo*, range $78\text{--}192 \times 21\text{--}67 \mu\text{m}$; on average $117 \times 33 \mu\text{m}$ in protargol preparations, range $94\text{--}142 \times 20\text{--}47 \mu\text{m}$). Shape elongated, cylindrical, transverse constriction at level of macronucleus, anterior cell end broadly tapered, posterior end transversely truncate with short peristomial overtube, peristomial projection indistinct since gradually merges with body proper. Macronucleus broadly ellipsoidal, long axis oriented transversely, karyophore at right and left ends of macronucleus. Aggregation of cytoplasmic platelets between macronucleus and anterior end of cell (possibly amylopectin).

Somatic cilia limited to approximately anterior 44% of cell length. About 15 ciliated free transverse kinetofragments,

comprising five basal bodies each, occupy right suture in anterior half of cell (Fig. 18D). Circumperistomial kineties only sparsely ciliated.

Oral ciliature as for the family. Adoral zone extends about 48% of cell length, composed of about 47 membranelles on average (range 36–56).

Remarks: Regarding measured characteristics ([Supporting Information, Tables S15–S21](#)), our populations are slightly larger than Kidder's (1937) isolates, very closely match those of Albaret (1975), and are similar to measurements published by Yamasaki (1939) and Pecina and Vdačný (2020b). Individual populations are very similar except for the number of adoral membranelles, which ranges from mean 39 (PAA) to mean 52 (STH population 1). Pecina and Vdačný (2020b) found similar variability in adoral membranelle number in their Thai population. The only visible shape variation can be seen in STH population 2, which lacks the typical constriction and has a slender, more acutely pointed anterior end, differing somewhat from the typical phenotype (Fig. 19C).

Rhynchoclevelandella nipponensis (Kidder, 1937)

(Fig. 21D–F, J, K; [Supporting Information, Table S22](#))

Rhynchoclevelandella nipponensis was detected and morphologically characterized in a single host population (PAC), never in high abundance.

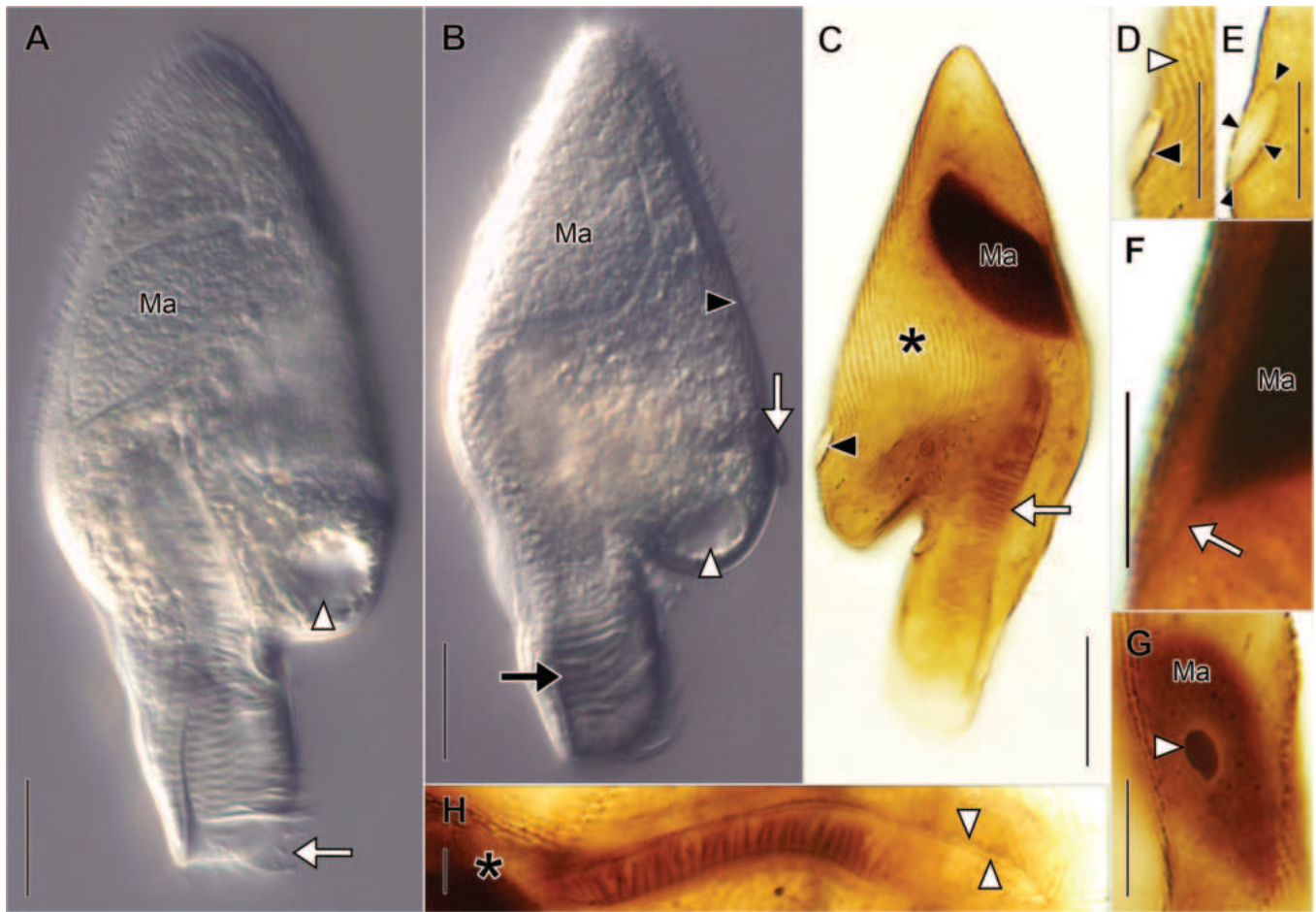


Figure 15. *Clevelandella sidi* sp. nov. *in vivo* (A, B) and after protargol impregnation (C–H). From SRU (A–H). A, ventral view showing contractile vacuole (white arrowhead) and cilia of adoral membranelles (white arrow). B, ventral view showing contractile vacuole (white arrowhead), adoral membranelles (black arrow), and elevated margin of the cytoproct (white arrow). C–E, the same cell in dorsal view: C, showing somatic infraciliature (asterisk), cytoproct (black arrowhead), and adoral membranelles (white arrow); D, showing cytoproct (black arrowhead) and dorsal kineties (white arrowhead); E, optical section showing excretory antrum within the left protuberance (black arrowhead). F, detail of the macronucleus showing the karyophore (white arrow). G, detail showing the micronucleus (white arrowhead). H, detail of the paroral membrane showing both files of equal length and extending nearly to the posterior end of the peristomial projection (white arrowheads), a feature unique to this species. Asterisk marks the anterior end. Ma, macronucleus. Scale bars: 20 μm (A–D), 10 μm (E–G), 5 μm (H, I).

Description based on PAC population: Medium-sized Clevelandellidae (on average $82 \times 33 \mu\text{m}$ *in vivo*, range $67\text{--}100 \times 27\text{--}42 \mu\text{m}$; on average $65 \times 25 \mu\text{m}$ in protargol preparations, range $47\text{--}83 \times 20\text{--}31 \mu\text{m}$). Slender spade-shaped (L/W about 2.6) with long posterior peristomial projection (about 31% of cell length). Anterior right and left margins of body proper straight or concave. Macronucleus broadly ellipsoidal (Fig. 21J, K) to broadly teardrop-shaped (Fig. 21D, F), obliquely oriented to right of midline, prominent numerous globular nucleoli *in vivo*. Karyophore extremely inconspicuous, attached to posterior of macronucleus (Fig. 21K). Micronucleus ellipsoidal (Fig. 21F, K), relatively large, about $5.5 \mu\text{m}$ across. Somatic cilia limited to approximately anterior 64% of cell length. Circumperistomial kineties only sparsely ciliated. Free right sutural kinetofragments not identified, presence of inconspicuous kinetofragments not completely excluded due to suboptimally oriented cells in protargol preparations. Adoral zone extends about 47% of cell length on average, composed of an average of 23 membranelles (range 20–25). POM unstudied.

Remarks: Overall morphology corresponds with original description and drawing by Kidder (1937) except (i) the ciliature: while Kidder (1937) depicts the cell as fully ciliated, we show that only the anterior two-thirds of the cell are ciliated and (ii) the macronuclear shape varies from broadly ellipsoidal (Fig. 21K) to broadly teardrop-shaped (Fig. 21D, F, J).

Regarding measured characteristics (Supporting Information, Table S22), our population is slightly shorter than that of Kidder (1937) and Yamasaki (1939).

A notch sometimes forms at the base of peristomial projection (Fig. 21F, J)—rarely *in vivo* but frequently in protargol-stained cells, creating a *R. hastula*-like appearance. However, *R. nipponensis* can be mostly distinguished from *R. hastula* by: length (approx. $65 \mu\text{m}$ vs. $85 \mu\text{m}$), length/width ratio (approx. 2.6 vs. 3.4), number of adoral membranelles (approx. 23 vs. 26), and characteristic curvature of the most posterior third of peristomial opening in larger cells of *hastula* (Fig. 21L, M). Very small cells of both species (e.g. Fig. 21D, N) can be rather indistinguishable.

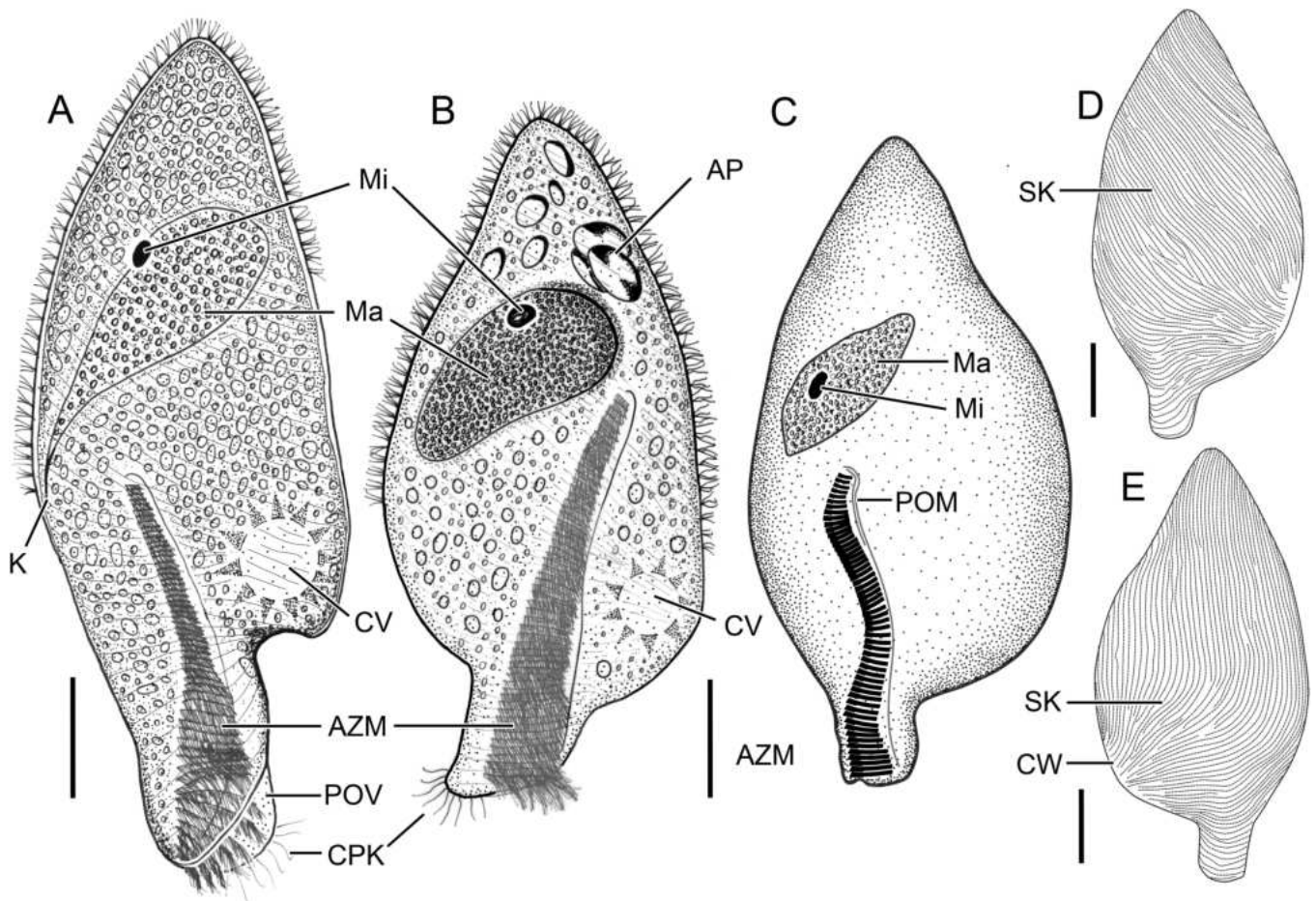


Figure 16. Schematic drawings of *Clevelandella fryntai* sp. nov. from life (A, B) and after protargol impregnation (C–E). From SRU. A, ventral view. B, ventral view. C, ventral view. D, ventral infraciliature. E, dorsal infraciliature. AP, cytoplasmic granules, possibly amylopectin; AZM, adoral zone of membranelles; CPK, cilia of circumperistomial kineties; CV, contractile vacuole; CW, ciliary whorl of left protuberance; K, karyophore; Ma, macronucleus; Mi, micronucleus; POM, paroral membrane; POV, peristomial overtire; SK, somatic kineties. Scale bars: 20 μm .

Rhynchoclevelandella sp. 2

(Fig. 21A–C, G–I; Supporting Information, Table S23)

Rhynchoclevelandella sp. 2 was detected and morphologically characterized in a single host population (ASS), with a very low abundance—no more than 10 individuals in a single host.

Rhynchoclevelandella sp. 2 is morphologically very similar to *R. nipponensis*, albeit a bit smaller: 73 (excluding one unusually large individual) vs. 82 μm *in vivo* and 56 vs. 65 μm in protargol preparations with larger micronucleus (7×4.8 vs. 5.5×3 μm). A single gargantuan cell (146 $\times$ 58 μm) was seen (Fig. 21A) and molecularly characterized.

Rhynchoclevelandella hastula (Kidder, 1937)

(Figs 21L–T, 22; Supporting Information, Table S24)

Rhynchoclevelandella hastula was detected and morphologically characterized in one host population (PGW). The ciliate was present in every dissected PGW individual and moderately abundant.

Description based on PGW population: Small- to medium-sized Clevelandellidae (on average 86×31 μm *in vivo*, 80×28 μm excluding single unusually large (142 $\times$ 53 μm)

individual, range 62–142 $\times$ 23–53 μm ; on average 82×24 μm in protargol preparations, range 64–94 $\times$ 18–31 μm). Slender spear or spade shape (L/W 2.5–3.4 *in vivo*, 2.9–4.2 in protargol preparations), left-sided notch at base of peristomial projection (Fig. 21L, M, P), inconspicuous or absent in smaller cells (Fig. 21N, Q). Posterior peristomial projection conspicuously elongated (about 40% of cell length on average), typically curves rightward (Fig. 21L, M, N, P, Q), in large cells visibly curved in last third (Fig. 21L, M). Macronucleus broadly ellipsoidal, posterior end sometimes pointed (Fig. 21P, S). Karyophore rarely visible, attached to posterior end of macronucleus (Fig. 21O, S). Micronucleus ellipsoidal, about 5.1 μm across. Somatic cilia limited to approximately anterior 60% of cell length. Dorsal kineties almost straight in protargol preparations (Fig. 21R). Free right sutural kinetofragments present (Fig. 21P). Adoral zone extends about 44% of cell length on average, composed of an average of 26 membranelles (range 23–28).

Regarding measured characteristics (Supporting Information, Table S24), our population is slightly broader than that of Kidder (1937) (L/W 2.8 *in vivo*, 3.4 in protargol preparations vs. 3.8).

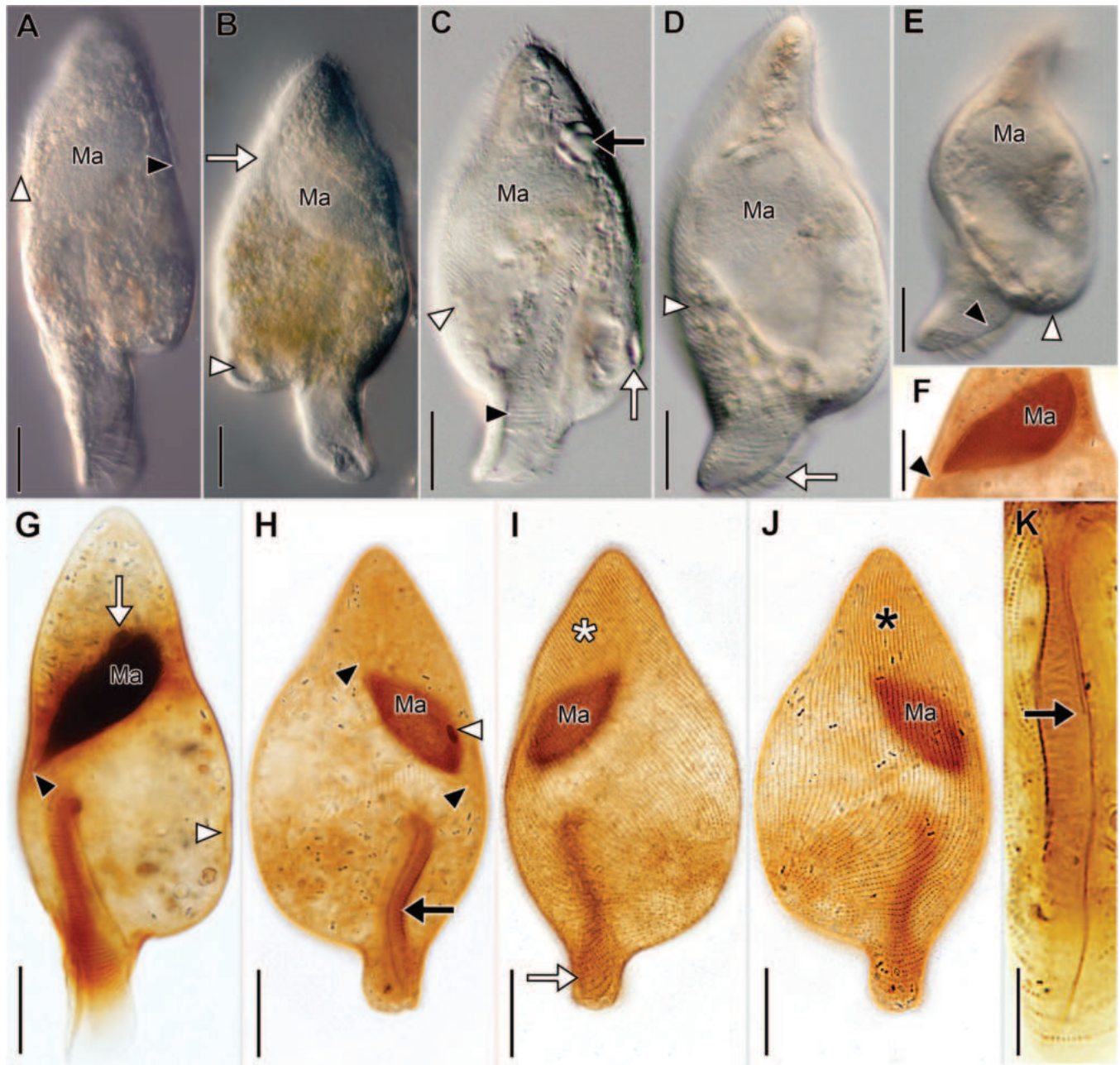


Figure 17. *Clevelandella fryntai* sp. nov. from life (A–E) and after protargol impregnation (F–K). From SRU. (A, B, F, G, K) slender morphotype, (C–E, H–J) broad morphotype. A, ventral view showing posterior extent of ciliation on the right margin (white arrowhead) and left margin (black arrowhead). B, dorsal view showing contractile vacuole (white arrowhead) and straight dorsal kineties (white arrow). C, ventral view (optical section) showing cytoplasmic granules, possibly amylopectin (black arrow), right kinetal suture (white arrowhead), adoral membranelles (black arrowhead), and cytoproct (white arrow). D, ventral view showing left-hand spiralling kineties (white arrowhead) and cilia of adoral membranelles (white arrow). E, ventral view showing the margin of the peristomial overtube (black arrowhead) and bulbous left protuberance extending over left cell margin (white arrowhead). F, detail showing karyophore (black arrowhead). G, ventral view (optical section) showing the karyophore (black arrowhead), micronucleus (white arrow), and cytoproct (white arrowhead). H, dorsal view showing karyophore (black arrowheads), micronucleus (white arrowhead), and adoral membranelles (black arrow). I, ventral infraciliature showing left-hand spiralling kineties (asterisk) and looping kineties of the peristomial projection (white arrow). J, dorsal infraciliature showing straight dorsal kineties (asterisk). K, detail view showing diplostichomonad paroral (black arrow). Ma, macronucleus. Scale bars: 20 µm (A–E, G–J), 10 µm (F, K).

Paraclevelandia brevis Kidder, 1937

Synonym: *Paraclevelandia simplex* Kidder, 1937.

Paraclevelandia brevis and *Paraclevelandia simplex* as described by Kidder (1937) represent two forms of a single species (see Discussion), *Paraclevelandia brevis*. For simplicity the two morphotypes are hereafter called ‘brevis’ and ‘simplex’ forms.

Paraclevelandia brevis in ‘brevis’ form

(Figs 23A–G, 24A, B, D, E;
Supporting Information, Tables S25–S29)

When present, the ‘brevis’ form was highly abundant.

Description based on ASS, PAA, PAC, PRG, STH *in vivo*, and PRG and STH *in protargol* preparation: Small Clevelandellidae

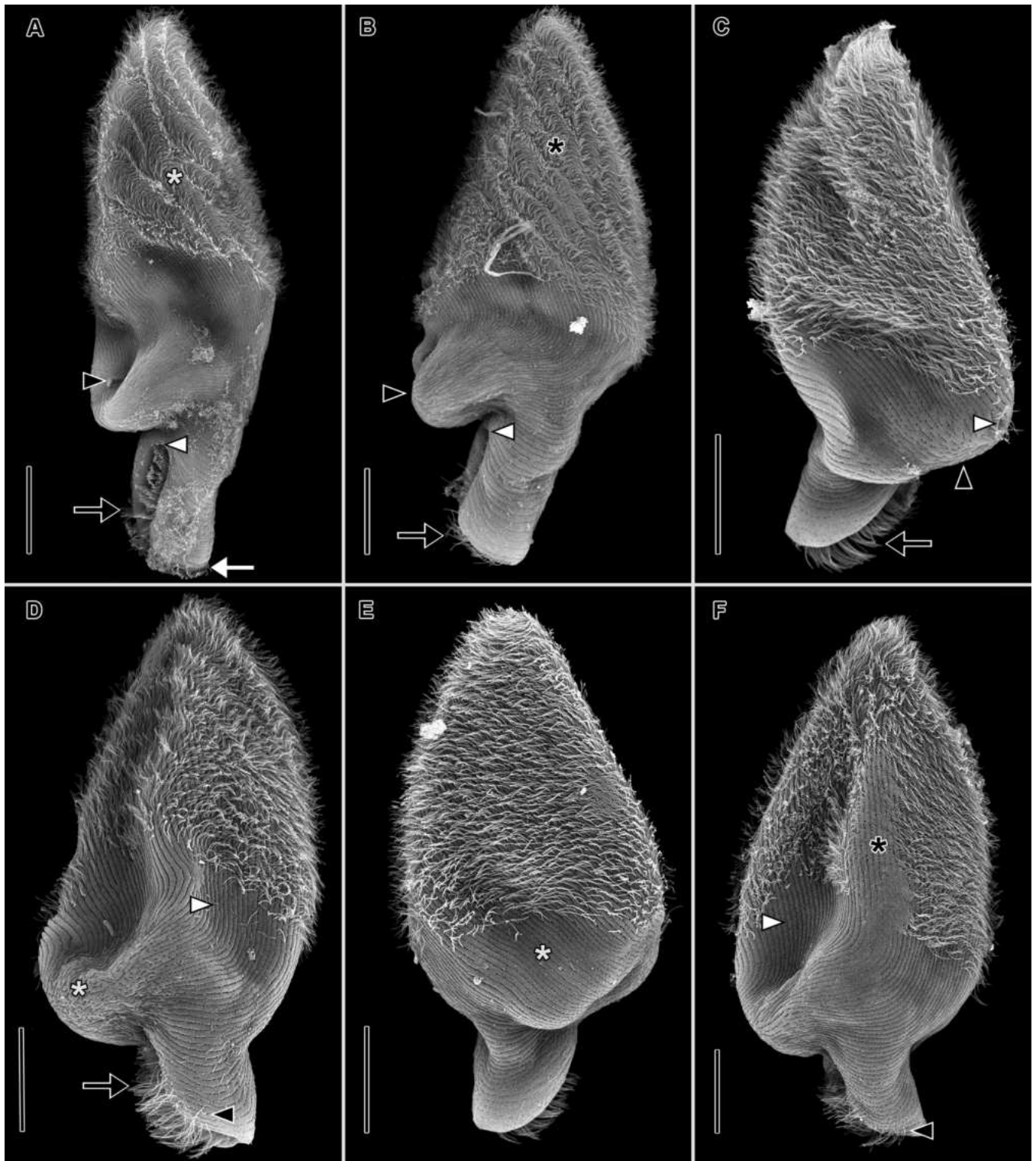


Figure 18. *Clevelandella fryntai* sp. nov. in the scanning electron microscope. From SRU. (A, B) slender morphotype, (C–F) broad morphotype. A, dorsal view showing cytoproct on left protuberance (black arrowhead), anterior margin of peristomial overture (white arrowhead), circumperistomial kineties (white arrow), cilia of adoral membranelles (black arrow), and metachronal ciliary waves (asterisk). B, dorsal view showing the left protuberance (black arrowhead), metachronal ciliary waves (asterisk), the anterior margin of the peristomial overture (white arrowhead), and cilia of the circumperistomial kineties (black arrow). C, ventral view showing characteristic lateral twisting of left protuberance also seen *in vivo* (black arrowhead, compare Fig. 17E), the cytoproct (white arrowhead) and cilia of the adoral membranelles (black arrow). D, dorsal view showing straight course of the dorsal somatic kineties (white arrowhead), the left protuberance (white asterisk), cilia of the circumperistomial kineties (black arrowhead), and cilia of the adoral membranelles (black arrow). E, ventral view showing left-hand spiralling ventral somatic kineties (white asterisk). F, dorsal view showing straight dorsal somatic kineties (white arrowhead), cilia of circumperistomial kineties (black arrowhead), and area of somatic cilia matted during preparation (black asterisk). Scale bars: 20 μ m.

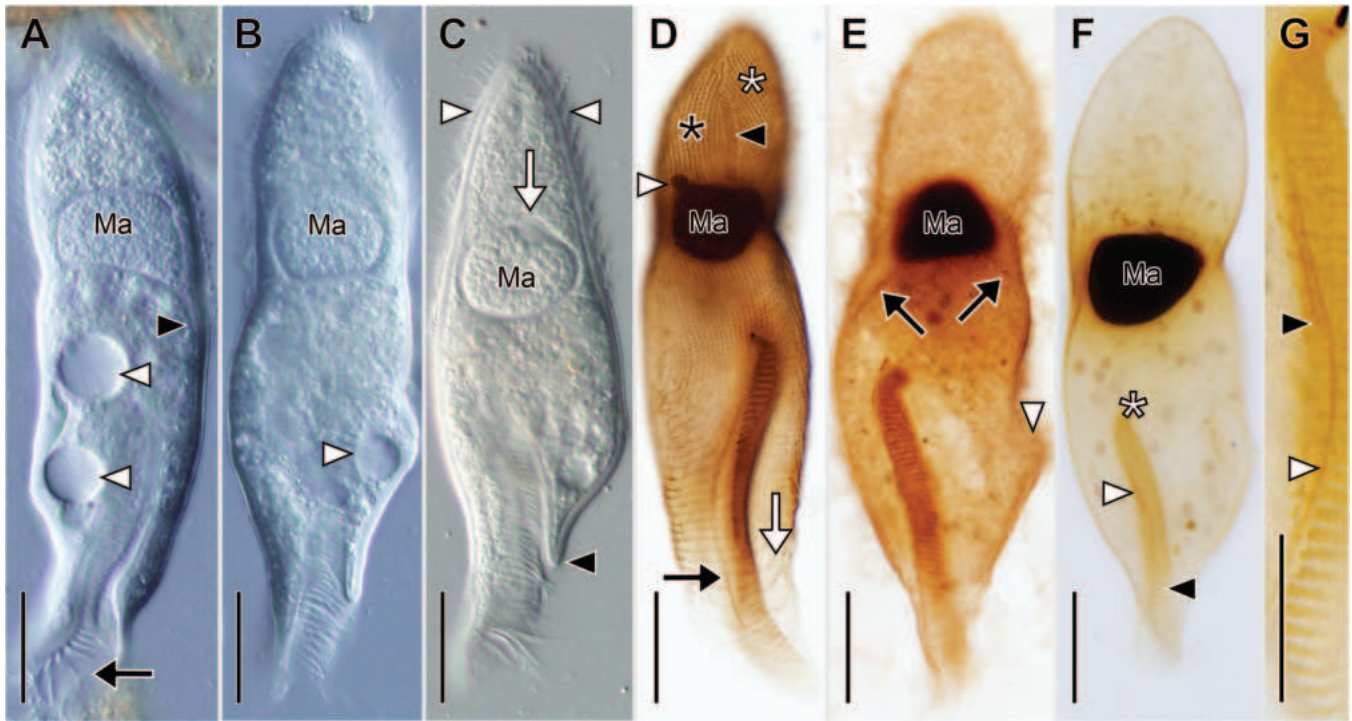


Figure 19. *Anteclelandella constricta* in vivo (A–C) and after protargol impregnation (D–G). From PAA (A), STH population 1 (B) from STH population 2 (C), from ASS (D), from STH population 1 (E), and from SRU (F, G). A, left lateral view (optical section) showing two large contractile vacuole collecting vesicles (white arrowheads), adoral membranelles (black arrow) and left part of the karyophore (black arrowhead). B, ventral; view (optical section) showing contractile vacuole on the small left protuberance (white arrowhead). C, ventral view (optical section) showing shape variation with more narrowly tapered anterior end (white arrowheads), the micronucleus (white arrow), and the margin of the peristomial overture (black arrowhead). D, right lateral view showing kinetofragments in suture between ventral (white asterisk) and dorsal (black asterisk) somatic kineties, micronucleus (white arrowhead), paroral membrane (black arrow), and looping kineties on the peristomial projection (white arrow). E, ventral view (optical section) showing the transverse orientation of the macronucleus with right and left parts of the karyophore (black arrows) and slightly pronounced left lobe (white arrow). F, ventral view showing position of the cytostome (white asterisk), paroral membrane (white arrowhead), and adoral membranelles (black arrowhead). G [same cell as (F)], detail view of the long (white arrowhead) and short (black arrowhead) files of the paroral membrane. Ma, macronucleus. Scale bars: 20 μm (A–F), 10 μm (G).

(on average $44 \times 28 \mu\text{m}$ in vivo, range $34\text{--}58 \times 21\text{--}48 \mu\text{m}$; on average 34×21 in protargol preparations, range $26\text{--}47 \times 15\text{--}28 \mu\text{m}$). Broadly ovate, slightly dorsoventrally flattened, anterior end tapers to rounded point, posterior end transversely truncate. Posterior peristomial projection absent. Peristomial overture on left posterior margin of cell. Macronucleus elongated ellipsoidal, chromatin coarsely granular. Micronucleus ellipsoidal, relatively large ($4\text{--}6 \mu\text{m}$ across), dorsal to macronucleus (Fig. 23D). Karyophore not detected. A sack-like structure formed between anterior margin of macronucleus and anterior end of cell as reported by Kidder (1937, 1938), not evident in protargol preparations (Fig. 23A). Cortex with refractile interkinetal granules, probably mucocysts (Fig. 23B). Somatic kineties strongly spiralled leftward (Fig. 24B). Somatic cilia absent on right, ventral, left parts of posterior cortex (Fig. 24A, B, E), extend to posterior cell margin dorsally. Kinetal furrows prominent. Two ciliated circumperistomial kineties. About 10 ciliated kinetofragments in midportion of right suture, increase in length from posterior to anterior (Figs 23E, F, 24B, D). Organization of oral ciliature as for the family except, in absence of peristomial projection, oral structures are situated in body proper (Fig. 23A, B, D, H, J). Adoral zone narrows from posterior to anterior, extends about

50% of cell length on average, composed of an average of 15 membranelles (range 14–17).

Paraclelandia brevis in 'simplex' form

(Figs 23H–P, 24C, F; Supporting Information, Tables S30–S33)

When present, the simplex form was present in low to moderate abundance.

Description based on ASS, PAA and STH in vivo, and PAA, STH, and SRU in protargol preparations: Medium-sized Clelandellidae (on average $84 \times 47 \mu\text{m}$ in vivo, range $70\text{--}101 \times 38\text{--}58 \mu\text{m}$; on average 76×45 in protargol preparations, range $61\text{--}98 \times 37\text{--}53 \mu\text{m}$). Ovate to almost broadly fusiform, anterior end tapers to rounded point, posterior end broadly rounded, peristomial projection absent as in 'brevis' form; distinct left lobe absent. Macronucleus elongated, cylindrical, situated longitudinally in anterior half of cell near right cell margin. Micronucleus oval, near midportion of macronucleus, smaller compared to brevis form (3.4 vs. $5.3 \mu\text{m}$). A sack-like structure formed between anterior margin of macronucleus and anterior end of cell

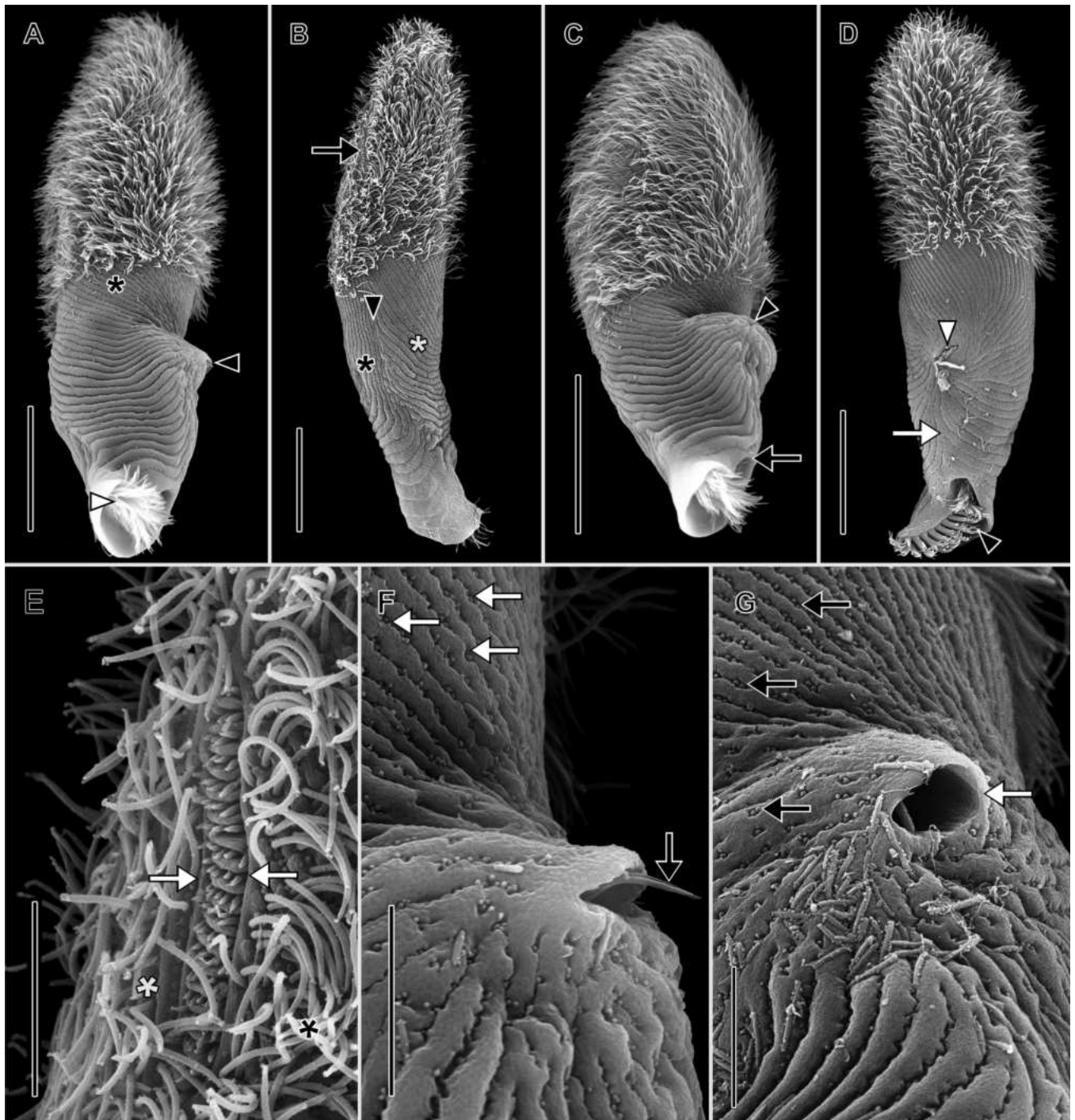


Figure 20. *Anteclevelandella constricta* in the scanning electron microscope. From PAA. A, ventral view showing cilia of the adoral membranelles protruding from the peristomial overtube (white arrowhead), the unciliated posterior body part (black asterisk), and cilia protruding from cytoproct on the left lateral protuberance (black arrowhead). B, dorsal view showing the right kinetal suture (black arrowhead) between the dorsal kinetias (black asterisk) and ventral kinetias (white asterisk) right suture, and sutural kinetofragments (black arrow). C, left ventrolateral view showing position of cytoproct on left posterior protuberance (black arrowhead) and the anterior margin of the peristomial overtube (black arrow). D, left lateral view showing the location of the cytoproct (white arrowhead), short left kinetal suture (white arrow), and adoral membranelles (black arrowhead). E, detail of same cell as (B) showing short transverse kinetofragments in anterior part of right suture (white arrows) between dorsal (black asterisk) and ventral (white asterisk) kinetias. F, showing the leftward-spiralling ventral kinetias which lack normal cilia (white arrows) and a cilium protruding from the cytoproct (black arrow). Scale bars: 20 μ m (A–D), 5 μ m (E–G).

as reported by Kidder (1937, 1938), visible both *in vivo* (Fig. 23H, I) and protargol preparations (Fig. 23J). Karyophore inconspicuous, not visible *in vivo*, sometimes visible in protargol

preparations (Fig. 23J, O), attached to both anterior and posterior end of macronucleus. Cortex with refractile interkinetal granules, probably mucocysts (Fig. 23I). Somatic ciliature in

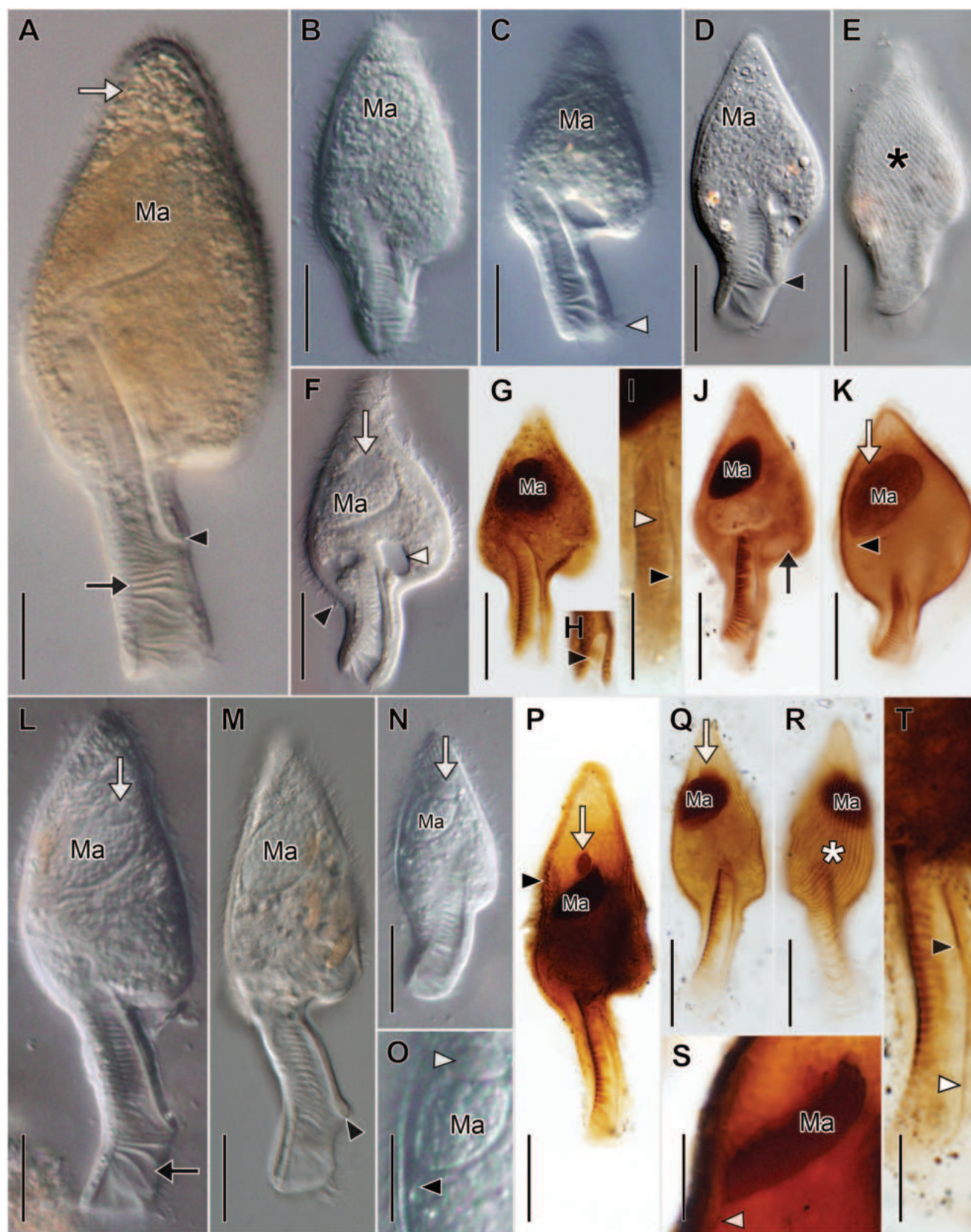


Figure 21. *Rhynchoclevelandella* sp. 2 (A–C, G–I) from ASS. *Rhynchoclevelandella nipponensis* (D–F, J, K) from PAC. *Rhynchoclevelandella hastula* (L–T) from PGW. In vivo (A–F, L–O) and after protargol impregnation (G–K, P–T). A, ventral view (optical section) showing margin of the peristomial overtura (black arrowhead) and adoral membranelles (black arrow). B, ventral view. C, ventral view showing cilia of circumperistomial kineties (white arrowhead). D, ventral view showing anterior margin of peristomial overtura (black arrowhead). E, same cell showing ventral kineties (asterisk). F, ventral view showing contractile vacuole (white arrowhead), the relatively large micronucleus



Figure 22. *Clevelandella hastula* in the scanning electron microscope. From PGW. A, ventral view showing collapsed left lobar protrusion (black arrow), right margin of the peristomial overture (black arrowhead), left-hand spiralling unciliated part of ventral kineties (white arrowhead), and cilia of adoral membranelles (white arrow). B, dorsal view showing left lobar protrusion (black arrow) and unciliated parts of dorsal somatic kineties (black arrowhead) on the characteristically elongated peristomial protrusion (white arrow). C, left lateral view of posterior part of cell showing cilia protruding from the cytoproct on the dome of the left lobar protrusion (black arrow), the anterior margin of the peristomial overture (black arrowhead), cilia of the adoral membranelles (small black arrow), cilia of the circumperistomial kineties (white arrow), and the inconspicuous left posterior suture (white arrowhead) between dorsal kineties (black asterisk) and ventral kineties (white asterisk). Scale bars: 20 µm.

same pattern and distribution as ‘brevis’ form. Kinetal furrows conspicuous. Up to 20 free kinetofragments in right suture (Fig. 23K, P). Organization of oral ciliature as for the family with same exceptions as ‘brevis’ form. Adoral zone narrows from posterior to anterior, extends about 50% of cell length on average, composed of an average of 28 membranelles (range 25–32).

Clevelandella elongata (Kidder, 1937)

(Figs 25–27; Supporting Information, Table S34)

Clevelandella elongata was detected and morphologically characterized in one population (SRU). When present, *C. elongata*

was moderately abundant (20–30 cells per host) with the other ciliate species being more abundant.

Description based on SRU population:

Largest member of Clevelandellidae (on average $285 \times 54 \mu\text{m}$ *in vivo*, range $245\text{--}321 \times 43\text{--}71 \mu\text{m}$; on average $220 \times 61 \mu\text{m}$ in protargol preparations, range $184\text{--}259 \times 43\text{--}75 \mu\text{m}$). Elongated, almost vermiform *in vivo*, slightly dorsoventrally flattened, anterior end bluntly pointed, posterior end truncate and slightly flared, ventral margin convex, dorsal margin concave (Figs 25C, 27C). Cells shrink asymmetrically in protargol preparations (see Supporting Information, Table S50) and long axis straightens

(white arrow), and the posterior extent of ciliation on the right margin (black arrowhead). G, H, the same cell in ventral view, with detail of peristomial overture (black arrowhead). I, detail view of the long file (black arrowhead) and short file (white arrowhead) of the paroral membrane. J, ventral view (optical section) showing excretory antrum within left protuberance (black arrow). K, ventral view showing micronucleus (white arrow) and karyophore (black arrowhead). L, ventral view (optical section) showing micronucleus and adoral membranelles (black arrow). M, ventral view (optical section) showing anterior margin of the peristomial overture (black arrowhead). N, ventral view showing relatively large micronucleus (white arrow). O, detail of same cell as (N) showing micronucleus (white arrowhead) and karyophore (black arrowhead). P, ventral view showing micronucleus (white arrow). Q, ventral view showing micronucleus (white arrow). R, dorsal view of same cell as (Q) showing straight dorsal kineties (asterisk). S, detail of karyophore (white arrowhead). T, detail view of the long file (white arrowhead) and short file (black arrowhead) of the paroral membrane. Ma, macronucleus. Scale bars: 20 µm (A–H, J–N, P–R), 10 µm (I, O, S, T).

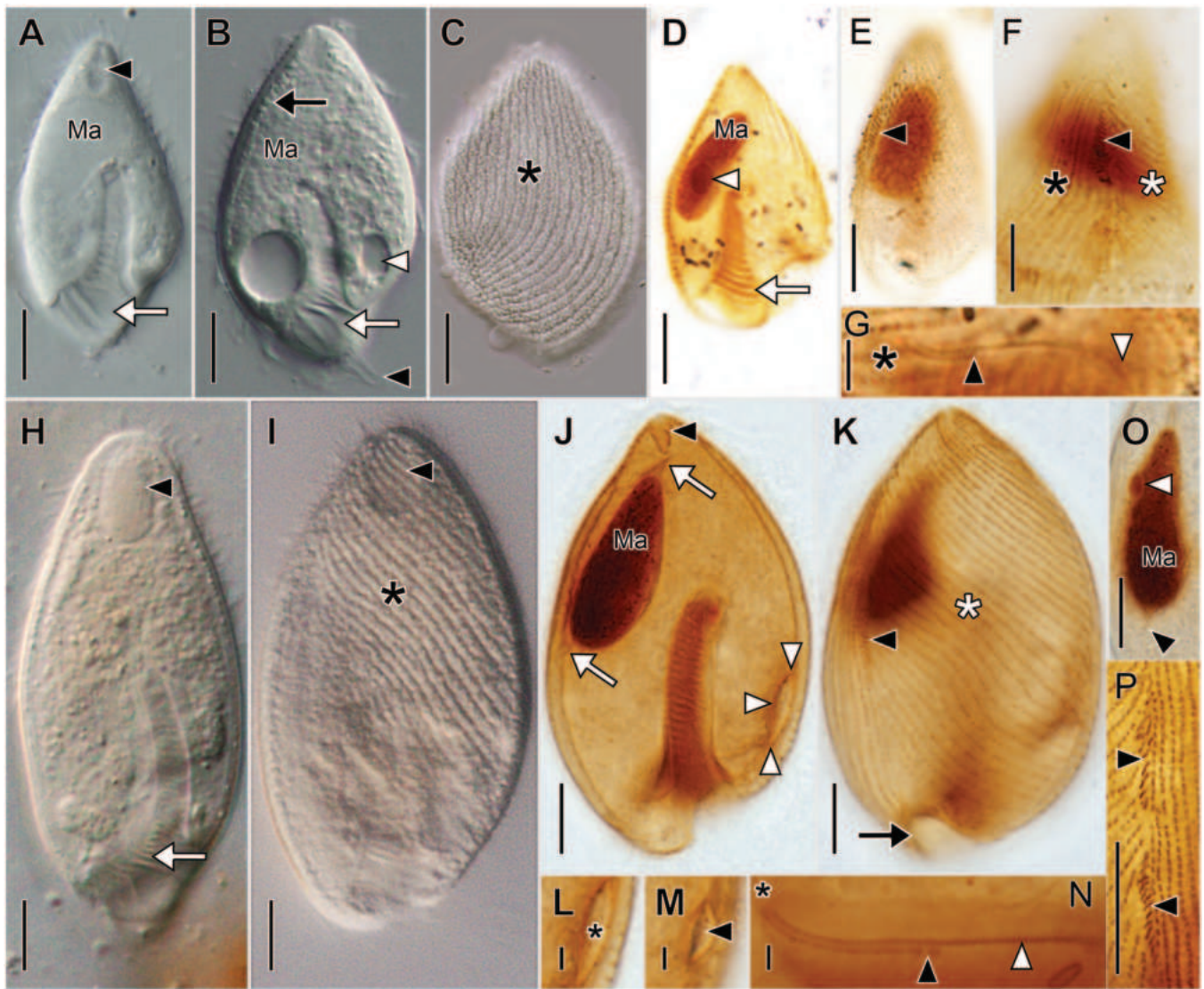


Figure 23. *Paraclevellandia brevis*, from life (A–C, H, I) and after protargol impregnation (D–G, J–P). Small ‘brevis’ morphotype (A–G), large ‘simplex’ morphotype (H–P); from ASS (A, H, I), SRU (J–M, K, O, P), STH (B, N), PAA (C), PGW (D–G). A, ventral view (optical section) showing the apical sac-like structure (black arrowhead) and adoral membranelles (white arrow). B, ventral view showing the subcortical layer of globules (probable mucocysts, black arrow), contractile vacuole (white arrowhead), adoral membranelles (white arrow), and circumperistomial cilia (black arrowhead). C, dorsal view showing relatively straight course of somatic kineties (asterisk). D, Ventral view (optical section) showing the micronucleus (white arrowhead) and adoral membranelles (white arrow). E, right ventrolateral view showing sutural kinetofragments (black arrowhead). F, right lateral view showing sutural kinetofragments (black arrowhead), ventral (white asterisk) and dorsal (black asterisk) kineties. G, detail view showing long (white arrowhead) and short (black arrowhead) files of the paroral membrane, asterisk indicates direction to anterior end of cell. H, ventral view (optical section) showing adoral membranelles (white arrow) and apical sac-like structure (black arrowhead). I, ventral view showing left-hand spiralling somatic kineties (asterisk) and apical sac-like structure (black arrowhead). J–M, the same cell in ventral view: J, optical section showing inconspicuous anterior and posterior karyophore (white arrows), the separate apical sac-like structure (black arrowhead), and elliptical excretory antrum (white arrowheads); K, surface view showing sutural kinetofragments (black arrowhead), left-hand spiralling ventral somatic kineties (asterisk), and peristomial overture (black arrow); L, excretory antrum (asterisk); M, elliptical cytoproct on left protuberance (black arrowhead). N, detail view showing long (white arrowhead) and short (black arrowhead) files of the diplostichomonad paroral membrane. Asterisk indicates anterior end. O, detail of macronucleus and micronucleus (white arrowhead) and karyophore (black arrowhead). P, kinetofragments in right suture (black arrowheads). Ma, macronucleus. Scale bars: 5 μ m (A–F), 1.25 μ m (G), 10 μ m (H–K, O, P), 2 μ m (L–N).

compared with more sinuous living specimens. Peristomial projection merges gradually with body proper hampering precise length measurement but, on average 20% of cell length (range 14–23%). Macronucleus broadly lenticular, situated slightly obliquely in anterior half of cell, large relative to cell

(on average $51 \times 24 \mu$ m), karyophore at anterior and posterior ends of macronucleus, usually distinct *in vivo* and in protargol preparations but sometimes indiscernible. Micronucleus large, globular (on average 7 μ m diameter), adjacent to anterior end of macronucleus. Somatic cilia arranged in very closely spaced

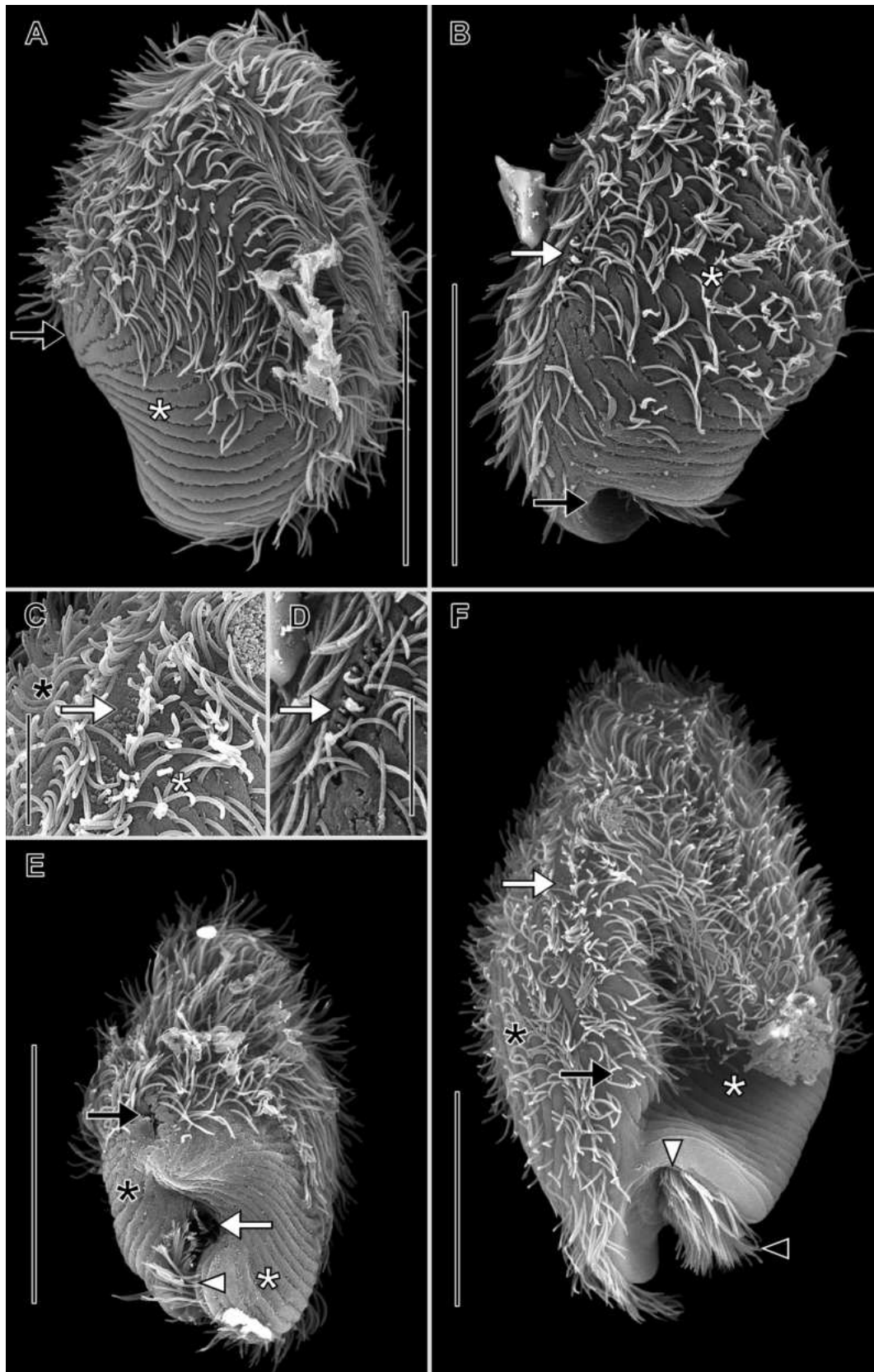


Figure 24. *Paraclevelandia brevis* in the scanning electron microscope. From PAC. Small 'brevis' morphotype (A, B, D, E), large 'simplex' morphotype (C, F). A, dorsal view of showing unciliated posterior parts of dorsal somatic kineties (white asterisk) and convergence of somatic kineties (whorl) on inconspicuous left posterior protuberance (black arrow). B, right ventrolateral view showing right sutural kinetofragments (white arrow) and margin of the peristomial overture (black arrow). C, detail view of right sutural kinetofragments (white arrow) between dorsal (black asterisk) and left-spiralling ventral (white asterisk) somatic kineties, from same cell as (F). D, detail view of same cell as (B)

kineties, somatic cilia limited to approximately anterior 50% of cell, usually a short 'tail' of ciliated cortex extends posteriorly (Fig. 27A, B, D). Right sutural kinetofragments absent. Circumperistomial kineties very densely ciliated (Fig. 27A, B, D, E). Adoral zone extends about 45% of cell length on average, composed of an average of 94 membranelles (range 75–120). POM diplostichomonad only in anterior quarter (Fig. 26A, E).

Remarks: Individuals of our population were longer and distinctly more slender *in vivo* than indicated by Kidder's diagnosis for the species. Kidder (1937) did not specify whether his diagnosis for *C. elongata* was based on measurements from *in vivo* observations or fixed material, although the latter possibility seems most plausible given the technology available at that time.

Clevelandella parapanesthiae (Kidder, 1937)

(Figs 28, 29; Supporting Information, Tables S35–S40)

Four populations (ASS, PGW, PAC, and MP) were morphologically characterized by both *in vivo* observations and in protargol preparations and two (PT and STH) only *in vivo*. The ciliate was moderately to highly abundant.

Description based on ASS, PGW, PAC, and MP populations: Medium-sized *Clevelandella* (on average $99 \times 55 \mu\text{m}$ *in vivo*, range $66\text{--}119 \times 31\text{--}71 \mu\text{m}$; on average $86 \times 46 \mu\text{m}$ in protargol preparations, range $70\text{--}114 \times 33\text{--}56 \mu\text{m}$). Spade-shaped with distinct left notch and protrusive left lobe; peristomial projection short, broad, about 24% of body length. Macronucleus from slender inverted teardrop-shape (Fig. 28A, G, H) to scimitar-like (Fig. 28C, E, F), obliquely oriented, situated right of midline, posterior end extends into posterior part of body proper, karyophore at posterior end of macronucleus, visible only inconsistently *in vivo* and in protargol preparations. In MP population cells with S-like (Fig. 28B) macronucleus (as well as normal teardrop-shaped) were observed. Micronucleus ellipsoidal, inconspicuous. Somatic cilia limited to approximately anterior 67% of cell. About 10 obliquely oriented, free right sutural kinetofragments in the right suture (Fig. 28D). Adoral zone extends about 56% of cell length on average, composed of an average of 38 membranelles (range 35–42). POM diplostichomonad only in anterior quarter (Fig. 28F).

Clevelandella kidderi Mandal and Nair, 1974

(Figs 30, 31; Supporting Information, Tables S41, S42)

One population (ASS) was morphologically characterized by both *in vivo* observations and in protargol preparations and one (PA) only *in vivo*. The ciliate was moderately to highly abundant.

Description based on ASS and PA populations: Medium-sized *Clevelandella* (on average $128 \times 58 \mu\text{m}$ *in vivo*, range $101\text{--}168 \times 44\text{--}72 \mu\text{m}$; on average $94 \times 50 \mu\text{m}$ in protargol

preparations, range $84\text{--}107 \times 42\text{--}59 \mu\text{m}$). Spade-shaped, peristomial projection about 32% of body length, conspicuous left-sided notch at junction of peristomial projection and body proper, left lobe well developed. Macronucleus inverted teardrop-shape with finely granular chromatin, obliquely oriented, situated right of midline in anterior half of body proper. Karyophore at posterior end of macronucleus, visible inconsistently (Fig. 30E). Micronucleus globular near anterior margin of macronucleus. Somatic cilia limited to approximately anterior 60% of cell. Right sutural kinetofragments probably developed (Fig. 30C), but better oriented and stained cells would be needed to know for sure. Adoral zone extends about 58% of cell length on average, composed of an average of 47 membranelles (range 45–49). POM as described for the family (Fig. 30D).

Clevelandella panesthiae (Kidder, 1937)

(Figs 32–34; Supporting Information, Tables S43–S47; Video S1)

Three populations (ASS, PAA, and STH) were morphologically characterized by both *in vivo* observations and in protargol preparations and two (PAC and PT) only *in vivo*. When present, the ciliate was highly abundant.

Description based on ASS, PAA, STH, PAC, and PT populations: Large-sized *Clevelandella* (on average $133 \times 62 \mu\text{m}$ *in vivo*, range $109\text{--}168 \times 43\text{--}84 \mu\text{m}$; on average $114 \times 56 \mu\text{m}$ in protargol preparations, range $93\text{--}157 \times 39\text{--}89 \mu\text{m}$). Broadly spade-shaped, distinct left-sided notch inconspicuous (Figs 32A, 34D, E) or absent (Figs 32B–E, 34A, B). Macronucleus relatively large, slender, inverted teardrop-shape, obliquely oriented in right half of body proper, chromatin finely granular, karyophore not clearly identified *in vivo* or in protargol preparations. Micronucleus ellipsoidal (about $4.5 \times 2 \mu\text{m}$). Somatic cilia limited to approximately anterior 60% of cell. Right sutural kinetofragments absent. Circumperistomial kineties only sparsely ciliated (Figs 32A, 34B). Adoral zone extends about 60% of cell length on average, composed of an average of 47 membranelles (range 42–52). Paroral membrane not studied.

Clevelandella sp. 4

Clevelandella sp. 4 was detected in both dissected STR hosts in which it was highly abundant.

Description based on STR population: Large-sized *Clevelandella* (*in vivo* on average $152 \pm 20 \times 63 \pm 10 \mu\text{m}$, range $127\text{--}185 \mu\text{m}$, $N = 9$), spade-shaped, and dorsoventrally flattened. Left cell margin conspicuously notched at the base of peristomial projection, the lobe does not overhang the peristomial projection. The peristomial projection constituted approx. one-third to one-quarter of cell length.

showing sutural kinetofragments (white arrow). E, left lateral view showing ciliated cytoproct (black arrow), unciliated parts of the dorsal (black asterisk) and ventral (white asterisk) somatic kineties, the peristomial overture (white arrow) and cilia of the circumperistomial kineties (white arrowhead). F, right lateral view showing the right suture (black arrow) between dorsal (black asterisk) and ventral (white asterisk) somatic kineties and sutural kinetofragments (white arrow, compare Fig. 23P), the margin of the peristomial overture (white arrowhead), and the cilia of the adoral membranelles (black arrowhead). Scale bars: 20 μm (A, B, E, F), 5 μm (C, D).



Figure 25. *Clevelandella elongata* in vivo. From SRU. A, ventral view showing adoral membranelles (black arrow), cytostome (white arrow), contractile vacuole (white arrowhead), and hyaline subcortical layer (black arrowheads). B, left ventrolateral view (optical section) showing micronucleus (white arrow), karyophore (black arrows), contractile vacuole (white arrowhead), and posterior extent of ciliation (black arrowheads). C, left ventrolateral view showing micronucleus (white arrow), karyophore (black arrow), and cilia of the circumperistomial kineties (white arrowheads). D, detail of same cell as (C) showing peristomial overture (black arrowhead) and cilia of adoral membranelles (white arrow). Ma, macronucleus. Scale 20 μ m.

Morphological analyses of cells

A NMDS (non-parametric multi-dimensional scaling) analysis of 455 morphologically characterized protargol-stained cells showed a significant grouping of cells based on their affiliation

to particular species (Fig. 35). Two clusters were clearly separated along the first NMDS axis, based on the presence/absence of the peristomial projection. The first cluster consisted of *Anteclevelandella constricta*, *C. klobasa*, *P. brevis* in 'brevis'

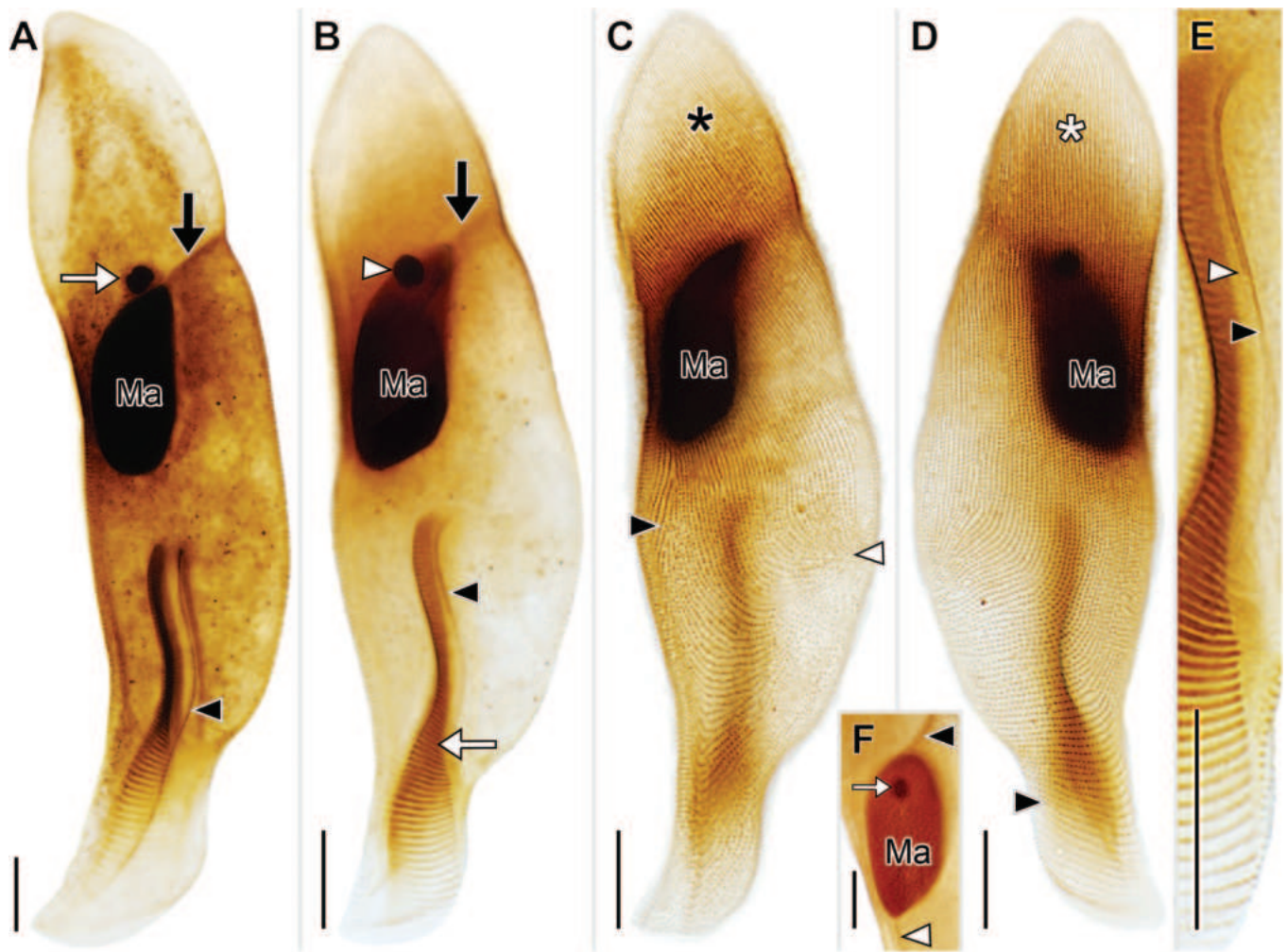


Figure 26. *Clevelandella elongata* after protargol impregnation. From SRU. A, ventral view (optical section) showing micronucleus (white arrow), anterior part of the karyophore (black arrow), and paroral membrane (black arrowhead). B–E, the same cell in ventral view: B, (optical section) showing anterior part of karyophore (black arrow), micronucleus (white arrowhead), adoral membranelles (white arrow), and paroral membrane (black arrowhead); C, ventral infraciliature showing right kinetal suture (black arrowhead), left-hand spiralling ventral kineties (asterisk), and kineties converging to form ciliary whorl on inconspicuous left protuberance (white arrowhead); D, dorsal infraciliature showing straight course of dorsal kineties (asterisk) and looping kineties of peristomial projection (black arrowhead); E, detail view of the long file (black arrowhead) and short file (white arrowhead) of the paroral membrane. F, detail showing anterior (black arrowhead) and posterior (white arrowhead) parts of the karyophore and the micronucleus (white arrow). Ma, macronucleus. Scale bars: 20 μm.

form and *P. brevis* in ‘simplex’ form; the second cluster contained remaining species. The second NMDS axis was related to peristomial projection length, macronucleus length, and body width (variable scores -0.054, 0.047, and 0.040, respectively). The linear discriminant analysis (LDA) resulted in the model accuracy of 0.892, meaning that 89.2% of the cells were correctly assigned to the appropriate species based on the morphological traits only. The mean accuracy for all the species was 85%. According to the classification matrix (Supporting Information, Table S49), the lowest correct discrimination levels were recovered in *Rhynchoclevelandella* sp. 2 (14.3%) and *C. kidderi* (55.6%).

The mean shrinking during protargol preparations for all Clevelandellidae species combined is $18.6 \pm 7.4\%$ for length and $16.6 \pm 10.9\%$ for width. However, close observation on shrinking of individual species (Supporting Information, Table S50) uncovered the striking difference among species, with length

shrinkage ranging from 4.2% in *R. hastula* to 29.2% in the long morph of *C. hromadkai* and width shrinkage ranging from 1.1% in *C. fryntai* to 31.9% in the long morph of *C. hromadkai*. In addition, there is an apparent disparity between shrinkage of the cell length and cell width in some species. The most notable example is *C. elongata*, whose length decreases in protargol preparations by 22.9%, but its width increases by 12.5%. The GLM model revealed the significant effect of species ($F_{16,840} = 361.26$, $P < 0.001$), cell condition ($F_{1,840} = 343.92$, $P < 0.001$), and their interaction ($F_{16,840} = 4.5$, $P < 0.001$) on the cell length. The effect of species ($F_{16,827} = 143.98$, $P < 0.001$), cell condition ($F_{1,827} = 232.94$, $P < 0.001$), and their interaction ($F_{16,827} = 4.85$, $P < 0.001$) on the cell width were also significant. As shrinkage is calculated directly from the cell measurements (i.e. cell length and width), the GLM model clearly indicates that the rate of shrinkage differs among species considerably. RMA regression did not reveal significant relationship

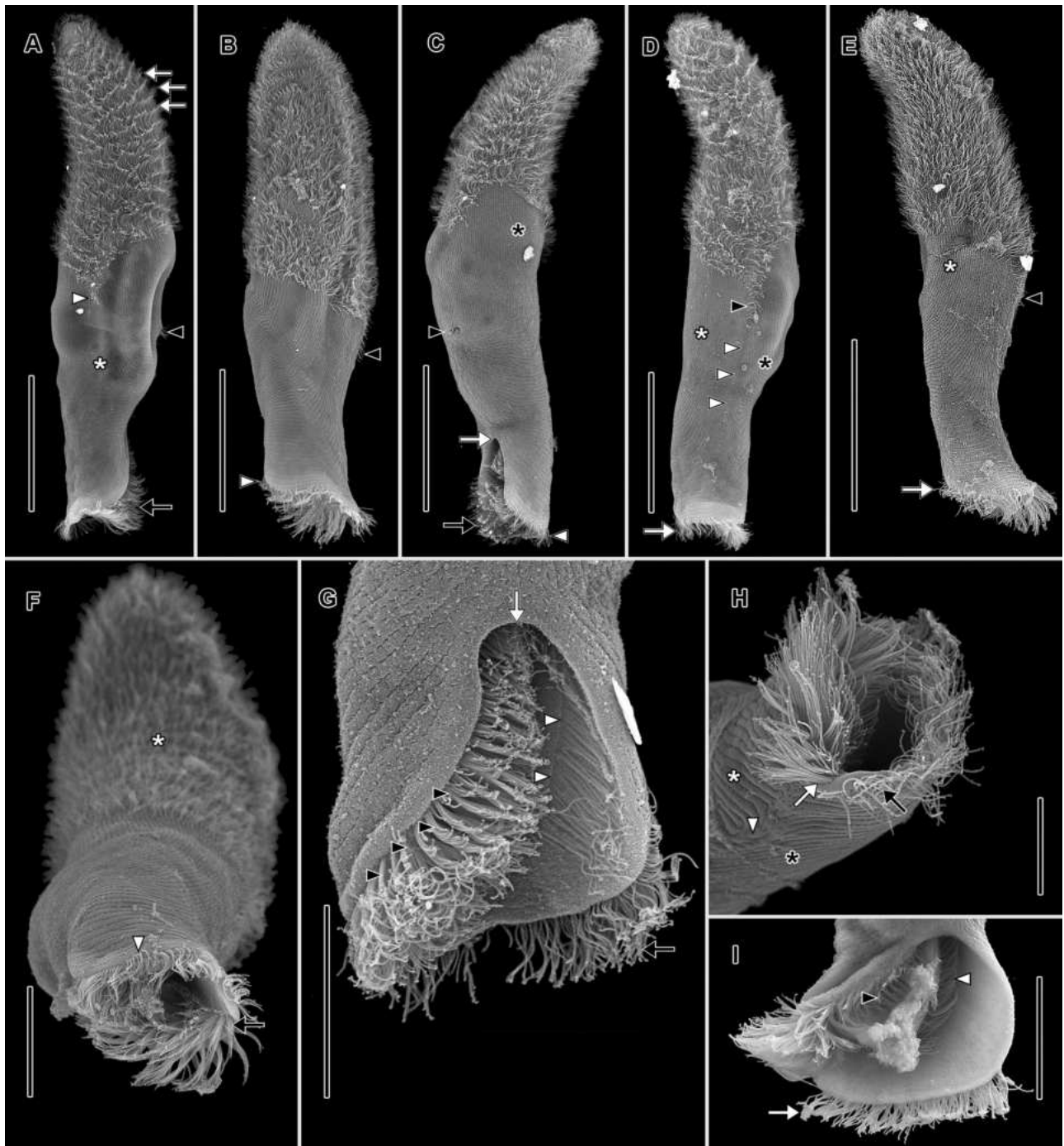


Figure 27. *Clevelandella elongata* in the scanning electron microscope. From SRU. A, right ventrolateral view showing metachronal ciliary waves (white arrows), and 'tail' of ciliated basal bodies extending posteriorly on right ventrolateral surface (white arrowhead) near the right suture (white asterisk). Cilia of the adoral membranelles protrude from the buccal overture (black arrow). Cilia of the collecting chamber protrude from the cytoproct (black arrowhead). B, dorsal view showing 'tail' of ciliated basal bodies extending posteriorly on right ventrolateral surface (black arrowhead) and cilia of circumperistomial kineties (white arrowhead). C, left dorsolateral view showing the cytoproct (black arrowhead), dorsal kineties (black asterisk), proximal margin of buccal overture (white arrow), cilia of the adoral membranelles (black arrow), and circumperistomial kineties (white arrowhead). D, right dorsolateral view showing posteriorly extending 'tail' of cilia (black arrowhead), right suture between dorsal kineties (white asterisk) and ventral kineties (black asterisk), and densely ciliated circumperistomial kineties (white arrow). E, dorsal view showing straight dorsal kineties (white asterisk), the ciliary 'tail' (black arrowhead), and cilia of circumperistomial kineties (white arrow). F, posterodorsal view showing convexity of the right dorsolateral cell surface (white asterisk), cilia of circumperistomial kineties (white arrowhead), and cilia of adoral membranelles (black arrow). G, left lateral detail view showing proximal margin of buccal overture (white arrow), circumperistomial cilia (black arrow), adoral membranelles (black arrowheads), and paroral membrane (white arrowheads). H, posterolateral detail view of the buccal overture showing posteriormost adoral membranelle (white arrow), circumperistomial cilia (black arrow), right suture (white arrowhead) between ventral kineties (black asterisk) and dorsal kineties (white asterisk). I, posterior view of buccal overture showing adoral membranelles (black arrowhead), paroral membrane (white arrowhead), and circumperistomial cilia (white arrow). Scale bars: 50 μ m (A–E), 20 μ m (F–I).

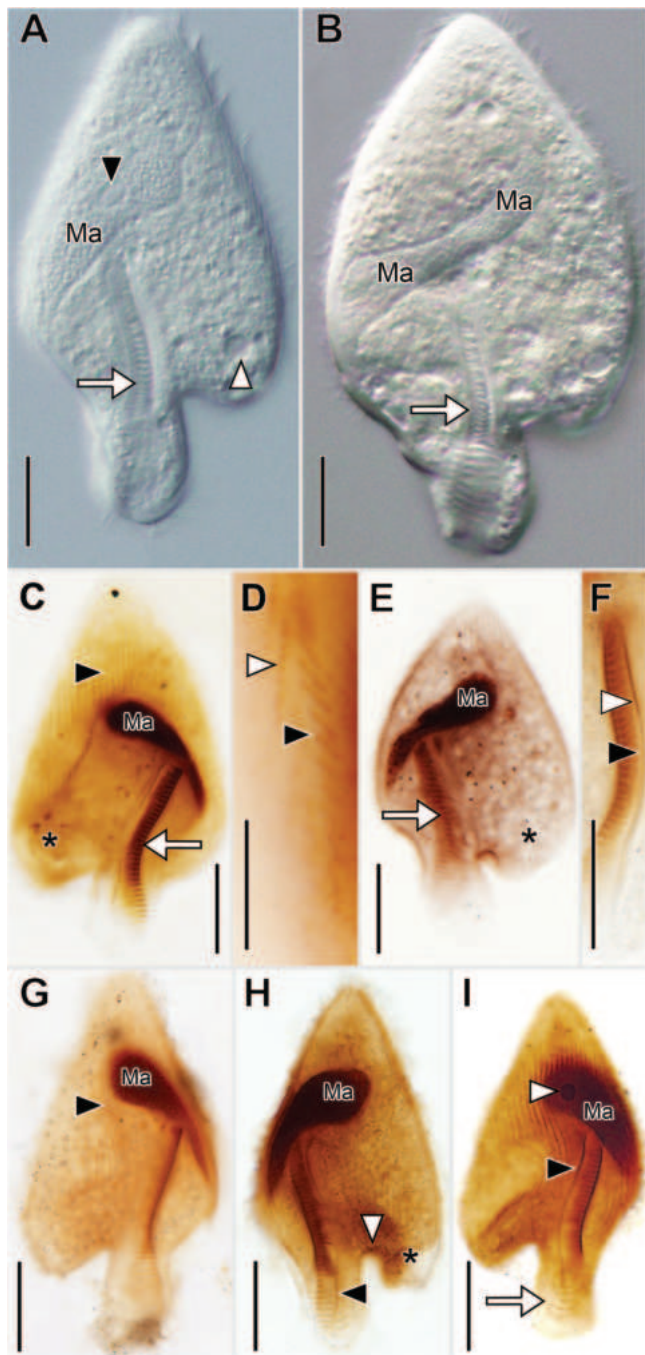


Figure 28. *Clevelandella parapanesthiae*. In vivo (A, B) and after protargol impregnation (C–H). From PGW (A), MP (B, F), ASS (C, D), PT (E), and PGW (G, H). A, ventral view showing adoral membranelles (white arrow), contractile vacuole (white arrowhead), and micronucleus (black arrowhead). B, ventral view showing atypical macronuclear ‘S’-shape and adoral membranelles (white arrow). C, dorsal view (optical section) showing straight dorsal kineties (black arrowhead), adoral membranelles (white arrow), and left protuberance (asterisk). D, right lateral detail view of sutural kinetofragments (black arrowhead) and dorsal kinety (white arrowhead). E, ventral view (optical section) showing adoral membranelles (white arrow) and left protuberance (asterisk). F, detail view of paroral membrane showing short (white arrowhead) and long (black arrowhead) files. G, dorsal view showing straight dorsal kineties (black arrowhead). H, ventral view showing paroral membrane (black arrowhead), prominent notch (white arrowhead)

between shrinkage and cell size either in length ($R^2 = 0.036$, $P = 0.466$) or width ($R^2 = 0.143$, $P = 0.133$). Nevertheless, a weak positive relationship between shrinkage and cell length (Supporting Information, Fig. S6) and a weak negative relationship between shrinkage and cell width (Supporting Information, Fig. S7) was found.

DISCUSSION

Morphologic peculiarities of Clevelandellidae

The Clevelandellidae all share several similar morphologic peculiarities that have been previously unmentioned or inconsistently and, sometimes, incorrectly described and/or depicted. The most important of these are discussed below.

Somatic ciliature: Kidder himself (1937) and others (Lynn 2008, Pecina and Vďačný 2020a, b) have described or depicted members of the Clevelandellidae as having complete (i.e. holotrichous) somatic ciliation (Fig. 36A, C, H). However, all Clevelandellidae actually share what we term a ‘hemitrichous’ distribution of somatic cilia (the ciliature of *C. contorta* has not been assessed since its original description). Both basal bodies of somatic dikinetids are ciliated in the (approximately) anterior half of the cell, while in the posterior part of the cell the dikinetids are barren, except for several ciliated circumperistomial kineties at the terminal margin of the peristomial overtube (e.g. Figs 5C, 27G, 29C, 31A). Although the circumperistomial kineties have previously been overlooked, Albaret (1975) recognized the otherwise partial somatic ciliation of the three Clevelandellidae species he redescribed, and Mandal and Nair (1974) recognized the restriction of somatic cilia to the anterior half of the cell in *C. kidderi* (Fig. 36I; here considered as a senior subjective synonym of *C. lynni*). Yamasaki (1939) recognized the restriction of somatic cilia to half of the cell in the three species he described, but he considered the half to be posterior (Fig. 36F, J). Albaret (1975) described ‘short’ cilia in the posterior part of the cell, but his transmission electron photomicrograph and scanning electron microscopy studies indicate that the posterior somatic dikinetids are actually barren. Although Pecina and Vďačný (2020a, b, 2022) describe holotrichous (complete) ciliation in Clevelandellidae and depict it in their drawings, their photomicrographs clearly show the invariable restriction of somatic cilia to the anterior part of the cell in the species they consider (Fig. 36D, G; Pecina and Vďačný 2020a: fig. 2E–G; 2020b: figs 3F, G, 5G, H, 8E–G, 11E–G, 14B, C).

The somatic cilia (including those of the circumperistomial kineties) of all Clevelandellidae are unusually short, i.e. about 5 μm long in both living and fixed states (e.g. Figs 4A, 7A, 10B, 23A). Somatic ciliary length in ciliates is highly variable, ranging from 6 to 100 μm (Grain 1984). The somatic cilia of Clevelandellidae may be near the lower limit of length for motile cilia since the minimum length required for organized ciliary beating of motile

between peristomial projection and left protuberance (asterisk). I, dorsal view showing micronucleus (white arrowhead), paroral membrane (black arrowhead), and looping kineties of peristomial projection (white arrow). Ma, macronucleus. Scale bars: 20 μm (A–C, E–I), 10 μm (D).

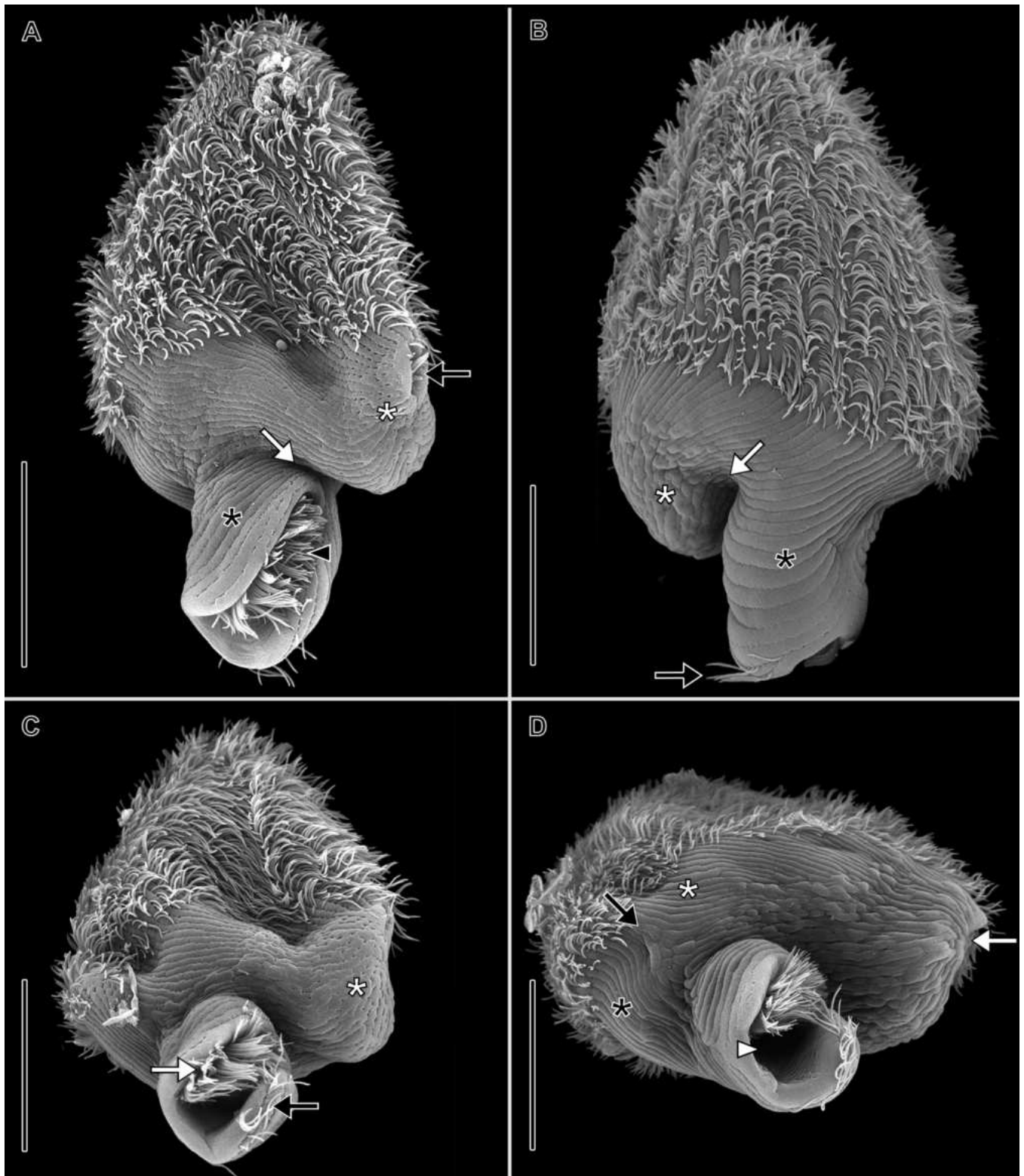


Figure 29. *Clevelandella parapanesthia* in the scanning electron microscope. From PAC. A, ventral view showing notch (white arrow) between left protuberance (white asterisk) and peristomial projection (black asterisk), cilia protruding from cytoproct (black arrow), and cilia of adoral membranelles (black arrowhead). B, dorsal view showing notch (white arrow) between left protuberance (white asterisk) and peristomial projection (black asterisk), and cilia of circumperistomial kineties (black arrow). C, posteroventral view showing posterior protuberance (white asterisk), cilia of adoral membranelles (white arrow), and cilia of circumperistomial kineties (black arrow). D, posterior view showing dorsoventral flattening, right kintal suture (black arrow) between ventral kineties (white asterisk) and dorsal kineties (black asterisk), position of cytoproct (white arrow), and peristomial overture (white arrowhead). Scale bars: 20 μm (A–D).

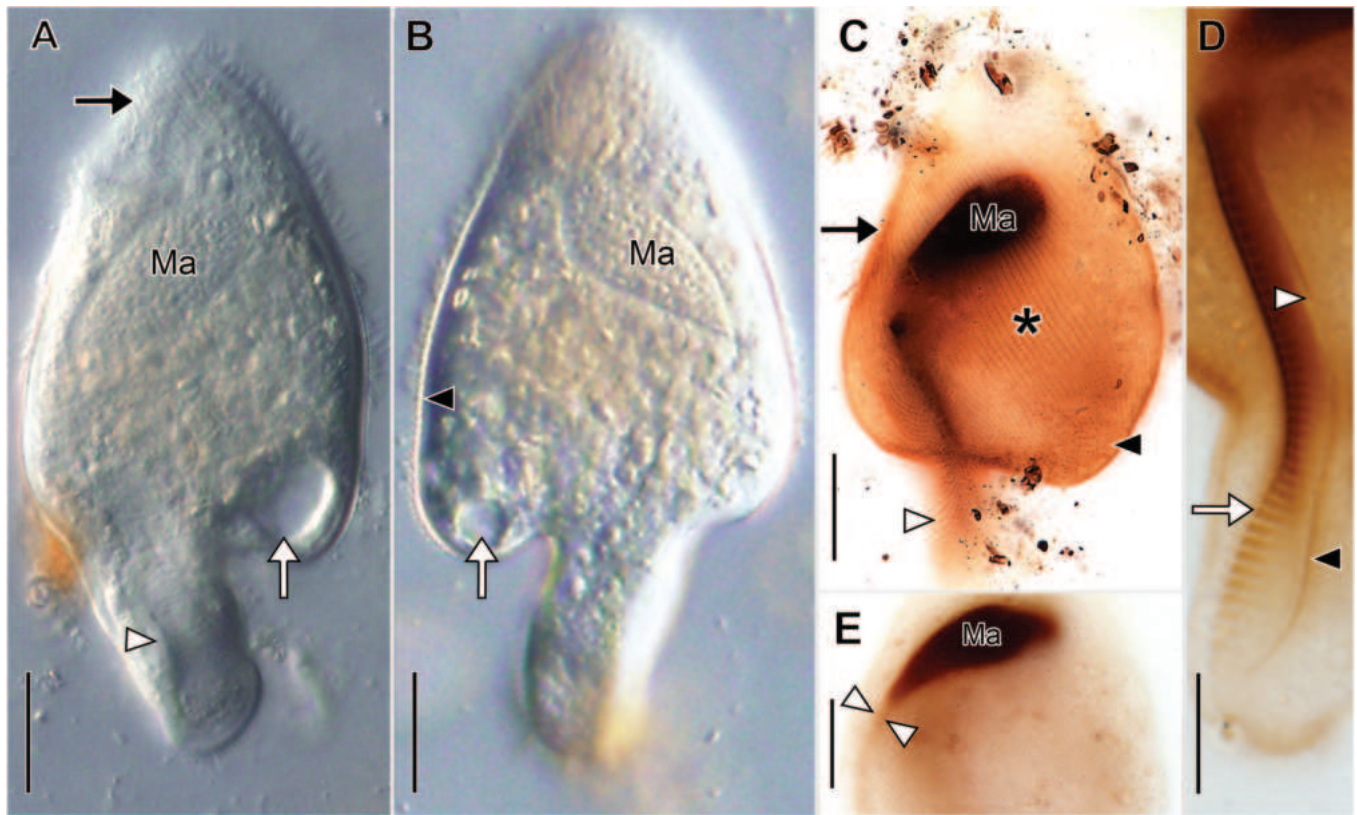


Figure 30. *Clevelandella kidderi* in vivo (A, B) and after protargol impregnation (C–E). From ASS (A–E). A, ventral view showing contractile vacuole (white arrow), ventral kineties (black arrow), and margin of peristomial overtube (white arrowhead). B, dorsal view showing contractile vacuole (white arrow) and posterior extent of ciliation on the left margin (black arrowhead). C, ventral infraciliature showing right sutural kinetofragments (black arrow), left-hand spiralling ventral kineties (asterisk), and looping kineties of the peristomial projection (white arrowhead). D, detail view of paroral membrane showing short (white arrowhead) and long (black arrowhead) files, and adoral membranelles, (white arrow). E, detail of macronucleus, ventral view showing inconspicuous karyophore (black arrow). Ma, macronucleus. Scale bars: 20 μ m (A–C), 10 μ m (D).

cilia is about 2–4 μ m (Bottier et al. 2019). A reduction of both cell surface ciliation and somatic ciliary length are possibly adaptations to the viscous content of the host gut. In mucous media, a short ciliary length (5–7 μ m) is necessary for effective ciliary propulsion (Satir and Sleight 1990). At least some *Nyctotherus* species inhabiting cockroaches, although holotrichous, also bear similarly short somatic cilia (Pecina and Vdačný 2020a: figs 4A, 5E). The somatic kineties of all Clevelandellidae have a left-handed spiral arrangement, i.e. toward the right side of the cell (Kidder 1937, Mandal and Nair 1974, Albaret 1975, Jankowski 2007). The depictions of right-handed-spiralling kineties (with respect to the viewer) in *Antecelelandella constricta* (Fig. 36C, D, E; Pecina and Vdačný 2020b: figs 1A, M, N, 3F, G) are misinterpretations. All *Antecelelandella constricta* cells from the current report, Kidder (1937), and Albaret (1975), as well as all other species depicted by Pecina and Vdačný (2020b), show left-hand-spiralling of somatic kineties (Fig. 36A, B, H, I, K). The small sutural kinetofragments recognized by Albaret (1975) in *Antecelelandella constricta*, *C. parapanesthiae*, and the ‘simplex’ form of *Paracelelandia brevis*, and confirmed in *Antecelelandella constricta* by transmission electron microscopy, were also found in six of the species we studied (*Antecelelandella constricta*, *R. hastula*, *Paracelelandia brevis*, *C. kidderi*, *C. parapanesthiae*, and *C. philipi* sp. nov.). In seven

species (*C. ananiasi* sp. nov., *C. elongata*, *C. fryntai* sp. nov., *C. hromadkai* sp. nov., *C. klobasa* sp. nov., *C. panesthiae*, and *C. sidi* sp. nov.) this feature was clearly absent and in two (*R. nipponensis* and *Rhynchoclevelandella* sp. 2) it could not be determined.

The ciliated excretory antrum: Describing the general morphologic features of the Clevelandellidae, Kidder (1937) stated: ‘Just ventral to the contractile vacuole and near the whorl of peripheral cilia occurs a slit-like cytoproct’ (sometimes referred to as the ‘cytopyge’). ‘Through this permanent slit the contractile vacuole empties its contents. No canal can be detected, but the contractile vacuole comes in contact with the interior edge of the cytopyge just before systole.’ Lynn (2008) mentions, without a citation, that ‘...in clevelandellids [the cytoproct] may open to the outside by a cilia-lined channel’. Albaret (1975) does not describe the cytoproct of Clevelandellidae in detail, but depicts it as a short, narrow canal adjacent to the contractile vacuole. The actual morphology of the clevelandellid excretory system (contractile vacuole, cytoproct, and intervening structures) is quite unusual in addition to its location on the lateral aspect of the cell. In all species studied, we identified what we term the ‘ciliated excretory antrum’, a distinct, permanent, ciliated chamber situated between the contractile vacuole and the slit-like cortical cytoproct (e.g. Figs 2A, C, 15D, E, 17C, 23L, M;

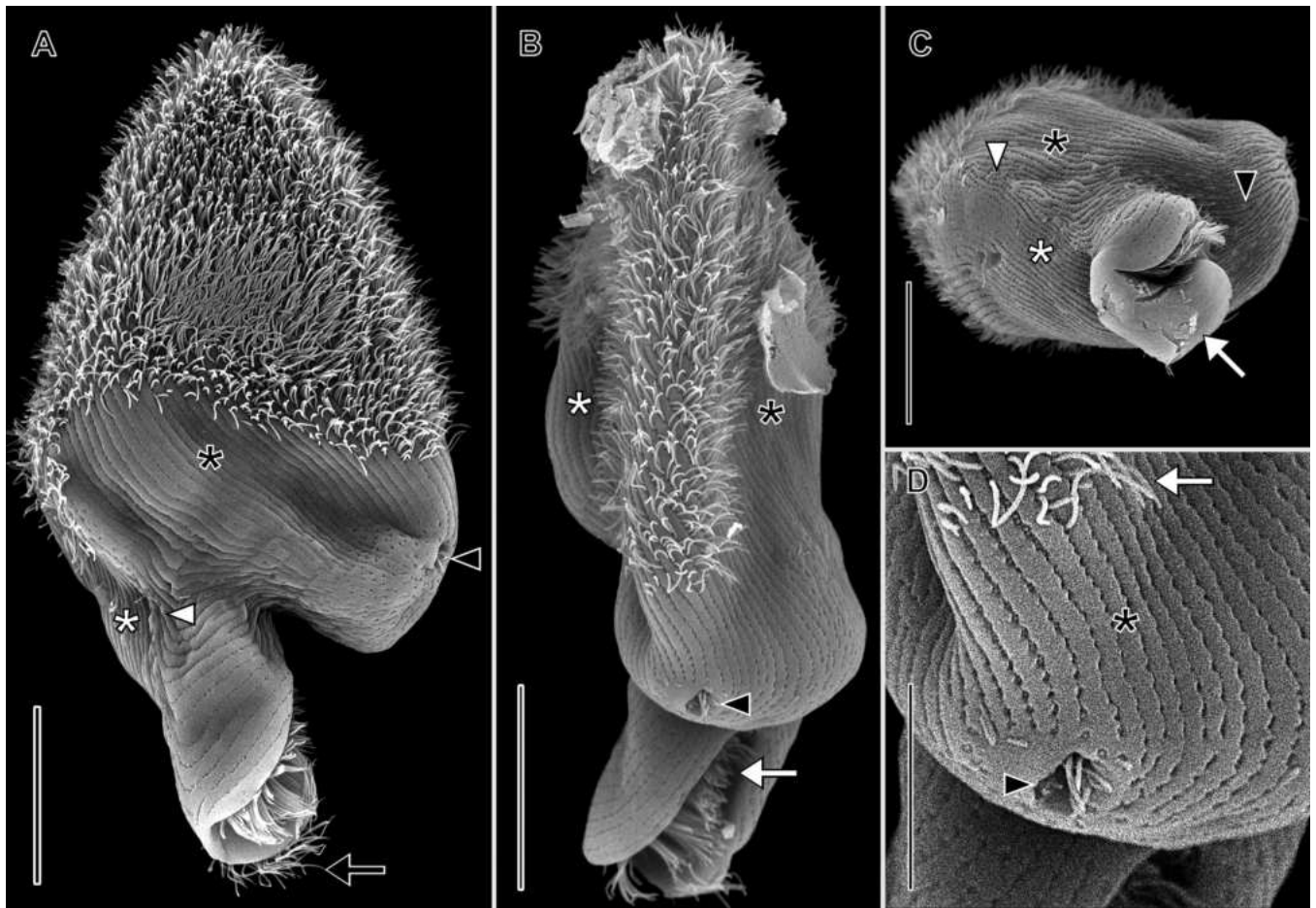


Figure 31. *Clevelandella kidderi* in the scanning electron microscope (A–D). From ASS. A, ventral view showing left-hand-spiralling ventral kineties (black asterisk) meeting dorsal kineties (white asterisk) to form right kinetal suture (white arrowhead), cilia protruding from cytoproct (black arrowhead), and ciliated circumperistomial kineties (black arrow). B, left lateral view showing unciliated parts of dorsal kineties (black asterisk) and ventral kineties (white asterisk), cilia protruding from cytoproct (black arrowhead), and cilia of adoral membranelles seen in peristomial overture (white arrow). C, posterior view showing dorsoventral flattening and right kinetal suture (white arrowhead) between ventral kineties (black asterisk) and dorsal kineties (white asterisk), and dorsal margin of the posterior peristomial projection (white arrow). D, detail of the same cell as (B) showing cilia protruding from cytoproct (black arrowhead) on the left protuberance (asterisk), and posterior extent of somatic ciliation (white arrow). Scale bars: 20 µm (A–C), 10 µm (D).

Supporting Information, Video S1). The contents of the contractile vacuole and faecal material are collected in this chamber and propelled from it, through the cytoproct by long, fine, cilia arising from the wall of the chamber. In SEM images these cilia can often be seen protruding from the cytoproct (e.g. Figs 11F, 20F, 22C, 33D). The antrum itself is rather inconspicuous and likely to be overlooked without careful *in vivo* study by DIC illumination. It is also quite difficult to discern even in optimally developed protargol preparations since the basal bodies of the chamber are very fine and irregularly distributed and obscured by overlying somatic kineties (Figs 15E, 23L). The cytoproct of the nyctotherids as described by Geiman and Wichterman (1937), McKean (1972), and Albaret (1975) differs in that it is situated in a more or less invaginated area of the posterior cell cortex. However, a very thorough ultrastructural investigation of *Nyctotherus ovalis* Leidy, 1894 showed a structure at the posterior end of the cell probably homologous to the ciliated excretory antrum of Clevelandellidae, namely a 20–25 µm long ‘expulsion vesicle canal’ lined by cilia, some of which protruded from the

cytoproct (McKean 1972: figs 3, 81). In the class Armophorea, these complex structures are unique to the order Clevelandellida suggesting that a ciliated excretory chamber was probably present in the last common ancestor.

Morphology of the paroral: The morphology of the paroral of all Clevelandellidae is invariably diplostichomonad, i.e. composed of two parallel single files of ciliated basal bodies, one usually longer than the other, separated by a cortical ridge (e.g. Fig. 7I). In all species we studied, both files extend posteriorly from the cytostome, the left file being longer and usually reaching the posterior margin of the peristome (e.g. Figs 5D, 7I, 26A, 30D). The right file is always shorter, with the exception of *C. sidi* sp. nov. in which both files are of the same length (Fig. 15H). The paroral of the nyctotherids is also diplostichomonad (de Puytorac and Grain 1976, Takahashi and Imai 1989). A diplostichomonad paroral occurs in various metopid lineages and may be more widely distributed in this group than assumed, since the shorter proximal file might be obscured within the buccal cavity and thus

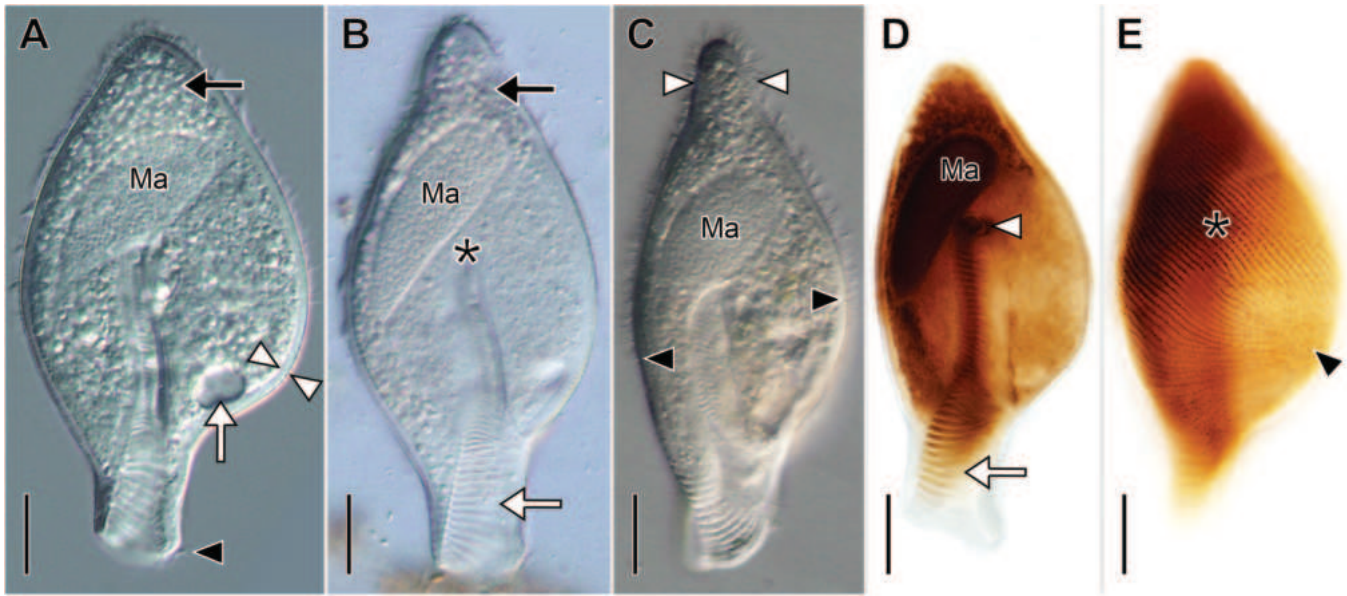


Figure 32. *Clevelandella panesthiae* in vivo (A–C) and after protargol impregnation (D, E). From ASS (A), PAA (B, D, E), and PT (C). A, ventral view (optical section) of typical form showing the contractile vacuole (white arrow), cytoplasmic granules, possibly amylopectin (black arrow), circumperistomial kineties (black arrow), and hyaline subcortical layer (white arrowheads). B, ventral view (optical section) showing location of the cytostome (asterisk), adoral membranelles, (white arrow), and cytoplasmic granules, possibly amylopectin (black arrow). C, ventral view (optical section) showing posterior extent of ciliation on the right margin and left margin (black arrowheads), and unusually tapered anterior end of cell (white arrow heads). D, E, the same cell in ventral view: D, optical section showing cytostome (white arrowhead) and adoral membranelles (white arrow); E, surface view showing ventral infraciliature showing left-hand spiralling ventral kineties (asterisk), disordered basal bodies on left protuberance (black arrowhead). Ma, macronucleus. Scale bars: 20 μ m.

easily overlooked (Bourland and Wendell 2014, da Silva-Neto *et al.* 2016, Bourland *et al.* 2018, 2020). The caenomorphids also have a diplostichomonad paroral (Lynn 2008). The possibility that the diplostichomonad character state is plesiomorphic in Armophorea should be considered. Our findings differ from a previous report by Pecina and Vdačný (2020a, b) who claim that both files of the paroral are always of the same length and that the paroral terminates more anteriorly at the proximal margin of the buccal overture.

Position and polarity of oral structures: The most striking characteristic of the Clevelandellidae is the posteriorization of oral structures. Other armophorean lineages (e.g. *Brachonella* spp.) also have posteriorized oral structures. However, in these cases the adoral membranelles are oriented in the same direction (i.e. with the shortest file of basal bodies anterior), as in taxa with anterior buccal cavities, and the membranelles have the same left-sided location with respect to the paroral. Thus, in these cases, the posteriorization can be accounted for by a simple posterior displacement of both structures without rotation. However, the orientation of the individual elements in Clevelandellidae is entirely different from the latter examples. Terms of orientation in the Clevelandellidae have been inconsistent, even with regard to the true anterior and posterior ends of the cell (Yamasaki 1939, Albaret 1975). As in all other ciliate taxa, the polarity of somatic dikinetids in Clevelandellidae obeys the rule of desmodexy (i.e. the kinetodesmal fibre projects to the right side of the cell from the posterior kinetosome near microtubular triplets 5 and 6), thus the somatic kineties of Clevelandellidae are not reversed (Chatton and Lwoff 1935, Albaret 1975, Lynn 1975, 2008). The

peristomial opening is thus considered to be ‘posteriorized’, as are the adoral membranelles, in which the shortest file of basal bodies faces posteriorly and not anteriorly as is typical of spirotrich, heterotrich, and metopid ciliates (Tuffrau and Fleury 1994). The selective pressures and structural constraints that resulted in this remarkable reorientation are unknown.

Duality of morphotypes: Several Clevelandellidae species exhibit two very distinct morphs that differ not only in size, but also in shape and other characteristics (e.g. number of adoral membranelles). The difference in appearance can be striking enough that two morphs of *Paraclevelandia brevis* (Kidder 1937)—‘brevis’ and ‘simplex’—have been identified as separate species. Similarly, prior to obtaining molecular data, we assumed ‘slender’ and ‘broad’ morphotypes of *C. fryntai* to be separate species. In the aforementioned cases, both morphotypes frequently co-occur in a single host. Furthermore, in *Paraclevelandia brevis* the large ‘simplex’ morphotype seems to never occur in the absence of the smaller ‘brevis’ morphotype. Conversely, the ‘short’ and ‘long’ morphotypes of *C. hromadkai* seem to never co-occur in the same host. The relationships between these morphotypes, and the factors leading to their formation, are unknown. It is probable that, in the case of *Paraclevelandia brevis*, the two morphotypes, which are strikingly different in size, number of adoral membranelles, and nuclear morphology, may represent different life stages. Morphotypes of *C. fryntai* have a different shape, but their metrics overlap strongly. It is thus possible that the difference is caused by the nutritional state of the cell or other environmental factors. In *C. hromadkai* the ‘long’ morph can be found mostly in adult male hosts, while the ‘short’ morph is found in females and larvae. Since

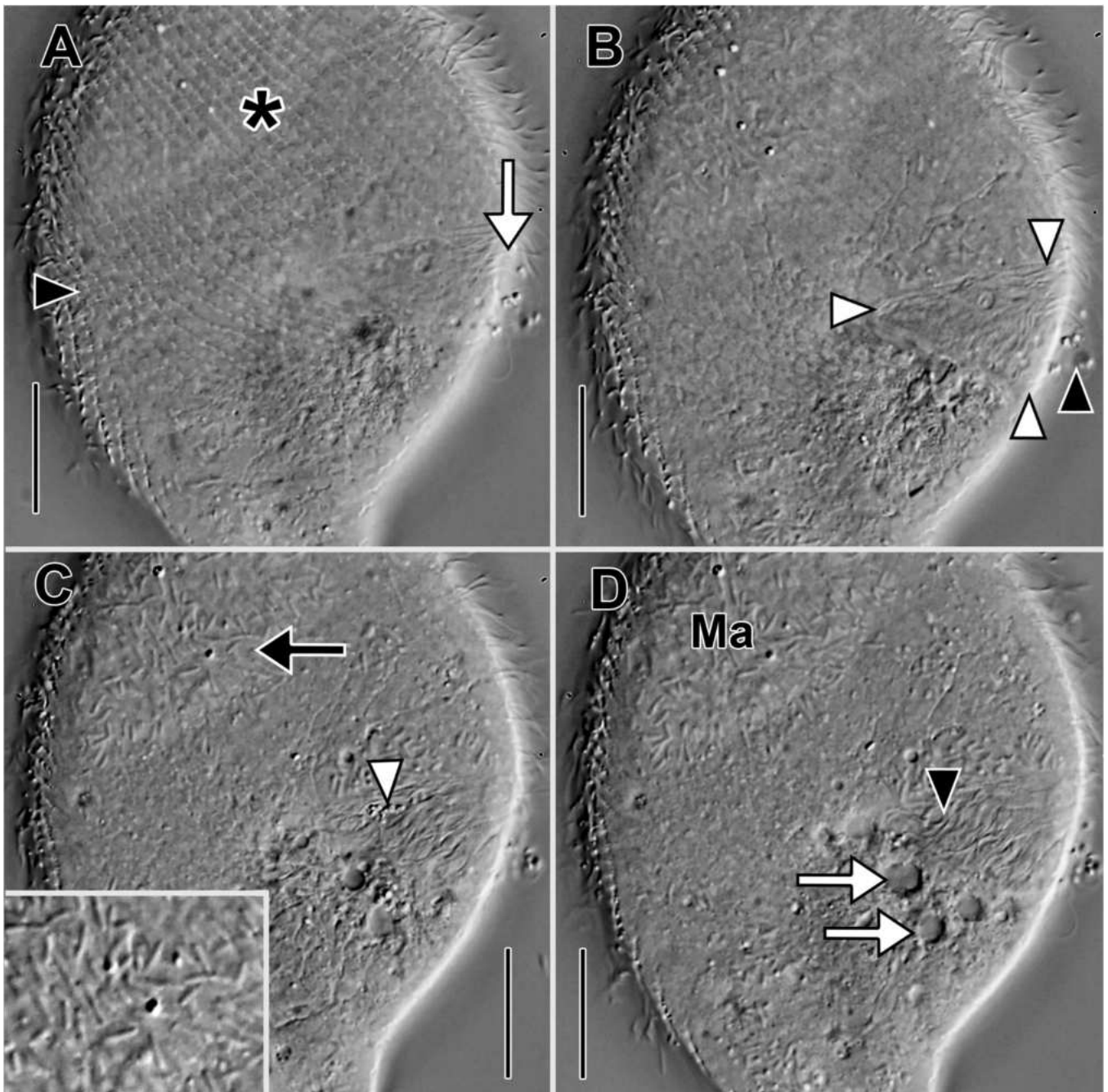


Figure 33. *Clevelandella panesthiae* in vivo (optical sections of same cell). From ASS. A, surface view showing left-hand spiralling ventral kineties (asterisk), right kinetal suture (black arrowhead), and position of the cytoproct (white arrow). B, slightly deeper focal plane showing conical ciliated excretory antrum within left protuberance (white arrowheads) and defecated material (black arrowhead). C, slightly deeper focal plane than (B) showing faecal material transiting the ciliated excretory antrum (white arrowhead) and numerous rod-shaped perinuclear symbionts (black arrow and inset). D, approximately same focal plane as (C) showing collecting vesicles of contractile vacuole (white arrowhead) which will coalesce and empty into the excretory antrum and the dense, fine cilia lining the antrum (black arrowhead). Ma, macronucleus. Scale bars: 20 µm.

the male cockroaches are of smaller or similar size as females and larger larvae, the host size is probably not the determining factor. However, the environment in the gut of the adult male is more stable than that of females and larvae. The insect larvae go through a number of ecdyses during ontogeny, first emptying their hindgut and then also shedding their hindgut cuticular lining (we have witnessed hindguts full of encysted ciliates in pre-ecdysis larvae).

Female cockroaches undergo repeated reproductive cycles accompanied by behavioural changes, including the period of drastic reduction in food intake while provisioning nutrients to egg case (Gore and Schal 2005, Varadínová *et al.* 2015). It is possible that, unlike the short-lived hindgut environment of larvae and females, the relatively stable environment of the male hindgut allows *C. hromadkai* to achieve the greater size of the 'long' morph.

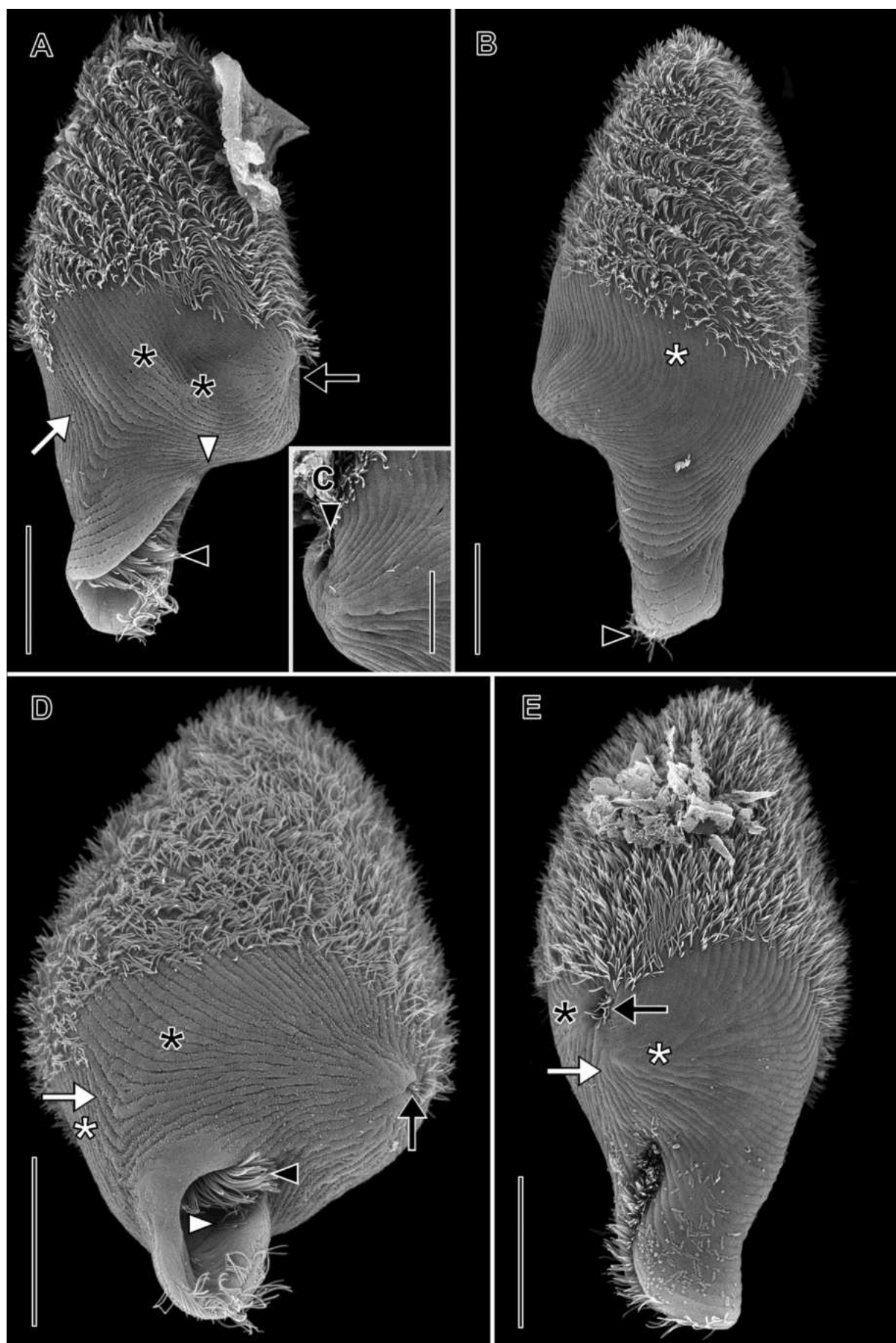


Figure 34. *Clevelandella panesthiae* in the scanning electron microscope. From ASS. A, ventral view showing right kinetal suture (white arrow) and short left suture (white arrowhead), cilia of adoral membranelles (black arrowhead), and ciliated cytoproct (black arrow).

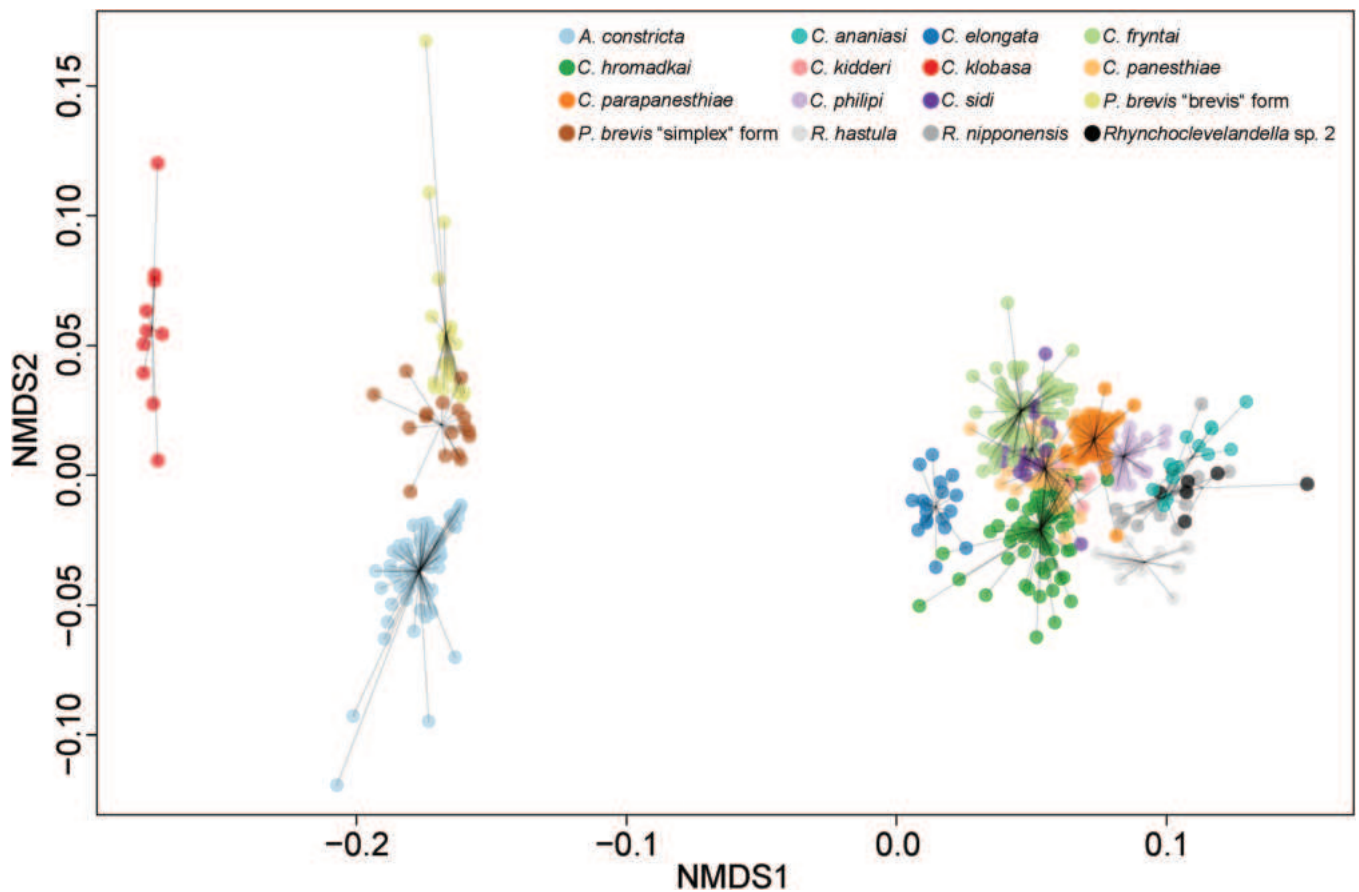


Figure 35. Non-metric multidimensional scaling (NMDS) plot based on the morphological analysis of traits measured on 455 scales. The cells are colour-coded and clustered based on their affiliation to 16 investigated strains.

Congruence of morphological and molecular data in taxonomy of Clevelandellidae

In order to test the congruence of morphological and molecular data, Pecina and Vďačný (2020b) employed a three-way integrative approach incorporating morphometric measurements, cell geometrical information (both obtained from their protargol preparations), and 18S rRNA gene sequences derived mostly from their own data (Pecina and Vďačný 2020a, b) but also from Lynn and Wright (2013). They had shown that the clevelandellid morphospecies, as defined by Kidder (1937), are fully consistent in both morphometric and geometrical analyses. However, they suggested that the morphospecies [based on their own data as well as *in vivo* data from Lynn and Wright (2013)] are not consistent with the 18S rRNA gene phylogeny, with *Anteclevelandella constricta*, *Paraclevelandella brevis*, and *R. hastula* being monophyletic, but *C. panesthiae* and *C. parapanesthiae* morphospecies comprising more or less phylogenetically disparate genotypes. This led to the conclusion that ‘morphology

and genetics are rather inconsistent’ in Clevelandellidae and that the morphospecies are complexes of cryptic taxa. Expanding on this idea, the authors decided that the majority of the studied genotypes represent separate species and tried to employ CBC concept to find species-delimiting characters for them (Pecina and Vďačný 2022). The concept is based upon supposed correlation between the presence of a double-sided base change of a nucleotide pair in a helix of the ITS2 rRNA gene region (a so-called compensatory base change—CBC), and the inability of strains to sexually cross. It was initially proposed for Volvocacean green algae (Coleman 2000) and was later used for ciliates as well (e.g. Coleman 2005), but was also shown numerous times to fail in delimiting species (Caisová *et al.* 2011, 2013, Assunção *et al.* 2012, Škaloud and Rindi 2013). However, Pecina and Vďačný did not find a compensatory base change in any of their proposed species and they concluded that ‘the ITS2 molecule per se failed to determine multiple clevelandellid species, as its primary sequence structure transcended several species boundaries’

Note absence of notch between posterior peristomial projection and left posterior protuberance. B, dorsal view showing unciliated posterior parts of somatic kineties (white asterisk) and cilia of circumperistomial kineties (black arrowhead). C, detail of cilia protruding from cytoproct (black arrowhead). D, posteroventral view showing right kinetal suture (white arrow) between ventral kineties (black asterisk) and dorsal kineties (white asterisk), cilia of adoral membranelles (black arrowhead), single file of ciliated basal bodies comprising posteriormost end of paroral membrane (white arrowhead), and cilia protruding from the cytoproct (black arrow). E, left dorsolateral view showing ventral kineties (black asterisk) and dorsal kineties (white asterisk) converging to form ciliary whorl on left protuberance, and kineties forming inconspicuous left posterior suture (white arrow). Scale bars: 20 μm (A, B, D, E), 10 μm (C).

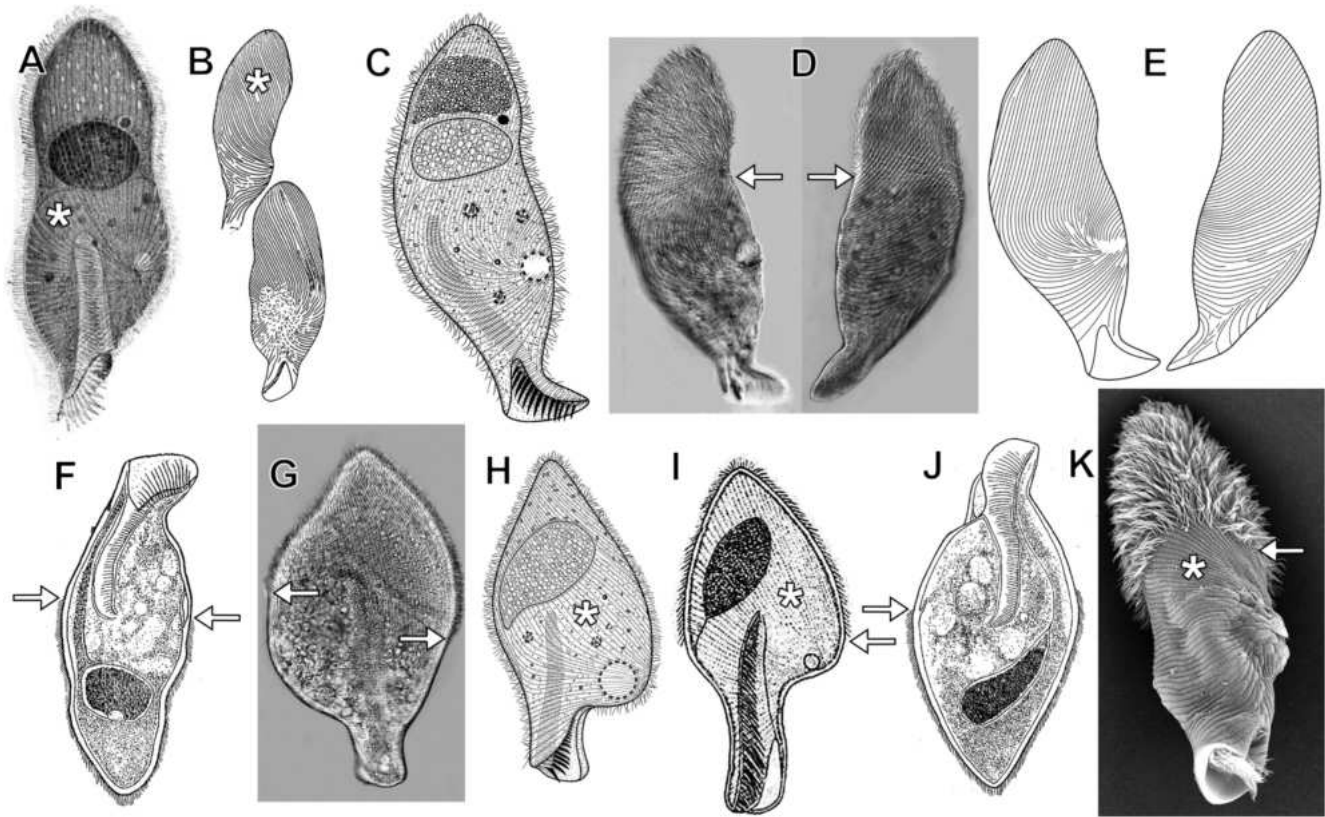


Figure 36. Various depictions of Clevelandellidae from the literature (A–J) and our picture (K). A, *Anteclevelandella constricta*, ventral view [modified from Kidder (1937), with permission]. B, *Anteclevelandella constricta* ventral view [modified from Alabaret (1975), with permission]. C–E, *Anteclevelandella constricta*, ventral view (C, viewer's left D, E), dorsal view (viewer's right D, E; modified from Pecina and Vďačný, 2020). F, *Emmaninius plantiformis*, now *Anteclevelandella constricta* [after Yamasaki (1939: fig. 8)]. G, H, *Clevelandella panesthiae*, dorsal and ventral view respectively (modified from Pecina and Vďačný, 2020). I, *C. kidderi* ventral view [modified from Mandal and Nair (1974), with permission]. J, *Emmaninius papilloris*, now *Clevelandella panesthiae* [after Yamasaki (1939: fig. 2)]. K, *Anteclevelandella constricta* under the scanning electron microscope, ventral view. Asterisk indicate true orientation of ventral kineties. Arrows depict true posterior extent of somatic ciliation.

(Pecina and Vďačný 2022). Despite that, they still did opt to describe eight new species based on minor molecular differences. *Clevelandella bipanesthiae*, for example, is diagnosed by just two bases in the 18S rRNA gene, which, according to the authors, is sufficient to distinguish and describe a species.

The abandonment of morphology in favour of purely molecular taxonomy is problematic in our view. The main basis for considering morphology and molecular taxonomy as incongruous in Clevelandellidae is that isolates with *C. panesthiae* morphology represent several genotypes, which do not form a clade, but branch at four different positions in the tree: three genotypes occupy three different places within the *C. parapanesthiae* branch; the rest of the genotypes form a separate lineage, which is rather distantly related to *C. parapanesthiae* (referred to as *Clevelandella* sp. 3 in our study). Since cells of the *C. panesthiae* morphotype cluster within a group of cells with *C. parapanesthiae* morphotype, and *C. parapanesthiae* morphotype cells do not form a clade, four new species were erected, based on molecular data, without defining which of them is the original *C. parapanesthiae* or *C. panesthiae* (Pecina and Vďačný 2020b, 2022). Two problems confound the species delimitation:

- (i) A widespread problem of sample identity in the study of Lynn and Wright (2013) should be noted. While in a more recent study (Pecina and Vďačný 2020b) cells were assigned to morphotypes based mostly on observation of protargol impregnated cells, Lynn and Wright (2013) assigned morphotypes solely on the basis of *in vivo* observations, which is, in our experience, often misleading. The cells of *Clevelandella* sp. 3 supposed by Lynn and Wright (2013: figs 3, 4) to be *C. panesthiae* have a very different shape than Kidder's (1937) description, the description in our current study (Fig. 32), and that of Pecina and Vďačný (2020b), being much smaller and more symmetrical and possessing more broadly rounded anterior. Thus, they cannot be reconciled to the same morphotype. Then, the peculiar isolate CP18 from Lynn and Wright with supposedly *C. panesthiae* morphology is most probably erroneous misidentification or typo, because it nests within *C. parapanesthiae* lineage (Pecina and Vďačný 2020b; this study). Lastly, the authors state that they isolated *C. parapanesthiae* from the host. The isolate marked as *C. parapanesthiae*, however, nests in *Rhynchoclevelandella* (this study).

- (ii) Two genotypes within *C. parapanesthiae* are described as exhibiting the *C. panesthiae* morphotype but the precise genotype associated with the morphology shown is not specified (Pecina and Vdačný 2020b). One of these genotypes is later described as a new species, *C. bipanesthiae* (Pecina and Vdačný 2022). Moreover, the isolates of the second genotype were coupled with cells of the *C. parapanesthiae* morphotype (TH 30 Psp + TH 22 Psp) and described as a new species, *C. triparapanesthiae*. We have studied the morphology of four genotypes (P37_ASS_PNG, P132_PT_VN2, P40_PGW_NB, P113_MP_MA; see Fig. 1), which are genetically almost identical to *C. bipanesthiae*, and all exhibit a clear *C. parapanesthiae* morphotype (Fig. 28A, B, E–H), so it seems highly improbable that *C. bipanesthiae* would not. While we cannot 100% rule out the possibility of dual morphology in the *C. parapanesthiae* lineage, we have never observed such a case. All our isolates in the lineage are consistently of *C. parapanesthiae* morphology. This raises the possibility that in the previous work (Pecina and Vdačný 2020b) either the cell shape might have been confused during the isolation or sequences and morphotype might have been incorrectly associated, i.e. the molecular sequences obtained might not belong to the cells with *C. panesthiae*-like morphology. This possibility is further suggested by the fact that an organism formally described as *Clevelandella nova* was later reported from the same cockroach population but without morphologic data (Pecina and Vdačný 2022). Several populations studied by us are closely related to *C. nova*, including the genetically almost identical P132_PT_VN2 (Fig. 32C), and all clearly exhibit the *C. panesthiae* morphology (Fig. 32). We conclude that it is possible that the *C. panesthiae*-like cells coupled with gene sequences from isolates TH 11E Paa and TH 45 Paa (Pecina and Vdačný 2020b), and later erected as *C. bipanesthiae* (Pecina and Vdačný 2022), probably belong to isolates TH 24–26 Paa of *C. nova*—which, in turn, we consider a junior synonym of *C. panesthiae* (see below).

In the aforementioned studies, images of cells isolated for molecular characterization were not provided. We provide these data (Supporting Information, Figs S1–S3, Table S2) to document the morphology of the sequenced cells and recommend that such data be included in future studies to exclude the possibility of misidentification when dealing with samples including multiple quite similar organisms. Despite the monophyly of the *R. hastula* group, it was split into three species (*R. hastula*, *R. bihastula* Pecina and Vdačný, 2022, and *R. trihastula* Pecina and Vdačný, 2022), for which the morphology of only one (*R. hastula*) was shown (Pecina and Vdačný 2020b, 2022). Apart from considerations of the subjective topic of splitting, conflicting morphology is also problem here: genotype P71NI_PAC_VN1 from the current study, which is genetically very similar to *R. trihastula*, clearly exhibits the *R. nipponensis* morphology, raising the possibility that *R. trihastula* might as well be *R. nipponensis*. However, the absence of morphologic data for *R. trihastula* leaves this matter unresolved. To show that

morphology is a valid tool for discrimination of clevelandellids, we performed ordination via non-metric multidimensional scaling (NMDS) based on 12 morphological traits measured from protargol-impregnated cells from a large sample of genotypes. We found that the molecularly defined monophyletic species (mostly comprising numerous genotypes) were clearly grouped into visible clusters (Fig. 35). Moreover, our linear discriminant analysis (LDA) confirmed that Clevelandellidae cells can be correctly attributed to genetically defined species based solely on morphometrical data with 88% success (Supporting Information, Table S49). While some of the species are weaker in this regard (*C. ananiasi*, *C. kidderi*, and *Rhynchoclevelandella* sp. 2), they can be easily distinguished by qualitative characteristics, such as the cell or nucleus shape.

The basis for proposed synonymizations in Clevelandellidae

Anteclevelandella salganeae Pecina and Vdačný, 2022 and *Anteclevelandella macropanesthiae* Pecina and Vdačný, 2022 branch within a well-supported clade of isolates with *Anteclevelandella constricta* morphology (Fig. 1). We have inspected morphology of isolates with identical 18S rRNA gene sequences and almost identical ITS/28S rRNA gene sequences to *Anteclevelandella salganeae* (P100_SRU_CA, P136_SRU_CA) and isolate with almost identical 18S sequence as *Anteclevelandella macropanesthiae* (P59_ASS_PNG) and they all exhibit clear *Anteclevelandella constricta* morphology (Fig. 19D, F) and are indistinguishable from other *Anteclevelandella constricta* isolates. We thus consider *Anteclevelandella salganeae* and *Anteclevelandella macropanesthiae* junior subjective synonyms of *Anteclevelandella constricta*.

Isolates of *Paraclevelandia brevis* and *Paraclevelandia simplex* form a well-supported clade composed of a mix of both morphotypes. Moreover, we can see instances of both morphotypes belonging to the same genotype and coming from the same host individual (P49_STH_PNG, P17_PAA_PH, P40_PGW_NB). We thus regard *Paraclevelandia simplex* as a junior synonym of *Paraclevelandia brevis* and assume that both morphotypes probably represent two life stages of the same species.

Clevelandella biparapanesthiae Pecina and Vdačný, 2022, *C. triparapanesthiae* Pecina and Vdačný, 2022, and *C. bipanesthiae* branch within a well-supported clade of isolates having *C. parapanesthiae* morphology. The morphology of *C. biparapanesthiae* and *C. triparapanesthiae* isolates (Pecina and Vdačný 2020b) is highly similar to our various *C. parapanesthiae* isolates and consistent with the original description of this species by Kidder (1937). As mentioned above, the morphology published by Pecina and Vdačný (2020b) possibly does not represent the *C. bipanesthiae* isolates, and all four genotypes from the current study, which are genetically nearly identical to *C. bipanesthiae*, exhibit canonical *C. parapanesthiae* morphology. We thus consider *C. biparapanesthiae*, *C. triparapanesthiae*, and *C. bipanesthiae* junior synonyms of *C. parapanesthiae*.

Clevelandella kidderi matches *C. lynni* very closely in size, shape, and macronuclear morphology. Despite that, the authors of the latter description did not compare the species. Both organisms have the same spade-shaped silhouette and conspicuous left posterior lobe with a distinct notch, a feature not well illustrated but mentioned in the description of *C. kidderi*: ‘... left side

more arched and forms a notch where it joins the peristomial projection' (Mandal and Nair 1974). They also share the same broadly ovoidal macronucleus with a posterior karyophore and a peristomial overture occupying about two-thirds of the length of the peristomial projection. Fixed individuals of *C. kidderi* and *C. lynni* have a similar length (105 vs. 94 μm) and width (52 vs. 46 μm), and the peristomial projection occupies a similar portion of the cell body (33% vs. 34 %). Considering these facts, and after examination of the type material deposited by Pecina and Vdačný (2020a), we consider *C. lynni* a junior synonym of *C. kidderi*. The hemicyclia so characteristic of Clevelandellidae, and mentioned by Mandal and Nair (1974), is also present in *C. lynni* but was previously overlooked (Pecina and Vdačný 2020a: figs 2A, E–G, 3A).

Clevelandella nova Pecina and Vdačný, 2022 branches within a well-supported clade of isolates with *C. panesthiae* morphology. The isolates correspond with Kidder's (1937) original description and the clade includes isolates from the type host (PAA) from Philippines as was studied by Kidder (1937). Therefore, the isolates appear to be conspecific with the *C. panesthiae* population of Kidder (1937) and not with the Australian isolates of Lynn and Wright (2013) or the isolates of Pecina and Vdačný (2020b), the latter two having possibly been misidentified as discussed above. In addition, we have studied the morphology of isolate P132PA_PT_VN2 (Fig. 32C) genetically almost identical to *C. nova*, which exhibits typical *C. panesthiae* morphology. Moreover, if the morphology earlier described by Pecina and Vdačný (2020b) for isolates, which were later described as *C. bipanesthiae* (Pecina and Vdačný 2022), truly belongs to *C. nova* (as discussed above), *C. nova* is morphologically indiscernible from our *C. panesthiae* isolates. We thus consider *C. nova* a junior synonym of *C. panesthiae*.

Rhynchoclevelandella trihastula is genetically very similar to *R. nipponensis*. Similarly, *R. bihastula* is genetically very similar to *R. hastula*. But until their morphology is studied, we prefer to retain their current species status.

Comments on recently erected genera in Clevelandellidae

Genus *Anteclevelandella* was described as follows: 'AZM on the left wall and PM on the right wall of vestibulum' (Pecina and Vdačný 2022). This is probably a misinterpretation since, if one accepts the morphologic chirality of Clevelandellidae as depicted by Kidder (1937), Albaret (1975), and Mandal and Nair (1974), all Clevelandellidae have a right-sided adoral zone and a left-sided paroral (with respect to the organism). Neither *Clevelandella constricta* nor any other species deviate from this arrangement (Fig. 19A–D). It also has to be noted that the depiction of the infraciliature of *C. constricta* (Pecina and Vdačný 2020b) showing right-hand spiralling somatic kineties is inconsistent with all previous descriptions (Kidder 1937, Mandal and Nair 1974, Albaret 1975) and the data from the current study (Figs 19D, 20A–D). One possible explanation is inadvertent reversal of images (Fig. 36D; Pecina and Vdačný 2020b: fig. 3F, G) from which drawings appear to have been made (Fig. 36C, E; Pecina and Vdačný 2020b: fig. 1A, M, N); no deposition of voucher material is mentioned in the study, which could help resolve the problem. All Clevelandellidae have left-hand spiralling ventral somatic kineties and straighter (i.e. more or less longitudinal)

dorsal kineties (Albaret 1975, Kidder 1937, Mandal and Nair 1974; this study). We propose a new diagnostic character for the genus: constriction of the cell at the level of the macronucleus.

Genus *Rhynchoclevelandella* was morphologically defined by the absence of a karyophore (Pecina and Vdačný 2022). We, however, show, that its representatives do have a karyophore (Fig. 21K, O, S). We note that, in our experience, the presence or absence of the karyophore (anterior, posterior, or both) is a highly unreliable taxonomic character in Clevelandellidae. This leaves the genus without morphological characterization. Despite this we prefer to consider the genus as valid. This may seem like an inconsistency in light of the numerous species-level synonymizations we made above. However, the species we synonymize are not only morphologically indistinguishable, but also genetically almost identical with already existing species. *Rhynchoclevelandella*, on the other hand, is a distinct phylogenetic lineage. Furthermore, if we did not synonymize these species, many of the current species would become paraphyletic. Conversely, if we synonymize *Rhynchoclevelandella* with *Clevelandella*, then *Clevelandella* would become paraphyletic with respect to *Paraclevelandia*. Although *Rhynchoclevelandella* lacks a recognized morphological apomorphy, at the current state of knowledge, we prefer to retain it.

Purely molecular taxonomy as applied to Clevelandellidae

The abandonment of morphology and its replacement by purely molecular species diagnoses, as done by Pecina and Vdačný (2022), is hampered, at least in the case of clevelandellids, by possible incorrect assignment of genotypes to various morphotypes as discussed in detail above. In the current study, we found that morphological data are highly consistent with the molecular phylogenetic data. The employment of geometrical methods (Pecina and Vdačný 2020b) can be potentially useful if applied to error-less datasets, and also with the addition of more characters than just body shape alone (e.g. macronuclear shape). On the other hand, using purely molecular methods to describe new species in a group for which we know very little about the possible diversity or what the genetic boundaries among species might be or how to apply a barcode-based delimitation approach, seems fraught with difficulties as described above. Perils of such an approach can be best seen on the example of *C. nova*, which we consider a junior synonym of *C. panesthiae*, the type species of the genus.

A number of species have been described by just splitting the existing ones (*Anteclevelandella constricta*, *R. hastula*, and *C. parapanesthiae*), generally making every population a separate species regardless of similar morphology (Pecina and Vdačný 2022). If we adopt this approach, we would have to add approximately 30 species just from the data in the current study, resulting in the disruption of clevelandellid systematics by artefactual species inflation. However, as we were able to find and describe six new morphologically and molecularly distinct species of genus *Clevelandella* by inspecting just a small fraction of host diversity, there is probably a great diversity of clevelandellids yet to be explored and there is no need to erect new species inside already existing ones, especially if such species are diagnosed by just a few nucleotide bases. Ironically, providing DNA barcodes as species diagnoses, or what has been termed the 'minimalist

approach' (Zamani *et al.* 2022), was editorially roundly criticized (Engel *et al.* 2021) in the same journal in which just such an approach to the Clevelandellida was later published (Pecina and Vdačný 2022). This practice has, by no means, been limited to protists and its application in entomology has also been disputed (Zamani *et al.* 2022).

From a conceptual standpoint, providing only molecular sequences, to the exclusion of other characters, as species diagnoses could be considered the antithesis of so-called 'integrative taxonomy'. This integrative approach embraces not only morphologic and molecular characters, but also ecologic, metabolic, and behavioural characters, among others, in the determination of species boundaries (Dayrat 2005, Clamp and Lynn 2017, Vdačný 2017, Dupérré 2020). The practice of diagnosing species solely on the basis of nucleotide substitutions and deposition of DNA extracted from individual cells as holotypes seems to contradict the recommendation that: '...delineation of units of life's diversity needs to be reciprocally illuminated by an integrative approach that takes into account and critically weights both morphological and molecular data' (Vdačný 2017, Pecina and Vdačný 2022). Furthermore, this practice ignores (or rejects) the existence of intraspecies and intragenomic rRNA gene sequence variability (Alverson and Kolnick 2005, Gribble and Anderson 2007, Lindner *et al.* 2013, Weber and Pawlowski 2014, Wang *et al.* 2017).

Shrinkage of protargol-impregnated Clevelandellidae

It is well established that the extent to which preparation changes the size and shape of ciliates varies with species, the fixative used, and staining method applied (Jerome *et al.* 1993). Ciliate cells impregnated by the same protargol method have been estimated to shrink by 20–30% in the case of *Fuscheria terricola* (Berger *et al.* 1983), 10% in the case of *Protospathidium serpens* (Xu and Foissner 2005a), 10–20% for *Arcuospathidium cultriforme* (Xu and Foissner 2005b), and 20–40% for *Dileptus sphagnicola* (Vdačný and Foissner 2012). Recently, a conversion value of 15% has been more frequently chosen (Foissner 2016, Vdačný and Foissner 2017a, b, 2019).

In recent studies of Clevelandellidae, the published *in vivo* cell size was calculated from combined morphometric data of protargol preparations considering 15% preparation shrinkage combined with *in vivo* measurements (Pecina and Vdačný 2020a, b). The difference between dimensions of directly measured live cells and protargol preparations in the current study shows significant and variable deviation from the popular 15% value (Supporting Information, Table S50). While the mean of shrinking for all the species combined is roughly close to constants used in aforementioned studies ($18.6 \pm 7.4\%$ for length, $16.6 \pm 10.9\%$ for width), the high standard deviation indicates there is a striking variability in shrinkage among different species, e.g. change in length from 4.2% in *R. hastula* to 29.2% in the long morph of *C. hromadkai*. Moreover, shrinkage is not necessarily symmetric, the most striking example being *C. elongata* in which length decreases by 22.9%, but its width increases by 12.5% in protargol preparations. Since the advent of affordable and widely available digital imaging, there has been no reason to substitute estimated dimensions for actual direct measurements of live cells. We recommend abandoning the practice of publishing

estimated *in vivo* cell sizes for ciliates, based on *in vivo* measurements mixed with measurements of fixed cells adjusted by arbitrary conversion factors, as numbers obtained in such a way are highly unreliable.

Comparison of newly described species with similar species

All dimensions mentioned are the mean of measurements from protargol preparations, unless indicated otherwise. In the case of multiple populations of one species, the range of means is provided.

Clevelandella ananiasi, *C. philipi*, *C. sidi*, and *C. fryntai* share a similar overall shape (spade-shaped with a conspicuous left-sided notch at the base of the peristomial projection) with *C. parapanesthiae* and *C. kidderi*.

Clevelandella ananiasi can be distinguished from *C. philipi* by: posterior protrusion of left lobe of anterior body part (overhanging no more than one-fifth of peristomial projection vs. overhanging about one-third of peristomial projection), number of adoral membranelles (23 vs. 30 or 32). It can be distinguished from *C. sidi* and *C. fryntai* by length (55 vs. 117 μm and 129 or 158 μm). It can be distinguished from *C. parapanesthiae* by: length (55 vs. 79–90 μm), macronucleus (teardrop-shaped to ellipsoidal vs. scimitar-shaped), and number of adoral membranelles (23 vs. 36–39). It can be distinguished from *C. kidderi* by: length (55 vs. 94 μm) and number of adoral membranelles (23 vs. 48).

Both *C. ananiasi* and *C. philipi* are of similar size as *R. nipponensis*, *Rhynchoclevelandella* sp. 2, and *R. hastula*. However, they can be distinguished from them by different body shape, with *Rhynchoclevelandella* species being more slender (L/W ratio in *C. ananiasi* and *C. philipi* 1.8–1.9 vs. 2.6 in *R. nipponensis*, 2.2 in *Rhynchoclevelandella* sp. 2, and 3.4 in *R. hastula*), outline (right and left margins of anterior body part straight or convex vs. more or less concave), and posterior protrusion of the left lobe of the anterior body part (overhanging vs. not overhanging).

Clevelandella philipi can be distinguished from *C. sidi* by length (63–75 vs. 117 μm). *Clevelandella philipi* can be distinguished from *C. parapanesthiae* by: length (63–75 vs. 79–90 μm), macronucleus (teardrop-shaped to ellipsoidal vs. long scimitar-shaped), and number of adoral membranelles (30–32 vs. about 36–39). *Clevelandella philipi* can be distinguished from *C. kidderi* by: length (63–75 vs. 94 μm), peristomial projection (24–28% vs. 32% of the body), number of adoral membranelles (30–32 vs. about 47), and posterior protrusion of the left lobe of the anterior body part (strongly overhanging vs. slightly overhanging).

Clevelandella sidi can be distinguished from the rest of spade-shaped species by morphology of the paroral (both files equal length vs. right file shorter than left). It can be distinguished from *C. parapanesthiae* by: length (117 vs. 79–90 μm), L/W ratio (2.5 vs. 1.8–1.9), and macronucleus (broad inverted teardrop-shaped vs. scimitar-shaped). It can be distinguished from *C. kidderi* by: length (117 vs. 94 μm) and L/W ratio (2.5 vs. 1.9). It can be distinguished from *C. fryntai* by: length (114 vs. 129–158 μm) and outline (right and left margins of anterior body part straight vs. convex).

Clevelandella fryntai is the largest of the spade-shaped Clevelandellida (see above). *Clevelandella fryntai* can be distinguished from *C. parapanesthiae* by: length (129–158 vs. 79–90 μm), number of adoral membranelles (62–67 vs. 36–39),

and macronucleus (inverted teardrop-shaped to lenticular vs. scimitar-shaped). It can be distinguished from *C. kidderi* by: length (129–158 vs. 94 μm) and number of adoral membranelles (62–67 vs. 46).

Clevelandella hromadkai in short morph can be easily distinguished from all other Clevelandellidae species due to its unique shape and appearance. A long morph of *C. hromadkai* can be distinguished from *C. elongata* by: length (132–155 vs. 220 μm), number of adoral membranelles (58–62 vs. 94), overall shape (broadest in the central third of the cell vs. more worm-like narrow appearance), and posterior protrusion of the left lobe of the anterior body part (present vs. absent).

Clevelandella klobasa can be distinguished from *C. panesthiae* by: length (135 vs. 105–121 μm), macronucleus (sausage-like vs. teardrop-shaped), peristomial projection (inconspicuous vs. conspicuous), adoral zone (markedly curved vs. slightly oblique), and number of adoral membranelles (59 vs. about 45–48).

Genetic variability and host/geographic specificity of Clevelandellidae

The current study samples Clevelandellidae from the broadest range of hosts to date, enabling at least some assessment of intra-specific genetic variability, host specificity, and biogeography. For most species, our isolates from different hosts and/or localities show some degree of genetic variability (Fig. 1). This is in stark contrast with recent publications (Pecina and Vďačný 2020b, 2022) where *Anteclevelandella constricta*, *R. hastula*, *C. panesthiae* (referred to under a younger synonym *C. nova*), and *C. parapanesthiae* from different host populations and distant localities are genetically identical. While the current study is primarily based on material obtained directly from wild host populations and bred in controlled cultures at the Department of Zoology of Charles University, the aforementioned works included material exclusively from commercial breeders. It is, however, questionable whether the conditions in commercial cultures were sufficient to prevent contamination by symbionts. Since Clevelandellidae form cysts (Kidder 1937), they can very likely cross-contaminate different host populations when the hosts come into contact with each other's faeces. This may possibly lead to one dominant genotype among contaminated populations originating from different species and locations.

Genus *Rhynchoclevelandella* was, despite extensive host sampling (Pecina and Vďačný 2022; this study), never found in host genus *Salganea*, which is repeatedly retrieved as a sister-lineage to the rest of the Panesthiinae (including Geoscapheinae) (Lo et al. 2016, Beasley-Hall et al. 2021). Other than that, the host specificity does not seem to be an important factor for the Clevelandellidae symbionts. Although several species have so far been reported from a single host species, this most likely reflects under-sampling. The majority of species can be found in diverse hosts. However, there may be some degree of geographic specificity, both regarding species and intraspecific relationships. The *C. ananiasi*/*C. philipi* clade is so far exclusive to Papua New Guinea (PNG) and *Clevelandella* sp. 3 to Australia. Within *C. panesthiae*, two large clades were identified—one exclusive to PNG and the second comprising the remaining isolates from Vietnam (VN), Thailand (TH), and the Philippines (PH). Similarly, of the three *Rhynchoclevelandella* clades, one is exclusive to Australia, one to

PNG, and one to Asia. Interestingly, the exclusively STH clade of *Anteclevelandella constricta* isolates from PNG branches together with a group from various hosts inhabiting other islands—Borneo (NB), Philippines (PH), and Halmahera (HA). A more confident assessment of Clevelandellidae biogeography and host specificity will require even broader sampling and a more robust phylogenetic analysis based on more molecular markers. However, it is possible that in such an evolutionarily young ciliate group with small genetic distances and high potential for spreading of cysts-contaminated faeces, the final picture will be mostly stochastic with the ciliates forming separate lineages not according to the host, but rather according to geographical isolation of a particular locality.

Interestingly it seems that in natural populations different genotypes of a single species can coexist at one locality. We have detected two sister-genotypes of *Anteclevelandella constricta* in STH, two genotypes of *Paraclevelandia brevis* in STH, two genotypes of *Paraclevelandia brevis* in ASS, two sister-genotypes of *C. fryntai* in SRU, and, most interestingly, two distantly related genotypes of *Anteclevelandella constricta* in one individual from PAC.

Diversity of Clevelandellidae

Although the family Clevelandellidae was erected more than 80 years ago (Kidder 1937), only two additional species have been described on the basis of the morphology until recently: *Metaclevelandella termitis*, and *C. kidderi*. Recent works (using both morphological and molecular methods), excluding the current study, have surprisingly found no new morphologically distinct species (Pecina and Vďačný 2020a, b, 2022). There are two possible (and probably concurrent) reasons:

- (i) Low host diversity: the majority of all studies ever done on Clevelandellidae have dealt with symbionts exclusively from a few representatives of a single host genus *Panesthia* (mostly just subspecies of *Panesthia angustipennis*), which is the most common Panesthiinae in captivity. Although Pecina and Vďačný (2022) tried to explore more diverse hosts by including a few host individuals of the genera *Macropanesthia* and *Salganea*, no new morphologically distinct species were found. The subfamily Panesthiinae, however, comprise at least 169 species in 11 genera.
- (ii) The studies included hosts obtained exclusively from private breeders. It is possible that prolonged captivity of the hosts may have a negative impact on the diversity of symbionts, as mentioned above.

The fact that the same morphospecies of Clevelandellidae were found in hosts from Philippines (Kidder 1937), Japan (Yamasaki 1939), Andaman Islands (Mandal and Nair 1974), Australia (Lynn and Wright 2013, Pecina and Vďačný 2022), and also in Vietnam, Thailand, and Cambodia (Pecina and Vďačný 2020a, b) might give the misimpression that Clevelandellidae have low species diversity. The findings of the current study, however, show the opposite.

While we still chose to use five populations of genus *Panesthia* (PAA, PGW, PAC, PA, and PT), we broadened the sampling of hosts with genera *Ancaudellia* (ASS, AP, and AH), *Miopanesthia*

(MP), and *Salganea* (STH, STR, SRU, and SR). Six new species in genus *Clevelandella* were molecularly and morphologically characterized and one (*Clevelandella* sp. 4) has not yet been completely characterized. Six of them are found only in the newly included hosts. Lynn and Wright (2013) considered all the organisms they have found as identical to species described by Kidder (1937). But when we put their sequences into the context of broader molecular data, we see that they probably include two new species (*Rhynchoclevelandella* sp. 1 and *Clevelandella* sp. 3) with unknown morphology, possibly indigenous to Australia. It is thus anticipated that there is still a considerable diversity of Clevelandellidae to be found by exploring new host genera and populations from isolated localities.

Considerations for future studies

The Clevelandellidae provide a rich diversity of endocommensal ciliates for the study of biogeography, host specificity, ultrastructure, cell physiology, and evolution. Although the current study and previous studies (Pecina and Vďačný 2020a, b, 2022) shed further light on the biogeography and host specificity of Clevelandellidae, more remains to be learned necessitating even broader host sampling (e.g. wider sampling of Geoscapheinae). To date, there has been relatively little ultrastructural study of Clevelandellidae (Albaret 1975). The comparative ultrastructure of features such as the complex internalized ciliated excretory apparatuses of nyctotherids and Clevelandellidae would be of particular interest, especially with the advent of sophisticated techniques such as focused ion beam scanning electron microscopy (Kizilyaprak et al. 2014). More detailed morphological data will better inform possible evolutionary scenarios for the evolution of the ‘posteriorized’ Clevelandellidae from a *Nyctotherus*-like ancestor. In terms of cell physiology, the origin and composition of the cytoplasmic carbohydrate platelets and their contribution to cell metabolism require further study. Techniques such as Raman microscopy may provide specific information on the polysaccharide composition of these structures (Smith et al. 2016). Transcriptomic methods should provide information on metabolic pathways involved in the formation of these products and of their metabolic role at various stages of the cell cycle, and offer answers to the question, as to whether Clevelandellidae play any role in wood digestion. Such approaches may clarify the relative benefits in the symbiotic relationship of Clevelandellidae and cockroaches: is the relationship actually mutualistic or do Clevelandellidae enjoy a more one-sided benefit from the relationship?

Taxonomic summary

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Remark: The voucher material along with inventory numbers is summarized in Supporting Information, Table S51.

Taxonomic assignment: Ciliophora: Intramacronucleata: SAL clade: APM clade: Armophorea: Clevelandellida.

Family Clevelandellidae Kidder, 1938

Diagnosis: Small to large Armophorea with posteriorized oral structures. Adoral zone of membranelles on right and paroral

membrane on left side of peristomium. Somatic ciliature hemitrichous (possibly except in *Metaclevelandella*). Excretion via an internal ciliated excretory antrum.

Type genus: *Clevelandella* Kidder, 1938.

Included genera: *Anteclevelandella* Pecina and Vďačný, 2022; *Clevelandella* Kidder, 1938; *Metaclevelandella* Uttangi and Desai, 1963; *Paraclevelandia* Kidder, 1937; *Rhynchoclevelandella* Pecina and Vďačný, 2022.

Remarks: Although *Metaclevelandella termitis*, not reported since its original description, was described as holotrichously ciliated, the possibility that incomplete ciliation was overlooked (as was the case in Kidder’s descriptions of Clevelandellida) cannot be excluded. This question can only be resolved by a thorough redescription.

Genus *Anteclevelandella* Pecina and Vďačný, 2022

Diagnosis: Body elongate and nearly cylindrical, only slightly flattened dorsoventrally, constricted in the region of macronucleus. Macronucleus evenly ovoid, its long axis parallel to the transverse axis of the body. Peristomial projection inconspicuous, merges gradually with body proper.

Type species:

Clevelandia constricta Kidder, 1937.

Included species:

Anteclevelandella constricta (Kidder, 1937).

Anteclevelandella constricta (Kidder, 1937)

Synonyms: *Emmaninius plantiformis* Yamasaki, 1939; *Clevelandella plantiformis* (Yamasaki, 1939); *Anteclevelandella macropanesthia* Pecina and Vďačný, 2022; *Anteclevelandella salganeae* Pecina and Vďačný, 2022.

Genus *Clevelandella* Kidder, 1938

Amended diagnosis: Body broadly spade-shaped to elongate curved elliptical with posterior peristomial projection.

Type species: *Clevelandia panesthia* Kidder, 1937.

Included species: *Clevelandella ananiasi* sp. nov., *C. elongata* (Kidder, 1937); *C. fryntai* sp. nov.; *C. hromadkai* sp. nov.; *C. panesthia* (Kidder, 1937); *C. parapanesthia* (Kidder, 1937); *C. philipi* sp. nov.; *C. kidderi* Mandal and Nair, 1974; *C. klobasa* sp. nov., *C. sisi* sp. nov.

Clevelandella ananiasi sp. nov.

ZooBank registration: urn:lsid:zoobank.org:act:693D96F5-0E81-4648-BE2B-F5AC52FD31CB.

Diagnosis: Spade-shaped, dorsoventrally flattened *Clevelandella* with average length in protargol preparations 55 µm, mean 23 adoral membranelles, posterior protrusion of left lobe of anterior body part overhanging no more than one-fifth of peristomial projection, and with teardrop-shaped or ellipsoidal macronucleus.

Etymology: Named in honour of Mr Ananias Kamam, our collaborator from Papua New Guinea.

Type locality: Papua New Guinea, Wanang 3, S5.228, E145.080.

Type host: *Ancaudellia serratissima serratissima* (Brunner von Wattenwyl, 1865).

Type material: A protargol-impregnated holotype cell (Fig. 4D, E) marked in a black circle on a slide deposited in the collection of the National Museum of the Czech Republic, Prague, Czech Republic, inventory number P6E 5431.

Clevelandella fryntai sp. nov.

ZooBank registration: urn:lsid:zoobank.org:act:E84B29E8-A18E-44E3-92E3-663958980E9D.

Diagnosis: Spade-shaped, dorsoventrally flattened *Clevelandella* with average length in protargol preparations 143 µm, mean 65 adoral membranelles, and with teardrop-shaped or lenticular macronucleus.

Etymology: Named in honour of eccentric zoologist and ethologist Prof. Daniel Frynta who provided the material.

Type locality: Cambodia, near Mondulkiri province, N12.383, E107.176.

Type host: *Salganea rugulata* Saussure, 1895.

Type material: A protargol-impregnated holotype cell (Fig. 17H–J) marked in a black circle on a slide deposited in the collection of the National Museum of the Czech Republic, Prague, Czech Republic, inventory number P6E 5460.

Clevelandella hromadkai sp. nov.

ZooBank registration: urn:lsid:zoobank.org:act:EB15F99F-FD7D-49F0-9F63-6771F3697429.

Diagnosis: Dorsoventrally not flattened *Clevelandella* of cultriform shape, body proper curved in dorsal direction, with prominent left lobe and peristomial projection merging gradually with body proper. Average length in protargol preparations 127 µm in short form and 215 µm in long form.

Etymology: Named in honour of remarkable Czech cockroach enthusiast Mr Jiří Hromádka.

Type locality: Papua New Guinea, Wanang 3, N5.228, E145.080.

Type host: *Ancaudellia serratissima serratissima* (Brunner von Wattenwyl, 1865).

Type material: A protargol-impregnated holotype cell (Fig. 9D) marked in a black circle on a slide deposited in the collection of the National Museum of the Czech Republic, Prague, Czech Republic, inventory number P6E 5364.

Clevelandella klobasa sp. nov.

ZooBank registration: urn:lsid:zoobank.org:act:CC02115A-273A-43BA-9BCB-4F6783939788.

Diagnosis: Dorsoventrally flattened *Clevelandella*, broadly lanceolate, peristomial projection inconspicuous, peristomial opening broad. Macronucleus sausage-shaped. Average length in protargol preparations 170 µm.

Etymology: The species epithet is from *klobása* (n., feminine gender), the Czech word for sausage, referring to the sausage-shaped macronucleus.

Type locality: Papua New Guinea, Wanang 3, N5.228, E145.080.

Type host: *Salganea ternatensis hirsuta* Roth, 1979.

Type material: A protargol-impregnated holotype cell (Fig. 13D) marked by a black arrow on a slide deposited in the collection of the National Museum of the Czech Republic, Prague, Czech Republic, inventory number P6E 5380.

Clevelandella philipi sp. nov.

ZooBank registration: urn:lsid:zoobank.org:act:72561759-FBB1-4007-AF97-39630E48C436.

Diagnosis: Spade-shaped, dorsoventrally flattened *Clevelandella* with average length in protargol preparations 69 µm, mean 31 adoral membranelles, posterior protrusion of left lobe of anterior body part overhanging up to one-third of peristomial projection, and with teardrop-shaped or ellipsoidal macronucleus.

Etymology: Named in honour of Mr Philip Komong, our collaborator from Papua New Guinea.

Type locality: Papua New Guinea, Wanang 3, N5.228, E145.080.

Type host: *Salganea ternatensis hirsuta* Roth, 1979.

Type material: A protargol-impregnated holotype cell (Fig. 7C, D) marked in a black circle on a slide deposited in the collection of the National Museum of the Czech Republic, Prague, Czech Republic, inventory number P6E 5381.

Clevelandella sidi sp. nov.

ZooBank registration: urn:lsid:zoobank.org:act:312386D2-BE75-45F2-AEE7-E24D28C77719.

Diagnosis: Dorsoventrally flattened *Clevelandella* with average length in protargol preparations 142 µm, mean 52 adoral membranelles, broad, inverted teardrop-shaped macronucleus, and entirely diplostichomonad POM.

Etymology: Named in honour of exceptional terrain entomologist and leader of PNG expedition, Dr Petr 'Sidi' Stiblík.

Type locality: Cambodia, near Mondulkiri NP, N12.383, E107.176.

Type host: *Salganea rugulata* Saussure, 1895.

Type material: A protargol-impregnated holotype cell (Fig. 15C) marked in a black circle on a slide deposited in the collection of the National Museum of the Czech Republic, Prague, Czech Republic, inventory number P6E 5438.

***Clevelandella panesthiae* (Kidder, 1937)**

Synonyms: *Emmaninius papilloris* Yamasaki, 1939; *Clevelandella papilloris* (Yamasaki, 1939); *Clevelandella nova* Pecina and Vdačný, 2022.

***Clevelandella parapanesthiae* (Kidder, 1937)**

Synonyms: *Clevelandella bipanesthiae* Pecina and Vdačný, 2022; *C. biparapanesthiae* Pecina and Vdačný, 2022; *C. triparapanesthiae* Pecina and Vdačný, 2022.

***Clevelandella kidderi* Mandal and Nair, 1974**

Synonym: *Clevelandella lynni* Pecina and Vdačný, 2020

Genus *Paraclevelandia* Kidder, 1937

Diagnosis: As in Kidder (1937).

Type species: *Paraclevelandia brevis* Kidder, 1937.

***Paraclevelandia brevis* Kidder, 1937**

Synonym: *Paraclevelandia simplex* Kidder, 1937.

Genus *Metaclevelandella* Uttangi and Desai, 1963

Diagnosis: As in Uttangi and Desai (1963).

Type species: *Metaclevelandella termitis* Uttangi and Desai, 1963.

Genus *Rhynchoclevelandella* Pecina and Vdačný, 2022

Amended diagnosis: Clevelandellidae with slender, spade-shaped cells (L/W ratio >2), more closely related to *R. hastula* than to *Anteclevelandella constricta*, *C. panesthiae*, and *Paraclevelandia brevis*.

Type species: *Clevelandia hastula* Kidder, 1937.

Included species: *Rhynchoclevelandella bihastula* Pecina and Vdačný, 2022; *R. hastula* (Kidder, 1937); *R. nipponensis* (Kidder, 1937) (syn. *Emmaninius longicollis* Yamasaki, 1939; *Clevelandella longicollis* (Yamasaki, 1939); *R. trihastula* Pecina and Vdačný, 2022).

SUPPLEMENTARY DATA

Supplementary data are available at *Zoological Journal of the Linnean Society* online.

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CREDIT STATEMENT

Michael Kotyk: conceptualization, methodology, formal analysis, investigation, resources, writing—original draft, review and editing, visualization, project administration, funding acquisition. William A. Bourland: methodology, formal analysis, investigation, writing—original draft, review and editing, visualization. M. Soviš: investigation. Daniel Méndez-Sánchez: visualization. P. Škaloud: formal analysis, writing—original draft. Zuzana Kotyková Varadinová: formal analysis, resources, writing—original draft, review and editing. Ivan Čepička: formal analysis, funding acquisition, supervision, writing—original draft, review and editing.

CONFLICT OF INTEREST

None declared.

DATA AVAILABILITY

Sequence data underlying this article are available in GenBank at <https://www.ncbi.nlm.nih.gov/nucleotide/>, accession numbers OQ993372–OQ993504 (Supporting Information, Table S2). Permanent protargol slides are deposited in the collection of the National Museum of the Czech Republic, Prague, Czech Republic <https://www.nm.cz/en> with catalog numbers (Supporting Information, Table S51).

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ORIGINAL ARTICLE



Morphology, morphogenesis, and molecular characterization of *Castula specialis* sp. nov. (Ciliophora, Armophorea, Metopida)

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Abstract

The morphology, morphogenesis, and molecular phylogeny of a new metopid ciliate, *Castula specialis* sp. nov., comprising three strains from geographically distant (China, Mexico, Czech Republic) anoxic freshwater habitats, were studied based on microscopic observation of live and protargol-stained specimens as well as SSU rRNA gene sequence data. The new species is characterized as follows: size in vivo 105–220 × 25–70 μm, body oblong to elongated ellipsoidal and asymmetrical; preoral dome distinctly projecting beyond the body; 32–46 adoral membranelles; 31–52 somatic kineties; and 4–7 setae. This study brings the first morphogenetic investigation of a member of the genus *Castula*. The morphogenesis of the type population (China) of the new species proceeds as in *Metopus* spp. comprising drastic changes in body shape and a pleurotelokinetal stomatogenesis; however, the main difference is the origin of the opisthe's paroral membrane that derives from all perizonal rows and some adjacent dome kineties. Phylogenetically, the genus *Castula* is paraphyletic.

KEY WORDS

anaerobic ciliates, binary fission, Metopidae, phylogeny, SSU rRNA gene

INTRODUCTION

WITH the growing interest in discovering the diversity and important ecological function of anaerobic archaea, anaerobic ciliates as their main hosts also have become a matter of great concern recently (Bourland et al., 2017a; Li et al., 2020; Rotterová et al., 2022; Seitz et al., 2019; Zhang et al., 2021; Zhuang et al., 2021). The order Metopida Jankowski, 1980, consisting of three families, i.e. Apometopidae Foissner, 2016, Metopidae Kahl, 1927, and Tropidoatractidae Rotterová et al., 2018, is the most speciose taxon within the class Armophorea Lynn, 2004, and Metopida usually inhabits anoxic and hypoxic terrestrial, freshwater, or marine environments (Bourland et al., 2020; Bourland & Wendell, 2014; Hu, 2014; Li et al., 2017).

Recently, some nominal species of the genus *Metopus* Claparède & Lachmann, 1858 have been transferred to several new metopid genera, and even families based

on cell morphology and SSU rRNA gene sequences (Bourland et al., 2017a, 2018, 2020; Foissner, 2016; Rotterová et al., 2018; Vďačný & Foissner, 2017). Despite the advances in the taxonomy of metopids, some known species still lack a proper redescription and many others might still need to be discovered.

Castula Bourland et al., 2020 is a newly erected genus with *Metopus setosus* Kahl, 1927 as a type species (Bourland et al., 2020). *Castula* resembles *Metopus*, except for its long setae which are interspersed in the posterior quarter to a third of the cell with ordinary somatic cilia (Bourland et al., 2020). So far, *Castula* includes only three species, i.e. *Castula setosa* (Kahl, 1927) Bourland et al., 2020, *C. flexibilis* Bourland et al., 2020, and *C. fusca* (Kahl, 1927) Bourland et al., 2020. In this study, a new congener is added.

Ontogenetic studies may provide valuable information for the analyses of ambiguous phylogenetic relationships

between ciliate taxa (Corliss, 1968; Lynn & Small, 2002). However, morphogenetic data are only available for four metopid species: *Metopus inversus* (Jankowski, 1964) Foissner & Agatha, 1999, *Lepidometopus platycephalus* Vďačný & Foissner, 2017, *M. hasei* Sondheim, 1929, and *M. boletus* Vďačný & Foissner, 2019 (Foissner & Agatha, 1999; Vďačný & Foissner, 2017, 2019). While molecular data and phylogenetic analyses are missing for the former two species, the latter two species fall phylogenetically into the “IAC” clade (Bourland et al., 2020). Consequently, the cell division mode of metopids remains widely unknown.

In this report, we describe a new *Castula* species, using morphological and phylogenetic analyses based on light and scanning microscopy and SSU rRNA gene sequences, respectively. In addition, we provide the first morphogenetic investigation of the genus *Castula*, further contributing to our understanding of the diversity and life cycle in metopids.

MATERIALS AND METHODS

Sample collection

Castula specialis sp. nov. was isolated from three distant freshwater localities: (1) from the upper 5 cm sedimentary layer of a pond (36°03'59" N, 120°20'43" E) in Qingdao, China, in September 2020, where the water temperature was 23°C (QD0901 strain); (2) from sediment of the freshwater marsh Atarasquillo in Lerma municipality, Estado de México (19°20'37" N, 99°30'09" W), Mexico, in September 2019 (LERMETZ strain); and (3) from sediment of a small agricultural water reservoir in Žinkovy (49°28'32" N, 13°29'23" E), Czech Republic, in August 2021 (LIPKY2 strain). These three strains were all established as non-clonal cultures. Three additional *Castula* and one *Pileometopus* species were isolated, but their morphology was not studied in detail. *Castula fusca* (LERMONSTR strain) and *Castula* sp. (LERM10C strain) were obtained from the same site as the Mexican LERMETZ strain of *Castula specialis* sp. nov.; *Castula* sp. (LIB2C strain) was obtained from sediment of a small pond in Libický luh (50°06'48" N, 15°10'34" E), Czech Republic. A population of *Pileometopus* sp. (MOKOTPIL strain) was isolated from raw samples from a freshwater pond adjacent to the Vltava River in Prague (49°59'25" N, 14°24'04" E), Czech Republic, in January 2023.

The culture medium for the Chinese population (QD0901 strain) was made, using straw medium (Carolina™ Protozoan Pellets, Carolina Biological Supply Company). Specifically, one pellet of the medium was added to 1 L of sterilized freshwater before being inflated with nitrogen to induce anoxic conditions. About 10 mL of the sample were inoculated in 60 mL of the medium and maintained at 26°C. To promote bacterial growth, a sterilized wheat grain was also added.

Subcultures were made at approximately 2-week intervals and no other metopids besides the newly described species were observed in the cultures. For the remaining strains, 1–2 mL of each sample was inoculated in 10 mL of Sonneborn's *Paramecium* medium (ATCC medium 802) and kept at room temperature as previously described in Bourland et al. (2017b).

Morphological methods

Living ciliates were observed and measured at magnifications of 40–1000× with bright field and differential interference contrast microscopy. Cells were stained, using synthesized protargol (Pan et al., 2013), according to Wilbert (1975) in the Chinese population and following the “Procedure A” of protargol methods in Foissner (2014) for the remaining strains. Measurements were performed at 1000× magnification. Besides, the Hoechst 33342 solution was used to show the nuclear apparatus in the Chinese population (Yang et al., 2007). Cells from the Mexican and Czech strains were studied under a fluorescence microscope (Olympus BX61), using a UV light filter with excitation at 420 nm to reveal potential intracellular methanogens (Doddema & Vogels, 1978). Scanning electron microscopy of the Mexican strain was done as follows: the cells were fixed for at least 30 min in a 1:1 solution of 5% glutaraldehyde and 2% aqueous osmium tetroxide and then processed according to Foissner (2014). Specimens were investigated at an accelerating voltage of 15 kV in a JSM-6380 scanning electron microscope (JEOL LV, USA). All illustrations of individuals were drawn based on microphotographs.

DNA extraction, amplification, cloning, and sequencing

To extract genomic DNA of *Castula specialis* sp. nov., the three additional *Castula* species, and *Pileometopus* sp., ciliate cells were picked from the cultures or raw samples and washed five times, using 0.22 µm-filtered mineral water to minimize contamination. Individuals were stored at –20°C or added directly to 30 µL of DNA/RNA Shield (Zymo Research). The genomic DNA was extracted, using the DNeasy Blood & Tissue Kit (QIAGEN), following the instructions of the manufacturer. The SSU rRNA gene sequences were amplified, using the following primer combinations: 18S-11F-Karyo and 5.8SR (Gao et al., 2012; Zhuang et al., 2022) for the Chinese population or Medlin A and Medlin B (Medlin et al., 1988) for the Czech and Mexican populations. A high-fidelity DNA polymerase, the Q5 Hot Start High-Fidelity 2× Master Mix (New England BioLabs), or a taq DNA polymerase (PPP Master Mix, Top-Bio) were used for PCR (Polymerase Chain Reaction) amplification (Wang et al., 2023). The PCR products were

purified, using the EasyPure®Quik Gel Extraction Kit (TransGen, EG101, Beijing) or the Gel/PCR DNA Fragments Extraction Kit (Qiagen). The purified PCR product (about 1800 bp) from the Chinese population was cloned, using the pEASY®-T1 cloning kit (TransGen, CT101) according to the manufacturer's instructions, transferred into competent *Escherichia coli* DH5α cells, and then sequenced in both directions by five reactions (M13R, 18SR, +B, 900F, 900R) in TSINGKE (Qingdao, China; Zhao et al., 2015). The purified PCR products from the remaining populations were sequenced, using three (for LERM10C and LERMONSTR) or four internal primers (Elwood et al., 1985). Contigs were assembled by Seqman 7.1.0.44 (DNASTar) or Geneious 2020.0.5.

Phylogenetic analyses

To determine the phylogenetic position of *Castula specialis* sp. nov., the three obtained sequences, plus the new sequences of *C. fusca*, *Castula* spp., and *Pileometopus* sp. were aligned along with 85 SSU rRNA gene sequences of seven ciliate classes obtained from GenBank, consisting of Armophorea (Clevelandellida, Metopida, Armophorida), Litostomatea, Odontostomatea, Parablepharisma, Muranotrichea, and Cariacotrichea. Two heterotrichean species (*Spirostomum subtilis* and *Stentor roeselii*) and three karyorelictean species (*Geleia sinica*, *Kentrophoros gracilis*, and *Loxodes striatus*) were used as outgroups.

Alignments were constructed, using MAFFT under the G-INS-i algorithm (Kato et al., 2002). The original alignment consisted of 1896 positions. The alignment was manually edited in BioEdit 7.0.5.2 (Hall, 1999), trimming the ends, mostly the primers, resulting in a matrix of 1778 positions. Phylogenetic trees were reconstructed by maximum likelihood (ML) and Bayesian inference (BI) methods. The uncorrected genetic distances were calculated in MEGA 6.0 (Tamura et al., 2013), and gaps and missing data were treated, using pairwise deletion.

Maximum-likelihood analysis was implemented on the CIPRES Science Gateway server (<http://www.phylo.org>), using RAXML-HPC2 version 8.2.12 (Stamatakis, 2014) under the GTRGAMMAI model. Support values for the maximum-likelihood analyses came from 1000 bootstrap pseudoreplicates, using heuristic searches.

A Bayesian inference analysis was carried out, using MrBayes v. 3.2.7a on XSEDE (Ronquist et al., 2012) on the CIPRES Portal. The GTR+I+Γ model was selected by the Akaike Information Criterion in MrModeltest 2.2 (Nylander, 2004) as the best substitution model, with support from four simultaneous Markov chain Monte Carlo (MCMC) chains run for ten million generations with sampling every 100 generations. The first 25% of sampled trees were considered as burn-in; convergence was assessed, using Tracer v1.7.2 (Rambaut et al., 2018). MEGA 6.0 (Tamura et al., 2013) was used to visualize the tree topologies.

Following Hillis and Bull (1993), we consider bootstrap values ≥ 95 as high, from 71 to 94 as moderate, from 50 to 70 as low, and < 50 as no support. For Bayesian posterior probabilities, we consider values ≥ 0.95 as high and values < 0.95 as low following Alfaro et al. (2003).

Terminology

General terminology mainly follows Lynn (2008), Foissner and Agatha (1999), Foissner (2016), Vďačný and Foissner (2017), and Bourland et al. (2020). In metopids, “setae” refer to the long cilia that can stiffen and are originated over the posterior one-quarter to one-third of the cell, different from caudal cilia that originate from the rear cell (Bourland et al., 2020).

Cell division is a gradual process, and the timing is basically in the same as described by Vďačný and Foissner (2017). Accordingly, we consider those “late early dividers” whose perizonal stripe elongates to the posterior body portion (Figures 4E–G and 5E–G) and specimens whose opisthe is slightly longer than the proter as “early late dividers” (Figures 6C,D and 8A).

RESULTS

Description based on type population from China

Cell size in vivo $105\text{--}180 \times 25\text{--}60 \mu\text{m}$, length:width ratio 3.8:1 on average, ranging from 3.2:1 to 4.6:1 ($n=19$); protargol-stained specimens $108\text{--}180 \times 31\text{--}56 \mu\text{m}$ in size ($138 \times 44 \mu\text{m}$ on average), length:width ratio on average 3.2:1 ($n=31$; Table 1). Body outline elongated elliptical in ventral view, anterior half of cell twisted leftwards, posterior end rounded, widest in middle cell portion (Figures 1A and 2A–C). The preoral dome distinctly projected from the left margin of the anterior body portion, inclined about 60° to the horizontal axis of the cell, occupying near 50% of body length in vivo (Figures 1A and 2A). Nuclear apparatus in mid-body and left of cytostome, composed of one macronucleus and one micronucleus. Macronucleus about $40 \times 20 \mu\text{m}$ in size, usually reniform, with nucleoli scattered and usually about $0.4\text{--}2.1 \mu\text{m}$ across in both protargol-prepared and Hoechst 33342-stained specimens (Figures 1A,E and 2A,B,I). Micronucleus globular to ellipsoidal, up to about $5 \mu\text{m}$ across after Hoechst 33342 staining, in an invagination or pouch near the middle of the macronucleus (Figures 1A,E and 2A,B,I). Contractile vacuole distinct, obconical, and positioned terminally (Figures 1A and 2C,E). Cytoplasm colorless. Some food vacuoles, about $8 \mu\text{m}$ in diameter, packed with bacteria. Cortical granules globular and colorless, less than $0.5 \mu\text{m}$ across in vivo, arranged in six or seven loose, but relatively regular rows between adjacent ciliary rows (Figure 2F). Prokaryotic methanogenic symbionts were not studied in

TABLE 1 Morphometric characteristics for *Castula specialis* sp. nov. Chinese strains (QD0901 from the type population, the Mexican strain LERMETZ, and the Czech strain LIPKY2).

Characteristics ^a	Strain	Mean	<i>M</i>	SD	CV	Min	Max	<i>n</i>
Body, length in vivo	QD0901	133.6	133.0	21.8	16.3	105.0	180.0	23
	LERMETZ	176.1	175.0	23.7	13.5	140.2	220.0	23
	LIPKY2	136.9	140.2	11.6	8.4	108.8	153.7	14
Body, width in vivo	QD0901	41.8	35.0	5.8	13.9	25.0	60.0	19
	LERMETZ	52.8	53.9	6.3	12.0	41.6	69.9	23
	LIPKY2	37.4	37.6	5.0	13.4	26.6	44.7	14
Body, length:width, ratio in vivo	QD0901	3.8	3.9	0.4	10.2	3.2	4.6	19
	LERMETZ	3.3	3.2	0.3	8.8	2.9	3.8	23
	LIPKY2	3.7	3.6	0.3	7.5	3.2	4.2	14
Body, length	QD0901	138.4	138.0	12.0	8.7	108.0	180.0	31
	LERMETZ	142.1	141.9	11.8	8.3	115.5	165.8	26
	LIPKY2	118.8	118.7	12.1	10.2	96.7	135.5	11
Body, width	QD0901	43.9	44.0	5.8	13.2	31.0	56.0	31
	LERMETZ	35.8	34.5	4.3	12.1	29.3	45.6	26
	LIPKY2	31.1	31.3	4.6	14.8	24.0	40.3	11
Body, length:width, ratio	QD0901	3.2	3.0	0.5	14.5	2.6	4.6	31
	LERMETZ	4.0	4.1	0.4	9.4	3.3	4.7	26
	LIPKY2	3.9	3.8	0.5	12.6	2.8	4.7	11
Anterior pole to distal end of adoral zone, distance	QD0901	7.2	7.0	2.0	27.8	2.0	12.0	28
	LERMETZ	8.2	7.7	2.0	24.4	5.2	13.3	26
	LIPKY2	6.5	5.8	2.1	32.1	4.0	10.5	11
Anterior pole to distal end of adoral zone, % of body length	QD0901	5.3	5.4	1.5	28.2	1.5	8.8	28
	LERMETZ	5.8	5.6	1.4	24.7	3.7	8.8	26
	LIPKY2	5.5	5.6	1.6	28.8	3.0	8.5	11
Anterior pole to anterior end of macronucleus, % of body length	QD0901	32.4	32.0	2.1	6.6	26.4	36.8	20
Anterior pole to posterior end of macronucleus, distance	QD0901	84.8	85.0	4.5	5.3	79.0	92	20
	LERMETZ	71.6	71.0	6.5	9.1	60.2	86.8	26
	LIPKY2	56.5	59.1	4.9	8.8	46.6	62.2	11
Anterior pole to posterior end of macronucleus, % of body length	QD0901	62.3	63.0	2.3	3.7	56.0	66.0	20
	LERMETZ	50.5	50.1	3.4	6.8	45.1	58.2	26
	LIPKY2	47.8	46.9	3.8	7.9	43.5	56.4	11
Anterior pole to proximal end of adoral zone, distance	QD0901	71.8	72.0	5.6	7.8	60.0	80.0	28
	LERMETZ	63.1	63.2	4.8	7.5	55.2	72.0	26
	LIPKY2	56.1	54.7	4.9	8.8	47.3	65.4	11
Anterior pole to proximal end of adoral zone, % of body length	QD0901	52.9	53.0	3.0	5.7	48.0	60.0	28
	LERMETZ	44.5	44.2	3.4	7.7	39.7	53.9	26
	LIPKY2	47.4	45.6	3.9	8.2	42.9	54.6	11
Adoral zone, length	QD0901	65.0	65.5	5.8	9.0	53.0	74.0	28
	LERMETZ	54.9	55.3	4.5	8.3	47.4	63.7	26
	LIPKY2	49.6	48.3	5.0	10.0	42.5	57.4	11
Adoral zone, % of body length	QD0901	47.6	48.0	3.2	6.8	41.9	54.8	28
	LERMETZ	38.8	37.8	3.1	8.0	33.0	46.1	26
	LIPKY2	42.0	41.3	4.3	10.1	34.4	48.7	11

TABLE 1 (Continued)

Characteristics ^a	Strain	Mean	<i>M</i>	SD	CV	Min	Max	<i>n</i>
Macronucleus, length	QD0901	40.3	40.5	3.9	9.7	32.0	47.0	28
	LERMETZ	40.6	40.1	5.3	13.0	31.3	52.7	26
	LIPKY2	28.3	28.4	2.6	9.3	22.6	32.0	11
Macronucleus, width	QD0901	19.8	19.5	1.9	9.4	16.0	23.0	28
	LERMETZ	17.1	16.6	2.5	14.5	13.6	22.0	26
	LIPKY2	11.6	11.1	2.1	18.3	8.6	15.9	11
Adoral membranelles, number	QD0901	37.8	38.0	2.0	5.2	33.0	41.0	34
	LERMETZ	42.0	42.0	2.0	4.7	38.0	46.0	26
	LIPKY2	38.0	37.0	3.2	8.5	32.0	42.0	9
Somatic kineties ^b , number	QD0901	37.8	38.0	3.2	8.6	31.0	50.0	33
	LERMETZ	47.0	47.0	2.6	5.6	40.0	52.0	26
	LIPKY2	43.0	43.5	3.3	7.9	36.0	46.0	8
Preoral dome kineties, number	QD0901	11.2	11.0	2.0	18.1	7.0	17.0	33
	LERMETZ	13.0	13.0	1.6	12.1	10.0	16.0	26
	LIPKY2	11.0	11.0	1.3	12.1	9.0	12.0	5
Postoral kineties, number	QD0901	26.6	27.0	3.2	11.9	20.0	36.0	33
Largest adoral membranelle, width	QD0901	13.0	13.0	2.2	16.3	8.0	16.0	34
Perizonal ciliary stripes, number	QD0901	5.0	5.0	0.0	0.0	5.0	5.0	34
	LERMETZ	5.0	5.0	0.0	0.0	5.0	5.0	26
	LIPKY2	5.0	5.0	0.0	0.0	5.0	5.0	10
Paroral membrane, length	QD0901	39.0	38.0	3.8	9.9	31.0	49.0	30
	LERMETZ	26.0	26.5	2.5	9.7	20.4	30.8	25
	LIPKY2	24.0	23.4	2.5	10.6	20.0	29.0	9

Note: Data are from protargol-stained specimens, except those labeled as “in vivo”.

Abbreviations: CV, coefficient of variation (%); *M*, median; Max, maximum; Min, minimum; *n*, number of specimens examined; SD, standard deviation.

^aMeasurements in μm .

^bExcluding the perizonal ciliary stripe rows.

the type population. Swims moderately fast by rotating about the cell's longitudinal axis.

Somatic cilia about 10 μm long in vivo, except for slightly longer perizonal cilia (11–18 μm long) and 4–6 setae about 15–30 μm long (Figures 1A and 2D,E). Somatic kineties composed of dikinetids having cilia associated with both basal bodies (Figures 1F,G and 2L,M). Excluding the five perizonal rows, the somatic ciliature comprises 31–50 narrowly spaced kineties, including 20–36 postoral kineties. Perizonal stripe invariably composed of sequentially elongated rows, which never form false kineties. Perizonal stripe rows 1 and 2 more closely spaced than rows 3–5. Perizonal row 5 2.4–3.9 μm apart from dome kinety 1 in protargol preparations. Perizonal stripe rows 1–4 proximally shorter than adoral zone of membranelles, while row 5 extends beyond proximal end of the adoral zone (Figures 1B,F and 2J,L).

The adoral zone composed of 33–41 membranelles, commences at the anterior dorsal side, extends obliquely, and spiralized towards the ventral surface, performing a turn of about 180°, and occupying about half of the body length in protargol-stained specimens. The width of the membranelles in the adoral zone decreases

towards both zone ends with a maximum width of about 13 μm . The middle individual membranelle composed of three files of four basal bodies each anteriorly and more than 10 inclined files of three basal bodies posteriorly; proximal and distal membranelles more or less cuneiform or rectangular, the others typically L-shaped (Figures 1D,F,H and 2H,L,M). Paroral membrane bipartite, comprising two files of basal bodies in proximal part and a single file in distal part, up to 49 μm long after protargol staining, which extends posteriorly to cytostome (Figures 1F and 2K,L).

Notes on the LERMETZ (Mexico) and LIPKY2 (Czech Republic) strains (Table 1; Figure 3; Video S1). Both populations are highly similar to the Chinese population in shape, size in vivo as well as in the numbers of adoral membranelles and somatic kineties. They possess prokaryotic methanogenic symbionts. Their paroral membranes seem to be composed of a single row of basal bodies; further protargol-stained material or preferentially transmission electron microscopy have to confirm the paroral's structure in all three strains.

Mexican specimens (LERMETZ strain): cell size 140–220 $\times$ 40–70 μm in vivo, 116–166 $\times$ 29–46 μm after

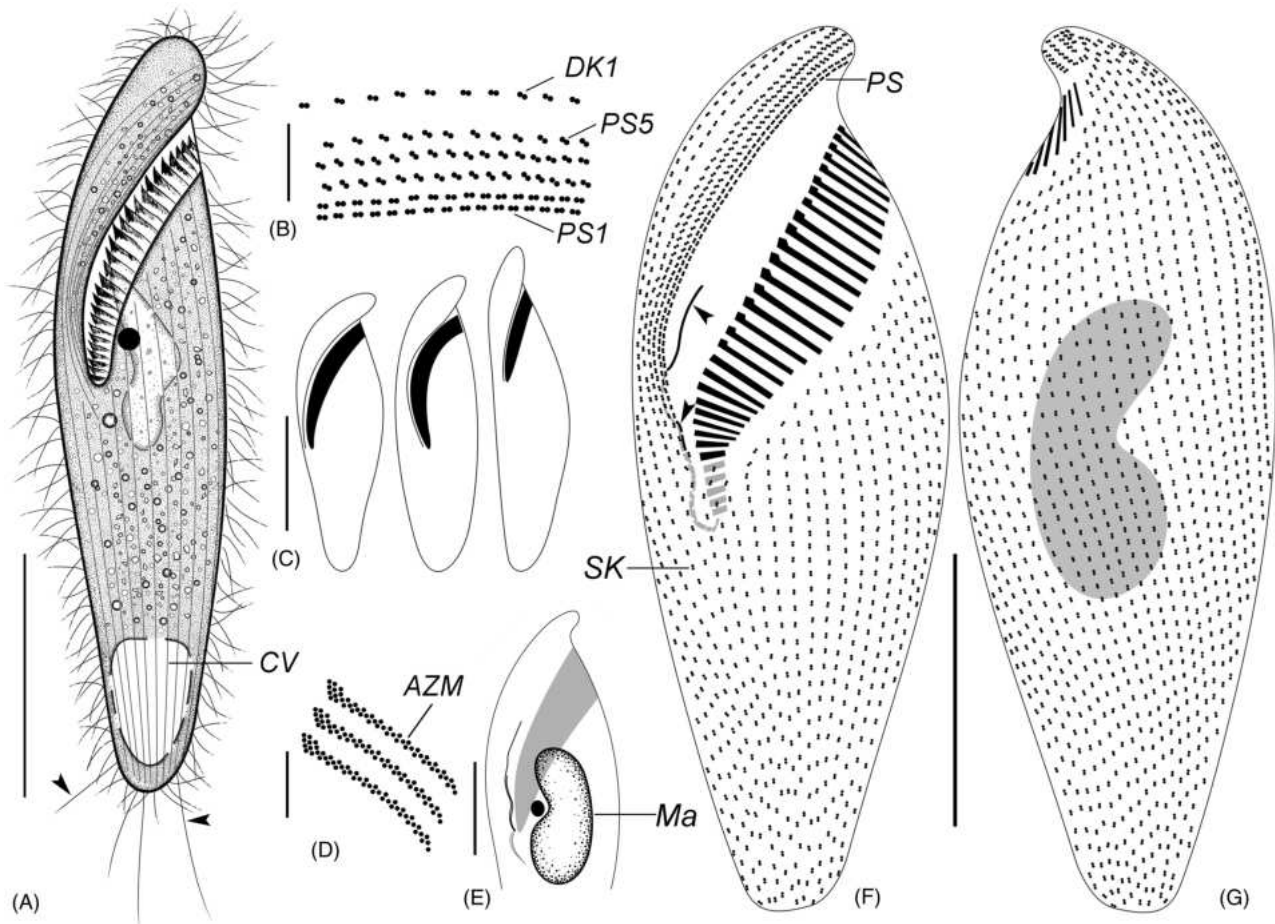


FIGURE 1 The Chinese population of *Castula specialis* sp. nov. from life (A, C) and after protargol staining (B, D–G). (A) Ventral view of a representative individual showing, inter alia, the body shape, the position of the contractile vacuole, and the elongated genus-specific setae (arrowheads). (B) Structure of the perizonal stripe and dome kinety 1. (C) Different body shapes and sizes. (D) Details of membranelles in the middle portion of the adoral zone. (E) Anterior cell portion showing the shape and position of the nuclear apparatus. Ventral (F) and dorsal (G) views of the holotype, showing the somatic kineties, the adoral zone of membranelles, the paroral membrane (arrowheads), and the perizonal stripe (double-arrowhead). AZM, adoral zone of membranelles; CV, contractile vacuole; DK1, dome kinety 1; Ma, macronucleus; PS, perizonal stripe; PS1, 5, perizonal stripe rows 1 and 5; SK, somatic kineties. Scale bars: 45 μ m (A, F, G), 5 μ m (B, D), 70 μ m (C), 35 μ m (E).

protargol staining; about 42 adoral membranelles; about 47 somatic kineties, including about 13 preoral dome kineties; 4 or 5 setae 20–37 μ m long.

Czech specimens (LIPKY2 strain): cell size 108–154 $\times$ 27–45 μ m in vivo and 97–136 $\times$ 24–40 μ m in protargol-stained specimens; 38 adoral membranelles on average; about 43 somatic kineties, including about 11 preoral dome kineties; 4–7 setae 20–35 μ m long.

Morphogenesis of the Chinese population

Generally, the ontogenesis of *Castula specialis* sp. nov. follows the metopid mode with some modifications. Binary fission occurs in freely motile conditions. The very early dividers move at normal speed, while later division stages move slowly or do not rotate about the main body axis possibly due to changes in cell shape and ciliary pattern.

Changes in cell shape

The preoral dome and the perizonal stripe undergo modification in dividers with a fundamental change in cell shape. Protargol-stained early dividers are 93–123 $\times$ 38–68 μ m in size, and their length:width ratio ranges from 1.8:1 to 2.9:1 ($n=9$), i.e. they are wider than morphostatic specimens, owing to their fusiform or broadly ellipsoidal shape (Figures 4A–E and 5A,D,E). In early dividers, the preoral dome becomes less vaulted, but still distinctly projects beyond the body proper as in vegetative cells (Figure 4A–D). In late early dividers, the perizonal stripe elongates posteriorly, terminating subterminally in the cells being about a quarter wider than vegetative ones (Figures 4E and 5E,G).

Middle dividers are conspicuously broad and 104–140 $\times$ 45–78 μ m in size with a length:width ratio of about 1.9:1 ($n=9$). The cell's outline becomes broadly elliptical with the widest portion in the mid-body (Figure 6A,B).

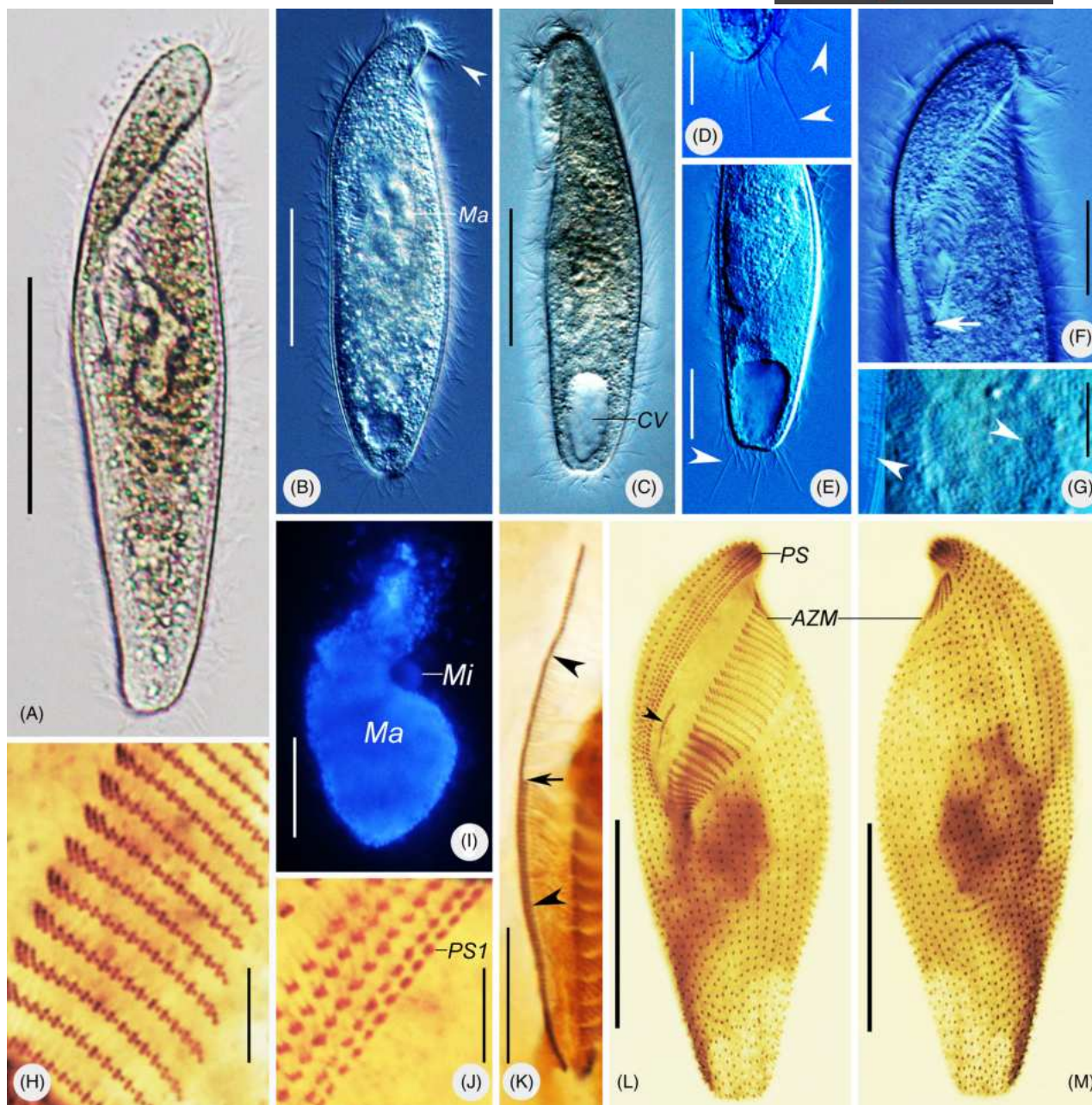


FIGURE 2 Photomicrographs of *Castula specialis* sp. nov. (Chinese type population) from life, after Hoechst 33342 (I), and protargol (H, J–M) staining. (A) Ventral view of a representative individual. (B) Ventral view of a slightly squeezed specimen showing the macronucleus and the elongated cilia of the perizonal stripe (arrowhead). (C) Dorsal view showing the large contractile vacuole. Posterior cell portions of two specimens in ventral (D) and left ventrolateral (E) views, showing the elongated setae (arrowheads). (F) Anterior body half showing the proximal end of the adoral zone (arrow). (G) Detail of cell periphery showing the cortical granules (arrowheads). (H) Details of adoral membranelles. (I) Macronucleus and micronucleus. (J) Detail of perizonal rows 1–5. (K) Detail of paroral membrane (arrowheads); arrow marks the transition from the single- to the two-rowed portion. Ventral (L) and dorsal (M) views of the holotype, showing the somatic kineties, the adoral zone of membranelles, the paroral membrane (arrowhead), and the perizonal stripe. AZM, adoral zone of membranelles; CV, contractile vacuole; Ma, macronucleus; Mi, micronucleus; PS, perizonal stripe; PS1, perizonal stripe row 1. Scale bars: 45 μ m (A–C), 10 μ m (D, G, K), 20 μ m (E, F, I), 5 μ m (H, J), 60 μ m (L, M).

The preoral dome still slightly projects from the left anterior body margin in early middle dividers (Figures 5J,L and 6A), whereas it disappears in late middle dividers.

Late dividers are 102–164 $\times$ 30–56 μ m in size with a length:width ratio of on average 2.9:1 ($n=11$). Just before the separation of proter and opisthe, the daughter

cells are broadly elliptical in outline without any sign of a preoral dome (Figures 6C,D, 7C,E–H, and 8A,C,D). In early late dividers, the opisthe is slightly longer than the proter (Figures 6C,D and 8A). With the constriction of the division furrow in the mid-body, the opisthe and proter become similar in size and the two daughter cells finally

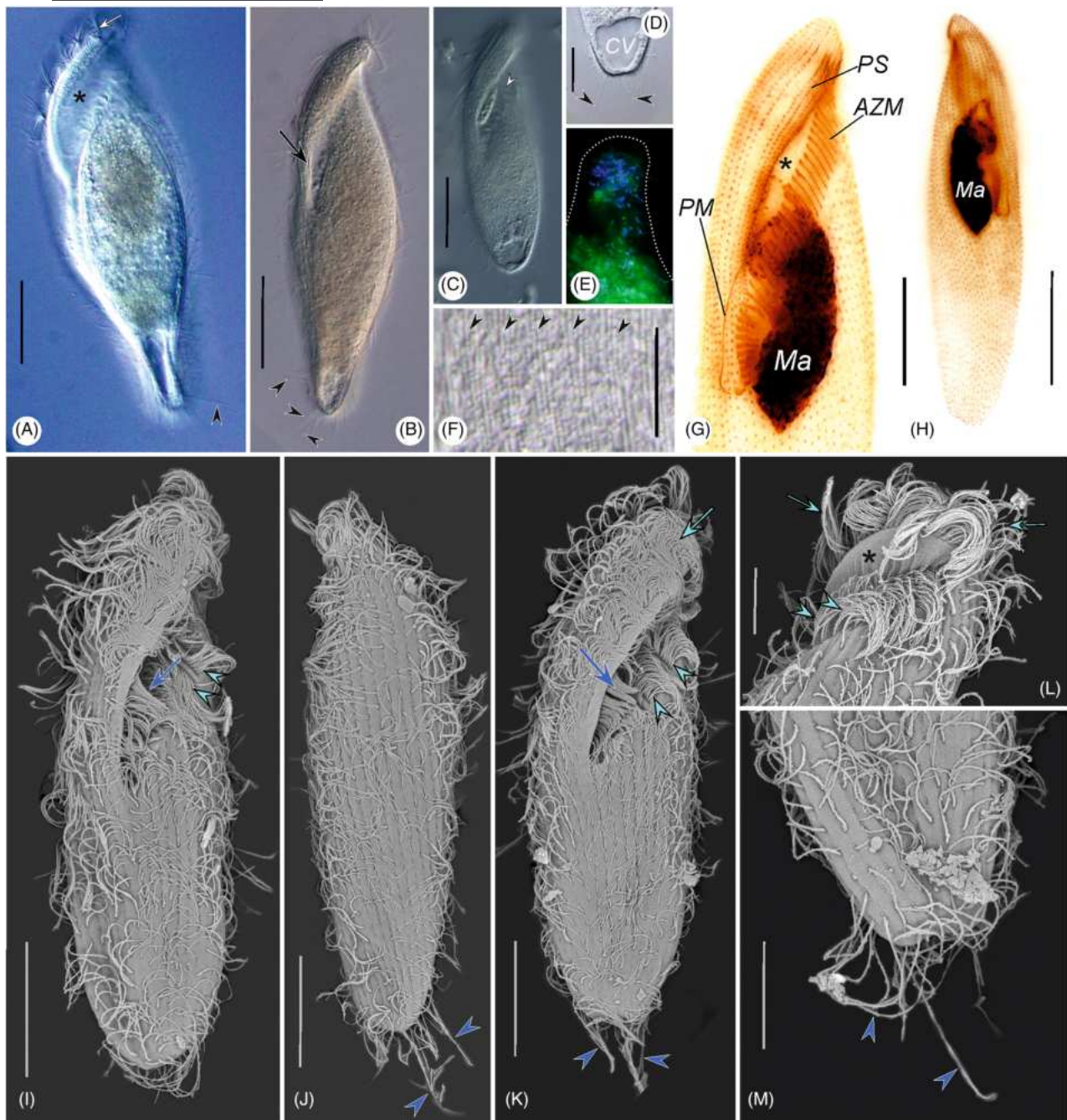


FIGURE 3 *Castula specialis* sp. nov., the Mexican strain LERMETZ (A, B, E, G–M) and the Czech strain LIPKY2 (C, D, F) from life (A–D, F), in autofluorescence (E), after protargol staining (G, H), and in the scanning electron microscope (I–M). Different individuals showing the perizonal stripe (white arrow; A), the peristome (asterisk; A), membranelles (white arrowhead; C), the paroral membrane (black arrow; B), and setae (black arrowheads; A, B). (D) Posterior cell portion showing the contractile vacuole and setae (black arrowheads). (E) Cytoplasmic rod-shaped methanogens (blue). (F) Detail of cell periphery showing the arrangement of the cortical granules (arrowheads). (G) Anterior body portion showing the somatic and oral ciliature, as well as the peristome (asterisk). (H) Dorsal view showing the somatic ciliature and the macronucleus. Ventral (I, K) and dorsal (J) views showing the somatic ciliature, the paroral membrane (blue arrows; I, K), membranelles (turquoise arrowheads; I, K), the perizonal stripe (blue arrow; K), and setae (blue arrowheads; J, K, M). (L) Anterior cell portion showing the peristome (asterisk), membranelles (arrowheads), and the perizonal stripe (arrows). (M) Posterior cell portion showing the setae (black arrowheads). AZM, adoral zone of membranelles; CV, contractile vacuole; Ma, macronucleus; PM, paroral membrane; PS, perizonal stripe. Scale bars: 40 μ m (A–C, G–K), 20 μ m (D, M), 10 μ m (F, L).

separate. Post-dividers are oblong with a slight anterior torsion, namely, the preoral dome appears but just slightly projects beyond the cell, twisting leftwards (Figure 8F). Accordingly, post-dividers differ from vegetative cells.

Development of adoral membranelles

Stomatogenesis is pleurotelokinetal, namely, the oral primordium originates from several somatic kineties

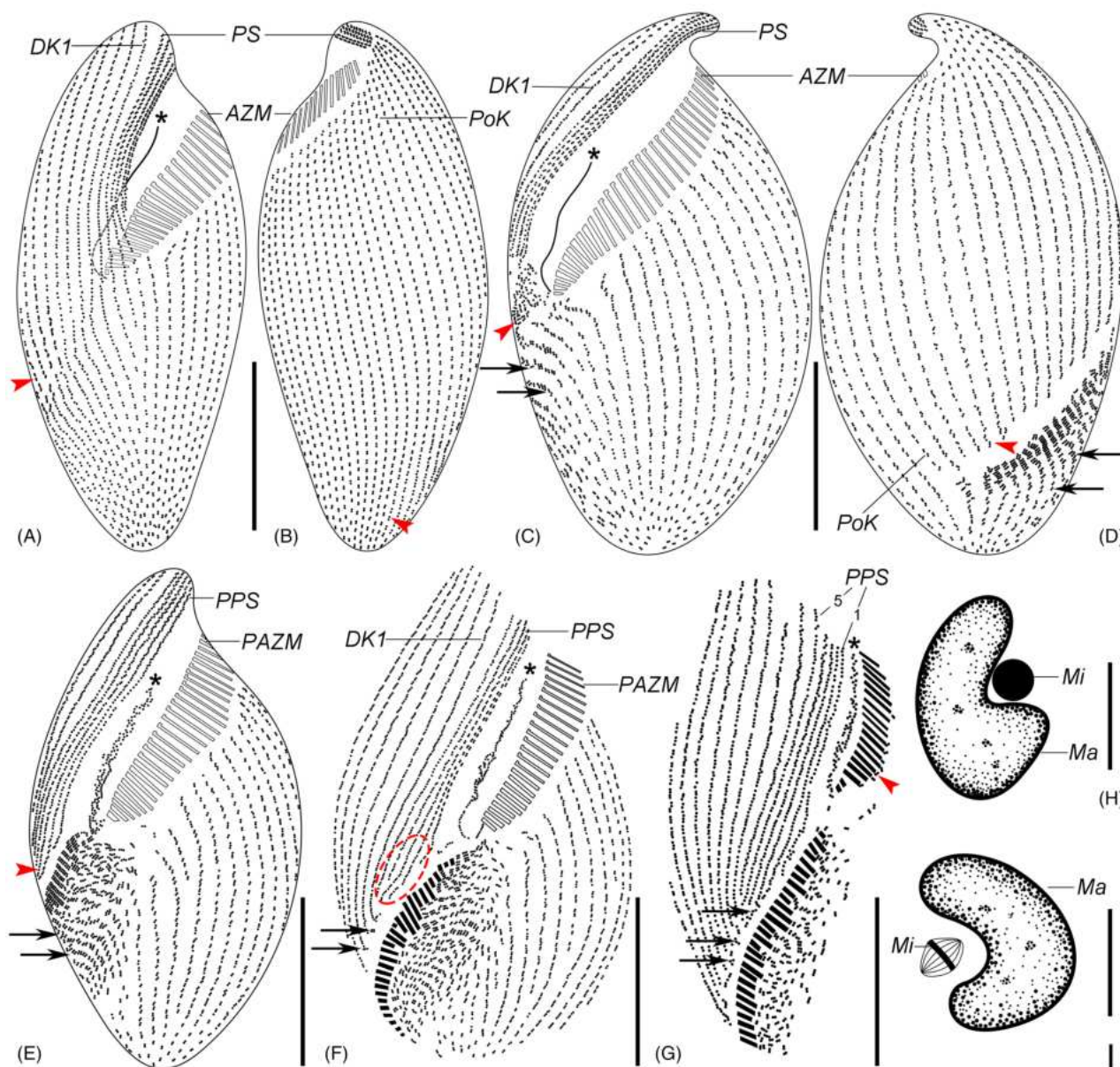


FIGURE 4 Early dividers of *Castula specialis* sp. nov. (Chinese type population) after protargol staining. Asterisks mark the parental paroral membrane (A, C) or anlage for paroral membrane of the proter (E–G). Ventral (A) and dorsal (B) views of the same specimen, showing the replication of dikinetids, which begins posterior to the cell equator (arrowheads). Ventral (C) and dorsal (D) views of the same individual, showing the formation of the adoral membranelles in the opisthe (arrows), the dedifferentiation of the perizonal stripe in its posterior portion (arrowhead in C), and the postoral kineties which are involved in the formation of the adoral membranelles (arrowhead in D). (E) Ventral view, showing the rearrangement and proliferation of kinetids in the parental perizonal rows (arrowhead), the dedifferentiation of the parental paroral membrane (asterisk), and the continuous formation of new adoral membranelles (arrows). (F) The elongated perizonal rows (red circle) and some dome kineties are involved in the formation of the paroral membrane of opisthe (arrows). (G) Arrows mark the ends of the parental perizonal rows 1–5 and some dorsal or dorsolateral kineties which are involved in the formation of opisthe's paroral membrane. The arrowhead marks the parental adoral zone which starts to reorganize. (H, I) Nuclear apparatus in early dividers. AZM, adoral zone of membranelles; DK1, dome kinety 1; Ma, macronucleus; Mi, micronucleus; PAZM, proter's adoral zone of membranelles; PoK, postoral kineties; PPS, proter's perizonal stripe; PS, perizonal stripe. Scale bars: 40 μ m (A–E), 30 μ m (F, G), 20 μ m (H, I).

in the posterior cell half. Stomatogenesis commences within several postoral kineties and dome kineties. In very early dividers, about three postoral kineties and fourteen dome kineties but no perizonal stripe rows are involved in the stomatogenesis. Further, the proliferation of basal bodies commences in the posterior portion of the kineties, progressing anteriorly and posteriorly,

generating the oral primordium (Figure 4A,B). Next, more postoral kineties become involved in the formation of the oral primordium. Interestingly, these somatic kineties and the opisthe's adoral membranelles do not show a simple proportional relationship. All dome kineties (11 on average) and several postoral kineties form the opisthe's adoral membranelles (37 on

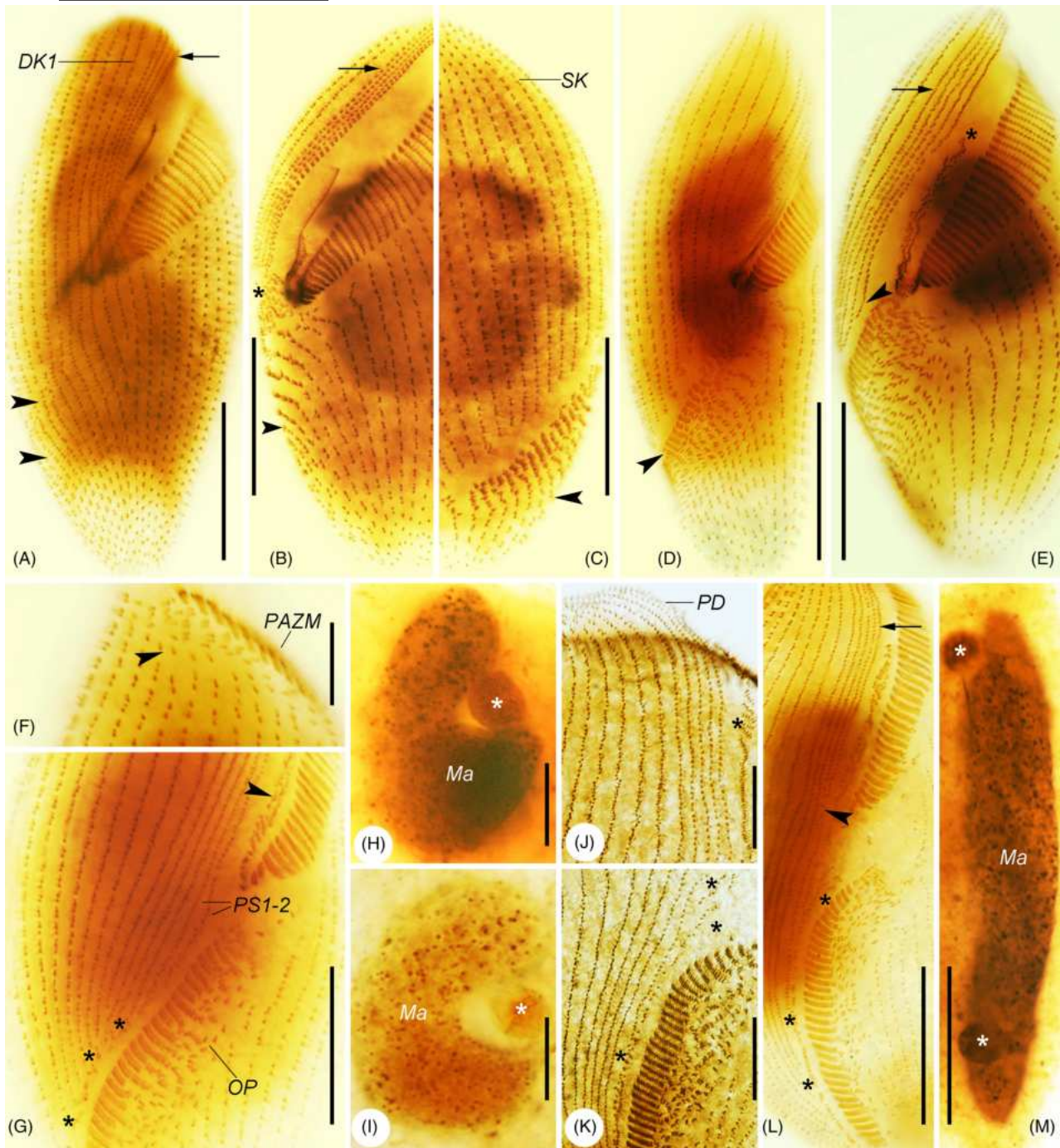


FIGURE 5 *Castula specialis* sp. nov. (Chinese type population), early (A–I) and middle dividers (J–M) after protargol staining. Arrows mark the parental perizonal stripe (A, C, E, L). (A) Ventral view of a very early divider, showing the proliferation of dikinetids (arrowheads). Ventral (B) and dorsal (C) views of the same specimen, showing the forming adoral membranelles (arrowheads), and the dedifferentiation of perizonal stripe in its posterior portion (asterisk). (D) Right lateral view of an early divider, showing the newly formed adoral membranelles (arrowhead). (E) Ventral view. Arrowhead and asterisk mark the proliferation of the parental perizonal rows and the dedifferentiation of paroral membrane of proter, respectively. (F) Left lateral view showing the proliferation of kinetids in the dome kineties (arrowhead). (G) Asterisks mark the ends of the parental perizonal rows 1–5 and some dorsal or dorsolateral kineties which are involved in the formation of the opisthe's paroral membrane. The arrowhead marks the dedifferentiation of parental paroral membrane. (H, I) Nuclear apparatus. The asterisks denote the micronucleus. Ventral views of the proter (J) and opisthe (K) in the same specimen, showing the preoral dome, the proter's paroral membrane (asterisk; J), and the opisthe's paroral membrane (asterisks; K). (L) Ventral view showing the parental perizonal rows 1 and 2 (arrow), which split (arrowhead) in mid-body to form the opisthe's paroral membrane (asterisks). (M) Nuclear apparatus. The asterisks mark the daughter micronuclei. AZM, adoral zone of membranelles; DK1, dome kinety 1; Ma, macronucleus; OP, oral primordium; PAZM, proter's adoral zone of membranelles; PD, preoral dome; PS1–2, perizonal stripe rows 1 and 2; SK, somatic kineties. Scale bars: 40 μ m (A–D), 30 μ m (E, G, L), 10 μ m (F, H, I), 20 μ m (J, K, M).

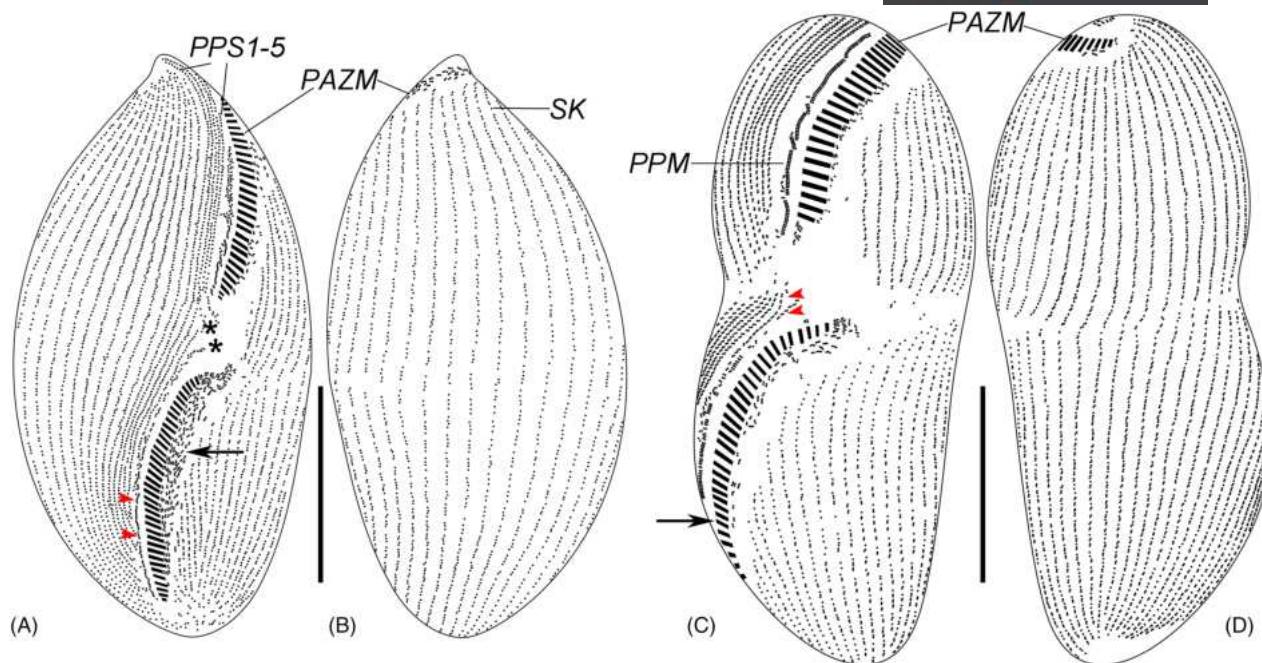


FIGURE 6 *Castula specialis* sp. nov. (Chinese population), a middle divider (A, B) and an early late divider (C, D) after protargol impregnation. Ventral (A) and dorsal (B) views of the same specimen, showing the posterior (arrowheads; A) and the anterior (asterisks; A) portions of the opisthe's paroral membrane and newly formed kineties left of the opisthe's adoral zone (arrow; A). Ventral (C) and dorsal (D) views of the same individual, showing the opisthe's paroral membrane (arrowheads; C) and the newly formed adoral zone (arrow). PAZM, proter's adoral zone of membranelles; PPM, proter's paroral membrane; PPS, proter's perizonal stripe; SK, somatic kineties. Scale bars: 40 μ m (A–D).

average). The newly formed short and scattered kinetofragments, which consist of more than two dozen basal bodies each originate from all dome kineties and several postoral kineties and arrange almost perpendicular to the kinty axes at the posterior portion of the cell and represent the future adoral membranelles (Figures 4C,D and 5B,C).

In middle dividers and late dividers, kinetofragments still migrate posteriorly. In late dividers, the opisthe's adoral zone of membranelles is shorter (the width of the largest adoral membranelle, 3–7 μ m vs. 8–16 μ m) and wider (these basal bodies of files that make up the individual membranelle are aligned vs. oblique) than that of morphostatic individuals (Figures 1D, 6C, 7B,C,G, and 8A,D).

The reorganization of the parental adoral zone, which begins in late early dividers, possibly involves only the original adoral membranelles, and these membranelles reorganize in situ, these original basal bodies seem to proliferate, migrate, and be resorbed. (Figures 4G and 5F,G). In later dividers, the assembly of the parental adoral zone gradually completes, and the basal bodies are rearranged to form rectangular membranelles rather than L-shaped membranelles (Figures 6C, 7B,C,E, and 8A,C).

The adoral zones of proter and opisthe have a similar morphology in late dividers, i.e. these membranelles are short and wide (Figures 6C, 7B,C,E,F, and 8A,C,D). In post-dividers, the adoral zones of both

proter and opisthe obtain the species-specific pattern, i.e. redundant basal bodies are probably resorbed and some kinetofragments join in reshaping the adoral membranelles into the metopid pattern (Figure 8F). Unlike in morphostatic cells, the adoral zones in both late dividers and post-dividers occupy a large proportion (85%) of the body length (Figures 6C, 7B,C,E,F, and 8A,C,D,F).

Development of perizonal stripe and paroral membrane

The opisthe's perizonal stripe and paroral membrane originate from both the parental perizonal stripe and some dome kineties.

In the early dividers, dikinetids in the posterior half of the parental perizonal stripe rows 1–5 move slightly apart (Figures 4A and 5A). The posterior portions of rows 1–5 dedifferentiate forming some disordered kinetofragments in early dividers (Figures 4C and 5B). The destruction of these kinetofragments and an intrakinetal proliferation of kinetids in the parental perizonal rows cause the posterior elongation of the perizonal rows onto the glabrous area above the opisthe's adoral zone in early dividers, with a change in cell shape (becoming round and short; Figures 4E,F and 5E,G). The formation of the opisthe's paroral membrane commences at the ends of the parental perizonal rows 1–5 and some dome kineties

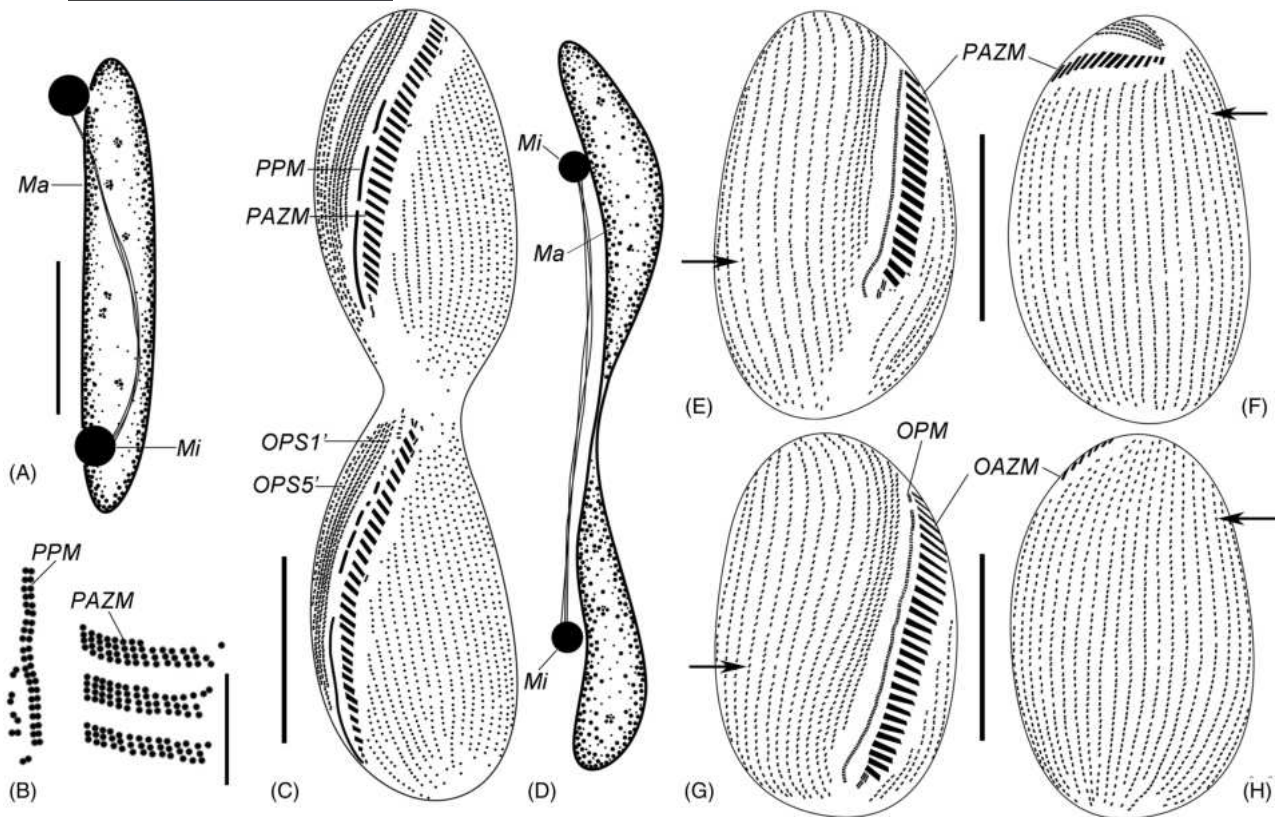


FIGURE 7 *Castula specialis* sp. nov. (Chinese type population), a middle divider (A) and late dividers (B–H) after protargol impregnation. (A) Nuclear apparatus. (B) Detail of the proter's adoral zone of membranelles. Ventral view (C) and nuclear apparatus (D) of the same individual. Ventral and dorsal views of proter (E, F) and opisthe (G, H) of the same divider; the arrows denote dikinetids. Ma, macronucleus; Mi, micronucleus; OAZM, opisthe's adoral zone of membranelles; OPM, opisthe's paroral membrane; OPS, opisthe's perizonal stripe; PAZM, proter's adoral zone of membranelles; PPM, proter's paroral membrane. Scale bars: 35 μm (A), 5 μm (B), 30 μm (C, D), 40 μm (E–H).

(about nine kineties) in late early dividers. The new paroral membrane is assembled from the right to the left and parallel to the new adoral zone (Figures 4G and 5G).

In middle dividers, the perizonal rows 1 and 2 split near mid-body, and most fragments of these two rows generate the anterior portion of the opisthe's paroral membrane, the posterior of which is formed by perizonal rows 3–5, and some dome kineties adjacent to row 5 (Figures 5L and 6A). Next, the anterior ends of perizonal rows 1 and 2 of the opisthe remain ordered in early late dividers and the fragments of the opisthe's paroral membrane arrange in the late divider (Figures 6C and 8A). In the early late dividers, the perizonal rows 3–5 remain essentially unchanged but split, forming with their posterior fragments the perizonal rows 1'–3' in the opisthe (Figures 6C and 8A). Eventually, the opisthe's dome kineties 1 and 2 possibly are shifted leftwards, becoming the opisthe's perizonal rows 4' and 5' (Figures 7C,G and 8C). The number of the opisthe's somatic kineties remains unchanged due to the formation of new somatic kineties on the left of the opisthe's adoral zone of membranelles, i.e. a number of parental somatic kineties (about 16) generate kinetofragments which form new somatic kineties (Figures 5G,L and 6A). In late dividers, the paroral membranes in the proter and opisthe

assemble successively, a process which is completed earlier in the proter (Figures 7C,E,F and 8C,D).

With the elongation of the parental perizonal stripe, the proter's paroral membrane reorganizes which commences in late early dividers, and is composed of dikinetids throughout in late dividers (Figures 4E,F,G, 5E,G,J,L, 6A,C, 7C,E, and 8A,C). The typical metopid pattern of the paroral membrane is probably obtained only after the separation of proter and opisthe. Very likely, the surplus kinetids of the paroral membranes disintegrate resulting in an anterior shortening of the proter's and opisthe's paroral membranes (Figure 8F). Likewise, the paroral membrane of the proter reorganizes in situ, and it is not involved in the occurrence of other structures.

Development of remaining somatic ciliature

The replication of dikinetids begins in the posterior portion of all somatic kineties and progresses anteriorly and posteriorly. The basal body replication starts with the generation of a new basal body anteriorly to the dikinetid, resulting in a triad; finally, a new basal body is generated between the dikinetidal basal bodies, producing a tetrad. The color of newly generated basal bodies is

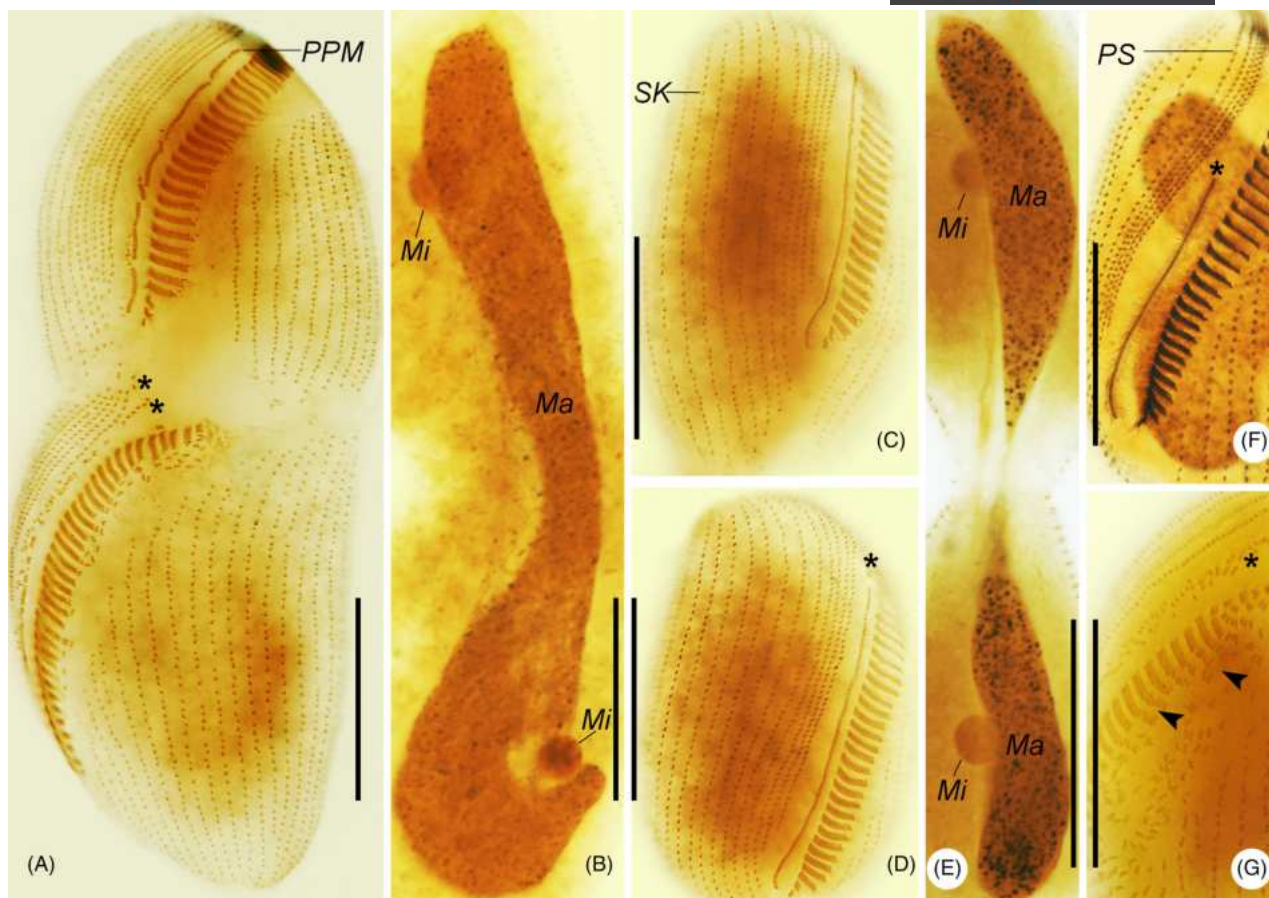


FIGURE 8 Photomicrographs of *Castula specialis* sp. nov. (Chinese type population), late dividers (A–E), a post-divider (F), and a reorganizer (G) after protargol impregnation. Asterisks mark the paroral membrane (A, D, F, G). (A) Ventral view showing the paroral membrane, whose anterior part is mainly formed by the perizonal rows 1 and 2. (B, E) Ventral views showing the elongated macronucleus and the two micronuclei. Proter (C) and opisthe (D) of the same divider, showing the rectangular adoral zone of membranelles and dikinetid somatic kineties. (F) Ventral view showing the rather distinct preoral dome and the paroral membrane. (G) Detail view showing the reorganization of the adoral membranelles (arrowheads) and the paroral membrane (asterisk). Ma, macronucleus; Mi, micronucleus; PD, preoral dome; PPM, proter's paroral membrane; PS, perizonal stripe; SK, somatic kineties. Scale bars: 40 μ m (A, C, D, G, F), 30 (B, E).

lighter than that of the old dikinetids in protargol preparations. The new basal bodies become ciliated and the dikinetids move away from each other, resulting in an elongation of the kineties and their longitudinal orientation. The replication of dikinetids is not simultaneous and newly formed kinetofragments will participate in the formation of the opisthe's adoral zone, the opisthe's paroral membrane, the opisthe's perizonal stripe, and new ordinary somatic kineties (see above; Figures 4A–G, 5A–G, J–L, 6A–D, 7C, E–H, and 8A, C, D).

Nuclear division

In the very early dividers, the macronucleus is similar to that of morphostatic cells, i.e. it is reniform and situated in the cell center (Figures 4H and 5H). Next, it enlarges and becomes twisted. It further elongates and obtains a characteristic oblong shape in middle dividers (Figures 5M and 7A). In the late dividers, the macronucleus is dumbbell-shaped with a conspicuous

constriction in the middle third and occupies about 81% of cell length (Figures 7D and 8B, E). Finally, the macronucleus divides, and its division products condense and move to their species-specific positions (Figure 8F).

The micronucleus remains in an indentation of the macronucleus, where it enlarges, reaching up to 8.7 μ m in diameter and showing distinct spindle microtubules in early dividers (Figures 4I and 5I). The micronucleus divides incompletely in middle dividers, i.e. the division products remain connected by a distinctly narrowed strand in middle and late dividers (Figures 5M, 7A, D, and 8B, E). In very late dividers, the micronuclei move to the right margin of one macronucleus each (Figures 7D and 8E).

Physiological reorganization based on the Chinese population

Although only a few individuals in the phase of physiological reorganization were found in the protargol slides, several features can be summarized. The process

shows similar characteristics to the proter's morphogenesis. The adoral zone of membranelles, the paroral membrane, and the perizonal stripe are reorganized, but unlike dividing cells, the reorganization does not generate the new structures mentioned above (Figure 8G).

Molecular phylogeny

Three partial SSU rRNA gene sequences of *Castula specialis* sp. nov. were obtained, namely, from the Chinese strain: QD0901 (GenBank: ON923867) with 1669 bp and G/C content of 43.0%; from the Mexican strain LERMETZ (GenBank: OP034700) with 1677 bp and G/C content of 43.0%; and from the Czech strain LIPKY2 (GenBank: OP034701) 1692 bp and G/C content of 43.0%. In addition, one sequence of the Mexican strain LERMONSTR of *Castula fusca* (1305 bp, G/C content 42.3%), two sequences of *Castula* spp. (Czech strain LIB2C 1676 bp, G/C content 34.1%; Mexican strain LERM10C 1306 bp, G/C content 42.1%), and one sequence of *Pileometopus* sp. (Czech strain MOKOTPIL 1695 bp, G/C content 43.2%) were obtained. In the present analyses, the family Metopidae includes at least six well-supported clusters. One of them contains the genera *Castula* and *Pileometopus* that cluster together in a robust “PiCa” clade (ML 100/ BI 1.00). *Castula specialis* sp. nov. and five GenBank sequences from morphologically uncharacterized but molecularly nearly identical ciliates (MN604061-64 and KY855532) cluster together in ML analysis (Figure 9). Other species of *Castula*, i.e. *C. fusca*, *C. flexibilis*, *C. setosa*, and *Castula* spp. (LIB2C, LERM10C) cluster together with *Pileometopus* sequences (ML 96/BI 1.00). A genetic distance matrix (uncorrected *p*-distances) was built for all the members of the “PiCa” clade (Table S1). The genetic distances between our three SSU rRNA gene sequences of *Castula specialis* sp. nov. and the undetermined metopids (MN604061-64 and KY855532) were similar (0.000–0.010). On the contrary, the genetic distances were higher with 0.014 when compared to the sequences of *Castula* spp., *C. fusca*, *C. setosa*, *C. flexibilis*, and *Pileometopus* species.

According to the current phylogenetic analyses, the “PiCa” group, is the sister to the clade with *Bothrostoma* and *Planometopus* (BoPl clade) (ML 69/BI 1.00). Both clades are again sisters to the clade including *Heterometopus palaeformis*, *Longitaenia gibba*, *Atopospira* spp., *Parametopidium circumlabens*, *Idiometopus turbo*, and the Clevelandellida with high support (ML 98/BI 1.00).

DISCUSSION

Comparison of *Castula specialis* sp. nov. with related and similar species

The similarity and conspecificity of the three isolates (QD0901, LERMETZ, LIPKY2) are supported by both morphological and molecular data. They share

morphological features such as body shape (elongated ellipsoidal) and ciliary pattern including long posterior setae. The few slight differences (Table 1), e.g. the length of the paroral membrane, are interpreted as intraspecific variability, as most of the morphometric data significantly overlap. The assumption of conspecificity is corroborated by almost identical SSU rRNA gene sequences of the three populations (uncorrected *p* distances 0.003–0.009); the observed deviations are attributed to geographic variations. The genetic distance range between our populations and the ones from GenBank (MN604061-64 and KY855532) was 0.00–0.01. Since the morphology of these sequenced specimens was not studied in detail (Bourland et al., 2020), we tentatively regard these five strains (MN604061-64 and KY855532) as *Castula* spp. until further data are available.

Morphologically, *Castula specialis* sp. nov. can be easily distinguished from *C. flexibilis* and *C. setosa* by larger cell size in vivo (105–220 × 25–70 μm vs. 87–102 × 29–48 μm vs. 55–68 × 20–29 μm, respectively), and more adoral membranelles (32–46, 38, 38 or 42 for the three isolates on average vs. 27–33, 30 on average vs. 18–26, 23 on average, respectively). Furthermore, *Castula specialis* sp. nov. differs from *C. fusca* by fewer setae (3–6 vs. 15–20) and somatic kineties (31–52 vs. 80–108; Bourland et al., 2014, 2020).

Though easily distinguishable from other genera due to the unique presence of the elongated setae, *Castula specialis* sp. nov. has some similarities with some *Metopus* species. *Metopus es* (Müller, 1776) Lauterborn, 1916 resembles *C. specialis* sp. nov. in body shape, oral area pattern, and the number of adoral membranelles (Figure S1E) but can be distinguished by the number of somatic kineties (18–32 vs. 31–52; Bourland et al., 2017b; Li et al., 2021). Moreover, *C. specialis* sp. nov. has slightly more somatic kineties excluding perizonal stripe rows than British and Spanish populations of *M. es* (usually 38, 31–50 vs. 30–35) and more posterior setae or caudal cilia (3–6 long vs. 2 short; Esteban et al., 1995).

Regarding the large and asymmetrical body form, the morphology and location of the adoral zone, the presence of distinct setae or caudal cilia, and a perizonal stripe without false kineties, *Castula specialis* sp. nov. should be compared with *Metopus acidiferus* Kahl, 1932, *M. entorhipidioides* Jankowski, 1964, *M. laminarius* Kahl, 1927, and *M. major* Kahl, 1932 (Esteban et al., 1995; Jankowski, 1964; Kahl, 1927, 1932). Specifically, *C. specialis* sp. nov. differs from *M. entorhipidioides*, *M. laminarius*, and *M. major* in the number of somatic kineties (31–52 vs. 15–17 vs. about 20 vs. 50–55, respectively). Furthermore, *M. acidiferus* has conspicuous, rod-shaped extrusomes in vivo (Kahl, 1932) which are missing in the new species.

Morphogenesis based on the Chinese population

Armophorean ciliates are poorly known in terms of morphogenesis, i.e. the process has merely been described in four species: *Metopus hasei* Sondheim, 1929;

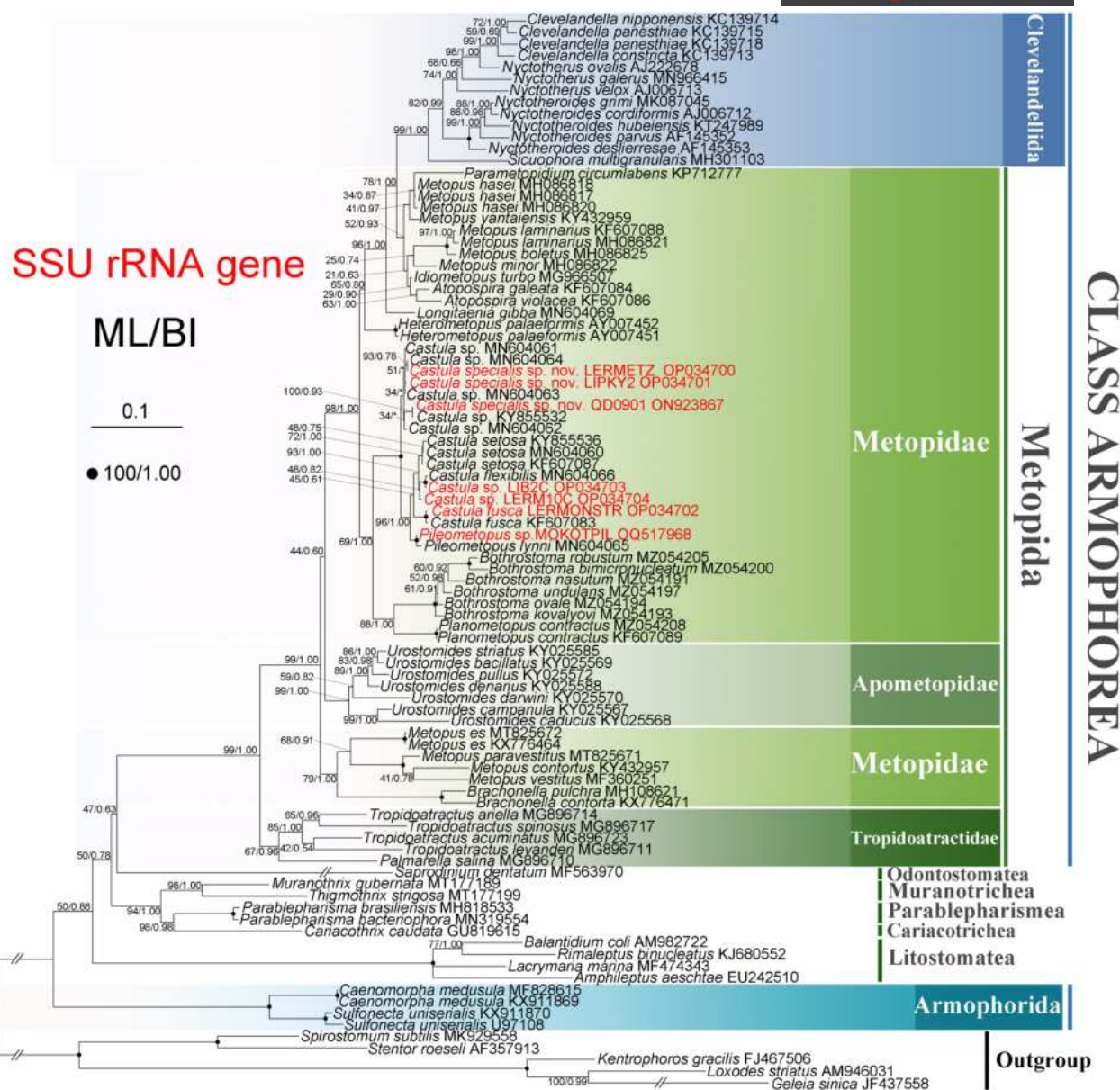


FIGURE 9 Maximum likelihood (ML) tree based on the small subunit rRNA gene sequences showing the positions of *Castula specialis* sp. nov. The sequences from the present study are marked by red. Numbers at nodes represent the bootstrap values of the Maximum Likelihood (ML) analysis and posterior probabilities of the Bayesian inference (BI), respectively. Black circles indicate full support (ML/BI, 100/1.00) in both analyses. Disagreements between ML and BI are shown by asterisks. The scale bar represents five changes per 100 positions.

M. inversus (Jankowski, 1964) Foissner & Agatha, 1999; *Lepidometopus platycephalus* Vďačný & Foissner, 2017; and *M. boletus* Vďačný & Foissner, 2019 (Foissner & Agatha, 1999; Vďačný & Foissner, 2017, 2019). The morphogenesis of *Castula specialis* sp. nov. is the first detailed study of the genus *Castula*. The ontogenesis of this species is similar to that of other metopids, sharing the following characteristics: (1) the body shape changes distinctly; (2) the stomatogenesis is pleurotelokinetal (Foissner, 1996); (3) the previous perizonal rows 1 and 2 participate with their posterior portions in the assembly of the opisthe's paroral membrane; (4) the opisthe's perizonal stripe originates from the posterior portions of the previous perizonal rows 3–5 and two dome kineties; (5)

the micronucleus divides initially incompletely, i.e. both products remain connected by a distinctly narrowed strand; (6) the ordinary somatic kineties proliferate to generate kinety fragments for proter and opisthe, to balance the loss of two rows due to the transformation of perizonal rows 1 and 2 into the paroral membrane, and to form the adoral membranelles of the opisthe; and (7) the parental paroral membrane is reorganized and becomes the proter's paroral membrane (Foissner & Agatha, 1999; Vďačný & Foissner, 2017, 2019).

However, the cell division pattern of *Castula specialis* sp. nov. differs in some details from that in other metopids (Foissner & Agatha, 1999; Vďačný & Foissner, 2017, 2019): (1) all dome kineties are involved in

the formation of the new adoral membranelles (vs. only some dome kineties in *Lepidometopus platycephalus* and *M. boletus*; although not explicitly stated, all dome kineties participate in this process in *Metopus hasei* and *M. inversus*). Here, the ratio between the number of dome kineties involved and the total number of dome kineties is assessed for the first time, as it might be of taxonomic significance. (2) The opisthe's paroral membrane does not completely derive from the perizonal rows 1 and 2. Actually, its posterior portion comes from kinetofragments at the ends of the perizonal rows 3–5 and some dome kineties adjacent to the perizonal stripe rows. (3) Compared with *L. platycephalus* and *M. boletus*, *C. specialis* sp. nov. does not complete the replication of somatic dikinetids until in late dividers instead of middle dividers (Vďačný & Foissner, 2017, 2019). (4) The micronucleus remains still close to the macronucleus during its division, while the micronucleus moves away from the macronucleus in the other metopids. (5) The whole parental paroral membrane of *C. specialis* sp. nov. dedifferentiates (Figures 6C and 8A) like in *L. platycephalus*, *M. boletus*, and *M. inversus*, in contrast to *M. hasei*, in which it is partially reorganized. Only in *C. specialis* sp. nov., the proter's paroral membrane is arranged in a discontinuous row of dikinetids in late dividers and post-dividers. (6) The opisthe's perizonal stripe probably comprises the posterior half of the parental perizonal rows 3–5 and the whole opisthe's dome kineties 1 and 2. (7) In early dividers, the posterior portions of the elongated perizonal rows 1–5 dedifferentiate, forming some disordered kinetofragments (Figures 4C and 5B). It remains unclear, whether this stage exists in all metopids or is typical of the genus *Castula*. (8) The stage during which the preoral dome disappears is possibly species- or even genus-specific. The preoral dome of the new species disappears in late middle dividers, while that of *M. hasei* disappears in early dividers (Foissner & Agatha, 1999). Besides, the perizonal rows 1–3 in *Metopus hasei*, *M. inversus*, *M. boletus*, and *Lepidometopus platycephalus* all form “false” kineties in contrast to those of *C. specialis* do not; hence, the morphogenetic significance of perizonal rows might differ, especially in the formation of the opisthe's paroral membrane (Foissner & Agatha, 1999; Vďačný & Foissner, 2017, 2019).

According to the current phylogenetic analyses, *Metopus hasei* and *M. boletus* are distantly related to *Castula* species, and *M. inversus* and *Lepidometopus platycephalus* lack molecular characterizations; therefore, correlations between the division mode and the phylogenetic placement cannot be inferred yet. Likewise, the scarcity of ontogenetic data on metopids prevents a reliable evaluation of the morphogenetic characters regarding species delimitations in metopids. Accordingly, more integrative studies considering the morphology, morphogenesis, and molecular phylogeny of metopids are pivotal.

Phylogeny and the paraphyly of *Castula*

Our phylogenetic analyses match previous findings (Bourland et al., 2020; Rotterová et al., 2018) in the detection of several robust clades (Figure 9). In the phylogenetic tree, sequences of four unidentified cultured *Castula* strains (MN604061–64) and sequence KY855532 of an uncultured metopid are assigned as undescribed metopids. They clustered together with *Pileometopus* and *Castula* spp., forming a fully supported “PiCa” clade, which also emerged in previous phylogenetic analyses (Bourland et al., 2020; Méndez-Sánchez et al., 2022).

Due to the placement of *Pileometopus lynni* and *Pileometopus* sp. (MOKOTPIL) among the *Castula* species, the latter genus is currently not monophyletic. Furthermore, *P. lynni* and *Pileometopus* sp. (MOKOTPIL) are closer related to three *Castula* species, viz., *C. setosa*, *C. fusca*, and *C. flexibilis*, than to the new congener *C. specialis* sp. nov. based on previously published phylogenetic trees and the present analyses (Bourland et al., 2020; Méndez-Sánchez et al., 2022; Figure 9). Therefore, more detailed investigations and a broader taxon sampling are required for a reliable taxonomic revision of this ciliate group.

We assign the new isolates to the genus *Castula* based on the following synapomorphies that are missing in *Pileometopus*: (1) the presence of setae; (2) an adoral zone of membranelles commencing on the dorsal side and twisting by less than 180° around the cell (nearly 360° in *Pileometopus*); and (3) an elongated ellipsoidal body shape (vs. turbinated in *Pileometopus*; Figure S2; Bourland et al., 2020).

Bourland et al. (2020) considered the structure of the paroral membrane genus-specific; however, *Pileometopus* spp. (Figure S2; Bourland et al., 2020) and *Castula specialis* sp. nov. share the same structure, namely, a bipartition into an anterior portion composed of a single row of basal bodies and a posterior portion consisting of two rows of basal bodies; hence this feature is not specific for *Pileometopus*.

Obviously, the clade comprising *Castula specialis* sp. nov. is more closely related to the clade of the other metopids (including many *Metopus* species) and Clevelandellida species than to the clade of “typical” *Metopus* species, i.e. the *Metopus-Brachonella* clade. Once again, the present study supports the hypothesis that *Metopus* is not monophyletic (Bourland et al., 2014; Li et al., 2016, 2021; Omar et al., 2017; Paiva et al., 2013; Rotterová et al., 2018; Silva-Neto et al., 2016; Vďačný et al., 2019; Zhuang et al., 2021).

TAXONOMIC SUMMARY

ZooBank registration

Present work: urn:lsid:zoobank.org:pub:D5FFE629-3770-495A-ACC8-52B5BF7D1C06.

Castula specialis sp. nov.: urn:lsid:zoobank.org:act:D4EC8891-EDC1-44CA-B68C-E8BE174D4365.

***Castula specialis* sp. nov.** (Table 1; Figures 1–8)

Diagnosis based on the Chinese population

Cell 105–220 × 25–70 µm in vivo, oblong to elongated ellipsoidal, twisted anteriorly. Macronucleus reniform; one globular to ellipsoidal micronucleus, centrally positioned. 31–52 somatic kineties; 4–7 setae, 15–30 µm long. The adoral zone composed of 32–46 membranelles, occupies on average 43% of body length.

Type locality. A freshwater pond in the Baihuayuan Park in Qingdao, China (36°03'56" N 120°20'41" E).

Specimens' deposition. The protargol slide from the Chinese QD0901 strain containing the holotype (Figures 1F,G and 2L,M) and some paratypes (registration number: FXC20200901-01) was deposited in the Marine Biological Museum, Chinese Academy of Sciences, Qingdao, China. Two further slides with paratypes (registration numbers: FXC20200901-02, FXC20200901-03) were deposited in the Laboratory of Systematic Taxonomy, Ocean University of China, Qingdao, China. The holotype and these paratypes were all marked by black ink circles on the back side of the slide.

Two slides with paratypes from the Mexican strain LERMETZ and the Czech strain LIPKY2 were deposited in the collection of the National Museum in Prague, Czech Republic, with the inventory numbers P6E 5304 and P6E 5305, respectively.

Etymology. The species-group name “*specialis*” (Latin adjective) refers to the distinctness of the new species regarding body shape and the position and length of its setae.

AUTHOR CONTRIBUTIONS

Xiaozhong Hu conceived and guided the study. Wenbao Zhuang, Ondřej Pomahač, and Daniel Méndez-Sánchez collected the samples. Xiaochen Feng, Wenbao Zhuang, Daniel Méndez-Sánchez, and Ondřej Pomahač conducted the laboratory work and molecular phylogeny analyses. Xiaochen Feng and Daniel Méndez-Sánchez drafted the manuscript. Johana Rotterová, Ran Li, Ondřej Pomahač, and Ivan Čepička as well as Xiaozhong Hu made further revisions. All the authors read and approved the final version of the manuscript.

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ORIGINAL ARTICLE



Bit by bit toward the diversity of metopids: Description of the genus *Pidimetopus* n. gen. (Ciliophora: Armophorea)

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Abstract

While metopids (Armophorea: Metopida) represent the most species-rich group of free-living anaerobic ciliates thriving in hypoxic environments, our understanding of their true diversity remains incomplete. Most metopid species are still characterized only morphologically. Particularly, the so-called IAC clade (named in the past after some of the taxa included, *Idiometopus*, *Atopospira*, and *Clevelandellida*), comprising free-living members as well as the endosymbiotic ones (order *Clevelandellida*), is in serious need of revision. In our study, we establish a new free-living genus in the IAC clade, *Pidimetopus* n. gen., with descriptions of two new species, *P. nanus* n. sp., and *P. permonicus* n. sp., using up-to-date molecular and morphologic methods. The genus is characterized by small cells (up to 75 µm long), not more than 10 adoral membranelles and eight somatic kineties, and usually, four long caudal cilia that can stiffen. In addition to morphologic and molecular characterizations, we also conducted a statistical morphotype analysis of the polymorphic species *P. nanus* n. sp. We discuss the relevance of the earlier morphologically described species *Metopus minor* as a putative collective taxon for several small metopids less than 50 µm long.

KEY WORDS

18S rRNA gene, Armophorea, Metopida, phylogeny, transmission electron microscopy

INTRODUCTION

METOPIDS (Armophorea: Metopida) are obligately anaerobic ciliates living in freshwater to marine habitats or soil. The order Metopida Jankowski, 1980, represents the most diverse group of free-living anaerobic ciliates in terms of species richness (Bourland et al., 2017a; Esteban et al., 1995; Jankowski, 1964a, 1964b; Kahl, 1927, 1932; Méndez-Sánchez et al., 2022; Rotterová et al., 2018; Vďačný & Foissner, 2017a, 2017b, 2019; Zhuang et al., 2022). Unfortunately, most metopid species have been studied based solely on observations of living or fixed but unstained cells (Jankowski, 1964a, 1964b, 2007; Kahl, 1927, 1932). It is evident that we are only beginning to scratch the surface of their diversity as new species have been reported regularly over the past few years (Bourland et al., 2017a, 2020; Feng et al., 2024; Li et al., 2021; Méndez-Sánchez et al., 2022; Rotterová et al., 2018; Vďačný & Foissner, 2017a, 2017b,

2019; Zhuang et al., 2021, 2022). About 40 species of metopids have been newly described or redescribed using up-to-date staining, microscopy, and molecular methods (e.g. Bourland et al., 2017a, 2018, 2020; Méndez-Sánchez et al., 2022; Rotterová et al., 2018). Recently, several new species were described based on detailed morphologic data, but molecular data were not included (Foissner, 2016a; Vďačný & Foissner, 2017a, 2017b). Regardless of the method used, most metopid species have been assigned to the nonmonophyletic genus *Metopus* Claparède and Lachmann, 1858 (Esteban et al., 1995; Foissner & Agatha, 1999; Jankowski, 1964a, 1964b, 2007; Kahl, 1927, 1932; Omar et al., 2017; Vďačný & Foissner, 2017a, 2017b, 2019; Zhuang et al., 2022). This conservative tendency to subsume most of the newly described species under the genus *Metopus* is a significant source of largely tangled metopid taxonomy.

In the class Armophorea, we recognize an interesting clade named in the past after some of the taxa included,

Idiometopus, *Atopospira*, and *Clevelandellida* (Bourland et al., 2018). The IAC clade comprises both free-living and endosymbiotic members, that is *Parametopidium circumlabens* (Biggar & Wenrich, 1932) Aescht, 1980, from sea urchins, and the order *Clevelandellida*, inhabitants of the intestinal tract of various vertebrate and invertebrate hosts. Free-living members of the IAC clade are thus perceived as the closest relatives of clevelandellids. They exhibit a remarkable morphologic diversity, even though they are notoriously understudied with only five species described with up-to-date morphologic and molecular methods (Bourland et al., 2018; Bourland & Wendell, 2014; Omar et al., 2017; Vďačný & Foissner, 2019). One of the historical conundrums of the IAC clade involves the taxonomy of small metopids that have most often been assigned to a single species, namely, *Metopus minor* Kahl, 1927, which has been redescribed several times (Čapar, 2007; Foissner, 1980; Foissner et al., 2002; Kahl, 1927, 1932). So far, there are only morphologic, often self-contradicting descriptions of *M. minor* and a single published sequence under this name, which is unfortunately not accompanied by morphologic data (Vďačný et al., 2019).

In this study, we provide the first molecular and morphologic descriptions of two new species in the new genus *Pidimetopus* n. gen. belonging to the IAC clade. In addition, we conducted a statistical morphotype analysis of the polymorphic species *P. nanus* n. sp.

MATERIALS AND METHODS

Sampling and culturing

Samples of hypoxic sediments and soils were collected, using 50-mL Falcon tubes and plastic ziplock bags. The samples were kept in closed sampling tubes or bags and inoculated into 10 mL of ATCC medium #802 (Ward's Science, USA) or 3% LB Broth medium (MilliporeSigma, USA). Successfully established cultures were kept in the dark at room temperature, and 1 mL of sediment from the culture tube was transferred into fresh media every 3 weeks and maintained with undetermined eukaryotes and prokaryotes as previously described (Bourland et al., 2017b).

Localities

KRUMLI, a small eutrophic pond bordered by forest, sediment, Český Krumlov, Czechia, spring 2021, 48°49'45" N 14°19'17" E, 548 MASL.

MALKALXC, a shore of drained Kala pond, sediment, Hostivice, Czechia, winter 2020, 50°04'18" N 14°15'05" E, 348 MASL.

VELKALXA, a shore of drained Kala pond, sediment, Hostivice, Czechia, winter 2020, 50°04'18" N 14°15'05" E, 348 MASL.

HOSLIR, a shore of Litovický pond, soil, Hostivice, Czechia, winter 2020, 50°04'20.1" N 14°14'50.6" E, 343 MASL.

VRZEN2A, a forest stream (Čečovický potok), sediment, Vrčeň, Czechia, summer 2021, 49°30'52.3" N 13°38'17.5" E, 452 MASL.

ANNZ, a mountain creek (Račí potok), sediment, Annín, Czechia, winter 2019, 49°10'29.0" N 13°31'13.9" E, 542 MASL.

HARR, a mountain meadow puddle, soil, Harrachov, Czechia, winter 2021, 50°46'23.5" N 15°25'29.4" E, 667 MASL.

DARTH, a forest park, leaf litter, Prague, Czechia, autumn 2022, 50°01'15.7" N 14°28'46.4" E, 317 MASL.

SLANIF, a dried small salt marsh next to a field, soil, Netřebská slaniska, Czechia, autumn 2020, 50°15'39.2" N 14°25'49.1" E, 171 MASL.

LIPKYIIS, a small pond next to a field, sediment, Žinkovy, Czechia, summer 2021, 49°28'31.6" N 13°29'23.3" E, 462 MASL.

RUM5R2, a small mountain pool, sediment, Romania, summer 2023, 47°34'19.4" N 24°49'07.9" E, 1733 MASL.

MILOT, a marsh, decomposing leaf matter, Kemerli, Riga, Latvia, summer 2023, 57°00'07.0" N 23°28'41.0" E, 0 MASL.

Morphologic characterization

Living cells were studied at 400–1000× magnifications with bright-field and differential interference contrast illumination, using an Olympus BX51 microscope equipped with an Olympus DP70 camera. Cells were fixed in 10% aqueous formalin or Bouin's solution and impregnated with protargol (Foissner, 2014) or fixed in 10% aqueous formalin and impregnated with silver carbonate to reveal the infraciliature and nuclear apparatus (Fernández-Galiano, 1976). Photomicrographs of uncompressed cells were used for in vivo measurements. Counts and measurements from protargol and silver carbonate preparations were made at 1000× magnification, using QuickPhotoCamera software. For transmission electron microscopy (TEM), cells were prefixed for at least 30 min in 2.5% glutaraldehyde, washed with distilled water, and postfixed with 1% osmium tetroxide overnight. Next, the cells were washed with distilled water and stained with 2.5% uranyl acetate for 2 h, dehydrated in an ethanol series, and embedded in resin. All steps were performed at room temperature. The ultrathin sections (50 nm) were cut, using a diamond knife on an Ultracut E ultramicrotome (Reichert), stained with lead citrate and a saturated solution of uranyl acetate, and examined using a JEOL JEM-1011 transmission electron microscope (JEOL LV, USA). For scanning electron microscopy (SEM), cells were fixed for at least 30 min in a 1:1 aqueous solution of 2.5% glutaraldehyde and 2% osmium tetroxide at room temperature and then processed according to Foissner (2014). The specimens were examined

at an accelerating voltage of 20kV in a JEOL JSM-6380 scanning electron microscope (JEOL LV, USA). The terminology is based on Bourland et al. (2017b), Foissner and Agatha (1999), and Lynn (2008), except as noted. We follow the terminology for morphotypes used in Esteban et al. (1995), that is cells with extreme size (length×width), which we refer to as “giant,” while cells with only extreme length we refer to as “elongated.” We newly define the extreme value of the characteristic (length×width, or length) as exceeding the two standard deviations (SD) above the average of all measured cells.

Morphotype analysis

Two samples of a long-term (>2years) culture of *Pidimetomus nanus* n. sp. (HARR) were inspected 1 and 4 weeks, respectively, after transfer into fresh media. Cells were chosen randomly from several drops for measurements (at least 118 cells per sample). Measurements of the length and width of cells were made on an Olympus BX51 microscope equipped with an Olympus DP70 camera, at 100× magnification, using the QuickPhotoCamera software. Calculations and plots were executed in Excel (Microsoft). Statistical tests were performed in R (<https://www.R-project.org>).

DNA extraction, amplification, and sequencing

One to three cells from all populations were hand-picked with glass micropipettes, washed in distilled water, and added into 30µL of DNA/RNA shield (Zymo Research). Genomic DNA was isolated, using the MasterPure™ Complete DNA and Purification Kit (LGC Biosearch Technologies) following the manufacturer's instructions. The 18S rRNA gene of all populations was amplified using the primers EukA (5'-AACCTGGTTGATCCTGCCAGT-3'), and EukB (5'-TGATCCATCTGCAGGTTACCT-3') (Medlin et al., 1988), using the PCR protocol according to Méndez-Sánchez et al. (2022). PCR products were verified by 1% agarose gel electrophoresis and purified, using EXOSAP (Applied Biosystems). Purified PCR products were directly sequenced, using four internal primers: 577F (5'-GCCAGCACCCGCGGT-3'), 577R (5'-ACCGCGGGTGCTGGC-3'), 1055R (5'-GGTGGTGCATGGCCG-3'), and 1055F (5'-CGGCCATGCACCAC-3') (Elwood et al., 1985). Sequencing was done at the Laboratory of DNA Sequencing (Charles University, Prague, Czech Republic) on an ABI PRISM 3100 sequencer (Applied Biosystems).

Phylogenetic analysis

We assembled an 18S rRNA gene sequence data set comprising 105 sequences: 12 newly determined and

93 obtained from GenBank (including four outgroup sequences: OP034689 *Muranthrix felix*, KX911870 *Sulfonecta uniserialis*, MH472608 *Caenomorpha medusula*, OP985792 *Legendrea pespelicani*). The sequences were aligned, using MAFFT (Katoh et al., 2002) under the G-INS-i algorithm. The alignment was manually edited, using Geneious Prime 2023.2.1 (Biomatters) to trim primer sequences and remove positions with single-nucleotide insertions within otherwise completely conserved positions. The final alignment included 1672 positions. Phylogenetic trees were constructed by maximum likelihood (ML) and Bayesian inference (BI) methods under the GTR+I+Γ as the best-fit model by ModelTest-NG 0.1.7 (Darriba et al., 2006) according to the Akaike Information Criterion (AIC). The ML analysis was performed in RAxML-HPC2 Workflow 8.2.12 (Stamatakis, 2014). Bipartition supports were statistically tested by 1000 bootstrap pseudoreplicates. The Bayesian analysis was performed in MrBayes 3.2.7a (Ronquist et al., 2012). Four Markov chains Monte Carlo were run for 10,000,000 generations with a sampling frequency of 1000 generations. The first 25% of sampled trees were removed as burn-in. Convergence was assessed, using Tracer v1.7.2 (Rambaut et al., 2018).

RESULTS

Description of *Pidimetopus nanus* n. sp. based on ANNZ (measurements) and HARR populations (Figures 1–4; Table S1; Video S1)

Cell size is about 52×15µm in vivo ($n=16$) and about 42×15µm in protargol preparations ($n=24$) (Table S1). The average length:width ratio in vivo is 3.5:1. The body is ellipsoidal to ovate, slightly elongated, leftward twisted anteriorly, and dorsoventrally flattened. Cells in older cultures are morphologically variable (Figures 1G,H,J, and 2F), including “giant” (Figure 1D,E) or extremely elongated forms (Figure 1K). The preoral dome is slightly convex, narrower than the mid-body, and inclined by 45° to the main cell axis (Figure 1F). The posterior end is rounded or obconical. The macronucleus is spherical to ellipsoidal, about 10µm across; in the anterior cell half, the chromatin is coarsely granular and forms multiple nucleoli (Figure 1A–D). The macronucleus is irregular in protargol preparations. The micronucleus is globular or slightly ellipsoidal, about 3µm across, and attached to the macronucleus. The somatic ciliature consists of dikinetids arranged in about eight longitudinal rows (Figure 2E). Somatic cilia are about 10µm long. In the anterior cell portion, both basal bodies of dikinetids are ciliated; the remaining dikinetids have only one basal body ciliated (Figures 1A,B, 2A, and 3B); in “giant” cells, all basal bodies in kineties can be ciliated. Two pairs of long caudal cilia (Figure 3D) that

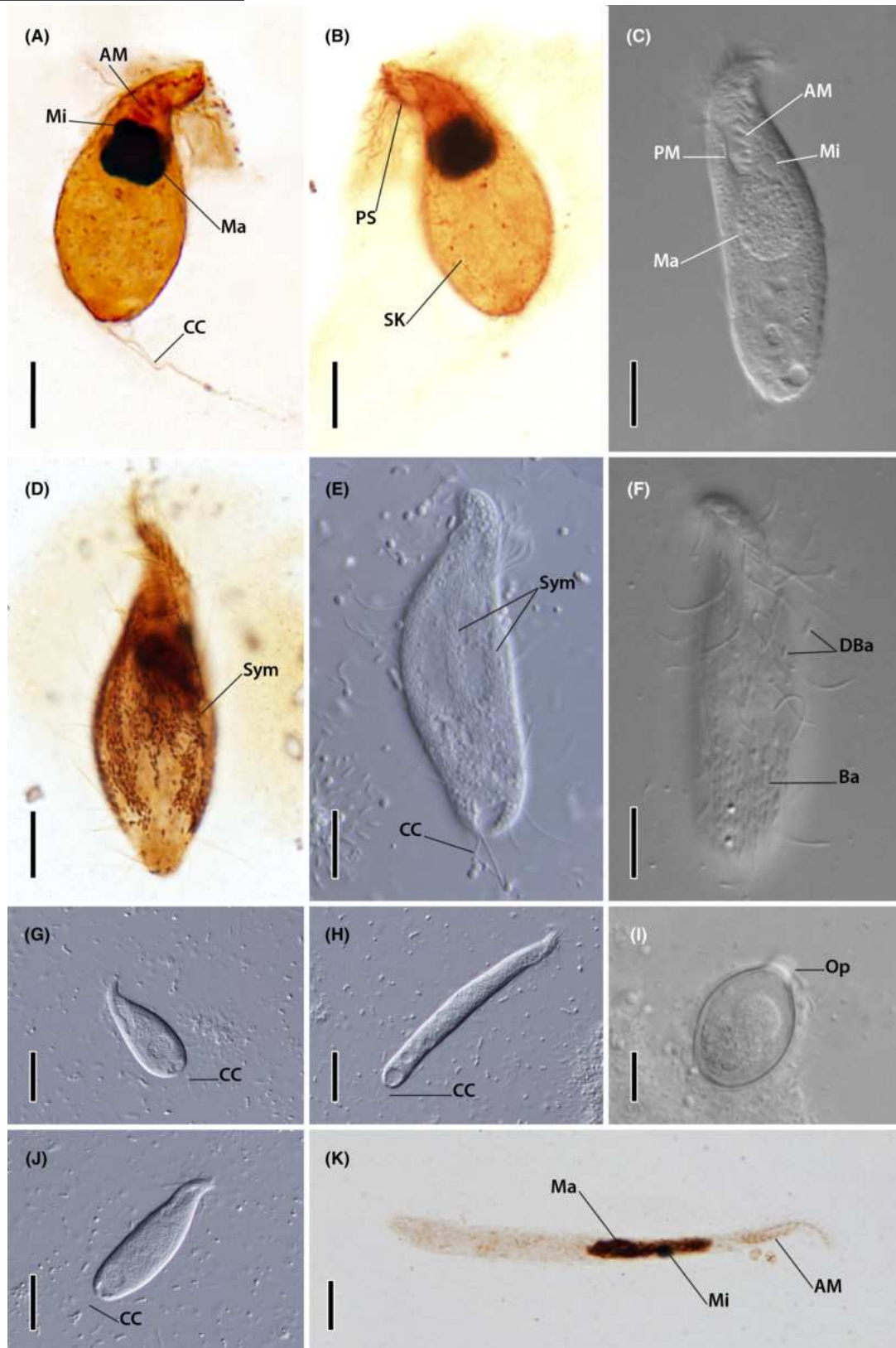


FIGURE 1 *Pidimetopus nanus* n. sp. (A–K) in vivo (C, E–J) and after protargol impregnation (A, B, D, K). (A, B) Ventral and dorsal views of an impregnated specimen. (C) Ventral view showing prokaryotic cells attached to the cell surface (Ba). (D, E) Dorsal views of the “giant” morphotype showing endosymbiotic prokaryotes (Sym). (F) Dorsal view showing prokaryotes in the process of detachment from the surface (DBa). (G, J) Normal morphotype. (H, K) Elongated morphotypes. (I) Typical metopid cyst of *P. nanus*. AM, adoral membranelles; CC, caudal cilia; Ma, macronucleus; Mi, micronucleus; Op, cyst operculum; PM, paroral membrane; PS, perizonal stripe; SK, somatic kineties. Scale bars: 10 μ m (A–C, F, I), 20 μ m (D, E, G, H, J, K).

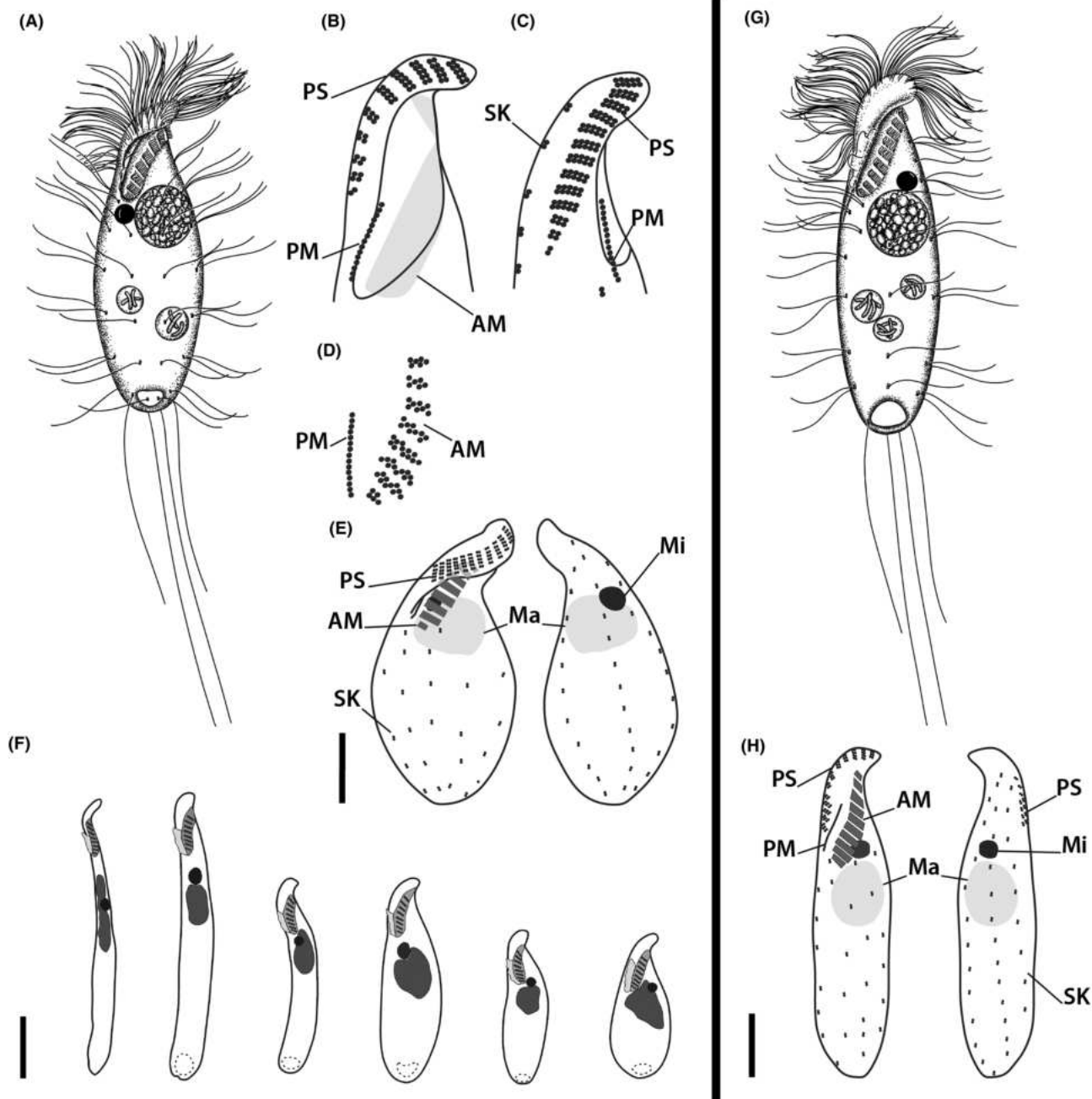


FIGURE 2 *Pidimetopus nanus* n. sp. (A–H), *P. permonicus* n. sp. (G–H) in vivo (A, F, G), and after protargol impregnation (B–E, H). (A) Typical cell shape in ventral view. (B) Anterior cell portion in ventral view. (C) Anterior cell portion in lateral view. (D) Adoral zone and paroral membrane. (E) Ventral (left) and dorsal (right) view showing infraciliature. (F) Different morphotypes of *P. nanus*. (G) Typical cell shape in ventral view. (H) Ventral (left) and dorsal (right) view showing infraciliature. AM, adoral membranelles; Ma, macronucleus; Mi, micronucleus; PM, paroral membrane; PS, perizonal stripe; SK, somatic kinety. Scale bars: 10 μ m.

can stiffen (Video S1): one pair very long (50–70 μ m) and the second pair shorter (20–30 μ m), originate in the posteriormost dikinetids of the somatic kineties 1–4 (Figure 3D). The perizonal stripe invariably comprises five rows arranged in about 12 false kineties (Figures 1D and 2C). The paroral membrane, composed of a single file, stretches from the buccal cavity at the proximal end of the adoral zone to the undersurface of the preoral dome up to about half of the length of the

adoral zone (Figures 1A, 2B–D, 3A,C, and 4A,B). The adoral zone is composed of about eight membranelles, rather straight and twisted only in its anteriormost portion (Figure 2A–C). The cytoplasm is colorless and hyaline; symbionts are not visible in vivo, while rod-shaped prokaryotes forming longitudinal rows in the cell periphery are recognizable in protargol-stained cells and TEM micrographs (Figures 1D,E, and 4A,D). The cytophyge is at the posterior end of the cell. The

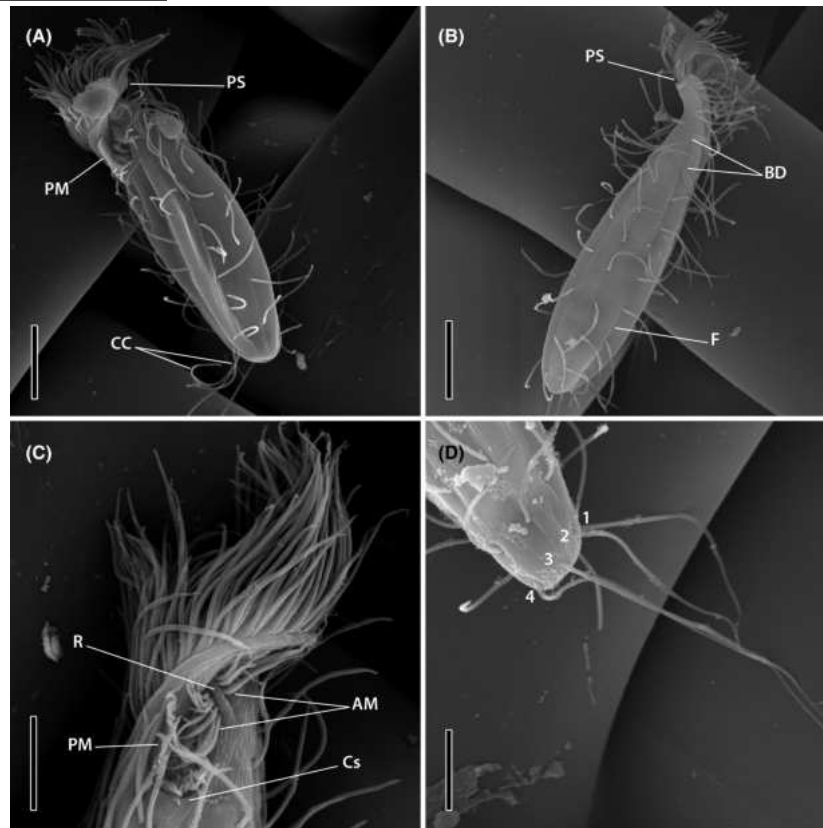


FIGURE 3 Scanning electron micrographs of *Pidimetopus nanus* n. sp. (A–D). (A) Ventro-lateral view. (B) Dorsal view. (C) Ventral view of the anterior cell portion. (D) Dorso-lateral view of the posterior cell portion showing four caudal cilia (1–4). AM, adoral membranelles; BD, biciliated dikinetids; CC, caudal cilia; Cs, cystostome; F, cortical furrow; PM, paroral membrane; PS, perizonal stripe; R, intermembranellar ridge. Scale bars: 10 μ m (A, B), 5 μ m (C, D).

contractile vacuole is terminal, spherical to obconical (Figure 1E). The cortex is flexible or with several deep furrows (Figure 3A,B). Mitochondrion-related organelles (MROs) are spherical, without apparent cristae (Figure 4A–C). Swims moderately to fast and rotates on the main cell axis (Video S1). Resting cysts ellipsoidal, about $25 \times 15 \mu$ m in size, with a typical papula closing the emergence pore (Figure 1I).

Description of *Pidimetopus permonicus* n. sp. based on VRCEN2A (measurements) and VELKALXA populations

Cell size is about $75 \times 20 \mu$ m in vivo ($n=21$) and about $47 \times 11 \mu$ m in protargol preparations ($n=17$) (Table S1). Length:width ratio on average 3.8:1 in vivo. The body is elongated ellipsoidal, leftward twisted anteriorly, and dorsoventrally flattened. The preoral dome is slightly convex, narrower than the mid-body, and inclined by 45° to the main cell axis. Posterior end rounded or obconical. The macronucleus is spherical or ellipsoidal, about 10μ m across, in the anterior cell half; the chromatin is coarsely granular and forms multiple nucleoli (Figures 2G,H, and 5A–C,E); in protargol preparations,

macronucleus irregularly shaped. The micronucleus is globular or slightly ellipsoidal, attached to the macronucleus. The somatic ciliature consists of about eight ciliary rows of dikinetids (Figure 2H). In the anterior cell portion, both basal bodies of a dikinetid are ciliated, and the rest of the dikinetids have only a second basal body ciliated (Figure 5D,F). Somatic cilia are about 10μ m long. Two pairs of long caudal cilia, one pair very long ($50\text{--}70 \mu$ m) and the second pair shorter ($20\text{--}30 \mu$ m), originate in the posteriormost dikinetids of the somatic kineties 1–4 (Figures 2G, and 5A–C,G,H; Video S2). The perizonal stripe invariably comprises five rows arranged in up to 14 false kineties. The paroral membrane, composed of a single file, stretches from the buccal cavity at the proximal end of the adoral zone to the undersurface of the preoral dome up to about half of the length of the adoral zone (Figures 2H, and 5A,C,D). The adoral zone is composed of about 10 membranelles rather straight and twisted only in its anteriormost portion. The cytoplasm is colorless and hyaline. The cytophyge is at the posterior end of the cell (Figure 5F). The contractile vacuole is terminal, spherical to obconical; an excretory pore was not observed. The cortex is flexible, with several deep furrows (Figure 5G). Cytopharyngeal fibers are not visible in vivo and extend longitudinally from the

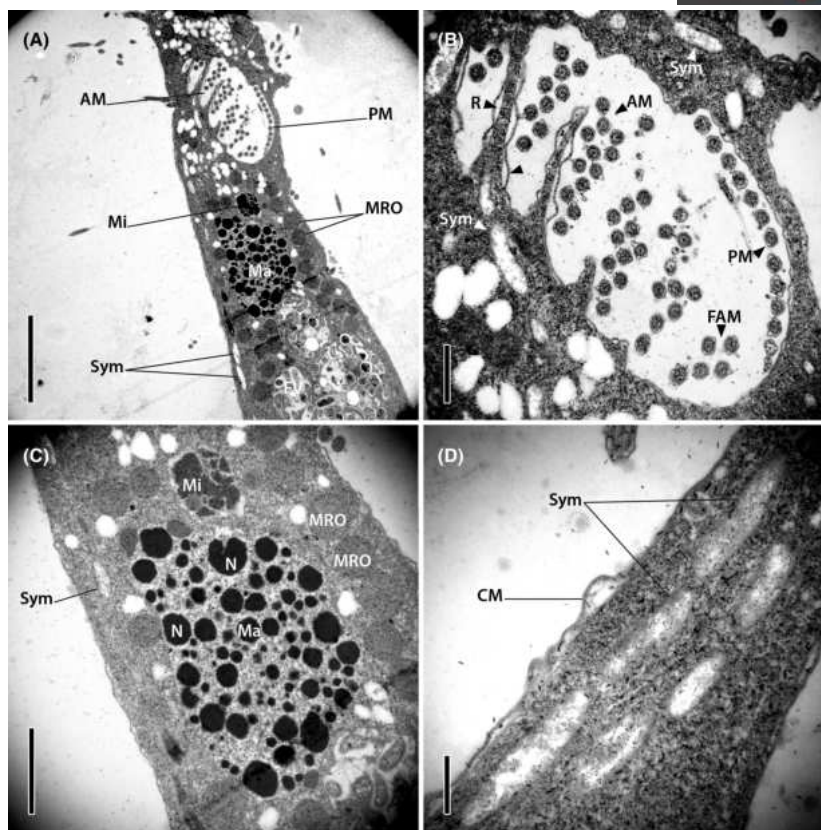


FIGURE 4 Transmission electron micrographs of *Pidimetopus nanus* n. sp. (A–D). (A) Longitudinal section of the anterior cell portion. (B) Longitudinal section of oral ciliature. (C) Longitudinal section of nuclear apparatus. (D) Longitudinal section showing detail of the anterior cell portion with the symbionts. AM, adoral membranelles; CM, cell membrane; FAM, first adoral membranelle; FV, food vacuole; Ma, macronucleus; Mi, micronucleus; N, nucleoli; MRO, mitochondrion-related organelle; PM, paroral membrane; R, intermembranellar ridge; Sym, symbiotic prokaryote. Scale bars: 5 μm (A), 1 μm (B), 2 μm (C), 500 nm (D).

cytostome into the cytoplasm in protargol-stained cells (Figure 5A). Swims moderately to fast and rotates on the main cell axis.

Remarks on *Pidimetopus* sp. based on MALKALXC (measurements) and KRUMLI populations

Cell size is about $38 \times 13 \mu\text{m}$ in protargol preparations ($n=5$). The body is ellipsoidal, leftward twisted anteriorly, and dorsoventrally flattened. The preoral dome is slightly convex, narrower than the mid-body, and inclined by 45° to the main cell axis. The posterior end is rounded or obconical. The macronucleus is spherical, at the level of the adoral zone of membranelles; the chromatin is coarsely granular and forms multiple nucleoli (Figure 6B,D). The somatic ciliature consists of six ciliary rows of dikinetids ($n=4$). Two pairs of long caudal cilia: longer pair about $50 \mu\text{m}$, shorter pair about $25 \mu\text{m}$. The perizonal stripe invariably comprises five rows. The paroral membrane, composed of a single file, stretches from the buccal cavity at the proximal end of the adoral zone to the undersurface of the preoral dome up to about half of the length of the adoral zone

(Figure 6A). The adoral zone has 6 membranelles ($n=2$) and is rather straight (Figure 6A,D). The cytoplasm is colorless and hyaline. The contractile vacuole is terminal, spherical to obconical. Swims fast and rotates on the main cell axis.

Remarks on the unnamed *Pidimetopus* species

From the other populations, we obtained only a few in vivo images; thus, we can only state that they share the morphologic characteristics of the genus. They all exhibit a nearly spherical macronucleus (Figure 6E,H), a reduced adoral zone (Figure 6E,G,H,J), and four (or at least some) long caudal cilia that can stiffen (Figure 6F,G,I,K).

Morphotype analysis of *Pidimetopus nanus* n. sp.

We measured two samples of the same culture of the polymorphic species *Pidimetopus nanus* n. sp. (HARR): the first sample—1 week, and the second sample—4 weeks after subculturing. We identified three morphotypes of

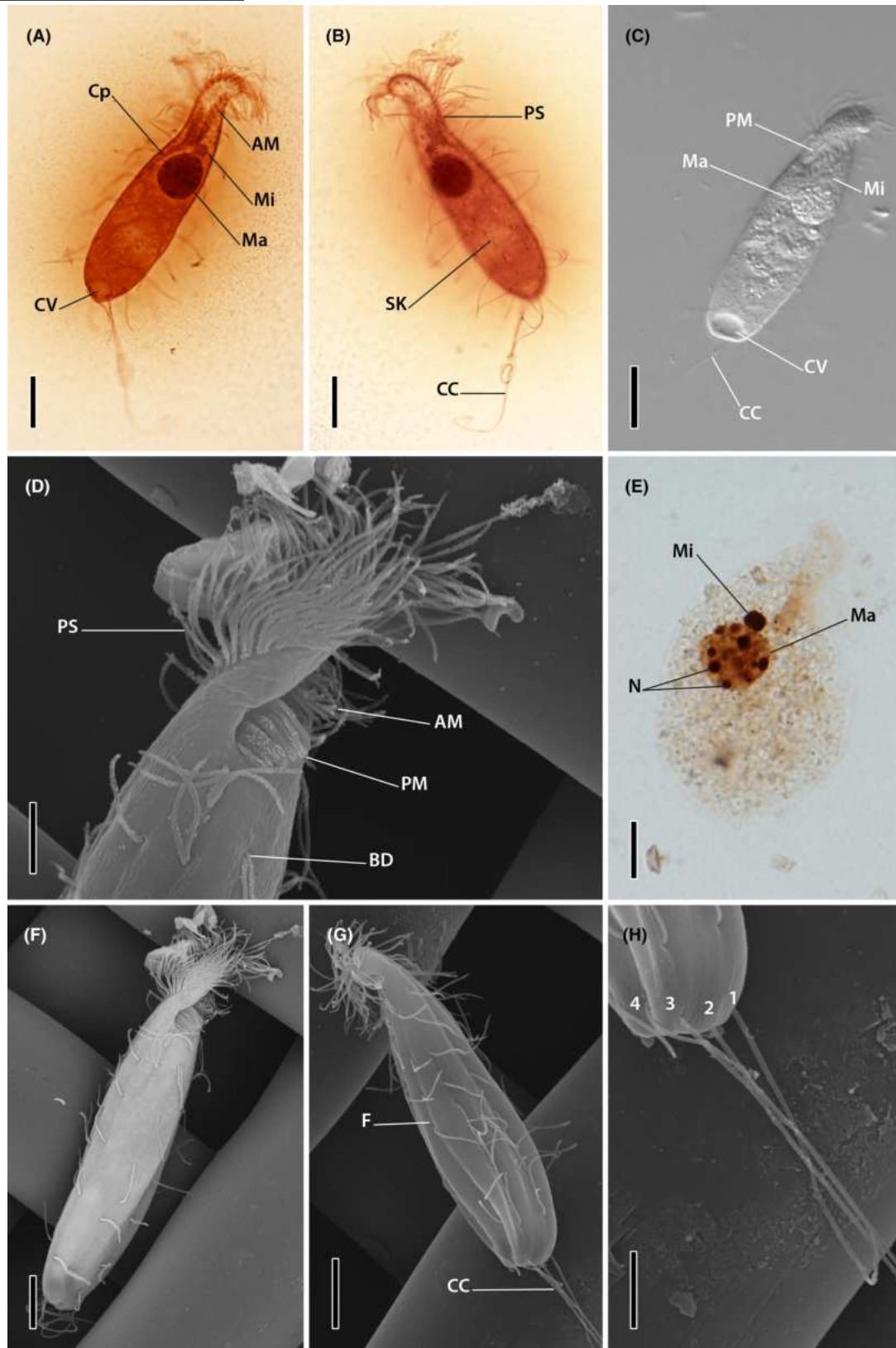


FIGURE 5 *Pidimetopus permonicus* n. sp. (A–H) in vivo (C), after protargol impregnation (A, B), after silver carbonate impregnation (E), and in the scanning electron microscope (D, F–H). (A, B) Ventral and dorsal views of the same specimen. (C, F) Ventral views. (D) Ventral view of the anterior portion. (E) Nuclear apparatus. (G) Dorso-lateral view. (H) Dorso-lateral view of the posterior portion showing the caudal cilia (1–4). AM, adoral membranelles; BD, biciliated dakinets; CC, caudal cilia; Cp, cytopharynx; CV, contractile vacuole; F, cortical furrow; Ma, macronucleus; Mi, micronucleus; N, nucleoli; PM, paroral membrane; PS, perizonal stripe. Scale bars: 10 μ m (A–C, E–G), 5 μ m (D, H).

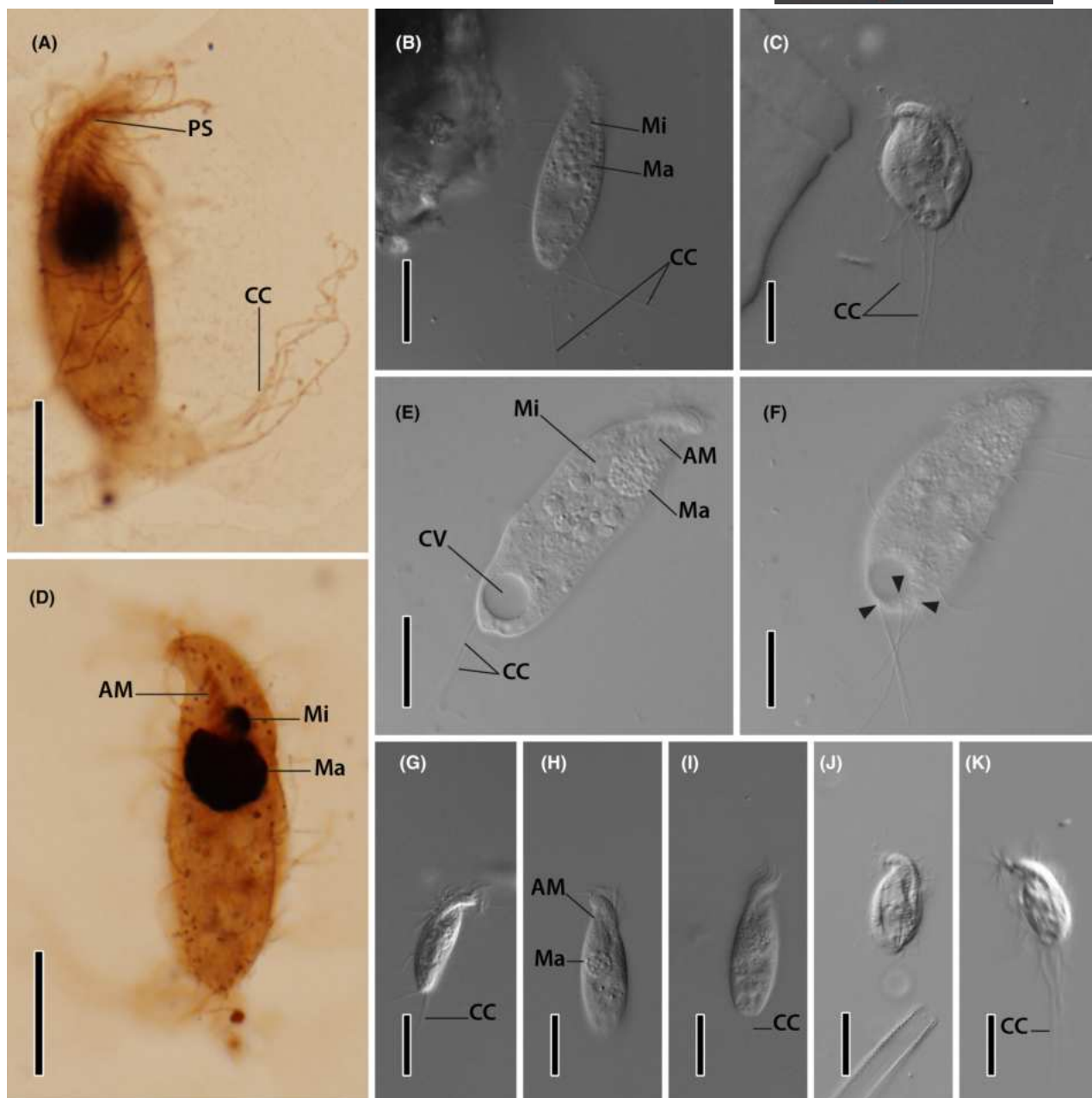


FIGURE 6 Different populations of *Pidimetopus* spp. (A–K) in vivo (B, C, E, F, G–K) and after protargol impregnation (A, D). (A, C, D) MALKALXC, (B) KRUMLI, (E, F) RUM5R2, (G) LIPKY11S, (H, I) SLAN1F, (J, K) MILOT. (A, E, G, I) Ventral views. (B, D, K) Dorsal views. (C, F, H, J) Optical cell sections (F; black arrowheads—caudal cilia). AM, adoral membranelles; CC, caudal cilia (stiffen in B); CV, contractile vacuole; Ma, macronucleus; Mi, micronucleus; PS, perizonal stripe. Scale bars: 20 μ m (A–D, G–K), 10 μ m (E, F).

trophozoites; “normal” cells and two extreme morphotypes: “giant” (long and wide) and “elongated” (long and slender) cells. The extreme morphotypes are defined by a value over two SD above average in the respective characteristic, that is length $\times$ width for “giants” and length for “elongated,” respectively. Approximately 7% of the measured cells in both samples exhibited an extreme morphotype. One week after subculturing, we mainly observed normal cells, and 6.3% of the cells were giant (likely predividers) (Figure 7A, blue dots). Four weeks after subculturing, we mainly found normal cells, and

7.6% of the cells were elongated (Figure 7A, red triangles). Fisher's exact test showed a significant difference in the ratio of the extreme morphotypes between the samples. Significantly more elongated than giant cells were observed 4 weeks after subculturing (ratio 9:2 for 4 weeks: 1 week, Fisher's exact test, p -value=0.026), and significantly more giant than elongated cells were observed 1 week after subculturing (ratio 9:1 for 1 week: 4 weeks, Fisher's exact test, p -value=0.025). The cells 1 week after subculturing were, on average, smaller in size (length $\times$ width) than those 4 weeks after subculturing

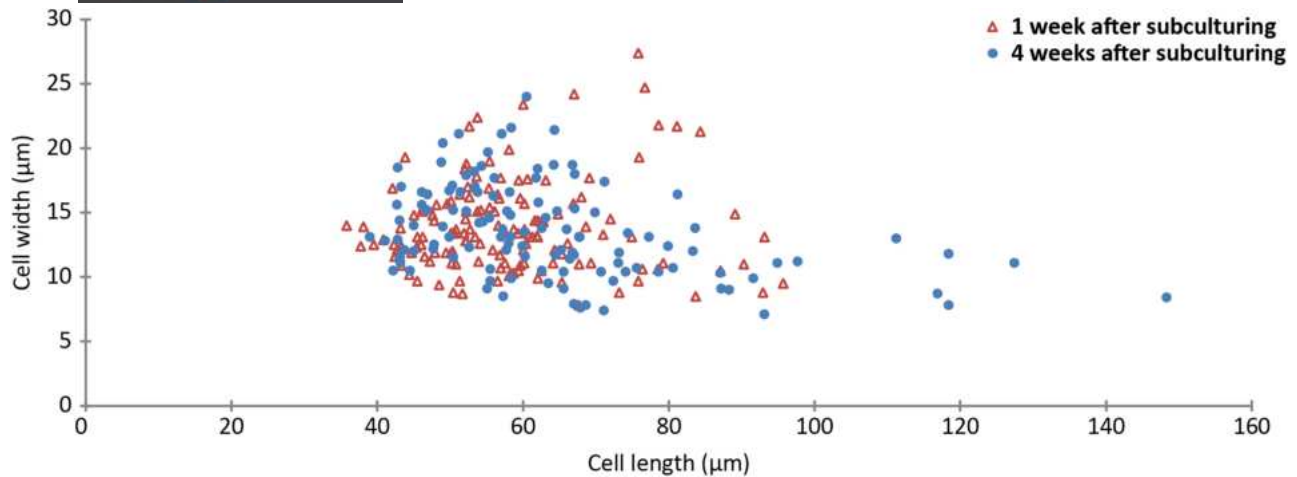


FIGURE 7 Morphotype analysis of *Pidimetopus nanus* n. sp. (HARR population). Measured cell width (y-axis) and cell length (x-axis) 1 week after subculturing (red triangles) and 4 weeks after subculturing (blue dots).

(808.5 vs. 867.3 μm^2). However, this difference was not significant (Mann–Whitney *U*-test, *p*-value=0.062). The average length was significantly lower in the cells 1 week after subculturing (56.5 vs. 60.1 μm ; Mann–Whitney *U*-test, *p*-value $<2.2\text{e}^{-16}$).

Phylogenetic analysis

The overall topology of the phylogenetic tree (Figure 8) is consistent with recent 18S rRNA gene-based analyses (e.g. Bourland et al., 2020; Li et al., 2023; Méndez-Sánchez et al., 2022; Zhuang et al., 2021, 2022). The backbone of the phylogenetic tree was mostly statistically unsupported, but the major clades, that is the main previously identified clades of the Metopida/Clevelandellida lineage: MAC (named after included taxa *Metopus*, *Atopospira*, Clevelandellida), HAC (*Heterometopus*, *Atopospira*, Clevelandellida), LAC (*Longitaeina*, *Atopospira*, Clevelandellida), IAC, and Clevelandellida (Bourland et al., 2018), were recovered, and fully or well supported. The position of the Apometopidae was not congruent in maximum likelihood and Bayesian inference analyses and was not statistically supported in both methods. The position of the IAC clade and the order Clevelandellida corresponds to previous analyses after the addition of 12 newly determined sequences and environmental sequences not used in previous analyses. The genus *Pidimetopus* n. gen. forms a robust clade fully supported by bootstrap and Bayesian inference inside the IAC clade. It appeared closely related to the Clevelandellida, though the position was weakly supported (53/1). Within the genus *Pidimetopus* n. gen., *P. permonicus* n. sp. is sister to all congeners with full support. *Pidimetopus nanus* and *Pidimetopus* spp. form a fully supported clade as well; however, the internal relationships of this clade remain unresolved. The intraspecific genetic uncorrected *p*-distances are in *P. nanus* and *P. permonicus* up

to 0.001; the environmental sequence HF568858 was not included due to its short length (Table S2).

DISCUSSION

The basis for the establishment of *Pidimetopus* n. gen. as a new genus

Owing to its small size, the genus *Pidimetopus* can hardly be confused with most other metopid genera. Species of the genus *Longitaeina* are similar in size to those of *Pidimetopus* and also possess long caudal cilia. In the case of *Pidimetopus*, however, the caudal cilia can stiffen, unlike those in *Longitaeina*. Furthermore, both genera also strongly differ in the number of adoral membranelles (about 16 vs. ≤ 10) and the number of false kineties (>40 vs. <15) (Bourland et al., 2020). Although some morphotypes of *Heterometopus palaeformis* are small or slender and hence superficially similar to *Pidimetopus*, this species has more adoral membranelles (about 19), its preoral dome lacks the typical metopid leftward torsion, and caudal cilia are missing (Esteban et al., 1995; Zhuang et al., 2024). *Pidimetopus permonicus* is similar to *Tropidoatractus ariella* Rotterová et al., 2018, in cell size, the number of somatic kineties (8 vs. 10), and the same maximum number of adoral membranelles (10); however, the two species differ in their overall shape and caudal cilia (straight vs. sigmoidal; caudal cilia present vs. absent; Rotterová et al., 2018). The genus *Castula* possesses setae (cilia that can stiffen) occupying the posterior third of the body, splayed when stiffened (Bourland et al., 2020; Feng et al., 2024), which is different from *Pidimetopus* whose caudal cilia can stiffen as setae, but originate only at the posterior end of the cell and do not splay. *Castula* is different from *Pidimetopus* in overall cell shape and size and has a higher number of somatic kineties and adoral membranelles (Bourland et al., 2020). According to old descriptions, *Metopus setifer* Kahl, 1932,

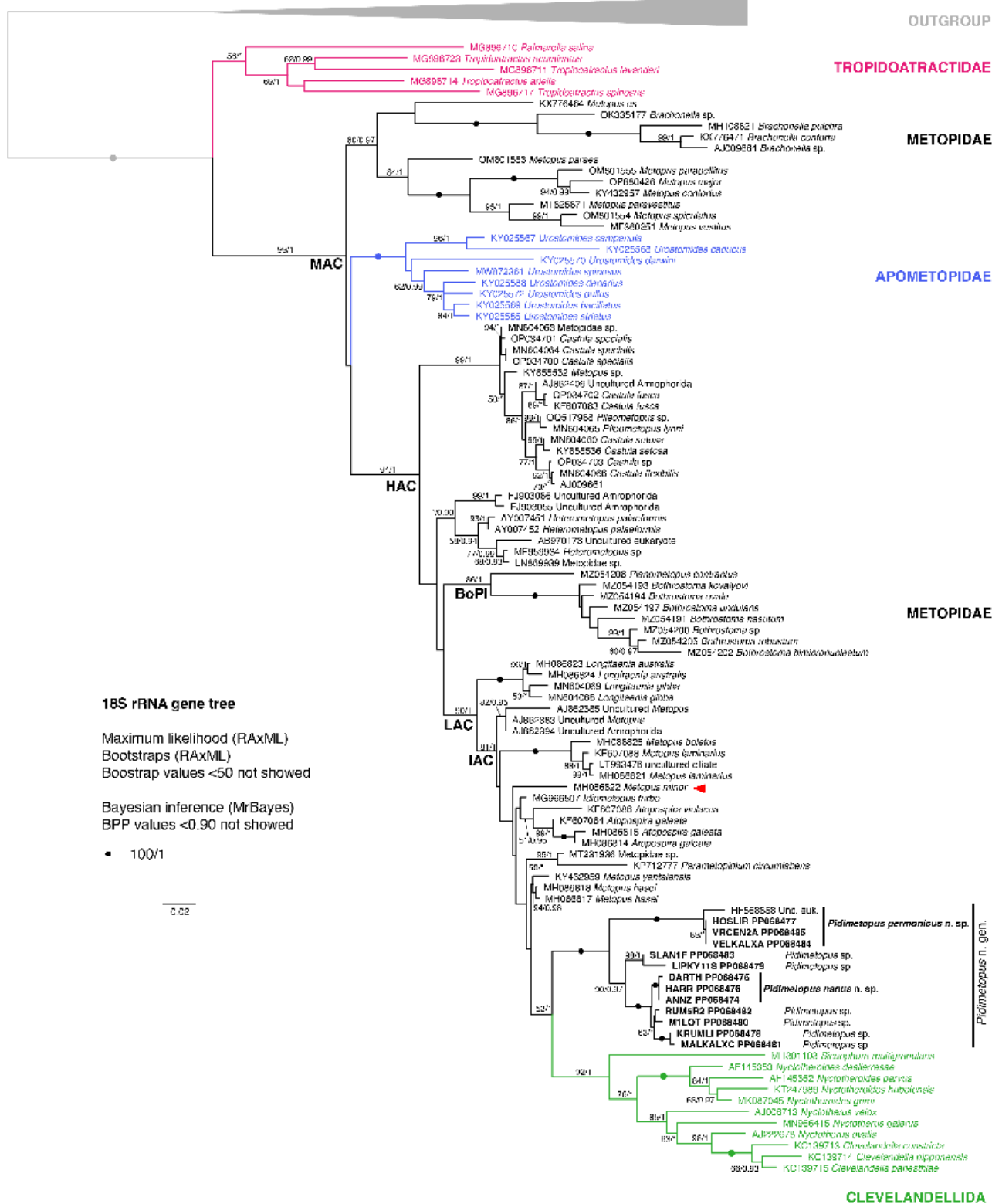


FIGURE 8 Phylogenetic tree of Metopida based on 18S rRNA gene sequences. The tree was constructed by the maximum likelihood method in RAxML (GTR+I+ Γ model). The values at branches represent the statistical support in bootstrap values (RAxML)/posterior probabilities (MrBayes). Support values below 50/0.90 are not shown or are represented by an asterisk (*). New sequences in bold.

resembles *Pidimetopus* in the four long caudal cilia and the cell size, but, based on the drawing in Kahl, it has a different cell shape and lacks the spherical macronucleus

typical for the genus *Pidimetopus* (Kahl, 1932). Likewise, the descriptions of *M. recurvatus* and *M. recurvatus* var. *pusillus* Vuxanovici, 1962, are very brief and ambiguous

(Vuxanovici, 1962b). Both species resemble the genus *Pidimetopus* in size (40 and 25 μm , respectively), exactly four caudal cilia depicted, and the overall shape of the cell. The number of somatic kineties (6) is problematic as it is not clear if Vuxanovici (1962b) refers to ventral kineties only or the kineties in total. The length of caudal cilia differs between the text (10–12 μm) and the line drawing (approx. 15–20 μm ; Vuxanovici, 1962b). *M. fusus* Vuxanovici, 1962, has a small size of 35–45 μm in vivo and four caudal cilia, but different number of somatic kineties than *Pidimetopus* species (10–12 (ventral side or in total) vs. ≤ 8). To summarize, all the discussed species described by Vuxanovici (1962a, 1962b) might be members of the genus *Pidimetopus* but differ from the species described in this study. The current data about other studied populations of pidimetopids (KRUMLI, MALKALXC, SLANIF, LIPKY11S, RUM5R2, and MILOT) is too incomplete to make any decision about possible conspecificity with these species.

Comparison of the species *Pidimetopus nanus* n. sp. and *P. permonicus* n. sp.

Pidimetopus permonicus differs from *P. nanus* in the average cell length (75 vs. 52 μm), the typical overall body shape (rather slender vs. rather ovate, 3.8:1 vs. 3.5:1), and the number of adoral membranelles (about 10 vs. about 8). Ciliates can be recognized in vivo as belonging to the genus *Pidimetopus* by the following characteristics: four long caudal cilia that can stiffen, a spherical macronucleus, and a reduced adoral zone (≤ 10 membranelles). However, in vivo identification at the species level is difficult due to their diminutive size and rather similar morphometry. *Pidimetopus nanus* and *P. permonicus* are mainly distinguished by the number of adoral membranelles (8 vs. 10).

Diversity and phylogeny of *Pidimetopus* n. gen.

Apparently, the true diversity of metopids is still underrated. We identified about six evolutionary lineages in the 12 populations studied. With regard to reports of small metopids from different environments ranging from moorlands through pond sediments to soils, and the occurrence on different continents (e.g. Foissner, 1980, 2016b; Foissner et al., 2002), we can expect a much higher diversity even in the genus *Pidimetopus*. Our phylogenetic analysis provides full support for the monophyly of the new genus *Pidimetopus* and its placement within the IAC clade, though its exact relationships are not resolved. *Pidimetopus permonicus* (HOSLIR, VELKALXA, VRCEN2A populations, and environmental sequence HF568858) clade is sister to all other species of the genus. The position of *Pidimetopus nanus* (ANNZ, HARR, and DARTH populations) is

also well supported. Other lineages, referred here to as *Pidimetopus* spp., need detailed morphologic examination. We obtained insufficient data for the description of these populations. Interestingly, the environmental sequence HF568858 from a groundwater sample in the former uranium mine in Rifle, Colorado, represents the first sequence of a putative *Pidimetopus* ever obtained (Holmes et al., 2013) and very likely belongs to *P. permonicus*. Despite the addition of new lineages, the IAC clade remains largely unresolved, and the position of clevelandellids is uncertain.

Morphologic variability of *Pidimetopus nanus*

In a previously studied population of *Heterometopus palaeformis*, Esteban et al. (1995) demonstrated its remarkable phenotypic plasticity. Based on cell length and width, they identified three forms: “normal,” “giant,” and “elongated” cells. Until now, this polymorphism has not been studied in other metopids, even though some level of polymorphism has been observed in *Tropidoatractus* and *Bothrostoma* (Méndez-Sánchez et al., 2022; Rotterová et al., 2018). We designed our measurement methodology for the cultured *P. nanus* so that the results could be compared to the study by Esteban et al. (1995). Surprisingly, we identified a pattern consistent with that found in their study (Figure 7). *Heterometopus palaeformis* is thus not the only species exhibiting such morphologic variability. Interestingly, both *H. palaeformis* and *P. nanus* belong to the HAC clade. However, we cannot decide whether the ability has been lost multiple times or evolved independently or whether it is widespread over all metopids in general. We elaborated on the analysis of Esteban et al. (1995), performing measurements 1 and 4 weeks after subculturing, respectively. Our results support the conclusion of the abovementioned authors that “elongated” cells appear significantly more frequently 4 weeks after subculturing, suggesting that starvation may be a factor. On the contrary, the “giant” cells (i.e. extreme in both length and width) were significantly more likely found 1 week after subculturing (Figure 7). Although the effect of culture conditions (diet, media, temperature, etc.) can play a role, the pattern implied by our results cannot be explained solely by a random occurrence of “untypical” cells due to culture conditions.

The case of *Metopus minor*

The history of the species *Metopus minor*, originally described by Kahl (1927) as *Metopus setosus* var. *minor*, illustrates the taxonomic difficulties associated with small metopid ciliates. Understandably, Kahl provided only limited information, for example an approximate length of 35 μm in vivo, a flattened and significantly leftward projected preoral dome, and several long

stiffened cilia (in the drawing, the caudal cilia are not located only posteriorly, but in the posterior third of the cell; Kahl, 1927). Kahl assumed this variety to be a stable form, but he also speculated that it might be a variety or even several different species. He refrained from establishing separate species for these “dwarfish forms” (Kümmerformen) and encouraged future research to resolve the question. Later, Kahl (1932) redescribed it as a variety, giving the previous characters but expanding its size range to 30–40 µm. Foissner (1980) studied a population of small metopids and, with reference to the size and the presence of the long caudal cilia like those of *Metopus setosus* var. *minor*, he redescribed it and elevated it to species rank as *Metopus minor* Kahl, 1927. He further synonymized the species with *M. recurvatus* and *M. recurvatus* var. *pusillus* Vuxanovici, 1962.

It is questionable what organism Kahl (1927) referred to. He pointed out a flattened and significantly leftward extended preoral dome, which is rather a character of the genus *Longitaenia* than of *Pidimetopus* (see Bourland et al., 2020). Also, in otherwise mostly accurate Kahl's drawings, the long posterior cilia appear more similar to the setae of *Castula* or the caudal cilia of *Longitaenia* than the caudal cilia of *Pidimetopus* (see Figures 3D and 5H; Bourland et al., 2020; Kahl, 1927, 1932). The Alpine population studied by Foissner (1980) and the Turkish population studied by Çapar (2007) might be conspecific based on the morphometry and drawings provided. In the Turkish population, there are more adoral membranelles than in *Pidimetopus* (max. 18 vs. max. 10) and up to eight long caudal cilia (neither of the characteristics is indicated in Foissner (1980)). However, both characteristics are more consistent with *Longitaenia*, although the nuclear apparatus in both is more similar to *Pidimetopus* (see Bourland et al., 2020; Çapar, 2007; Foissner, 1980). The taxonomic position of these *Metopus minor* populations thus remains uncertain. Based on the little information available, these populations are very likely not conspecific with the populations of *Metopus minor* studied by Foissner et al. (2002), particularly due to differences in the number of adoral membranelles and caudal cilia.

For the populations studied in Foissner et al. (2002), the small cell size, the presence of caudal cilia (number not specified), and a low number of adoral membranelles (6–9) were considered as the basis for conspecificity with *M. minor* (Foissner et al., 2002). Together with the low number of somatic kineties, the spherical macronucleus, and the four caudal cilia in at least one population (see Figure 405a-f of the Namibian population in Foissner et al., 2002), the populations studied by Foissner et al. (2002) strongly resemble the genus *Pidimetopus*, although they are not conspecific with hereby described species, *P. nanus* and *P. permonicus*. Additionally, the invariable numbers of adoral membranelles found in our populations of *Pidimetopus* suggest that the populations

of *M. minor*, which differ in this feature, likely represent several separate species. In particular, considering all data available on the number of adoral membranelles in the populations studied by Foissner et al. (2002), they found almost no variability within the particular population for this characteristic. On the contrary, there was a noticeable interpopulation variability, which suggests nonconspecificity. Unfortunately, there are currently not enough data on the two crucial characteristics (the number of adoral membranelles and the number of somatic kineties) to decide about the conspecificity of the previously studied *M. minor* populations and the undescribed *Pidimetopus* populations.

To conclude, *Metopus minor* is currently very likely a collective taxon, and the single published 18S rRNA gene sequence attributed to putative *M. minor* without morphologic data provided (Vďačný et al., 2019) belongs most likely to a different metopid than those studied by Foissner et al. (2002). Once the sequenced ciliate is morphologically studied in detail, its similarity to *Pidimetopus* or the populations of *Metopus minor* can be re-examined. On the other hand, it would be interesting to find that there is a morphological convergence in small metopids in the IAC clade, which cannot currently be excluded.

TAXONOMIC SUMMARY

Taxonomic assignment: Ciliophora: Intramacronucleata: SAL: Armophorea: Metopida: Metopidae Kahl, 1927.

ZooBank registration of this work: urn:lsid:zoobank.org:pub:28C2B76A-0590-4CBC-A9A6-38CB7FAFF2FE.

Pidimetopus n. gen.

Diagnosis: Small to very small Metopidae (≤75 µm) with four long caudal cilia that can stiffen: one pair >50 µm long, second pair ≥25 µm, arise from dikinetids at posterior ends of somatic kineties. In living cells, the nuclear apparatus usually comprises a spherical or slightly ellipsoidal macronucleus with prominent nucleoli and an attached almost spherical micronucleus. Ten or less adoral zone membranelles. Eight or less somatic kineties.

Etymology: *Pidimetopus* [pɪˈmɛtɒpʊs]. A composite of the Czech onomatopoeic prefix *pidi-* (derived from “píd” (span), meaning “tiny,” “dwarfish”), and the genus name *Metopus*. Refers to the small size of cells. Masculine gender.

Type species: *Pidimetopus nanus* n. sp.

Included species: *P. nanus* n. sp.; *P. permonicus* n. sp.

Zoobank registration: urn:lsid:zoobank.org:act:DD4507BD-8F81-46A6-88D8-D5BBA65A1EF7.

Pidimetopus nanus n. sp.

Zoobank registration: urn:lsid:zoobank.org:act:65FFE5C4-D888-4359-8B41-9032F2EB7EEB.

Diagnosis: Cell on average 50 µm long with eight adoral membranelles and eight somatic kineties. Polymorphic species.

Type material: A slide with the protargol-impregnated holotype specimen and one voucher slide are deposited in the collection of the National Museum in Prague, Czech Republic, inventory numbers P6E 5490 (ANNZ) and P6E 5491 (HARR). Relevant specimens are marked with black ink circles. The holotype is in [Figure 1A,B](#).

Type locality: A mountain creek Račí potok, sediment, Annín, Czechia, 2019, 49°10'29.0" N 13°31'13.9" E.

Habitat: Freshwater anoxic sulfidic sediment or soil.

Etymology: The Latin adjective, *nanus* (dwarfish), refers to the small size of the cell.

Gene sequence: 18S rRNA gene sequences of the following populations are deposited in the GenBank database: ANNZ (Accession number: [PP068474](#)), DARTH (Accession number: [PP068475](#)), HARR (Accession number: [PP068476](#)).

Species: *Pidimetopus permonicus* n. sp.

Zoobank registration: urn:lsid:zoobank.org:act:0EB55E4E-DCB6-4FB3-9336-11E7E4346561.

Diagnosis: Cell on average 75 µm long with 10 adoral membranelles and eight somatic kineties.

Type material: A slide with the protargol-impregnated holotype specimen and one voucher slide are deposited in the collection of the National Museum in Prague, Czech Republic, inventory numbers P6E 5488 (VELKALXA) and P6E 5489 (VRCEN2A). Relevant specimens are marked with black ink circles. The holotype is in [Figure 5A,B](#).

Type locality: A shore of drained Kala pond, sediment, Hostivice, Czechia, 2020, 50°04'18" N 14°15'05" E.

Habitat: Freshwater anoxic sulfidic sediment or soil.

Etymology: The adjective *permonicus* is a Latinized version of the Czech word “permoník” referring to mine dwarf or ghost, as the first environmental sequence of the species was obtained from groundwater in a mining area. Masculine gender.

Gene sequence: 18S rRNA gene sequences of the following populations are deposited in the GenBank database: VELKALXA (Accession number: [PP068484](#)), HOSLIR (Accession number: [PP068477](#)), VRCEN2A (Accession number: [PP068485](#)).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

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Methanogenic symbionts of anaerobic ciliates are host and habitat specific

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Abstract

The association between anaerobic ciliates and methanogenic archaea has been recognized for over a century. Nevertheless, knowledge of these associations is limited to a few ciliate species, and so the identification of patterns of host–symbiont specificity has been largely speculative. In this study, we integrated microscopy and genetic identification to survey the methanogenic symbionts of 32 free-living anaerobic ciliate species, mainly from the order Metopida. Based on Sanger and Illumina sequencing of the 16S rRNA gene, our results show that a single methanogenic symbiont population, belonging to *Methanobacterium*, *Methanoregula*, or *Methanocorpusculum*, is dominant in each host strain. Moreover, the host's taxonomy (genus and above) and environment (i.e. endobiotic, marine/brackish, or freshwater) are linked with the methanogen identity at the genus level, demonstrating a strong specificity and fidelity in the association. We also established cultures containing artificially co-occurring anaerobic ciliate species harboring different methanogenic symbionts. This revealed that the host–methanogen relationship is stable over short timescales in cultures without evidence of methanogenic symbiont exchanges, although our intraspecific survey indicated that metopids also tend to replace their methanogens over longer evolutionary timescales. Therefore, anaerobic ciliates have adapted a mixed transmission mode to maintain and replace their methanogenic symbionts, allowing them to thrive in oxygen-depleted environments.

Keywords: anaerobiosis, archaea, endosymbionts, methane, symbiosis, syntrophy, transmission mode

Introduction

Symbiotic relationships between unicellular eukaryotes, or protists, and archaea or bacteria are prevalent across the major supergroups of eukaryotes. However, little is known about the biology, ecology, physiology, and evolution of many of these complex interactions [1, 2]. Ciliates, a well-known group of protists, have independently established multiple associations with a wide range of eukaryotes [3, 4] and prokaryotes [1, 3, 5] during their evolutionary history. Although most ciliates are aerobic, most of their major lineages (classes) include free-living or endobiotic taxa that have secondarily transitioned to an anaerobic lifestyle [6]. These transmissions have involved the modification of their mitochondria into mitochondrion-related organelles (MROs) and the acquisition of archaeal and/or bacterial symbionts that facilitate their fermentative metabolism [6–13]. This is assumed to be a syntrophic partnership in which symbiotic prokaryotes, commonly methanogens or sulfate-reducing bacteria, utilize the fermentation end-products of the host's MROs, mainly H₂, during methanogenesis or sulfate reduction, yielding a higher energetic efficiency for the host and resulting in methane or hydrogen sulfide production, respectively [6, 9, 13–20]. In some anoxic environments, the methanogenic symbionts of protists can

significantly contribute to biogenic methane production, which is a potent greenhouse gas [14, 21–26].

Though it is assumed that methanogens form partnerships with most, if not all, anaerobic ciliates, only a few known symbionts associated with a limited number of ciliate species have been characterized using autofluorescence, transmission electron microscopy (TEM), fluorescence in situ hybridization (FISH), marker gene sequencing, and/or genomic analysis. These ciliates belong to the classes Armophorea and Plagiopylea, the subclasses Trichostomatia and Haptoria (Litostomatea), the family Anaerocyclidiidae (subclass Scuticociliatia), and the karyorelictid genus *Parduczia* [6, 8, 12, 16, 17, 27–44]. However, methanogenic endosymbionts have been observed in many more host species, predominantly in the obligate anaerobic, free-living, and species-rich order Metopida (Armophorea), using the characteristic autofluorescence of the methanogen F420 coenzyme [45–53].

Existing evidence suggests that each ciliate species harbors a single methanogen population [11, 12, 28, 29, 38, 44]. Although it is generally assumed that the symbionts are transmitted vertically during host division [11, 12], this evidence is limited to two ciliate species [54, 55]. Additionally, host and methanogen phylogenies do not show co-diversification patterns [6, 11, 12, 30], which would

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be expected in the case of strict vertical transmission [56]. In contrast, the horizontal acquisition of methanogenic symbionts is also presumed, as experimentally demonstrated in a cultivated aposymbiotic strain of *Trimyema* (Plagiopylea) that was successfully reinfected with two strains of symbiotic *Methanobacterium* [57, 58]. Overall, understanding of the methanogen–ciliate association remains largely unresolved, confounding our ability to discern, over both ecological and evolutionary timescales, drivers of fidelity and specificity between the host and the symbiont, as well as the transmission modes and mechanisms of maintenance of the symbiont.

Here, we present a comprehensive evaluation of host–symbiont partnerships within and among phylogenetic clades of host taxa, in the context of habitat type and host phylogeny. We studied the archaeal diversity associated with 54 strains of 32 anaerobic ciliate species, mostly metopids isolated from soil, marine/brackish, and freshwater sediments, via microscopy and Sanger and Illumina amplicon sequencing. In addition, we surveyed artificial co-cultures of several ciliate species to determine the potential for symbiont exchange between co-occurring pairs of ciliates.

Materials and methods

Source of anaerobic ciliates

Fifty-four anaerobic ciliate strains from long-term stable polycyclic cultures were used in this study, 42 from freshwater sediments, 11 from brackish/marine sediments, and one from soil (Table S1). All cultures were maintained in 10 ml of culture medium, American Type Culture Collection (ATCC) #802 for freshwater or #1525 for brackish/marine species. The cultures were polyxenic containing ciliates and unidentified prokaryotes in the sediment at the bottom of the tube. The sediment (ca. 1 ml) was subcultured biweekly into a new tube with fresh medium. Tubes were tightly closed and kept in darkness at room temperature (24°C) [49, 59]. One culture contained three ciliate species, five had two species each, and the remaining 41 had a single ciliate species (Table S1). Each ciliate strain was identified using 18S rRNA gene sequences amplified from individual cells (see below) (Table S1). Thirty-two species were examined: 30 metopids (Armophorea, Metopida), one *Caenomorpha* (Armophorea, Armophorida), and one *Trimyema* (Plagiopylea, Plagiopylida).

Co-cultivation experiments

To test the potential for horizontal transmission of methanogenic symbionts between different ciliate species and the stability of the symbionts, pairs of ciliates from already established cultures were mixed and cultivated together by adding 1 ml of each culture into 9 ml of medium. These artificial co-cultures were subcultured every 1 or 2 weeks (as described above). Seven mixed cultures were successfully established (Table S1). The species from the cultures were randomly examined at various intervals (Tables S1, S2).

Detection of methanogenic archaea through microscopy

Symbiont autofluorescence via methanogen-specific coenzyme F420 was determined in living and fixed cells for most of the ciliate strains using a Zeiss Axioskop 2 plus microscope (Carl Zeiss Microscopy) with a UV filter (Zeiss filter set 01) according to [53] or a confocal microscope Leica TCS SP8. Symbiont-targeted FISH was performed using Cy3-labeled probes: ARC915 for archaea and MG1200b for order Methanomicrobiales [60–62] (Supplementary methods). TEM was implemented to localize the

putative methanogenic symbionts in the host cells of several strains (Supplementary methods).

Molecular identification of the ciliates and their methanogenic symbionts

Cell picking, DNA extraction, and sample preparation for SSU rRNA gene sequencing

For each ciliate strain, including the artificially mixed cultures, ~15 cells were handpicked with glass micropipettes, washed by transferring several times through sterile media, and usually starved for 30 min or up to 27 h [27, 63, 64]. Cells were finally transferred into 30 µl of UV-treated DNA/RNA Shield (Zymo Research, Irvine, CA, USA) and frozen at –20°C. Replicates (additional pools of cells) were collected for several strains (Table S2). Additionally, for most of the cultures, ciliates were removed from 30 µl of the culture using sterile glass micropipettes to obtain the culture medium containing only the free-living prokaryotic community (hereinafter control medium), which was dispensed into a tube with UV-treated DNA/RNA Shield and frozen at –20°C (Supplementary methods). Ciliates from the artificially mixed cultures were examined after up to 13 months of cultivation (Table S2).

PCR amplification and sequencing of host and symbiont SSU rRNA genes

DNA from picked cells and control medium was extracted using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's directions. The identity of the ciliates and their methanogenic symbionts was assessed via amplification and Sanger sequencing of the 18S and partial 16S rRNA genes, respectively, using the eukaryotic primers Euk A and Euk B [65] for the ciliates, and the archaeal primers Arc915F and ArcR1326 [12, 34] for the methanogens. In parallel and using the same isolated DNA from each sample, amplicons for Illumina sequencing were prepared using archaea-specific barcoded primers Arc915F and ArcR1326. To obtain enough DNA and reduce PCR bias, PCR was done in triplicate for each sample. PCR products were subsequently pooled and purified, and then sequenced on a MiSeq system (Illumina 2x250bp) (Supplementary methods).

Identification and taxonomic assignment of amplicon sequence variants

Demultiplexing of the raw paired-end 16S rRNA gene reads was performed with BBMap [66] and then reads were imported into QIIME2 [67]. 16S rRNA gene amplicon reads were initially processed using QIIME2 and the DADA2 plugin. Primer sequence trimming, quality trimming, sequence denoising, chimera filtering, singleton removal, and sequence dereplication were performed with DADA2 [68]. Because paired-end reads could not be merged due to the poor quality of the forward reads, only the reverse reads were utilized in our analysis. For the taxonomic assignment, we utilized a Naive Bayes classifier pre-trained on the SILVA 138 99% OTUs (i.e. operational taxonomic units) full-length database and the q2-feature-classifier plugin [69, 70].

Amplicon sequence variants (ASVs), whose relative abundance was not >4% in any single sample, were assumed to represent contaminants and discarded in subsequent analyses [71, 72]. The remaining ASVs that were taxonomically assigned to a methanogen group were named according to their assigned genus or otherwise lowest assigned taxonomic rank. Sample metadata information is available in Table S3. Count tables and taxonomic assignments of ASVs after filtering and renaming are available in Tables S4–S6. ASV relative abundance per sample was computed

after these filtering steps and fractional abundance plots were created with the PHYLOSEQ package in R [73]. Symbiont ASV and Sanger sequences that were obtained from the same ciliate strain were aligned, and pairwise nucleotide sequence identity distance matrices were computed using Clustal Omega (Table S7) [74] in Geneious Prime 11.0.9 + 11.

Phylogenetic analysis of host 18S and symbiont 16S rRNA gene sequences

A dataset containing 18S rRNA gene sequences of the 54 ciliate strains used in this study was created and aligned using MAFFT (G-INS-i algorithm) [75]. The alignments were manually trimmed to the primer regions, using AliView v1.28 [76] (Supplementary methods).

The 131 16S rRNA sequences of methanogenic symbionts obtained from Sanger sequencing were de-replicated with vsearch (—derep_fulllength). A total of 32 unique sequences were identified and aligned along with reference and outgroup sequences obtained from GenBank, using the SILVA SINA aligner 1.2.11 [77], and manually trimmed to the Arc915F/ArcR1326 primer region using AliView v1.28. In parallel, all the obtained 16S rRNA gene sequences of *Methanobacterium* (65), *Methanoregula* (56), and *Methanocorpusculum* (10) were separately aligned with the reference and outgroup sequences as above. Pairwise distance matrices (Tables S8 and S9) were computed using Clustal Omega [74] in Geneious Prime 11.0.9 + 11.

The 16S and 18S rRNA gene phylogenetic trees were generated using the maximum likelihood method in RAxML 8.2.12 under the GTRGAMMAI model with 1000 bootstrap pseudoreplicates [78] in the web server The CIPRES Science Gateway V. 3.3 (<https://www.phylo.org>).

We conducted a full and partial Mantel test with the Vegan and NCF packages in R [79, 80] to identify relationships between the host and symbiont genetic distances and geography based on Spearman rank correlations. Pairwise genetic distances were calculated from both 18S rRNA gene sequences of the host and 16S rRNA gene sequences of the symbionts based on Kimura's two-parameter model [81]. Geographic distances were determined by calculating the geodesics between sample sites with the GeoSphere package in R [82]. Host and symbiont sequences from *Trimyema* and *Caenomorphia* were excluded from this analysis because these ciliate hosts are phylogenetically divergent from the rest.

Results

Ultrastructure and localization of presumable methanogenic symbionts in ciliates

Methanogenic endosymbionts were detected by autofluorescence of the F420 coenzyme in 50 out of 54 of the studied strains (Figs. 1B, G, I, J, N, and S1; Table S10).

FISH using archaea- or Methanomicrobiales-specific oligonucleotide probes confirmed the presence of the methanogenic symbionts and further proved their distribution throughout the cytoplasm for selected strains of the host species *Tropidotractus levanderi*, *Urostomides bacillatus*, *Bothrostoma undulans*, and *Metopus es* (Fig. 1C, D, H, K, O). Importantly, FISH experiments indicated the presence of the order Methanomicrobiales in the cytoplasm of *T. levanderi* since both the general archaeal probe ARC915 and MG1200b probe specific to order Methanomicrobiales showed a similar pattern (Fig. 1C, D), likely corresponding to its symbiont *Methanoregula* (Methanomicrobiales). Additionally, TEM revealed the ultrastructure and localization of the methanogenic

symbionts in the cytoplasm of some strains belonging to the ciliates *T. levanderi*, *Bo. undulans*, and *M. es*. In all studied strains, the symbionts were found in close association with the ciliates' MROs, and they were never surrounded by a host membrane (Fig. 1E, L, M, P). We observed thin rod-shaped symbiont cells with electron-dense centers in *T. levanderi* (KUCR13) (Fig. 1E) and wider, more electron-dense, symbiont cells in *Bo. undulans* (LERMA1) and *M. es* (TRIANGLE) (Fig. 1L, M, P). Dividing methanogens were also observed in *Bo. undulans* and *M. es* (Fig. 1M, P).

A single methanogen dominates the archaeal community of each anaerobic ciliate

Methanogenic symbionts of all examined ciliate species, except *Palmarella salina*, were identified through Sanger (131 samples) and/or Illumina amplicon sequencing of the 16S rRNA gene (62 samples) (Supplementary methods). The Sanger sequencing resulted in clean, high-quality chromatograms, indicating the presence of a single dominant methanogenic archaeal genotype in each of the 131 samples, corresponding to 65 *Methanobacterium*, 56 *Methanoregula*, and 10 *Methanocorpusculum* sequences (Figs. S2, S3, and S4). All the Sanger sequences originated from the control medium were of poor quality, and with noisy background, e.g. ambiguous nucleotides and double peaks (not shown). The results from the amplicon Illumina sequencing of the ciliate samples were highly consistent with those from Sanger. Fifty-one amplicon samples were dominated by a single ASV corresponding to either *Methanoregula*, *Methanobacterium*, or *Methanocorpusculum*, each representing >70% of the amplicon reads, up to 100% in six samples (Figs. 2 and S5; Table S2). These dominant ASVs were identical to the Sanger sequence obtained from respective ciliate strains and consistent among replicates from the same strain (Table S7). Although the remaining amplicons recovered two or more ASVs, their dominant ASV, and in two cases the second dominant, was consistent with their respective Sanger sequences (Figs. 2 and S5; Table S2). Only one case, *Palmarella salina* WH5, the ASVs from the amplicon sequencing were incongruent with the Sanger sequencing results (*Methanomethylophilaceae* sp. vs. *Methanocorpusculum*, respectively, not shown).

From the 131 Sanger sequences, a total of 32 unique sequences were identified. These were considered lineages of *Methanoregula* (15), *Methanobacterium* (11), and *Methanocorpusculum* (6), corresponding to the 15 dominant ASVs recovered from amplicon sequencing—10 of *Methanoregula*, three of *Methanobacterium*, and two of *Methanocorpusculum* (Figs. 3, S2–4, S6, and S7). Based on the results generated by the Sanger and amplicon Illumina sequencing methods, we define those unique methanogen lineages (32) and their respective dominant ASVs (15) as putative symbionts for each strain and ciliate species (Figs. 3 and S6), except for *P. salina*, where the results were incongruent.

Symbiont specificity within a host species

Based on the sequencing results, all the identified methanogenic symbionts from the replicate samples that originated from one ciliate strain were genetically identical (Figs. S2, S3, S4, and S8A; Table S12). The methanogens from different strains of a particular ciliate species, except for *M. es*, always belonged to the same methanogen genus, but their 16S rRNA Sanger sequences usually differed (Figs. 3, S6, and S8B, D). The methanogen lineages were 100% identical across all studied strains only in *Bo. undulans*, *Heterometopus palaeformis*, and *U. pullus* (Figs. 3 and S6). Eight of the identified symbiont lineages (four *Methanoregula*, one *Methanocorpusculum*, and three *Methanobacterium*) were shared between different strains, species, or genera of anaerobic ciliates. Remarkably,

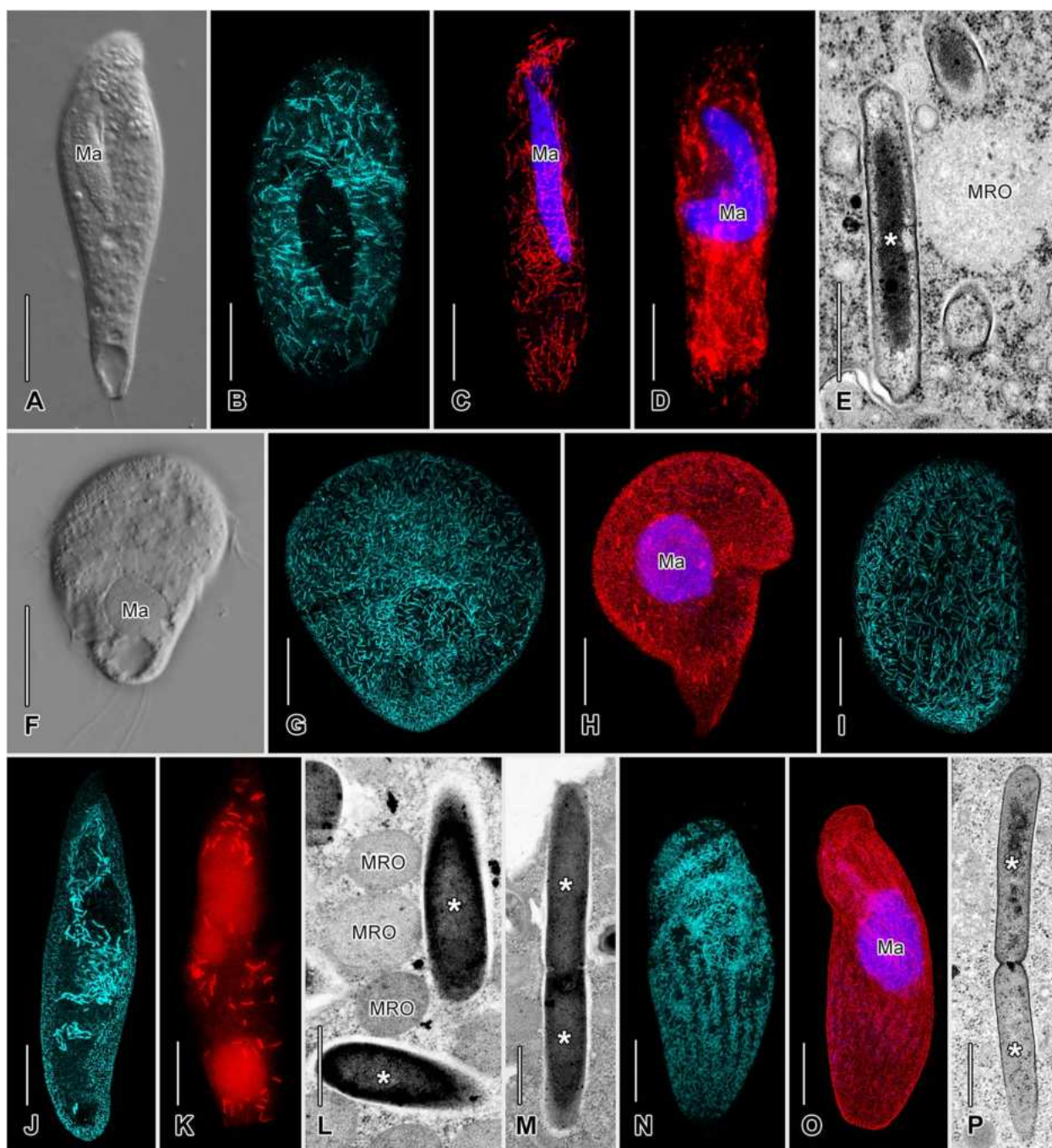


Figure 1. A–P, Representatives of studied anaerobic ciliates and their respective methanogenic endosymbionts. Ciliates shown *in vivo* (A, F), symbionts detected by FISH (C, D, H, K, O), micrographs showing methanogen-specific autofluorescence (B, G, I, J, N), and transmission electron micrographs showing the ultrastructure of the symbionts and host mitochondria (E, L, M, P). A–E, *Tropidoatractus levanderi* KAENG (B, C), RIOT (A, D), and KUCR13 (E) and its *Methanoregula* symbionts; methanogen-specific autofluorescence (B), cell hybridized with archaeal (C) and Methanomicrobiales-specific (D) probes, and ultrastructure of the symbionts in close proximity to host mitochondrion (E). F–H, *Urostomides bacillatus* (BOPAT) (F) and its *Methanobacterium* symbionts showing methanogen-specific autofluorescence (G) and hybridized with archaeal probe (H). I, Methanogenic symbionts of *Brachionella pulchra* (BOPAT) visualized by methanogen-specific autofluorescence. J–L, Methanogenic symbionts of *Bothrostoma undulans* (LERMA1); methanogen-specific autofluorescence (J), symbionts hybridized with archaeal probe (K), close association of the symbionts and host mitochondria (L), and dividing symbiont (M). N–P, Symbionts of *Metopus* es; symbionts of strain GDUKABAM showing methanogen-specific autofluorescence (N), hybridized with archaeal probe (O), and ultrastructure of strain TRIANGLE showing a diving symbiont (P). Macronucleus in FISH preparations was counterstained using DAPI. Ma, macronucleus; MOR, host's mitochondrion-related organelles; methanogenic symbionts marked by asterisks. Scale bars: E, L, M, P 500 nm; A–D, F–K, N, O 20 μ m.

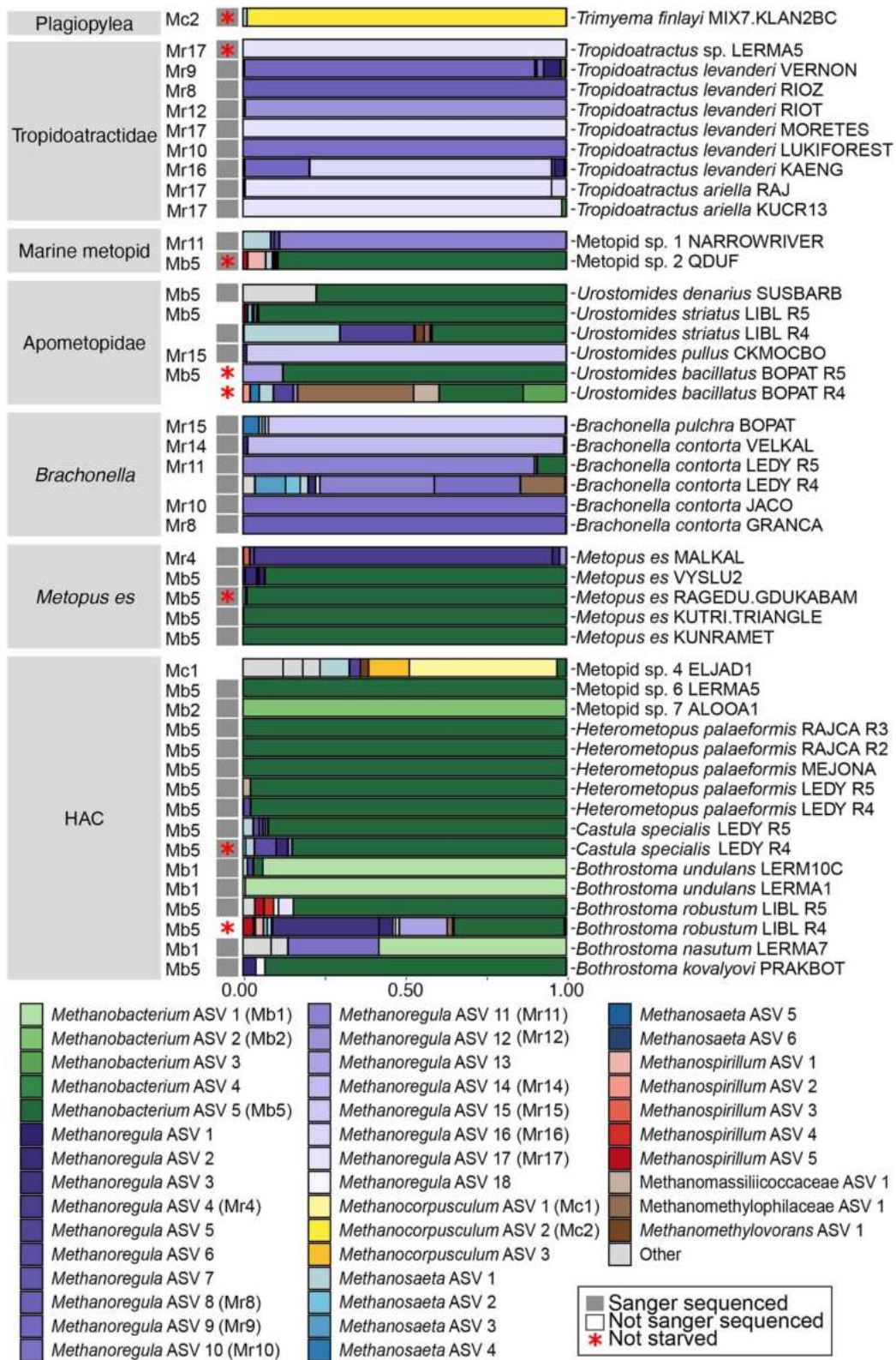
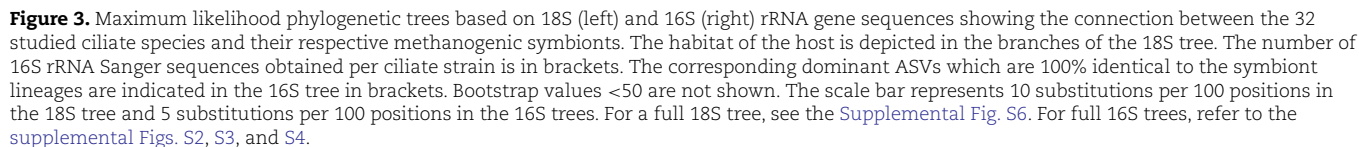


Figure 2. Barplot showing the relative abundances of the methanogen ASVs recovered from 16S rRNA gene amplicon sequencing from each ciliate sample. A dominant ASV is observed in most of the samples, which putatively represents the symbiotic methanogenic archaea. The dominant ASV (70% relative abundance) for each sample is indicated to the left of the bars, and these abbreviations are included in the legend as well. Mc: *Methanocorpusculum*, Mr: *Methanoregula*, and Mb: *Methanobacterium*. Non-methanogen ASVs are labeled "other." Samples are grouped according to the host clade.



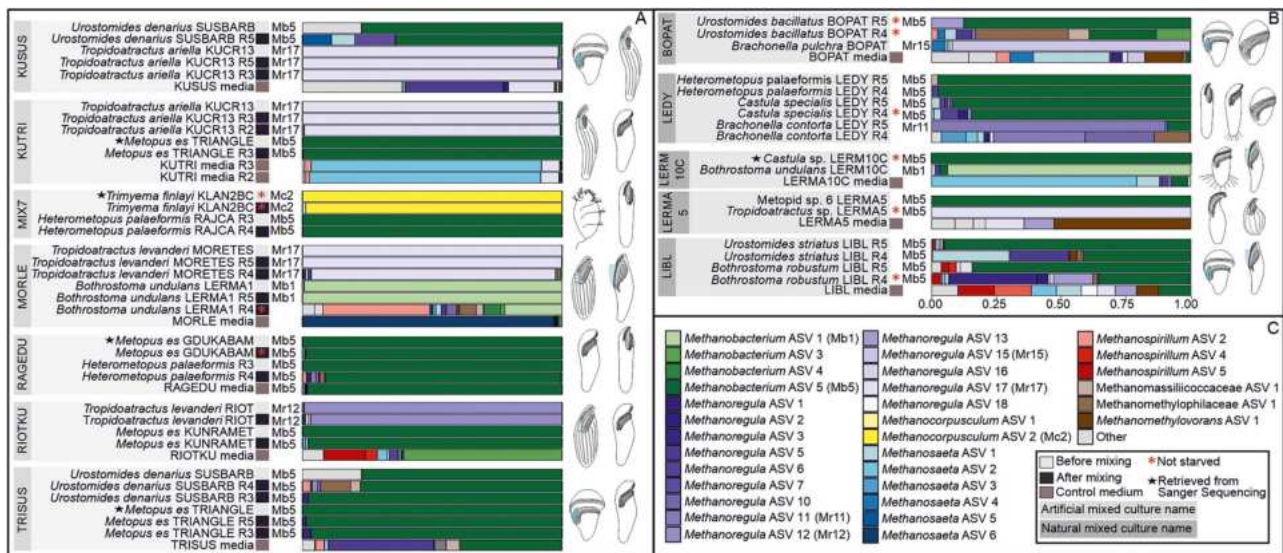


Figure 4. A, Barplot showing the relative abundances of methanogen ASVs recovered from 16S rRNA gene amplicon sequencing in the artificially mixed cultures, including the control medium. In each mixed culture, the combined ciliate species retained the same methanogen ASV before and after being mixed. Samples are grouped by mixed culture and boxes to the left of the barplots indicate whether the time the sample was taken (before or after mixing). B, Relative abundances of methanogen ASVs in natural mixed cultures which contained naturally co-occurring ciliate species. Samples are grouped by the culture name. Stars indicate that there was no amplicon sequencing performed on the sample, but that the results from Sanger sequencing (the ASV which is identical to the 16S rRNA gene sequence obtained from Sanger) are represented in the barplot instead. Asterisks indicate that host cells were not starved during sample processing.

the remaining 24 symbiont lineages were exclusively found in a single ciliate strain (Figs. 3 and S6).

Methanogen genera across the ciliate host phylogeny and environment

We studied anaerobic ciliates from two classes—Armophorea, with 31 species, in two orders (Armophorida and Metopida), and Plagiopylea, represented by a single species (*Trimyema finlayi*). The 30 Metopida species form six clades, here referred to as Tropidoatractidae, “marine metopid clade,” Apometopidae, *Brachonella* clade, *M. es* clade, and HAC clade (Figs. 3 and S6). The Armophorida are represented by *Caenomorphus medusula*.

The distribution of methanogenic symbionts across phylogeny (clade/genus) and habitat (marine/brackish vs. freshwater) of the host showed a degree of specificity (Figs. 3, S6, and S8D). *Methanobacterium* and *Methanoregula* symbionts were detected in all the freshwater armophorean species (metopids and *C. medusula*), in the single soil metopid (ALOOA1), and in two marine/brackish species from the “marine metopid clade” (NARROWRIVER and QDUF). In contrast, *Methanocorpusculum* was found in the rest of the marine/brackish metopid species, except in *P. salina* (Tropidoatractidae), and in the freshwater plagiopylid *Trimyema finlayi*.

In most cases, a particular methanogen genus was consistently observed across all species of a particular ciliate genus or clade (Fig. 3). For instance, *Methanoregula* was present in all the examined species and strains of Tropidoatractidae (Tropidoatractidae), *Brachonella* (*Brachonella* clade), *Longitaenia* (HAC clade), and *Caenomorphus* (*Caenomorphidae*), whereas *Methanobacterium* was observed in almost all the strains within the HAC clade, e.g. *Heterometopus*, *Castula*, and *Bothrostoma*. The exceptions were the genus *Urostomides* (Apometopidae), where the examined species possessed different methanogens (*Methanobacterium* in *U. denarius* and *U. striatus*, and *Methanoregula* in *U. pullus*) and the species *M. es*, in which one strain (MALKAL) harbored *Methanoregula*, while the remaining four harbored *Methanobacterium* (Figs. 3, S6, S7, and S8D).

The degree of specificity that we observed between host and symbiont was further supported statistically—full and partial Mantel tests indicated a weak, but significant, positive correlation between host and symbiont genetic distances ($r=0.1529$, $P=0.001$), whereas there was no observed association between symbiont genetic distances and geography (Table S13). This suggests that host specificity is more important for symbiont variation than geography.

Symbiont switches do not occur in experimentally co-cultured ciliates

A total of seven artificially mixed cultures, each containing a pair of different ciliate species, were successfully established. Five of them contained species that harbored different methanogenic symbionts (*Methanobacterium* vs. *Methanoregula*) before mixing (Table S1, Figs. 4 and S8C). During our experiments, the sequencing results revealed that the dominant methanogenic symbiont ASV associated with each ciliate taxon remained the same, even when grown in the presence of another ciliate and its associated symbionts (Fig. 4A). Due to sparse read numbers obtained during amplicon sequencing of *Trimyema finlayi* KLAN2BC and *M. es* TRIANGLE prior to mixing, we instead compared the Sanger sequences obtained from both species with the dominant ASV obtained after they were mixed, resulting in 100% identity between Sanger sequences and ASVs. Despite our observation that *M. es* harbors either *Methanobacterium* or *Methanoregula*, after mixing a *Methanobacterium*-hosting strain of *M. es* with *Methanoregula*-specific ciliate species (e.g. *Tropidoatractus* spp.), no switches of methanogens and ASVs were observed in either partner.

Except for the MIX7 culture, the free-living archaeal community from the control medium of these mixed cultures (Fig. 4A) as well as for four naturally mixed cultures (BOPAT, LERMA5, LERM10C, LIBL; Fig. 4B) was also screened to determine the methanogen community persisting outside the ciliates (Fig. 4). For the RAGEDU culture, a single dominant ASV (*Methanobacterium*

sp. ASV5) was observed in both the control medium and the two mixed ciliates. In the remaining cultures, the archaeal communities and their relative abundance of the ciliates differed from those in the control medium, e.g. the ASVs belonging to the genus *Methanosaeta* were dominant in KUTRI and MORLE. The presence of the symbiont ASV in the medium, i.e. in RAGEDU culture, might be possibly attributed to its persistence or its accidental presence after being released from dead host cells.

Discussion

Anaerobic ciliates maintain stable populations of dominant methanogenic symbionts

Based on our and previous observations [11, 45], metopid cells typically host hundreds to thousands of symbiotic methanogens (Table S10). We observed the symbionts living adjacent to the host's MROs (Fig. 1E, L) [27, 35, 36], which supports the hypothesis that they are metabolically integrated through hydrogen-based syntrophy [6, 7, 54]. Previously, the symbionts of only a limited number of ciliate populations were identified mostly via Sanger sequencing [12, 28–32, 35, 37, 38]. Here, we found that each ciliate strain (except for *Palmarella salina* WH5) hosts an archaeal community, which is dominated (>70% relative abundance) by a single methanogen ASV, and this methanogen ASV is identical to the 16S rRNA gene sequence obtained via Sanger sequencing from the same ciliate strain. These results strongly corroborate previous suggestions that a particular anaerobic ciliate population is generally colonized by a single, dominant methanogen genotype [12, 28, 29].

Most of the symbiotic methanogens of free-living ciliates and other anaerobic protists belong to *Methanobacterium*, *Methanoregula*, and *Methanocorpusculum* [6, 12, 18, 28–30, 35, 37, 38, 44]. Besides being intracellular symbionts of anaerobic protists, members of these genera are also found as luminal symbionts in the guts of various invertebrates and vertebrates, as well as free-living in a wide range of anoxic environments [22, 33, 83–88]. These genera belong to different euryarchaeote classes, *Methanobacteria* (*Methanobacterium*) and *Methanomicrobia* (*Methanoregula* and *Methanocorpusculum*), but share a hydrogenotrophic metabolism, i.e. they all are dependent on H₂ intake and, therefore, are likely to play a syntrophic role dependent on the use of H₂ from ciliate fermentation [9, 13, 18, 19, 22, 36, 38, 88–90].

Interestingly, the methanogenic symbionts identified here and those from other studies [18, 27, 30, 32, 36] are different from their free-living relatives. However, they cluster among them in a non-monophyletic manner (Figs. 3 and S7), implying that methanogen acquisition from the environment by anaerobic protists is possible, as suggested previously [11, 12] and experimentally demonstrated with the successful acquisition and integration of a *Methanobacterium* into an aposymbiotic strain of the ciliate *Trimyema compressum* through ingestion without digestion [57].

The host lifestyle and habitat are crucial drivers of the ciliate–methanogen specificity

Although evidence of co-diversification between anaerobic ciliates and their methanogenic endosymbionts has not been observed [6, 12, 30] (Fig. 3), our extensive taxon sampling has identified host lifestyle and habitat as factors which appear to influence the specificity of these partnerships. The methanogens from endobiotic ciliates are different from those of the free-living ciliates [12, 22, 91]. Two unrelated lineages of endobiotic ciliates, *Trichostomatia* (rumen ciliates) and *Clevelandellida* (e.g. *Nyctotherus*), harbor *Methanobrevibacter* (*Methanobacteriales*) [12,

91, 92]. *Methanobrevibacter* is also a predominant methanogen found in the gut, intestines, and rumen [91, 93]. As a symbiont of free-living ciliates, it has been only found in *Trimyema compressum* (*Plagiopylea*) [43]. Interestingly, the free-living close relatives of both, *Clevelandellida* (i.e. metopids) and *Trichostomatia* (i.e. *Legendrea* and *Dactylochlamys* [37]), invariably host *Methanoregula*, *Methanocorpusculum*, *Methanosaeta*, or *Methanobacterium*. This suggests that when an anaerobic ciliate becomes endobiotic, a symbiont switch takes place, in this case to *Methanobrevibacter* (Fig. S8D).

We also observed specificity patterns in methanogenic symbionts of free-living metopids isolated from two contrasting environments. We found that freshwater metopids are specific to *Methanobacterium* or *Methanoregula*. Based on environmental sequencing, *Methanoregula* and *Methanobacterium* are typically freshwater and rare in marine environments [84, 85, 87, 94]. This underscores the presence of these methanogens as symbionts of anaerobic protists in freshwater ecosystems [12, 18, 27, 30, 37]. In contrast, most of the marine strains from the “marine metopid clade,” including the previously studied *Metopus contortus* [28, 36, 44] and the only marine metopid strain (ELJAD1) from the predominantly freshwater HAC clade, specifically host *Methanocorpusculum* (Figs. 3, S4, S6, S7, and S8D). Moreover, *Methanocorpusculum*, absent in the freshwater metopids, apparently dominates as a symbiont of marine/brackish metopids and plagiopylids [44]. Nevertheless, exceptions exist—three metopids in the “marine metopid clade,” two of these originating from marine/brackish environments and one from granular sludge [32], host *Methanobacterium* or *Methanoregula* (Fig. 3). Despite the observed exceptions, and the potential absence of methanogens in *P. salina*, our data strongly support the idea that the environment is involved in the ciliate–methanogen specificity.

Other available yet scarce data suggest that the role of habitat (i.e. freshwater or marine/brackish) in metopid's specificity for a particular methanogen may not apply to other lineages of anaerobic ciliates [30–32, 35, 37, 43]. For instance, *Methanocorpusculum* was found as a symbiont of two unrelated freshwater ciliate species: *Trimyema finlayi* (*Plagiopylea*) [31, 35] and *Dactylochlamys pisciformis* (*Litostomatea*) [37], but their closest relatives, which are also freshwater species, host a different methanogen, i.e. *Methanobrevibacter* in *Trimyema compressum* [43], and *Methanobacterium* or *Methanosaeta* in *Legendrea* spp. [37].

The taxonomy of the host also influences the methanogen specificity

So far, co-diversification has not been observed between anaerobic protists and their methanogenic symbionts, suggesting a lack of strict vertical transmission [6, 11, 12, 30, 32]. However, we have found via a broad taxon sampling that in metopids there is considerable partner specificity [95] at the broader host taxonomy level. Although the phylogeny of metopids has not yet been completely resolved [96, 97], 18S rRNA gene phylogenies consistently recover the main metopid groups [96] (Fig. S6). Here, we divided the metopid species into six robust clades and assessed the identity of the methanogenic symbionts of multiple isolates within each clade. When the strong effect of the environment (i.e. freshwater vs. marine/brackish metopids) was filtered out and only freshwater metopids were considered, an interesting pattern suggesting specificity at the host clade level became apparent. Five of the six clades seem to be highly specific to a particular methanogen genus: all freshwater strains of *Tropidotractidae* and *Brachonella* hosted *Methanoregula*, whereas *M. es* and the HAC clade show clear preference for *Methanobacterium* (Figs. S7 and S8D).

The methanogen specificity at a higher taxon level might also be corroborated in future studies by determining the identity of symbionts of so-far unstudied metopid genera (e.g. *Planometopus* and *Atopospira*). Interestingly, the studied metopids from the mixed cultures, as well as those from naturally co-occurring cultures (Table S1), demonstrated fidelity, and, therefore, specificity at the clade level, for certain methanogen genera since both partners retained their methanogens during the experiment (Fig. 4). The situation in *Urostomides* (Apometopidae), and likely *Castula* (HAC), is less clear and we cannot discard the possibility that these genera are more opportunistic, certainly being good candidates for further studies (Figs. S6, S7, and S8D).

Lack of methanogen specificity at the ciliate species level

Despite a strong fidelity [95] between host genera/clade, the situation differs considerably at the host species level. The behavior of symbioses between ciliates and prokaryotes varies from almost strict co-diversification, as seen in the ciliate *Kentrophoros* and its thiotrophic symbionts [56], to frequent symbiont replacement, such as in *Euplotes* ciliates and their multiple symbionts [98–100]. Our results show that within a ciliate species, the methanogen symbionts are closely related but genetically divergent (Fig. 3). Similar results have been reported in metopids and plagiopylids [44]. However, a few promiscuous methanogen lineages, which associated with a large diversity of ciliate hosts, were also observed in ciliate species belonging to the same genus (*Tropidoatractus*) or even from different genera (marine metopids, *Tropidoatractus*, *Brachonella*, *Urostomides*). Notably, ciliate hosts that shared a particular methanogen lineage came from different sampling sites, with some originating even from different continents. Inversely, different metopid species retrieved from the same sampling site and at the same time harbored distinct methanogen lineages (<https://shorturl.at/IUuql>), e.g. ciliates from Atarasquillo, Mexico. Similar findings were already documented [12] though in a considerably lower number of taxa. This implies that ciliates likely do not acquire the most abundant methanogen from the environment and suggests some level of symbiont–host fidelity and specificity. This is supported by the Mantel test results (Table S13), where we found no significant correlation between symbiont genetic distances and the geographic distances between samples. We did find that host and symbiont genetic distances are positively correlated (full test $r = 0.1689$, $P = 0.001$; partial test controlling for geography $r = 0.1529$, $P = 0.001$). Taken together, these results suggest that host specificity is more important for symbiont variation than geography and supports our hypothesis of host–symbiont specificity at some, but not all, host taxonomic levels.

Three of our retrieved symbiont lineages were identical to already published symbiont sequences from two metopids (*Heterometopus* sp. CSS, *Metopus* cf. *contortus*) and one litostomatean (*Legendrea pespelicani*) [27, 36, 37]. Furthermore, one of the *Methanoregula* lineages found in *T. levanderi* and *B. contorta* was nearly identical (two nucleotide differences) to the endosymbiotic *Ca. Methanoregula pelomyxae* isolated from the archamoeba *Pelomyxa schiedti* [18] (Figs. 3 and S6). This alludes to the fact that there may be promiscuous methanogen lineages which can be acquired by distantly related anaerobic protists.

In the case of *H. palaeformis* and *U. pullus*, we observed a strict specificity pattern since all the strains of each species harbored a unique methanogen lineage. Interestingly, the symbiont lineage of herein studied *H. palaeformis* is identical to the methanogen symbiont of *Heterometopus* sp. CSS [27], but differs from the

symbiont previously detected in a different population of *H. palaeformis* [29]. On the other hand, the methanogen lineage found in the two examined strains of *U. pullus* was also found in *Brachonella pulchra*, hinting a lack of specificity at the intraspecies host level in metopid ciliates.

Strong specificity was also found in the case of *Trimyema finlayi* and its *Methanocorpusculum* symbiont since the obtained Sanger sequence from this study (Czech Republic) is highly similar (99.45% identity) to the one from the UK [31, 35]. Phylogenetically, the sequences of *Methanocorpusculum* symbionts are distributed in two clades: one of them comprises solely the symbionts of *Trimyema finlayi*, whereas the second clade clusters all the symbionts from marine/brackish metopids, including one of *Metopus* cf. *contortus* [36], together with some free-living methanogens (Figs. 3 and S4). Nevertheless, it was shown recently that the *Methanocorpusculum* symbionts of marine relatives of *Trimyema finlayi*, and *Plagiopyla*, are distributed in a non-monophyletic pattern and cluster within those of the marine metopids, suggesting a weak specificity [44].

Altogether, our data suggest that strict specificity patterns and co-diversification are uncommon between anaerobic ciliates and methanogens, fitting more into a symbiont replacement scheme in which methanogens are potentially replaced through horizontal transmission, like in the case of *Euplotes* [100].

The methanogen–ciliate association implies a mixed transmission mode

At short evolutionary timescales, vertical transmission during host division is a straightforward mechanism to maintain symbiont fidelity across generations, as previously demonstrated in the ciliate *Plagiopyla frontata* [55]. Moreover, we also observed dividing methanogenic symbionts within the host cytoplasm (Fig. 1M, P). This hypothesis is supported by the fact that symbiont sequences are identical across samples originating from a particular ciliate strain (Fig. S8A). Additionally, our co-cultivation experiments indicate that the ciliate–methanogen relationship is stable over short timescales since symbiont switches between host partners were not detected, and the methanogens from the environment apparently do not easily associate with the host, perhaps due to priority effect. This implies that the fidelity of a ciliate strain to its methanogenic symbiont is very strong.

Even so, occasional horizontal transmission of methanogens apparently also occurs among closely related hosts (e.g. within a species). Remarkably, the different methanogen lineages observed in each strain of a particular ciliate species, except for *M. es*, were closely related and always belonged to the same methanogen genus displaying not only the specificity of a ciliate species to a typically one methanogen genus, but also the capacity for flexibility and symbiont exchange or reacquisition through horizontal transmission as observed in the *Euplotes* symbiosis system [100]. Horizontal transmission would facilitate the substitution of a current symbiont lineage with a more optimal strain when environmental or other conditions change, the replacement of symbionts that have degenerated through genome erosion, and/or the reacquisition of symbionts upon loss of their partner [57, 58, 98–100]. Altogether, this implies a “mixed-mode” transmission system, where vertical transmission is common over short timescales, but horizontal transmission occurs occasionally [95, 101, 102].

Further considerations

Our extensive survey of anaerobic ciliates and their methanogens, focused on the order Metopida and including most of its

lineages, greatly expands our knowledge of this ecologically important symbiosis, elucidating specificity patterns across the host's phylogeny, environment, and lifestyle. Mechanisms involved in the establishment and maintenance of a dominant methanogen within a host ciliate have not been revealed but might include small methanogen population sizes during the initial stages of infection, symbiont population bottleneck during vertical transmission, recognition-based control of the infection by the host or symbiont leading to eradication of nonspecific symbionts, or incompatibility of nonspecific methanogens with the host's intracellular environment. Also, intra-host competition and priority effects should be considered since the volume of the host's cytoplasm is limited, as is the space around the MROs available for the methanogens. Already established symbionts may thus occupy all convenient spots and prevent colonization by other methanogen lineages.

Although the transmission modes of the methanogens are not yet fully understood, it is likely that a ciliate population maintains its symbiotic methanogen lineage through vertical transmission during the host cell division, which results in a strong partner fidelity. Additionally, the symbionts within a host species are genetically divergent without co-diversification, possibly due to occasional replacements through horizontal transmission, but showing a strong partner specificity.

The identified methanogenic symbiont lineages, along with previously sequenced symbionts, were genetically different yet similar to the "putatively" free-living methanogens, but clustered together without distinction on their lifestyle (Fig. 3). Regardless, the resolution of our phylogeny of the methanogens is weak due to a short, sequenced fragment of the 16S rRNA gene, which could obscure co-diversification patterns, if any exist. Phylogenomics may be necessary to fully resolve the relationships among closely related methanogenic symbionts of anaerobic ciliates. The acquisition/replacement of the methanogenic symbiont by a ciliate potentially may occur during an uptake from the environment, direct ingestion, or through eukaryovory. Conjugation is an alternative process in which ciliate partners may theoretically exchange their methanogenic symbionts, although evidence is unavailable. If it occurs, then it could be strong evidence of horizontal transmission, and, furthermore, evidence for a strong fidelity and specificity for a particular methanogen genus. Ciliates devoid of methanogens, naturally or experimentally, should also be studied using molecular tools to determine potential symbiont acquisitions through ingestion. The mechanisms involved in the symbiont replacement are unknown in anaerobic ciliates; however, the environment generally appears to play a large role since our results suggest that ciliates possibly switch their methanogens with those that are present in their niche, i.e. endobiotic, freshwater, or marine. Future studies focusing on monitoring ciliate-methanogen symbioses in natural populations through time could further unveil the level of host specificity.

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Author contributions

I.C., J.R., R.A.B., and D.M.S. conceptualized the work; D.M.S., A.S., J.R., and K.K. conducted the cell culturing and maintenance; D.M.S., A.S., J.R., and K.K. performed the sample preparation; D.M.S. and K.K. conducted the autofluorescence and electron microscopy investigation; D.M.S. and A.S. conducted the phylogenetic analyses and wrote the draft of the manuscript; A.S. conducted the microbiome analyses; K.K. performed the FISH; D.M.S., A.S., J.R., K.K., R.A.B., and I.C. performed the writing, reviewing, and editing; R.A.B. and I.C. acquired funding and supervised the work; all the authors discussed and interpreted the results.

Supplementary material

Supplementary material is available at *The ISME Journal* online.

Conflicts of interest

None declared.

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Data availability

Raw 16S rRNA gene amplicon sequences are available in the Sequence Read Archive (SRA) on NCBI (BioProject ID PRJNA1071061). The 18S rRNA gene sequences of the studied ciliates as well as their 16S rRNA from their methanogenic symbionts are deposited in GenBank under the accession numbers PP274949–PP274988, PP935522–PP935534, and PP250167–PP250287, respectively. Unaligned, aligned, and trimmed datasets used for the analyses are in the Supplementary files.

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An extensive morphological and molecular characterization of the neglected class Odontostomatea (Ciliophora)

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Running title: Diversity and systematics of Odontostomatea

**An extensive morphological and molecular characterization of the neglected class
Odontostomatea (Ciliophora)**

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Abstract

Odontostomatid ciliates, known for over a century, were historically classified within various taxonomic groups of Ciliophora until their reclassification into the class Odontostomatea. Despite recognizing 25 valid species, most descriptions predate the advent of silver impregnation and molecular methods. Consequently, many species were described based solely on observations of live specimens, leading to incomplete or ambiguous records. To date, only three species have been redescribed in detail, with 18S rRNA gene sequences available, and their evolutionary relationships remain unresolved. In this study, we investigated 32 populations representing 15 species—including three newly described—across the genera *Discomorphella*, *Epalxella*, *Limnomylestoma* gen. nov., *Mircalla* gen. nov., *Mylestoma*, *Pelodinium*, *Saprodinium*, and *Tostonella* gen. nov. Comprehensive analyses were conducted using light microscopy, protargol impregnation, and scanning electron microscopy. We also designed specific primers to amplify the partial 18S rRNA gene of various odontostomateans and retrieved additional 18S rRNA sequences from environmental metatranscriptomic and metagenomic datasets. This study represents the most extensive investigation of Odontostomatea to date, confirming the monophyly of the class by revealing the position of *Epalxella*, reconstructing its internal phylogeny, identifying two main odontostomatean lineages, and revealing its remarkable diversity.

Keywords Anoxic sediments · Discomorphellidae · Epalxellidae · Environmental sequences · Mylestomatidae · Phylogeny

39 Introduction

40 The anaerobic class Odontostomatea Fernandes et al., 2018 has received little attention regarding its
 41 taxonomy, unlike most other ciliate classes whose diversity has been widely studied using modern
 42 techniques. Odontostomeans are free-living anaerobic ciliates dwelling in anoxic/hypoxic
 43 freshwater, brackish, or marine environments. Perhaps their most outstanding characteristics include a
 44 complex morphology consisting of a rigid amour-like cortex equipped with spines, an epistomial
 45 fringe or frontal band, and a highly reduced somatic ciliature arising from cortical pits. Additionally,
 46 they possess mitochondrion-related organelles and prokaryotic endosymbionts (Kahl 1932a;
 47 Jankowski 1964a, b; Schrenk and Bardele 1991; Lynn 2008; Paiva et al. 2017; Fernandes et al. 2018;
 48 Méndez-Sánchez et al. 2023).

49 Since their discovery over a century ago, at least 25 species of Odontostomatea have been described
 50 and classified into a single order, three families, and six genera: *Atopodinium* Kahl, 1932,
 51 *Discomorphella* Corliss, 1960, *Epalxella* Corliss, 1960, *Mylestoma* Kahl, 1928, *Pelodinium*
 52 Lauterborn, 1908, and *Saprodinium* Lauterborn, 1908 (Roux 1899; Lauterborn 1901, 1908; Kahl
 53 1928, 1932a, b; Corliss 1960; Lynn 2008). Kahl (1928, 1932a, b) described the majority of the known
 54 species, some of which were revisited by Jankowski (1964a), but due to incomplete or brief
 55 descriptions, many species remain unclearly defined. Most odontostomean species have been
 56 characterized based solely on living or fixed cells, and only a few have been studied by modern
 57 methods (Klein 1930; Kahl 1932a; Jankowski 1964b, a; Schrenk and Bardele 1991; Tuffrau 1992;
 58 Paiva et al. 2017; Méndez-Sánchez et al. 2023).

59 The sole order of the class, Odontostomatida Sawaya, 1940, has been historically included by various
 60 authors in several ciliate classes, e.g., Spirotrichea Bütschli, 1889, Heterotrichea Stein, 1859, or
 61 Armophorea Lynn, 2004 (Jankowski 1964b, 2007; Corliss 1979). However, the classification of
 62 odontostomatid ciliates has changed with the complementation of molecular data. Based on an 18S
 63 rRNA gene sequence from a morphologically unidentified source organism, labeled in GenBank
 64 (EF014286) as *Epalxella antiquorum* (Penard, 1922) Corliss, the phylogenetic analysis of Stoeck et
 65 al. (2007), quite unexpectedly, suggested a position of odontostomeans within class Plagiopylea
 66 Small & Lynn, 1985. More recently, 18S rRNA gene sequences of three species, *Discomorphella*
 67 *pedroeneasi* Paiva et al. 2017, *Mylestoma* sp., and *Saprodinium dentatum* (Lauterborn, 1901)
 68 Lauterborn, 1908, have been partially or fully sequenced and all comprise a fully supported lineage
 69 closer to Armophorea or Litostomatea than Plagiopylea (Fernandes et al. 2018; Méndez-Sánchez et al.
 70 2023). Accordingly, Fernandes et al. (2018) proposed the class Odontostomatea. So-called “universal
 71 eukaryotic” or “ciliate-specific” primers are unsuitable for amplifying the divergent 18S rRNA gene
 72 of odontostomeans (Fernandes et al. 2018; Méndez-Sánchez et al. 2023). Consequently, the
 73 diversity of odontostomean ciliates is possibly underrepresented in many environmental analyses.

This study offers a comprehensive molecular and morphological characterization of 32 odontostomatean populations, representing eight known and three newly described species belonging to seven genera, three of which are novel. It provides a set of odontostomatean-specific primers for 18S rRNA gene amplification. Furthermore, our analysis of available environmental transcriptomic and metagenomic data reveals a substantial diversity of potential odontostomatean sequences, highlighting probable underestimation of odontostomatean diversity based only on limited 18S rRNA gene data.

Materials and Methods

Sampling

Hypoxic sediments from various localities (Table 1) were collected in Falcon tubes or plastic jars. Approximately 1 or 2 ml of the samples were inoculated into 9 ml of Sonneborn's *Paramecium* medium: American Type Culture Collection (ATCC) medium #802 for freshwater samples, ATCC medium #1525 for marine samples, or a 1:1 ratio of the two media for brackish samples, and maintained at room temperature (23 °C). Tubes were tightly closed to prevent rapid oxygenation. The inoculated tubes were subcultured biweekly or monthly, as previously described (Bourland et al. 2017; Rotterová et al. 2018). Short-term cultures were successfully obtained in a few cases, but most studied populations were directly studied from the raw sample (Table 1). A total of 32 populations were studied.

Morphological characterization

Living cells were studied at magnifications of 400–1000× with brightfield and differential interference contrast illumination (DIC) using an Olympus BX51 microscope equipped with an Olympus DP70 camera. Cells were fixed in 10% aqueous formalin (final concentration) and impregnated with protargol to reveal the infraciliature and nuclear apparatus, according to Pan et al. (2013) and Foissner (2014). The infraciliature of *Pelodinium reniforme* was studied using silver carbonate impregnation (Foissner 2014). In vivo measurements were made from photomicrographs of uncompressed, freely swimming cells. Counts and measurements from protargol preparations were made at 1000× magnification using calibrated software (QuickPhotoCamera, Promicra, s.r.o., Prague, CZ).

For scanning electron microscopy (SEM), cells were fixed for at least 30 min in an aqueous solution of 2.5% glutaraldehyde, washed, dehydrated in an ethanol series, and dried in a Bal-Tec CPD 030 critical point dry (Leica Microsystems, Vienna, AT). Specimens were mounted on adhesive carbon tabs on aluminum stubs and coated with Pt/Pd (80%,20%) in a Bal-Tec SCD 050 sputter coater. Specimens were examined at a 15-20 kV accelerating voltage in a JEOL IT200 scanning electron microscope (JEOL LV, USA). Morphological terminology is based on Kahl (1932a, 1932b), Schrenk

and Bardele (1991), Tuffrau (1992), Lynn (2008), Paiva et al. (2017), and new terminology where indicated (see Glossary).

DNA extraction, amplification, and sequencing

One to 30 cells from each of 27 odontostomatean populations were hand-picked with glass micropipettes, washed five times with distilled water or filtered culture medium, and added into 30 µl of DNA/RNA shield (Zymo Research, Irvine, CA, USA). Genomic DNA was isolated using the MasterPure™ Complete DNA and purification Kit following the manufacturer's instructions.

For amplification of the partial 18S rRNA gene sequence (about 1,500 bp), oligonucleotide primers (Table S1) were designed *in silico* for different odontostomatean genera, using eight sequences of *Mylestoma* sp., *Saprodinium dentatum*, and *Discomorphella pedroeneasi* already available in GenBank. Thermocycler conditions were as follows: 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 45 or 52 °C (annealing, Table S1), and 2 min at 72 °C, and a final extension cycle at 72 °C for 5 min. PCR products were verified by 1% agarose gel electrophoresis and purified using EXOSAP (Applied Biosystems) or the Gel/PCR DNA Fragments Extraction Kit (Qiagen, Hilden, Germany). In some cases, in order to increase the amount of PCR product, a reamplification of initial products was done using the same primers as for the first reaction.

The purified PCR products from *Saprodinium mimeticum* (strain BOPAT), *Mylestoma bipartitum* (strain KLAGOS), Odontostomatean sp. 3 (strain 3S6A), and *Discomorphella pectinata* (strain MOKOT) were cloned using the pGEM-T Easy Vector System I kit and JM109 competent cells of *Escherichia coli* (Promega). The plasmids were amplified using the primers T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-ATTTAGGTGACACTATAG-3'). Cloning procedures were done following the manufacturer's instructions. Four clones were sequenced from *S. mimeticum* BOTAN and three from the other two strains.

The purified PCR products were sequenced at the Laboratory of DNA Sequencing (Charles University, Prague, Czech Republic) on an ABI PRISM 3100 sequencer (Applied Biosystems) or at Macrogen (Amsterdam, Netherlands) on a 3730xl DNA Analyzer. Sequences were assembled in Geneious Prime® 2024.0.7, using *de novo* assembly options or mapped to a reference sequence (*Saprodinium dentatum* OP034694). All the partial sequences originating from clones of each species were assembled together. A total of 36 sequences were determined.

Phylogenetic analyses

Thirty of the obtained 18S rRNA gene sequences, representing unique species per population, were used in the phylogenetic analyses. The sequences and 104 sequences obtained from GenBank representing all ciliate classes, were aligned using the G-INS-i algorithm in MAFFT v7.520 (Katoh 2002). The alignment (1,944 positions) was manually edited using AliView (Larsson 2014) to trim

primer sequences and remove columns with singletons. The final dataset included 1,805 positions. Phylogenetic trees were constructed by maximum likelihood (ML) and Bayesian inference (BI) methods under the GTR + I + Γ as the best-fit model by ModelGenerator v0.85 (Keane et al. 2006) according to the Akaike Information Criterion. ML analysis was performed in RAXML HPC v8.2.4 (Stamatakis 2014). Node support was assessed by 1,000 bootstrap pseudoreplicates. Bayesian analysis was performed using MrBayes v3.2.7 (Ronquist et al. 2012). Four Markov chains Monte Carlo were run for 10 million generations with a sampling frequency of 1,000 generations. The first 25% of sampled trees were removed as burn-in; convergence was assessed using Tracer v1.7.2 (Rambaut et al. 2018); the average standard deviation of split frequencies was 0.003 after 10 million generations.

Environmental analyses

Unique sequences of odontostomateans (N=16) were queried in the PebbleScout web server (Shiryev and Agarwala 2024), using metagenomic and whole genome sequencing (WGS) databases with default parameters. A threshold of ≥ 90 PBScore was chosen, resulting in 42 environmental samples after dereplication between queries (Table S3). For the metatranscriptomic hits (N=41), assemblies were created using Trinity v2.15.1 and rnaSPAdes v3.15.4. For the single metagenomic hit, a publicly available assembly was downloaded. From all the assemblies, the 18S rRNA gene sequence was extracted by barrnap v0.9 (github.com/tseemann/barrnap), and the extracted sequences were added to a subset of a pan-eukaryotic dataset, which contained 88 sequences representing the broad eukaryotic diversity (not shown). The dataset was aligned using MAFFT v7.520 and trimmed (BMGE v1.12). The phylogenetic tree was constructed with IQ-TREE v2.3.4 (Minh et al. 2020) using a model selected by ModelFinder (Kalyaanamoorthy et al. 2017). The statistical support was tested with 1,000 ultrafast bootstraps (Hoang et al. 2018). The datasets were then progressively refined by deleting distant sequences and repeating the phylogenetic tree construction pipeline until sequences within the Odontostomatea clade were readily identifiable. Finally, the filtered sequences were checked for chimeras manually and automatically using VSEARCH v2.14.1 (Rognes et al. 2016). A final dataset comprising 2,001 positions was constructed by aligning the 263 odontostomatean environmental sequences, the 36 odontostomatean sequences, and 104 Ciliophora sequences from GenBank (see above) using the G-INS-i algorithm in MAFFT v7.520. The alignment was manually trimmed to remove primers and singletons, resulting in a final dataset with 1,890 positions. The minimum length of sequences was 419 bp. The phylogenetic tree was constructed in RAXML v8.2.12, using the model GTR + I + Γ and 100 standard bootstraps.

Results

Phylogeny of Odontostomatea based on the 18S rRNA gene sequences

Thirty-six 18S rRNA partial sequences were obtained from 28 populations of 14 odontostomatean species using different pairs of newly designed primers (Table S1). Odontostomatea are a fully

supported class, which branches sister to Litostomatea Small & Lynn, 1981 with low support (Fig. 1A). Odontostomatea comprises two fully supported clades, here referred to as the Clade I and II. Clade I includes two main branches, one with the fully supported family Epalxellidae Corliss, 1960, comprises the well-known genera *Saprodinium*, *Epalxella*, the new genus *Mircalla* gen. nov., and one population assigned as Epalxellidae sp. 1, and the other branch with Odontostomatea sp. 1 (GROSERDU). Clade II contains *Mylestoma* (Mylestomatidae Kahl in Doflein & Reichenow, 1929), *Discomorphella* (Discomorphellidae Corliss, 1960), and two new genera, *Tostonella* gen. nov. and *Limnomylestoma* gen. nov., as well as one undescribed odontostomatean (strain 3S6A) and one uncultured freshwater eukaryote (sequence AY821980) which are not assigned to any known odontostomatean family (Fig. 1A).

Environmental survey of odontostomateans

A total of 263 unique partial 18S rRNA sequences retrieved from available environmental metagenomic and metatranscriptomic data (Table S2, S3) were identified as potential odontostomatean sequences. The topology of the phylogenetic tree of environmental sequence, which includes our isolated populations, is consistent with the 18S rRNA gene sequence tree of Odontostomatea, showing the two main clades (Figs. 1C; S1). Fifty-three sequences are distributed within Clade I, and the remaining 210 in Clade II.

Within Clade I, Odontostomatean sp. 1 (strain GROSERDU) forms a supported clade with three environmental sequences and is sister to another well-supported clade containing 14 sequences. These two groups form a well-supported clade that is sister to the highly supported Epalxellidae clade. Epalxellidae is internally unresolved.

Clade II is divided into two major lineages: the well-supported Clade IIa, and the unsupported Clade IIb. Clade IIa contains two groups: a highly-supported clade with two freshwater environmental sequences and a weakly supported clade consisting of *Mylestoma bipartitum* and *Mylestoma monodontum* sp. nov., and environmental sequences from brackish and marine environments, distributed across four highly supported subclades.

Clade IIb contains three poorly supported clades. Two of them consist entirely of environmental sequences (41 and 17 sequences, respectively), while the third clade includes sequences from *Discomorphella* spp., *Limnomylestoma shuriken* sp. nov., *Tostonella uncinata* comb. nov., Odontostomatea spp., and 128 environmental sequences, which are not fully resolved internally.

General morphology of Odontostomatea

Odontostomatean morphology is exceptionally complex and is described with a rather specialized terminology (Schrenk 1989; Schrenk and Bardele 1991; Tuffrau 1992; Paiva et al. 2017) (see Glossary). The cell size varies, ranging from small (<30 µm), medium (30-50 µm), to large (>50 µm).

211 The cells are usually almost circular in outline, strongly laterally compressed, and show right and left-
 212 side asymmetry. The armor-like cortex is rigid, inflexible, and may bear *cortical slits*
 213 ("Fensterbildungen") and *cortical pits*. The anteroventral portion ends as a frontal spine (Fig. 2A), a
 214 hood-like structure (Fig. 2B), or a blunt shape (Fig. 2C), continuing posteriorly as a *dorsal keel* (Fig.
 215 2A-C). The posterior margin is rounded, with or without spines (Fig. 2F, H), or concave and
 216 surrounded by prominent to inconspicuous spines sometimes hidden in the posterior concavity (Fig.
 217 2G, I-J).

218 The nuclear apparatus consists of one to four macronuclear nodules and invariably a single spherical
 219 micronucleus. The cytoplasm is colorless and contains globules of various sizes. Food vacuoles
 220 contain prokaryotes and sometimes green algae. A single contractile vacuole is usually located in the
 221 center of the lateral right side or posteriorly. Mitochondria as mitochondrion-related organelles
 222 distributed in the cytoplasm. Endosymbiotic prokaryotes, including UV-autofluorescent methanogenic
 223 archaea, lie freely in the cytoplasm. The swimming pace varies from slow to fast, and the cell usually
 224 rotates around the long axis when swimming or crawls along debris on its right side.

225 The infraciliature is sparse and reduced to twelve or fewer kineties consisting of dikinetids in *ciliary*
 226 *sockets*. The dikinetids may have one or both basal bodies ciliated or may be barren. The kineties
 227 usually consist of three segments: an anterior ciliated segment, a non-ciliated middle segment, and a
 228 posterior ciliated segment often located on a spine. The non-ciliated segments are composed of
 229 *subsurface basal bodies* (barren dikinetids). There are usually two kineties on the ventral margin; the
 230 anterior and posterior ciliated segments of these kineties are called preoral kineties and ventral
 231 kineties, respectively. Between both segments, a row of a few supplementary ciliated ventral
 232 dikinetids may be present. The remaining kineties, which are on the lateral sides, are called somatic
 233 kineties (SK). The anterior ciliated segments of the first five kineties (SK1-5) constitute the epistomial
 234 fringe. The posterior ciliated segments of the somatic kineties usually end on the posterior spines and
 235 are called spine segments. On the left side, two caudal cirri, bearing long cilia, may emerge at the end
 236 of one or two somatic kineties. Additionally, an inverse kinety and two endoral kineties may be
 237 present. Usually, there are nine or ten adoral membranelles. The paroral membrane is absent. Four
 238 infraciliary patterns are recognized (see below).

239 The *ventral ridge* lies above the equator and bears the *epistomial fringe*. The *ventral ridge* may
 240 protrude as a *frontal awning*. Just posterior to it, two diagonal furrows usually contain the preoral
 241 kineties. The buccal cavity is located on the ventral margin in the posterior half of the cell, nearer to
 242 the left surface and just posterior to the preoral kineties. The buccal cavity is flanked by either a
 243 *ventrocaudal spine* (spine I) or a *ventral flap* on its right side, a rigid plate on its left side, and a buccal
 244 lip posteriorly. The ventrocaudal spine or ventral flap bears the ventral kineties and supplementary
 245 ventral dikinetids. The anterior portion of the left buccal plate usually folds outward, forming a *lapel-*

like structure that embraces the *inverse kinety*, which generally consists of two or three dikinetids (Fig. 2N). Based on transmission electron microscopy (TEM), the *inverse kinety* shows an inverse orientation with respect to the longitudinal cell axis. Inside the buccal cavity, up to 10 truncated adoral membranelles with inverted orientation and separated by septa are contained in two compartments, usually most membranelles in the outer compartment and usually one in the inner compartment.

The *epistomial fringe* is ventrally supported by the *ventral ridge* and consists of five ciliary rows of variable length that run horizontally above the equator. The epistomial ciliary rows form longitudinal *pectinelles* separated by notched interkinetal ridges (Fig. 2L) or *auricules* (Fig. 2M). The *epistomial fringe* commences on the left side of the cell, embracing the *ventral ridge*, and ending on the right side in a staggered manner. It may be continuous or comprise two fragments: a dextro-frontal and a left fragment, separated by an *epistomial fringe spacer*. *Papillary dikinetids* may be present at the end of the *epistomial fringe*.

Somatic infraciliary patterns

The *epistomial fringe* and the somatic kineties are arranged in at least four patterns: the *Epalxella* pattern (Fig. 2O), *Pelodinium* pattern (Fig. 2P), *Mylestoma* pattern (Fig. 2Q), and *Discomorphella* pattern (Fig. 2R).

The ***Epalxella* pattern** (Fig. 2O) invariably consists of nine somatic kineties (SK1-9). The SK1-6 are located on the right, and SK7-9 are on the left side. Five kineties (SK1-5) consist of an anterior ciliated segment (epistomial ciliary rows) and a posterior non-ciliated segment; SK1 ends on the posterior ventral margin, and SK2-5 end posteriorly. Three kineties (SK6, 7, and 9) consist of an anterior non-ciliated segment that ends as a posterior ciliated segment. One kinety (SK8) consists of an anterior ciliated segment, followed by a non-ciliated middle segment, and ending as a posterior ciliated segment. SK6 runs along the edge of the *dorsal keel*, whereas SK7 runs almost on the dorsal margin. The anterior segment of SK8 is located in the anterior third of the cell. The non-ciliated segments of SK7-9 begin in the middle third of the cell, extending as parallel rows posteriorly. The four posterior ciliated segments of SK6-9 are short, restricted to the spines, and separated by a short gap from the non-ciliated segments.

The ***Pelodinium* pattern** (Fig. 2P) consists of ten somatic kineties (SK1-10). The SK1-6 are located on the right, and SK7-10 are on the left side. Four kineties (SK2-5) consist of an anterior ciliated segment (epistomial ciliary rows) and a posterior non-ciliated segment ending posteriorly. Three kineties (SK6, 7, and 9) consist of an anterior non-ciliated segment that ends as a posterior ciliated segment. Two kineties (SK1 and SK8) consist of an anterior ciliated segment, followed by a non-ciliated middle segment, and ending as a posterior ciliated segment. One kinety (SK10) consists solely of a ciliated segment and is located in the ventral margin at the posterior third of the cell. SK6 runs

along the edge of the *dorsal keel*, whereas SK7 runs almost on the dorsal margin. The anterior ciliated segment of SK1 is an epistomial ciliary row. The anterior segment of SK8 is located in the anterior third of the cell. The non-ciliated segments of SK7-9 begin in the middle third of the cell, extend shortly as parallel rows, and are restricted to the middle third of the cell. The five posterior ciliated segments of SK1, 6-9, are long, commencing at or slightly above the cell equator, running posteriorly, ending on spines, and separated by a short gap from the non-ciliated segments.

The ***Mylestoma* pattern** (Fig. 2Q) consists of nine or fewer somatic kineties (SK1-9) distributed on the lateral sides and constantly two caudal cirri on the posterior left side. All of the kineties are non-ciliated. A short gap posteriorly separates one or two of the kineties on the left side from the caudal cirri. Two types of arrangement of the caudal cirri are recognized: a) amphikinetal pattern, where each caudal kinety is connected to a somatic kinety (Fig. 2Qi), and b) semikinetal pattern, where one caudal kinety is connected to a somatic kinety whereas the second one is disconnected or misplaced from a somatic kinety (Fig. 2Qii). Posterior marginal spines are reduced in *Mykestoma* species (Fig. 2Q).

The ***Discomorphella* pattern** (Fig. 2R) comprises around nine *non-ciliated kineties*. The SK1-4 are posterior to the dextro-frontal fringe on the surface of the vaulted right side. The SK5-7 are arched, starting anteriorly following the margin of the dorsal keel and ending posteriorly; the SK6-7 end below the posterior spine, whereas the SK7 continues on the posterior margin and possibly joins the end of the ventral kinety 2. On the left side, the SK8 is arched, running on the dorsal keel margin, anteriorly folds backward, and continues posteriorly, ending in one of the caudal cirri. The SK9 runs obliquely from the middle third of the cell, connecting to the most dorsal pectinelle of the left epistomial fragment, and continues posteriorly, possibly ending in the second caudal cirri. Scattered subsurface basal bodies are between the left and the dextro-frontal epistomial fragments (Klein 1930; Tuffrau 1992).

Occurrence and ecology

Odontostomateans are bacterivorous, although small flagellates can also be ingested. They are distributed worldwide in oxygen-depleted freshwater, brackish, or marine sediments. They are obligate anaerobes living in symbiotic syntrophic associations with prokaryotes, likely methanogenic archaea in freshwater (Foissner et al. 1992).

Descriptions of new and redescriptions of known odontostomatean species

***Saprodinium mimeticum* (Penard, 1922) Kahl, 1932**

Redescription based on IS7, BYMOKOT, and BOPAT populations (Figs. 3, 4; Table S5; Video S1):

Cells $49\text{--}55 \times 32\text{--}43 \mu\text{m}$ in vivo and $37 \times 30 \mu\text{m}$ on average in protargol-impregnated specimens. The shape is almost quadrangular in outline (Fig. 3A-H; 4A-C). Anteriorly, a short frontal spine ($4.5 \mu\text{m}$ on average), followed by a dorsal keel slightly folded rightwards, forming a shallow through ending subposteriorly (Fig. 3A-E, G; 4B, C, D). Seven slender, sharp, conspicuous spines surround a concavity at the posterior cell margin, three on the left and four on the right, including the conspicuous and prominent, sharp, curved, and large ventrocaudal spine (S1) (Figs. 3A-D, G, H; 4A, B, C, G). The lapel-like structure enfolds the two dikinetids of the inverse kinety (Figs. 3D, O; 4A, F). There are one to four (three on average) spherical macronuclei and a single spherical micronucleus (Fig. 3A-C, J, K, O-Q).

On average, the continual epistomial fringe comprises 24 pectinelles, eight on the left and 16 on the right (Figs. 3C-E, J-K, M-N; 4A, B, D). The two preoral kineties (PK1-2) have an average of 20 and 14 dikinetids, respectively (Figs. 3C-D, J-K, M-N; 4A, B, F). The two ventral kineties (VK1-2) have an average of six and 16 dikinetids, respectively, and are located on the ventrocaudal spine; the VK1 is more superficial than the VK2 (Figs. 3C-D, J-L, N; Fig. 4A-B, H). The oral cavity invariably has nine membranelles, eight within the outer compartment and the ninth in the inner (Fig. 3B, D, G, K, N, O).

The somatic kineties are arranged in an *Epilaxella* pattern (Figs. 3C-D, J-K, P-Q; 4B, G). The SK1 ends on the posterior ventral margin. The posterior ends of SK2-4 join at the base of the spine II. The SK5 ends in the spine III. The SK6 ends in the spine IV with 9 ciliated dikinetids (Figs. 3C, J; 4B). The SK8 begins with 13 ciliated dikinetids (Figs. 3D, F, K, Q; 4A, E). The SK7, 8, and 9 ends in the spines V, VI, and VII, respectively, with an average of 8, 7, and 6 dikinetids, respectively (Fig. 3D, K, Q; 4A, G).

Notes: The morphometric values given in the above description are based on the IS7 population. Refer to Table S5 for the morphometric values of the other two populations. The BOPAT and IS7 populations are highly similar to each other; the only notable difference between them is the number of dikinetids in the anterior ciliated segment of SK8 (21 vs. 13, on average, respectively) (Table S5). The data from BYMOKOT population were insufficient for a morphometric description.

Notes on 18S rRNA gene sequences: The genetic distance (uncorrected p distance) between the three studied populations (IS7, BYMOKOT, and BOPAT) ranges from 0 to 0.004 (Table S4).

***Saprodinium dentatum* (Lauternorn, 1901) Lauterborn, 1908**

Additional notes to the description of VLKOV population (Fig. 5; Table S6; Video S2): The shape is discoidal with a frontal spine and eight to nine posterior sharp spines. The dorsal keel is conspicuous. The lapel-like structure enfolds the three dikinetids of the inverse kinety (Fig. 5B, C, F, H). The two

ventral kineties (VK1-2) lie in the ventrocaudal spine, and a row of four supplementary ventral dikinetids runs anteriorly to the VK1 (Fig. 5B, C, G). The somatic kineties are arranged in an *Epalxella* pattern (Fig. 5A, B). The SK1 ends in the posterior ventral edge. The posterior ends of SK2-4 join at the base of the spine II. The SK5 ends in spine III (Fig. 5A). The SK6 ends in spine IV with 13 ciliated dikinetids (Fig. 5A). The SK7, 8, 9 end as a spine segment with 12, 12, and 10 ciliated dikinetids ending in the spine V, VI, and VII respectively (Fig. 5B). The SK8 with 94 ciliated dikinetids on average in its anterior segment.

Notes on 18S rRNA gene sequences: The genetic distance (uncorrected p distance) between the five studied populations (VLKOV, MOKOT, SLATINA, GROSERDU, and LERMA3) and the published population from Brazil and the previous VLKOV population (MF563970, OP034694) ranges from 0 to 0.01 (Table S4).

***Mircalla polidorii* gen. nov., sp. nov.**

Description based on MOKOTP1 population (Figs. 6, 7; Table S7; Video S3): Cells $30\text{--}36 \times 23\text{--}28$ μm in vivo and 26×19 μm on average in protargol-impregnated specimens. The shape is almost ellipsoidal in outline (Fig. 6A-E; 7A, B). The frontal spine is absent; the dorsal keel folds slightly rightwards, forming a shallow through ending subequatorially (Figs. 6A-D; 7A). About seven short, inconspicuous spines surround a concavity at the posterior cell margin; three of them are visible on the left side, whereas the remaining are on the right side and inwardly curved, except for the ventrocaudal spine, making the posterior right margin wavy (Figs. 6A-F; 7B, D-G). The lapel-like structure enfolds the two dikinetids of the inverse kinety (Figs. 6C, I; 7B, C, G). A conspicuous fang-like spine is on the ventral margin toward the left side, between the anterior end of the preoral kineties and the lapel-like structure (Figs. 6A-G; 7B, C). There are one or two (usually two) spherical macronuclei and a single spherical micronucleus (Fig. A, B, F, H, I, N).

On average, the continual epistomial fringe comprises 12 pectinelles, three on the left side and nine on the right (Figs. 6B, C, H-K; 7A, B). The two preoral kineties (PK1-2) have an average of nine and five dikinetids, respectively (Figs. 6B, C, H, K, L; 7C). The two ventral kineties (VK1-2) have an average of four and ten dikinetids, respectively, and lie in the ventrocaudal spine; the VK1 is more superficial than the VK2 (Figs. 6C, M, N; 7B, C, D, F, G). The oral cavity invariably has nine membranelles, eight within the outer compartment and the ninth in the inner (Fig. 6C, H).

The somatic kineties are arranged in an *Epalxella* pattern (Figs. 6B-E, H, I, J, N; 7B-G). The SK1 ends ventrally. The SK2-5 ends posteriorly, likely in the reduced spines. The SK6 ends in a reduced spine with five ciliated dikinetids (Figs. 6B, D, H; 7A, D-F). The SK8 begins with an anterior ciliated segment having 13 ciliated dikinetids (Figs. 6C, I; 7B). The SK7, 8, and 9 end in reduced spines with an average of 5, 4, and 2 dikinetids, respectively (Figs. 6C, I, N; 7B, C, G).

***Mircalla triangula* comb. nov.**

Redescription based on EPOPOLO population (Fig. 8; Table S7): Cells $32\text{--}37 \times 20\text{--}27 \mu\text{m}$ in vivo and $25 \times 16 \mu\text{m}$ on average in protargol-impregnated specimens. The shape is almost triangular in outline (Fig. 8A-D, I, J). The frontal spine is short and inconspicuous (Fig. 8B, D, I); the dorsal keel is folded slightly rightwards, forming a shallow through ending equatorially (Fig. 8C, E, G). About seven blunt, inconspicuous, inwardly curved spines surround a concavity at the posterior cell margin, except for the ventrocaudal spine, making the posterior margins wavy (Fig. 8C, D, F, H, I, J). The lapel-like structure enfolds the two dikinetids of the inverse kinety (Fig. 8D, K, L, P; 8I). An inconspicuous fang-like spine is on the ventral margin toward the left side, between the anterior end of the preoral kinety and the lapel-like structure (Fig. 8A, D, J). There are one or two spherical macronuclei and a single spherical micronucleus (Fig. 8C, H, K, M, P).

On average, the continual epistomial fringe comprises seven pectinelles, mostly on the right side (Fig. 8C, K, L). A single preoral kinety, PK, with an average of 13 dikinetids (Fig. 8C, D, K, L). The two ventral kineties (VK1-2) have an average of four and three dikinetids, respectively, and lie in the ventrocaudal spine; the VK1 is more superficial than the VK2. Two pairs of supplementary ventral dikinetids are anterior to the VK2 (Fig. 8C, D, K, M, P). The oral cavity invariably has nine membranelles, eight within the outer compartment and the ninth in the inner (Fig. 8C, D, K, L, N, P).

The somatic kineties are arranged in an *Epilxella* pattern (Fig. 8C, D, I-K, M, N, P). The SK1 ends ventrally. The SK2-5 ends posteriorly, likely in the reduced spines. The SK6 ends posteriorly with four ciliated dikinetids (Fig. 8C, F, K, M). The SK8 begins with seven ciliated dikinetids (Fig. 8D, I, J, O, P). The SK7, 8, and 9 end in reduced spines with an average of five, four, and two dikinetids, respectively (Fig. 8D, I, J, P).

***Epilxella exigua* (Penard, 1922) Corliss, 1960**

Redescription based on LAUFENJ population (Fig. 9; Table S8): Cells $33 \times 25 \mu\text{m}$ in vivo and $25 \times 17 \mu\text{m}$ in protargol-impregnated specimens on average. The shape is almost semicircular to ellipsoidal in outline (Fig. 9A-H). The frontal spine is absent; the ventro-frontal edge is blunt; the dorsal keel is fully folded rightwards, forming a deep through (Fig. 9B, C, E). On the left side, the cortex bears a conspicuous window-like cortical slit (Fig. 9A, B, D, G, H). Short, inconspicuous spines surround a concavity at the posterior cell margin; three of them are visible on the left side, whereas the remaining are on the right side and inwardly curved, except for the ventrocaudal spine, making the posterior right margin wavy (Fig. 9A-E, G). The lapel-like structure enfolds the two dikinetids of the inverse kinety (Fig. 9D, G, L). There are one or two spherical macronuclei and a single spherical micronucleus (Fig. 9A, B, C, I, P).

On average, the continual epistomial fringe comprises eight pectinelles, mostly on the right side (Fig. 9B, C, E, I-K). The two preoral kineties (PK1-2) have an average of five and three dikinets, respectively (Fig. 9D, F, L, N). One ventral kinety (VK) is located in the internal side of the ventrocaudal spine; the kinety is fragmented into two groups of three dikinets. Above the VK is one pair of supplementary ventral dikinets (Fig. 9D, G, L, M). The oral cavity invariably has nine membranelles, eight within the outer compartment and the ninth in the inner (Fig. 9C, D, F, I, L, O).

The somatic kineties are arranged in an *Epalxella* pattern (Fig. 9C, D, K, L, M). The SK1 ends ventrally. The SK2-5 ends posteriorly, likely in the reduced spines. The SK6 ends in a reduced spine with eight ciliated dikinets (Fig. 8C, E, I, K, L). The SK8 begins with eight ciliated dikinets (Fig. 9D, G, L). The SK7, 8, and 9 end in reduced spines with an average of eight, six, and four dikinets, respectively (Fig. 8D, G, M).

Notes on 18S rRNA gene sequences: The genetic distance (uncorrected p distance) between the two studied populations was 0.01 (Table S4).

Epalxella cf. antiquorum

Notes on the observed MOKOT populations (Fig. 10; Video S4): Cells about $64 \times 46 \mu\text{m}$ in vivo on average. The shape is ellipsoidal to quadrangular in outline. The Frontal spine is present (Fig. 10G, inset). Short, inconspicuous spines surround a concavity at the posterior cell margin; three or four are visible on the left side, whereas the remaining ones are inwardly curved on the right side, except for the ventrocaudal spine, making the posterior right margin wavy. The cortex bears window-like holes, four on the right and two on the left sides. The epistomial fringe is continuous, starting on the left side and ending with staggered rows on the right side. There are one to three macronuclei and a single micronucleus.

Notes on 18S rRNA gene sequences: Three sequences were obtained, two from MOKOT PQ699755, PQ699757), and one from ZLAKOR2A (PQ699763). The genetic distance (uncorrected p distance) between the two studied populations is 0.001 (Table S4).

***Pelodinium reniforme* Lauterborn, 1908**

Notes on the observed MOKOTPEL and GLENWOODP populations (Fig. 11): Cells about $50 \times 36 \mu\text{m}$ in vivo on average. The shape is ellipsoidal in outline. The Frontal spine is absent, and a ventral spine is present (Fig. 11A-F). Around six blunt spines border the posterior marginal heart-shaped concavity (Fig. 11 A-C, F). There are two preoral and two ventral kineties (Fig. 11G). There are 10 somatic kineties, including the epistomial rows (Fig. 2P), six of them (SK1, 6-10) end with long ciliated spine segments (Fig. 11G-I). The epistomial fringe is continuous, starting on the ventral margin and ending with staggered rows on the right side. The non-ciliated segments of the epistomial fringe (SK1-5) and the SK6 are folded posteriorly, forming an almost 90° angle (Fig. 11A, G). The

SK1 and SK6 end with long ciliated spine segments posteriorly, whereas the non-ciliated segments of SK2-5 end subposteriorly without ciliated segments. (Fig. 11G) The short SK10 is located on the ventral margin at the third posterior region of the cell and includes a ciliated segment solely (Fig. 11H, I). The inverse kinety comprises about two dikinetids (Fig. 11H). There are one or two macronuclei and a single micronucleus.

Odontostomatean sp. GROSERDU

Notes on the observed population (Fig. 12): Cells $32 \times 24 \mu\text{m}$ in vivo and $25 \times 17 \mu\text{m}$ in protargol-impregnated specimens on average. The shape is almost circular to ellipsoidal in outline (Fig. 12A-I). The frontal spine is absent. A concavity at the posterior cell margin is surrounded by short, inconspicuous spines on the left side; the posterior right margin is smooth (Fig. 12C, D, F). Details of the infraciliature could not be determined due to the suboptimal quality of the protargol preparations.

Notes on 18S rRNA gene sequences: Two sequences were obtained (PQ699741-42).

***Mylestoma bipartitum* (Gourret & Roeser, 1886) Kahl, 1928**

Redescription based on KLAGOS population (Fig. 13, Table S9): Cells $33\text{--}42 \times 24\text{--}31 \mu\text{m}$ in vivo and $30 \times 21 \mu\text{m}$ on average in protargol-impregnated specimens. The shape is semicircular in outline (Fig. 13A-H, N, O). Anteriorly, the cortex ends ventrally as a hood-like structure resembling a spine in lateral view and is continuous with the dorsal keel (Fig. 13B, D, N). The right side of the cell is distinctly vaulted with two conspicuous and two less conspicuous sharp spines on the posterior margin (Fig. 13B, D, J). The left side is more or less flattened, with a blunt spine on the posterior ventral margin. Ventrally, anterior to the buccal area, there are two conspicuous sharp spines, one on the right side and the other on the left side (Fig. 13D-F, H, J, K, P); two short spines protrude at the end of the ventral ridge and the base of the preoral kinety 1 (Fig. 13E, F, H). The lapel-like structure enfolds the two dikinetids of the inverse kinety (Fig. 13F, L, O). There is invariably one macronucleus and a single micronucleus (Fig. 13A, F, N inset).

On average, the continual epistomial fringe comprises eight pectinelles, which mostly lie on the left side (Fig. 13F, H, I, O). The two preoral kineties (PK1-2) have an average of 10 and seven dikinetids, respectively (Fig. 13F, I, O). One ventral kinety (VK1) is located in the rightmost plate of the buccal area having about four dikinetids plus one additional supplementary ventral dikinetid (Fig. 13F, J, P, Q). The oral cavity invariably has nine membranelles, eight within the outer compartment and the ninth in the inner (Fig. 13A, F, H, I, M, O, R). The right ventral spine bears at its base one pair of long cilia (Fig. 13J, P, Q).

The somatic kineties are arranged in a *Mylestoma* pattern consisting of nine non-ciliated kineties, five on the right and four on the left sides (Fig. 13D, E, F, N, O). Two caudal cirri, one each at the

posterior end of SK7 and composed of 10 kinetids each, bear long cilia (44 μm in vivo), forming a semikinetal pattern (Fig. 13A, B, E-G, H, R).

***Mylestoma monodontum* sp. nov.**

Additional notes to the description FARO population (Fig. 14; Table S9; Video 5): Anteriorly ending as a hood-like structure resembling a spine in lateral view. One conspicuous, fang-like spine emerges at the end of the epistomial fringe on the right ventrolateral side. An inconspicuous spine protrudes from the posterior ventral left side. The lapel-like structure is present (labeled as the oral spine in Mendez-Sanchez et al. 2023) and enfolds the two dikinetids of the inverse kinety (Fig. 14A-C). Five somatic kineties (SK1-5) on the right and two (SK6-7) on the left side are arranged in a *Mylestoma* pattern. Two caudal cirri, one each at the posterior end of SK7 and composed of six kinetids each, bear long cilia (25 μm in vivo), forming a semikinetal pattern (Fig. 14).

Notes on 18S rRNA gene sequences: The genetic distance (uncorrected p distance) between the FARO sequences and the previously published FRESHO (OP034695-9) ranges from 0 to 0.004 (Table S4).

***Limnomylestoma shuriken* gen. nov., sp. nov.**

Description based on SLATINA population (Figs. 15, 16; Table S9; Video S6): Cells 24–33 $\times$ 18–26 μm in vivo and 19 $\times$ 15 μm on average in protargol-impregnated specimens. The shape is semicircular in outline with two or three prominent spines (Figs. 15A-G; 16A-C). Anteriorly, the cortex ends ventrally as a hood-like structure resembling a spine in lateral view and is continuous with the dorsal keel, which is fused to the right lateral side (Figs. 15A-C, G, K; 16A-D, G). There is a ventral spine, which is an extension of the right lateral side of the cortex at the level of the epistomial fringe (Figs. 15A-C, F; 16A, B, G). The caudal spine arises on the right side and is sometimes inconspicuous (Figs. 15B, C, E, F; 16B, C). A fin-like projection is present on the posterior right margin (Figs. 15B, D; 16A-D). The lapel-like structure appears as a flattened lamina (e.g., no folded) and enfolds the two dikinetids of the inverse kinety (Figs. 15B, C; 16C-F). One spherical macronucleus and a single micronucleus (Fig. 15A, E, G-J).

On average, the continual epistomial fringe comprises four pectinelles on the ventral margin (Figs. 15A, B, C, I, J; 16C, D, G). Two preoral kineties, PK1 and PK2, with an average of five and three dikinetids, respectively (Figs. 15C, I, J; 16D, F, G). One ventral kinety, VK, is located on the rightmost plate of the buccal area having six dikinetids on average (Figs. 15C, I, J; 16A, C, E). The oral cavity invariably has nine membranelles, eight within the outer compartment and the ninth in the inner (Fig. 16C-E, I, J).

The somatic kineties are arranged in a *Mylestoma* pattern, consisting of seven non-ciliated kineties, five on the right and two on the left sides (Fig. 15B, C, K, L). Two caudal cirri, one each at the

posterior end of SK6 and SK7 and composed of eight kinetids each, bear long cilia (20 μm in vivo), forming an amphikinetal pattern (Figs. 15A-C, H, I, L; 16C, D, H).

***Tostonella uncinata* (Penard, 1922) comb. nov.**

Redescription based on BUL7A population (Figs. 17, 18; Table S10; Video S7): Cells 28–34 μm in diameter in vivo and 28 μm on average in protargol-impregnated specimens. The shape is circular in outline with three prominent posterior spines (Figs. 17A-G, I, J; 18A, C). Anteriorly, the cortex ends ventrally as a hood-like structure resembling a spine in lateral view (Figs. 17A, C, F; 18B, D), the dorsal keel is slightly folded rightwards, forming a shallow deep through and posteriorly ends as the backward-folded spine III (Figs. 17C, I; 18A, C, D). The right side of the cell is more or less flattened, whereas the left side is vaulted (i.e., strongly convex). Two parallel, posteriorly directed, sharp spines emerge from the posterior ventral cell margin (Figs. 17A-E, G; 18A, C, G). The lapel-like structure and the inverse kinety are absent. One spherical macronucleus and a single micronucleus (Fig. 17A, D, J, N).

On average, the continual epistomial fringe comprises five pectinelles on the ventral margin; the leftmost pectinelle is posteriorly displaced (Fig. 17D, H, M). Two preoral kineties, PK1 and PK2, with an average of seven dikinetids each, respectively (Fig. 17D, E, J, K, M). One ventral kinety, consisting of 10 dikinetids on average, is located on the rightmost side of the buccal area (Figs. 17C, J, K; 18E, G). The oral cavity invariably has 10 membranelles, nine within the outer compartment and the tenth in the inner (Fig. 17A, D, E, J, K, L).

Somatic kineties are not studied in detail, but apparently, there are three non-ciliated kineties on the right side and one on the left side (Fig. 17C, D). Two caudal cirri, with about seven kinetids each, lie on the leftmost posterior spine (S-I); one cirrus is apparently continuous with the somatic kinety on the left side; the caudal cirri bear long cilia (about 25 μm in vivo) (Figs. 17A-D, J; 18A, E, G).

Notes: The material obtained from the CEDARKOR population was insufficient to describe it. However, the cell shape was identical to that of the cells from the BUL7A population.

Notes on 18S rRNA gene sequences: The genetic distance (uncorrected p distance) between both populations is 0.

***Discomorphella pectinata* (Levander, 1894) Corliss, 1960**

Redescription based on MOKOT, BOTAN, and LAPLICE populations (Figs. 19, 20; Table S11; Video S8): Cells 67–79 μm in diameter in vivo and 63 μm on average in protargol-impregnated specimens. Shape circular in outline, the right side of the cell is vaulted, and the left side is flattened. Three long, sharp spines protrude from the cortex, one frontal spine and two spines on the right side: one above the end of the dextro-frontal epistomial fragment and one on the posterior region of the

548 vaulted surface (Figs. 19A-D, H, M; 20A, B). The lapel-like structure and the inverse kinety are
 549 absent. One spherical macronucleus and a single micronucleus (Fig. 19A, H, L).

550 The epistomial fringe resides on a conspicuously protruding hoop-like frontal awning and fragmented
 551 into a dextro-frontal and a left fragment linked by the epistomial fringe spacer (Figs. 19B, F, H, M;
 552 20A, B). Pectinelles are armored with auricles (Figs. 19E; 20E, I-K). The dextro-frontal fragment
 553 forms a frontal awning and bears 21 pectinelles, the left fragment with nine pectinelles, on average;
 554 each pectinelle in the left fragment bears four ciliated dikinets, whereas the dextro-frontal fragment
 555 starts with five and ends staggered with three (Figs. 19B, F, H, J, M; 20A, B, F, I-K). The auricle of
 556 the second or third pectinelle on the left fragment might extend forming a small spine, but not always
 557 present (Figs. 19G; 20I-K). The dextro-frontal fragment ends with about 11 papillary dikinets (Figs.
 558 19J; 20D). The preoral kinety is hook-like, sinistrally oriented below the frontal awning, with 25
 559 dikinets, which are armored with small pectinelles (Figs. 19E, I; 20C). Posterior to the frontal
 560 awning, in the most external part of the buccal cavity, two endoral kineties, outer and inner, lie in
 561 parallel to each other obliquely to the cell long axis and consist of 12 and 13 dikinets, respectively
 562 (Fig. 19K). Adoral zone with nine obliquely oriented membranelles (Fig. 19L) decreasing in length
 563 toward each end of the adoral zone. There are two supplementary ventral kineties slightly anterior to
 564 the cell equator (Fig. 19N). Posterior to, there is a serrated ventral flap in two sections, each bearing a
 565 ventral kinety consisting of 16 and 20 dikinets, respectively (Figs. 19B, M, N; 20B, G).

566 The somatic kineties are arranged in a *Discomorphella* pattern, with seven kineties on the right and
 567 three on the left side (Fig. 2R). Two caudal cirri, one each at the posterior end of SK9 or SK10 and
 568 composed of about seven kinets each, bear long cilia (20 μm in vivo) (Figs. 19F, M; 20B, H).

569 **Notes:** The morphometric values given in the above description are based on the MOKOT population.
 570 Refer to Table S11 for the morphometric values of the BOTAN population. The BOTAN and
 571 MOKOT populations are highly similar. Only a few differences were notable. For instance, the
 572 epistomial fringe spacer in the MOKOT population was longer than in the BOTAN population (25
 573 μm vs. 17 μm , on average).

574 **Notes on 18S rRNA gene sequences:** The genetic distance (uncorrected p distance) between the three
 575 studied populations ranges from 0 to 0.001 (Table S4).

576 Discussion

577 Odontostomatea from a phylogenetic point of view

578 Odontostomatean ciliates were historically considered to belong to several different groups, e.g.,
 579 Heterotrichida, Plagiopylea, and Armophorea (Kahl 1932b; Jankowski 1964b, 2007; Stoeck et al.
 580 2007; Lynn 2008; Adl et al. 2019), until the establishment of their own class (Fernandes et al. 2018).

581 The newly sequenced odontostomatean species strongly support the monophyly of the class
582 Odontostomatea (Fig. 1).

583 Our data provide clear evidence that the problematic genus *Epalxella* truly nests within
584 Odontostomatea since the partial 18S rRNA gene sequences of the species *E. exigua* and *E. cf.*
585 *antiquorum* branch within the Odontostomatea subtree of the ciliate phylogenetic tree, unlike the
586 previously published sequence published under the name *Epalxella antiquorum* that clustered with
587 plagiopylids (Stoeck et al. 2007). Since no morphological data were provided in the latter publication,
588 we conclude it was an artifact or a misidentified ciliate. Moreover, morphological affinities (e.g.,
589 infraciliature, body shape, and cortical architecture) between *Epalxella* and the other
590 odontostomateans are strikingly stronger than any morphological similarity to any plagiopylid ciliates
591 (Fernandes et al. 2018). Thus, the class Odontostomatea is monophyletic.

592 Unfortunately, misidentifications still occur. For instance, a few single-cell metagenome sequences
593 have recently been deposited in GenBank without associated morphologic data for the source
594 organism (Zhang et al. 2024a, b). After extraction of the 18S rRNA gene sequence from one of the
595 metagenomes under the name *Mylestoma anatinum*, we found that it, instead, belongs to *Saprodinium*
596 *dentatum*. Similar cases have also been recently pointed out for 18S rRNA gene sequences from other
597 groups (Rotterová et al. 2024; Pomahač et al. 2024).

598 18S rRNA gene sequences of the odontostomateans are highly divergent with respect to sequences of
599 other ciliates, which was a "feature" used for establishing the class Odontostomatea (Fernandes et al.
600 2018). Notably, sequences of particular odontostomatean species and genera are also highly divergent
601 from each other, which was not noted before due to the low number of published sequences. This
602 suggests that the 18S rRNA of odontostomateans has evolved relatively rapidly, usually preventing its
603 amplification with "universal eukaryotic" primers. Additionally, previously published "universal
604 eukaryotic" primers (Abraham et al. 2019) were mapped to the odontostomatean alignment, and only
605 the primers F-566 and R-1200, flanking the region V4 and V5 (Hadziavdic et al. 2014), matched
606 within the odontostomatean alignment, but only for members of the family Epalxellidae. As a result,
607 many environmental studies based on the 18S rRNA gene amplicons overlook odontostomatean
608 ciliates. However, the environmental odontostomatean sequences we identified from
609 metatranscriptomic and metagenomic data suggest a greater, as yet unexplored, diversity.

610 The phylogenetic position of Odontostomatea within Ciliophora remains unclear. In recent
611 phylogenomic analysis, Odontostomatea nest within Armophorea (Zhang et al. 2024b). This topology
612 is inconsistent with any previous analysis based on single gene phylogenies, where Odontostomatea
613 are sister to Armophorea or Litostomatea (Fernandes et al. 2018; Rotterová et al. 2020;
614 Méndez-Sánchez et al. 2023). Based on 18S rRNA gene analysis, our current phylogeny places
615 Odontostomatea sister to Armophorea or Litostomatea, although without support. However, it is likely

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3 616 that Odontostomatea are closely related to the class Armophorea since several morphological
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5 617 similarities between the two taxa are found, e.g., the likely homologous perizonal stripe and
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7 618 epistomial fringe, the basis for their previous inclusion in the class Armophorea (Jankowski 1964b;
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9 619 Da Silva-Neto et al. 2016; Fernandes et al. 2018; Adl et al. 2019).

10 620 **Environmental analysis reveals that Odontostomatea is unexpectedly diverse**

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12 621 The environmental odontostomatean 18S rRNA sequences retrieved from databases suggest that
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14 622 Odontostomatea are highly diverse, with clades representing potential new species, genera, or
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16 623 families, unlike the published metabarcoding environmental studies using standard eukaryotic
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18 624 barcoding primers (Karst et al. 2018; Burki et al. 2021). The discovered diversity covers a wide range
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20 625 of ecosystems from several localities around the globe (i.e., marine and freshwater sediments from
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22 626 wetlands, soil, estuaries, and creeks), recovering a broad range of diversity (Angle et al. 2017; Karst et
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24 627 al. 2018; Woodcroft et al. 2018; Peterson et al. 2020; Graf et al. 2021; Zhang et al. 2021; Dang et al.
25
26 628 2022) (Table S3). Our analysis showed that diversity in the 18S rRNA gene in odontostomateans
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28 629 coupled with their vast morphological diversity (Kahl 1932a, b).

29 630 Since all the environmental sequences (19) retrieved from marine samples (USA, Sweden, Baltic Sea)
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31 631 clustered with the marine *Mylestoma bipartitum* and *M. monodontum* into a poorly supported clade
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33 632 within Clade II, which is otherwise freshwater, we hypothesize that marine odontostomateans,
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35 633 particularly marine *Mylestoma*, have a freshwater ancestor.

36 634 We recognize that some environmental odontostomatean sequences may still contain artifacts or
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38 635 undetected chimeras, particularly when derived from assemblies of short reads in metagenomes or
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40 636 metatranscriptomes. These challenges can introduce noise into phylogenetic analyses, underscoring
41
42 637 the need for caution when interpreting the true diversity of Odontostomatea. Furthermore, the two
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44 638 major clades of Odontostomatea and their respective subclades remained well or highly supported and
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46 639 congruent with the phylogeny without environmental sequences.

47 640 Odontostomatea appear to be a relatively recent taxon within Ciliophora (Zhang et al. 2024b),
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49 641 begging the question of what caused their rapid diversification, which is more typical for
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51 642 endosymbiotic species (Vďačný 2018; Vďačný et al. 2019). One of the causes could be the
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53 643 acquisition of prokaryotic symbionts (Schrenk 1989; Schrenk and Bardele 1991; Paiva et al. 2017;
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55 644 Méndez-Sánchez et al. 2023), which are also common in anaerobic protists from anoxic sediments,
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57 645 similar to the case with their very diverse and closest armophorean relatives, the Metopida
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59 646 (Armophorea) (Kahl 1932b; Esteban et al. 1995; Rotterová et al. 2018; Treitli et al. 2023;
60
647 Schrecengost et al. 2024; Pomahač et al. 2024).

648 **Redefinition of the families Discomoprhehlidae, Epalxellidae, and Mylestomatidae and higher**
649 **taxonomic ranks of Odontostomatea**

The newly sequenced populations of various genera and species of odontostomateans provide the first internal phylogeny of the class Odontostomatea, demonstrating congruency with its current classification. Until now, the class consisted of a single order, Odontostomatida, and three families, Discomorphellidae, Mylestomatidae, and Epalxellidae (Wetzel 1928; Kahl 1932b; Corliss 1960; Lynn 2008; Fernandes et al. 2018). Our phylogenetic analysis strongly supports the monophyly of each of the taxa. However, odontostomatean sequences retrieved from environmental datasets, along with sequences of *Limnomylestoma* gen. nov., *Tostonella* gen. nov., and Odontostomatea sp. 2 and 3, suggest that additional families may exist within this class. Likewise, the genera *Pelodinium* and *Atopodinium*, which currently lack molecular data, may also represent distinct families. Since the genera *Limnomylestoma* and *Tostonella* differ morphologically, we hesitate to classify them into any family at the present state of knowledge.

Morphologically, the family Epalxellidae was defined by the presence of ciliated kineties, one anterior and four on the posterior rear, except in *Pelodinium*, which has six posterior ones (Kahl 1932a). Through a detailed examination of various epalxellid genera and species, i.e., *Saprodinium*, *Epalxella*, and *Mircalla* gen. nov., we identify and define the so-called *Epalxella* pattern, characterized by the ciliary arrangement of the ciliated and non-ciliated segments of the somatic kineties. This pattern is proposed as a synapomorphy of the family. Although *Pelodinium reniforme* was previously classified within Epalxellidae (Kahl 1932b, a; Jankowski 2007; Lynn 2008), its infraciliature pattern does not conform to the *Epalxella* pattern (Fig. 2P) (Wetzel 1928; Klein 1930; Kahl 1932a, b; Foissner et al. 1992). Thus, we recommend its exclusion from Epalxellidae and consider it as *incertae sedis* in Odontostomatea until molecular data are available. Due to a lack of morphological details, it remains unclear whether odontostomatean sp. 1 (GROSERDU strain), which clusters into Clade I, belongs to Epalxellidae. We, therefore, prefer not to classify it there and consider it as *incertae sedis* as well.

Based on morphology, Kahl (1932a) and Jankowski (1964a) suggested that Mylestomatidae and Discomorphellidae are more closely related to each other than to Epalxellidae, a relationship supported by our phylogenetic analysis. Additionally, *Tostonella* gen. nov. and *Limnomylestoma* gen. nov. also show a close phylogenetic relationship with these two families.

The strongly supported family Discomorphellidae currently contains a single genus, *Discomorphella*. The family has been well-defined by its peculiar morphological features, e.g., fragmented epistomial fringe and a hoop-like frontal awning (Kahl 1932a; Corliss 1960; Lynn 2008). Given the current data, it is premature to include *Tostonella*, *Limnomylestoma*, and the odontostomatean species 2 and 3 within Discomorphellidae.

Kahl (1932b, a) established the family Mylestomatidae, grouping freshwater and marine species of *Mylestoma* and the freshwater genus *Atopodinium*, based on similarities in body shape and ciliature. Based on the similarities of *Limnomylestoma* and *Mylestoma*, which are unrelated genera, and the

clustering of marine environmental sequences into a single clade, we are skeptical about the assignment of the freshwater *Mylestoma* species to Mylestomatidae. Thus, reexamining the remaining *Mylestoma* species is crucial to clarify the members of the family. The freshwater *Mylestoma* species may belong either to *Limnomylestoma* or another genus since a detailed morphological characterization is missing for most of them. It also remains unclear whether *Atopodinium* or any freshwater *Mylestoma* species corresponds to any of the freshwater environmental sequences associated with the marine clade. A thorough revision of the family is therefore needed.

Furthermore, the robustly supported Clade I and II may represent distinct orders within Odontostomatea. The possible synapomorphies for Clade I include a posterior marginal concavity bordered by spines and somatic kineties consisting of up to three segments: an anterior ciliated segment, a middle non-ciliated segment, and a terminal spine segment (Kahl 1932a, b; Jankowski 1964a). Whereas Clade II includes two caudal cirri along the left posterior cell margin, a reduction of ciliature to non-ciliated somatic kineties only, and a smooth, posterior cell margin without a concavity. However, we refrain from establishing these potential orders until other genera, e.g., *Atopodinium*, *Pelodinium*, are morphologically and molecularly characterized.

Establishment of new odontostomatean genera and comparison with congeners

Although the infraciliature of odontostomateans is reduced to several kineties, it remains complex due to the presence of ciliated and non-ciliated segments, which are only revealed with protargol or silver nitrate impregnation (Klein 1930; Schrenk and Bardele 1991; Tuffrau 1992). However, these techniques may miss finer structural details, leading to misinterpretations. Accurate identification also requires careful examination of the lateral aspects of the cortex, the structures of the ventral margin, and the number and arrangement of spines. Since these details are often not well seen in light microscopy, scanning electron microscopy is especially important for reliably distinguishing these structures and avoiding errors in species identification.

Odonstomatea comprises nine genera, distinguishable primarily by in vivo features, except for very similar *Mylestoma* and *Limnomylestoma*. *Saprodinium* has right and left side posterior marginal spines with thorn-like ends. In contrast, in *Epaxella* and *Mircalla*, the posterior spines are less conspicuous and more or less blunt or rounded; they are more prominent on the left side and inwardly curving on the right side, creating a wavy posterior edge (Kahl 1932a; Foissner et al. 1992; Foissner and Berger 1996). Despite similar body shapes, *Mircalla* and *Epaxella* form distinct clades within Odontostomatea, which are not closely related. *Mircalla* uniquely bears a fang-like spine between the buccal cavity and frontal ridge, distinguishing it from *Epaxella* and other genera. This fang-like spine, together with its phylogenetic position, supports the establishment of *Mircalla* as a separate genus. Note that this spine differs from the lapel-like structure of *Saprodinium*, *Epaxella*, *Mylestoma*, and *Limnomylestoma*, which appears like a spine and was previously regarded as an oral one but

instead is a fold of the anterior plate supporting the buccal cavity (Schrenk and Bardele 1991; Méndez-Sánchez et al. 2023).

Pelodinium differs from all genera of Epalxellidae by having a rather small heart-shaped posterior concavity and possessing a unique infraciliature, i.e., the *Pelodinium* pattern (described above) (Kahl 1932a, b; Jankowski 1964a; Foissner et al. 1992). *Discomorphella* stands out from other odontostomateans due to its discoidal shape, prominent spines, and fragmented epistomial fringe on a hoop-like frontal awning (Lauterborn 1901; Wetzel 1928; Tuffrau 1992; Paiva et al. 2017).

Despite their phylogenetic distance, *Mylestoma* and *Limnomylestoma* are morphologically quite similar to each other and cannot be easily distinguished without silver impregnation (Kahl 1932a, b). While not all *Mylestoma* species have been redescribed with the current methodologies, and *Limnomylestoma* is monotypic, our examination of impregnated specimens shows subtle differences: 1) *Limnomylestoma* displays an amphikinetal caudal cirral arrangement, while *M. monodontum*, *M. anatinum*, and *M. bipartitum* show a semikinetal arrangement; 2) *Mylestoma* has a pronounced lapel-like structure, whereas *Limnomylestoma* exhibits a thinner, lamina-like structure (Schrenk 1989; Méndez-Sánchez et al. 2023). The interesting morphological convergences of *Mylestoma* and *Limnomylestoma* warrant further investigation.

Kahl (1932a) suggested that the genus *Mylestoma* could possibly be further split into separate genera. Based on the morphology and phylogenetic position of *Tostonella uncinata* (original combination *Mylestoma uncinatum*), which does not cluster within Discomorphellidae, Mylestomatidae, or *Limnomylestoma*, we propose *Tostonella* as a new genus. It shares the discoidal body shape of *Discomorphella*, but differs from it by lacking the sharp spines, frontal awning, and fragmented epistomial fringe, characteristic features of *Discomorphella* (Lauterborn 1901; Tuffrau 1992; Lynn 2008; Paiva et al. 2017). *Tostonella* has an anterior hood-like structure originating from the dorsal keel, which covers the reduced epistomial fringe on the ventral surface, similar to *Mylestoma* and *Limnomylestoma* (Kahl 1932a; Méndez-Sánchez et al. 2023). Unlike *Mylestoma*, the dorsal keel is conspicuous in *Tostonella*. Additionally, one pectinelle of the epistomial fringe is displaced posteriorly in *Tostonella*, a feature not observed in other odontostomatean genera. *Tostonella* has three posterior spines, one originating from the dorsal keel, and the other two from the ventral side of the cortex (Penard 1922; Kahl 1932a). We do not discard the possibility that *Atopodinium* is closely related to *Tostonella* due to its discoidal shape and multiple posterior spines. However, this assumption is challenged by the absence of a ventral kinety and the presence of a single caudal cirrus (vs. 2) in *Atopodinium* (Kahl 1932b, a; Jankowski 1964a). Based on these morphologic differences we establish the genus *Tostonella* for the populations BUL7A and CEDARKOR. A thorough examination of *Atopodinium* is needed to clarify its phylogenetic and morphological relationships with *Tostonella*.

1
2
3 754 The current phylogenetic tree suggests that additional genera will likely be recognized in the future, as
4 755 Epalxellidae sp. 1, Odontostomatean sp. 1 (GROSERDU), and Odontostomatean sp. 2 and 3 may
5 756 represent new taxonomic ranks.

8
9 757 **The odontostomatean species and their comparison with congeners**

10
11 758 The genus *Saprodinium* comprises six valid species, with the type species, *Saprodinium dentatum*,
12 759 being the most well-known (Kahl 1932a, b; Schrenk and Bardele 1991; Méndez-Sánchez et al. 2023).
13 760 Reexamination of the previously described VLKOV population (Méndez-Sánchez et al. 2023),
14 761 revealed that *S. dentatum* has nine somatic kineties (excluding the preoral and ventral ones),
15 762 composed of ciliated and non-ciliated segments arranged in the *Epalxella* ciliature pattern. Previous
16 763 studies reported only eight somatic kineties (Klein 1930; Jankowski 1964a; Schrenk and Bardele
17 764 1991; Méndez-Sánchez et al. 2023); with one likely overlooked. While details on the infraciliature of
18 765 other *Saprodinium* species are limited, *S. dentatum* is distinguished from them by having 94 ciliated
19 766 dikinetids on average in the anterior segment of the SK8, and eight or nine caudal spines, including
20 767 the ventrocaudal one (Kahl 1932a; Schrenk and Bardele 1991; Méndez-Sánchez et al. 2023).
21
22 768 *Saprodinium mimeticum* is distinguished from other *Saprodinium* species by its quadrangular body
23 769 shape and long and conspicuous ventrocaudal spine. It is also smaller and has fewer dikinetids in the
24 770 ventral kinety 1 and anterior ciliated segment of the SK8 compared to *S. dentatum* (6 vs 21, and <26
25 771 vs. >80, respectively) (Jankowski 1964a; Schrenk and Bardele 1991; Foissner et al. 1992;
26 772 Méndez-Sánchez et al. 2023). *S. mimeticum* differs from *S. spinigerum* by lacking the long ventral
27 773 spine, from *S. integrum* by having shorter thorn-like posterior spines (vs. long), from *S. putrinum* by
28 774 having longer posterior spines (vs. short), from *S. triangulum* by its quadrangular body shape (vs.
29 775 triangular), and from *S. halophilum* by the presence of a frontal spine. Additionally, *S. halophilum* and
30 776 *S. integrum* were found in marine and brackish environments, respectively, while the other species are
31 777 freshwater (Lauterborn 1901, 1908; Kahl 1928, 1932b, a; Jankowski 1964a; Schrenk 1989; Kreutz
32 778 2024). Kahl (1932a, b) established two forms, *S. mimeticum* f. *obliquum* and *S. mimeticum* f. *simplex*.
33 779 Both forms require further investigation. *S. mimeticum* f. *simplex* might represent a different species
34 780 since its ventrocaudal spine is less conspicuous and prominent than in *Saprodinium mimeticum* (Kahl
35 781 1932a, 1932b; Kreutz 2024).

36
37 782 The genus *Epalxella* comprises seven valid species: *E. mirabilis*, *E. striata*, *E. elliptica*, *E. exigua*, *E.*
38 783 *antiquorum*, *E. bidens*, and *E. simplex*. Here, we redescribe *E. exigua* using modern morphologic
39 784 techniques and molecular data and provide the 18S rRNA sequence of *E. cf. antiquorum*. Both species
40 785 form a moderately supported clade and share the characteristic of window-like cortical slits (Kahl
41 786 1932a, b; Jankowski 1964a). The population of *E. exigua* we studied matches the description by
42 787 Penard (1922) and Kahl (1932b, a), particularly in the shape of the anterodorsal part of the cell, the
43 788 absence of a frontal spine, and the presence of a single sharply demarcated window-like cortical slit

on the left side. The only difference from the previous descriptions was the shorter length of the long cilia from the spine segment of SK9 in our population. *Epalxella exigua* has a blunt anteroventral margin and lacks the frontal spine, unlike *E. antiquorum*, *E. bidens*, and *E. mirabilis* (Kahl 1932a; Jankowski 1964a). *Epalxella elliptica*, *E. striata*, and *E. simplex* have a blunt frontal margin like *E. exigua*, but lack the window-like cortical slits. *Epalxella antiquorum* is a large species, possibly the largest in the genus or even in the class (Jankowski 1964a). The few cells observed in the sequenced populations resemble *E. antiquorum* in size and shape and displayed window-like cortical slits (four on the right side and 2 on the left), sufficiently distinctive features to identify it as *E. antiquorum*.

Mircalla gen. nov. contains two species, *M. triangula* comb. nov. (original combination *Epalxella triangula*) and *M. polidorii* sp. nov., both characterized by the presence of a ventral spine. *Mircalla polidorii* differs from *M. triangula* by the cell shape (ellipsoidal vs. triangular, respectively) and the presence of two preoral kineties (vs. one). Kahl (1932b, a) described *Epalxella triangula* as having a more or less triangular body shape with a small frontal spine, distinguishing it from *Epalxella striata*, which has a more rounded anterodorsal side and lacks a frontal spine. Kahl (1932b, a) studied two populations, one small and one large. The African population resembles the smaller one described by Kahl (1932b, a), so we consider it conspecific with *E. triangula*. *Mircalla polidorii* and *Epaxella triangula* are closely related species and have similar morphology. Thus, we transfer the species *Epalxella triangula* to the newly established genus *Mircalla*. Until now, all the studied odontostomatean species bear two preoral kineties, except *Mircalla triangula*, which possesses a single one.

Morphologically, *Mylestoma bipartitum*, *M. monodontum*, *M. anatinum*, *M. spinigerum*, *M. pusillum*, and *Limnomylestoma shuriken* are similar (Penard 1922; Kahl 1932a; Jankowski 1964a; Schrenk 1989; Méndez-Sánchez et al. 2023). Other species, such as *M. uncinatum*, *M. flagellatum*, and *M. discoideum*, differ from *Mylestoma anatinum*, *M. monodontum*, *M. bipartitum*, and *Limnomylestoma shuriken* by having a more discoidal and flattened body (Kahl 1932b, a; Kreutz 2024).

Limnomylestoma shuriken, *Mylestoma bipartitum*, and *M. monodontum* differ from *Mylestoma spinigerum* by lacking the long posterior spine on the right side and by having caudal cirri in the mid-portion of the margin, while in *M. spinigerum* are on the left side near the dorsal margin (Kahl 1932a). *Mylestoma pusillum* is distinctive due to its small size (15-20 μm vs., other species, which are $>20 \mu\text{m}$ long), smooth posterior margin (no spines), and widely separated caudal cirri with long cilia (Kahl 1932a; Schrenk 1989; Kreutz 2024).

Based on Kahl's descriptions (1928, 1932a, b), we identify the *Mylestoma* from KLAGOS as *Mylestoma bipartitum*, characterized by its large size, long epistomial fringe, two conspicuous posterior spines on the right side, and marine habitat. Although we could not determine the exact

number of dikinetids in the ventral kinety, Kahl (1928, 1932b, a) depicted three. Details of this particular structure need to be confirmed. *Mylestoma bipartitum* is larger and has more pectinelles in the epistomial fringe and somatic kineties compared to *M. monodontum*, *M. anatinum*, and *Limnomylestoma shuriken* (<40 µm, eight, and nine vs. >30 µm, four, and <8, respectively).

Mylestoma anatinum has a broad central concavity in the truncated posterior region and eight somatic kineties, in contrast to *M. monodontum* and *Limnomylestoma shuriken*, which both lack a posterior marginal concavity and each of which has seven somatic kineties (Schrenk 1989; Méndez-Sánchez et al. 2023; Kreutz 2024). The caudal cirri are semikinetal in *M. anatinum* and *M. monodontum* but amphikinetal in *L. shuriken* (Schrenk 1989). Additionally, *L. shuriken* has six dikinetids in the ventral kinety, whereas *M. monodontum* has four (Méndez-Sánchez et al. 2023). Additionally, *M. anatinum* and *Limnomylestoma shuriken* are freshwater species, while *M. monodontum* and *M. bipartitum* are marine.

The odontostomatean from the BUL7A population resembles *Mylestoma uncinatum* (Pernard, 1922) Kahl, 1932 (original combination *Epalxis uncinatum*). Despite lacking details for the infraciliature of *M. uncinatum*, the body shape, size, and spine number and arrangement support conspecificity with BUL7A (Kahl 1932a). Based on its phylogenetic position, we transfer *Mylestoma uncinatum* to the new genus *Tostonella*. *Tostonella uncinata* differs from *Mylestoma flagellatum* by lacking a ventral spine, having five pectinelles in the epistomial fringe (vs. ca. nine), and featuring a displaced pectinelle (Kahl 1932a). Accordingly, a population identified by Kreutz (2024) as *Mylestoma flagellatum* is more likely *Tostonella uncinata*.

Discomorphella contains three species: *D. pectinata*, *D. lauterborni*, and *D. pedroeneasi*. *Discomorphella lauterborni* differs from the other two species by having only two spines (one frontal and one posterior) and lacking the lateral spine present in *D. pectinata* and *D. pedroeneasi* (Levander 1894; Wetzel 1928; Paiva et al. 2017). *Discomorphella pedroeneasi* has a conspicuous fourth spine associated with the left fragment of the epistomial fringe, which is reduced or absent in *D. pectinata*. Additionally, *D. pectinata* has more pectinelles in the epistomial fringe (>21 in the dextro-frontal fragment and nine in the left fragment vs. 14 and 6 in *D. pedroeneasi*, respectively). *Discomorphella pedroeneasi* also has three precaudal dikinetids that are absent in *D. pectinata*.

Discomorphella pectinata is divided into two subspecies, distinguished by the number of short spines on the left fragment of the epistomial fringe: one in *D. pectinata pectinata* and two in *D. pectinata bidenticulata* (Lauterborn 1908; Kahl 1932a, b; Paiva et al. 2017). Paiva et al. (2017) suggested that populations lacking spines on the left fragment, as noted by Jankowski (1964a) and Tuffrau (1992), might represent a different subspecies. We sequenced the 18S rRNA gene of specimens with and without a spine from two *Discomorphella* populations. The sequences showed no genetic divergence (100% identity), indicating no subspecies differentiation. Thus, the presence or absence of a spine on

the left fragment is not a reliable character for species delimitation. Further study is needed to clarify the status of *D. pectinata bidenticulata*.

Conclusions

This study provides the first large-scale survey of class Odontostomatea, characterizing several species and genera and redefining the three known families from a morphological and molecular perspective. This group has been neglected for several decades, and some members were incorrectly classified. Due to the highly variable 18S rRNA gene of odontostomateans, specific primers were designed for this study, which will help to refine the phylogeny of the group in future studies. This study reveals the monophyly of Odontostomatea and clarifies the morphologic identification and molecular characterization of the previously problematic genus *Epalxella*. We also identify two main clades with morphological trends that could be used to create taxonomic orders. Additionally, new genera and species are now proposed. Based on the environmental analyses, odontostomatean diversity appears far greater than expected, with as yet undiscovered families and genera waiting to be described. This taxonomic revision will benefit future taxonomists when revisiting previously described or new species. Furthermore, the identity of prokaryotic symbionts, such as methanogenic archaea, in odontostomatean ciliates remains unknown. Since Odontostomatea seems to have emerged as a relatively recent taxon within Ciliophora, it would be interesting to study the timing of acquisition and the host specificity of these prokaryotic symbionts, and compare these with other closely related groups, e.g., Metopida. The true phylogenetic position of Odonstomatea within Ciliophora needs to be elucidated with multiple-taxon “omic” analyses.

Taxonomic summary

ZooBank registration of this work: urn:lsid:zoobank.org:pub:686C9F7C-68C1-4596-A9E4-50B8F6DBFEB2

Class Odontostomatea Fernandes et al., 2018

Improved diagnosis: Size small to medium-sized (20-90 µm) anaerobic Intramacronucleata; body discoid or semicircular to ellipsoidal or quadrangular and sometimes triangular, laterally compressed; armor-like cortex thick, rigid and with frontal, ventral, lateral, or posterior spines; alveoli reduced or absent; basic ciliary unit dikinetid; somatic cilia usually arising from cortical sockets; somatic infraciliature reduced, somatic kineties composed ciliated and/or non-ciliated segments; subsurface basal bodies present; kinetodesmal fibers absent; with or without caudal cirri; oral apparatus comprising two compartments; typically <10 adoral membranelles exhibiting inverted orientation; paroral membrane absent; one to four globular macronuclei; micronucleus spherical, usually one; free swimming; freshwater, brackish, and marine species.

Order Odontostomatida Sawaya, 1940.

892 Characteristics of the class. Three families: Epalxellidae Corliss, 1960; Discomorphellidae Corliss,
893 1960; Mylestomatidae Kahl in Doflein & Reichenow, 1929.

894

895 **Family Epalxellidae**

896 **Improved diagnosis:** Small to large odontostomatea; posterior marginal concavity present, bordered
897 by posterior spines; dorsal keel with or without a frontal spine; lapel-like structure present; inverse
898 kinety present; one or two preoral kineties; one or two ventral kineties; somatic kineties arranged in
899 *Epalxella* pattern; one to four macronuclei, single micronucleus; freshwater, brackish, or marine
900 habitat.

901 **Type and included genera:** *Epalxella* Corliss, 1960 (type genus); *Saprodinium* Lauterborn, 1908;
902 and *Mircalla* gen. nov.

903

904 **Genus *Saprodinium* Lauterborn, 1908**

905 **Improved diagnosis:** Posterior marginal concavity surrounded with spines; posterior spines with
906 thorn-like ends; without window-like holes on the cortex; freshwater and marine habitat.

907 **Species included:** *S. dentatum* (Lauternorn, 1901) Lauterborn, 1908 (type species); *S. mimeticum*
908 (Penard, 1922) Kahl, 1932; *S. integrum* Kahl, 1928; *S. putrinum* Lackey, 1925; *S. spinigerum*
909 Kahl, 1932; and *S. halophilum* Kahl, 1932.

910

911 ***Saprodinium dentatum*** (Lauternorn, 1901) Lauterborn, 1908

912 **Nomenclatural acts:** *Discomorpha dentata* Lauternorn, 1901 (original combination)

913 **Improved diagnosis:** Size 50-80 μm ; shape semicircular; eight or nine posterior spines including
914 ventrocaudal spine; >80 ciliated dikinetids in the anterior segment of somatic kinety 8; four
915 supplementary ventral dikinetids; inverse kinety with three dikinetids; >10 dikinetids in spine segment
916 of somatic kineties.

917 **GenBank sequence:** Five 18S rRNA gene sequences were obtained, one per studied population,
918 VLKOV (PQ699762), GROSERDU (PQ699743), MOKOTR (PQ699758), LERMA3 (PQ699751),
919 and SLATINA (PQ699761).

920

921 ***Saprodinium mimeticum* (Penard, 1922) Kahl, 1932**

- 922 **Nomenclatural acts and synonyms:** *Epalxis mimetica* Penard, 1922 (original combination);
923 *Saprodinium tortum* Kahl, 1926.
- 924 **Improved diagnosis:** Size 40-60 μm ; shape quadrangular; seven posterior spines including
925 ventrocaudal spine; ventrocaudal spine long and prominent; frontal spine short; 15-32 dikinetids in the
926 anterior segment of somatic kinety 8; <10 dikinetids in spine segment of somatic kineties.
- 927 **Voucher material:** Two voucher slides, one from IS7 and one from BOPAT populations, containing
928 protargol-impregnated specimens, are deposited in the National Museum of the Czech Republic,
929 Prague, Czech Republic (catalog numbers XXXX).
- 930 **GenBank sequence:** Five partial 18S rRNA gene sequences were obtained: three from IS7
931 (PQ699744-6), one from BYMOKOT (PQ699735), and one from BOPAT (PQ699730) populations.
- 932 **Remarks:** The forms *Saprodinium mimeticum* f. *obliquum* Kahl, 1932; and *Saprodinium mimeticum* f.
933 *simplex* Kahl, 1932, require further examination.
- 934
- 935 **Genus *Mircalla* gen. nov.**
- 936 **Zoobank registration:** urn:lsid:zoobank.org:act:7AD9396F-0ADD-488B-B711-430B113D0100
- 937 **Diagnosis:** Frontal spine absent or very reduced; posterior right margin wavy, with spines folded
938 inwards, and left margin with three spines; posterior spines without thorn-like ends; fang-like ventral
939 spine present; without window-like cortical slits; freshwater habitat.
- 940 **Included species:** *Mircalla polidorii* sp. nov. (type species); and *Mircalla triangula* comb. nov.
- 941 **Etymology:** Derived from one of the names of the lady vampire from the Gothic novella Carmilla by
942 Sheridan Le Fanu (1872), referring to the presence of a fang-like ventral spine; feminine gender.
- 943 **Remarks:** Based on current knowledge, we assigned *Mircalla triangula* comb. nov. and *Mircalla*
944 *polidorii* sp. nov. to the same genus due to their molecular phylogenetic position. *M. triangula*
945 requires further examination, especially using scanning electron microscopy, to confirm the presence
946 of the ventral spine. However, the possibility that *Mircalla triangula* represents a different genus
947 cannot be excluded.
- 948
- 949 ***Mircalla polidorii* sp. nov.**
- 950 **Zoobank registration:** urn:lsid:zoobank.org:act:496FD896-738E-4FC8-B2C7-B32AD33EEA3B

Diagnosis: Size 20-33 μm ; shape ellipsoidal; frontal spine absent; ventro-frontal edge blunt; epistomial fringe with 11-15 pectinelles; preoral kinety 1 and 2 with 8-10 and 4-5 dikinets, respectively; two ventral kineties with 4-5 and 7-11 dikinets, respectively; supplementary dikinets absent; spine segments of somatic kinety 6, 7, 8, and 9 with 4-5, 4-5, 2-5, and 2-3 dikinets, respectively.

Type locality: Small permanent pond adjacent to the Vltava river in Prague, Czech Republic, 49°59'24.9" N 14°24'04.1" E, freshwater.

Type material: One slide, containing the protargol-impregnated holotype (marked with a black ink circle) and several paratype specimens, is deposited in the National Museum of the Czech Republic, Prague, Czech Republic (catalog number XXXX).

GenBank sequence: One partial 18S rRNA gene sequence with accession number PQ699756.

Etymology: Eponym for the author John William Polidori, who wrote the first modern vampire story, "The Vampire," referring to the presence of a fang-like ventral spine.

***Mircalla triangula* (Kahl, 1932) comb. nov.**

Nomenclatural acts: *Epalxis triangula* Kahl, 1932 (original combination).

Improved diagnosis: Size 32-37 μm ; shape triangular; frontal spine highly reduced; epistomial fringe with 6-8 pectinelles; a single preoral kinety with 10-15 dikinets; two ventral kineties with 3-4 and 3 dikinets, respectively; 3-4 supplementary dikinets; spine segments of somatic kinety 6, 7, 8, and 9 with 4-6, 3-6, 3-5, and 2 dikinets, respectively.

Type locality: A freshwater pond in Bayanga, Central African Republic, 2°50'32" N, 16°27'56" E.

Voucher material: One voucher slide, with protargol-impregnated specimens, is deposited in the National Museum of the Czech Republic, Prague, Czech Republic (catalog number XXXX).

GenBank sequence: One partial 18S rRNA gene sequence with accession number PQ699738.

Genus *Epalxella* Corliss, 1960

Improved diagnosis: Posterior right margin wavy, with spines folded inwards, and left margin with three spines; posterior spines blunt, without thorn-like ends; cortex with or without conspicuous window-like cortical slits; ventral spine absent; freshwater genus.

Species included: *E. mirabilis* (Roux, 1899) Corliss, 1960 (type species); *E. antiquorum* (Penard, 1922) Corliss, 1960; *E. exigua* (Penard, 1922) Corliss, 1960; *E. striata* (Kahl, 1926) Corliss, 1960; *E. elliptica* (Penard, 1922) Corliss, 1960; *E. bidens* (Kahl, 1932) Corliss, 1960; and *E. simplex* Jankowski, 1964.

***Epalxella exigua* (Penard, 1922) Corliss, 1960**

Nomenclatural acts: *Epalxis exigua* Penard, 1922 (original combination)

Improved diagnosis: Size 30-37 × 23-29 µm; shape ellipsoidal; frontal spine absent; ventro-frontal edge blunt; a single and conspicuous window-like cortical slit on the left anterior surface; epistomial fringe with 7-9 pectinelles; preoral kinety 1 and 2 with 3-4 and 5-6 dikinetids, respectively; one ventral kinety with six dikinetids in two groups; two supplementary ventral dikinetids; spine segments of somatic kineties 6, 7, 8, and 9 with 7-8, 7-9, 6-7, and 4-5 dikinetids, respectively.

Voucher material: One voucher slide, with protargol-impregnated specimens, is deposited in the National Museum of the Czech Republic, Prague, Czech Republic (accession number XXXX).

GenBank sequence: Two partial 18S rRNA gene sequences were obtained, one per studied population, LAUFENJ (PQ699750) and ZLATOVASKA (PQ699764).

Family Mylestomatidae Kahl in Doflein & Reichenow, 1929

Improved diagnosis: Small to medium odontostomatea; with or without posterior concavity; posterior spine inconspicuous to well developed; epistomial fringe ventrally oriented; 7-9 non-ciliated kineties; one or two caudal cirri; one macronucleus and one micronucleus.

Type and included genera: *Mylestoma* Kahl, 1928 (type genus); and *Atopodinium* Kahl, 1932

Genus *Mylestoma* Kahl, 1928

Improved diagnosis: Shape semi-circular or circular; frontoventral margin ends as hood-like structure; with or without posterior concavity; posterior spines poorly developed; two preoral kineties and one ventral kinety; 7-9 non-ciliated kineties; two caudal cirri arranged in a semikinetal pattern; lapel-like structure present; inverse kinety present; one macronucleus and one micronucleus; freshwater, brackish, and marine habitat.

Species included: *M. bipartitum* (Gourret & Roeser, 1886) Kahl, 1928 (type species); *M. anatinum* (Penard, 1922) Kahl, 1932; *M. discoideum* (Penard, 1922) Kahl, 1932; *M. flagellatum* (Penard, 1922) Kahl, 1932; *M. monodontum* sp. nov.; *M. pusillum* Kahl, 1932; and *M. spinigerum* Kahl, 1932.

***Mylestoma bipartitum* (Gourret & Roeser, 1886) Kahl, 1928**

Nomenclatural acts: *Aspidisca bipartita* Gourret & Roeser, 1886 (original combination).

Improved diagnosis: Size, 33-42 × 24-31 μm; shape semicircular; right side vaulted; posterior cavity present; 7-9 epistomial pectinelles; several spines on the ventral edge and four on the posterior right edge; preoral kinety 1 and 2 with 8-11 and 6-9 dikinetids, respectively; ventral kinety present; nine non-ciliated somatic kineties; caudal cirri with 8-10 kinetids; nine membranelles; brackish habitat.

Voucher material: One slide, containing protargol-impregnated specimens, is deposited in the National Museum of the Czech Republic, Prague, Czech Republic (catalog number XXXX).

GenBank sequence: One partial 18S rRNA gene sequence with accession number PQ699747.

***Mylestoma monodontum* sp. nov.**

Zoobank registration: urn:lsid:zoobank.org:act:5147563F-23F4-447E-BDE5-CBB86B9FE2BC

Diagnosis: Size 18-25 × 14-21 μm; shape semicircular; posterior cavity absent; four epistomial pectinelles; one spine at the right edge of the ventral fringe, one inconspicuous spine on the posterior right side; preoral kinety 1 and 2 with 5-6 and 3 dikinetids, respectively; four dikinetids in ventral kinety; seven non-ciliated somatic kineties; 7-8 membranelles; brackish habitat.

Type locality: Fresh River, Falmouth, Massachusetts, USA, 41°32'30.804" N, 70°37'19.92" W, brackish.

Type material: One slide containing the protargol-impregnated holotype specimen (FRESHO population) marked with a black ink circle, and one slide with protargol-impregnated paratypes (FARO population), are deposited in the National Museum of the Czech Republic, Prague, Czech Republic (catalog numbers P6E 5306 and P6E 5307).

GenBank sequence: Two partial 18S rRNA gene sequences from the FARO population were obtained with accession numbers PQ699739-40

Etymology: Adjective, compound of the Greek prefix "mono-" (single) and "odontos" (tooth), referring to the single spine on the ventral ridge.

1039

1040 **Family Discomorphellidae Corliss, 1960**

1041 **Improved diagnosis:** Shape discoidal; dorsal keel flattened; one sharp frontal spine; one or two sharp
 1042 spines on the right lateral side; epistomial fringe segmented in dextro-frontal and left fragments on
 1043 ventral awning; pectinelles armored with auricles; papillary dikinetids present; one preoral kinety
 1044 armored with auricles; two endoral kineties; two ventral kineties with supplementary ventral
 1045 dikinetids; precaudal dikinetids may be present; non-ciliated somatic kineties arranged in
 1046 *Discomorphella* pattern; two caudal cirri present; lapel-like structure and inverse kinety absent;
 1047 freshwater habitat.

1048 **Type and only included genus:** *Discomorphella* Corliss, 1960

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1050 **Genus *Discomorphella* Corliss, 1960**

1051 **Nomenclatural acts:** Replacement name for preoccupied *Discomorpha* Corliss, 1960

1052 **Improved diagnosis:** Characteristics like in the family. See Paiva et al 2017.

1053 **Included species:** *D. pectinata* (Levander, 1894) Corliss, 1960 (type species); *D. pedroeneasi* Paiva
 1054 et al., 2017; and *D. lauterborni* (Wetzel, 1928) Corliss, 1960.

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1056 ***Discomorphella pectinata*** (Levander, 1894) Corliss, 1960

1057 **Nomenclatural acts:** *Discomorphea pectinata* Levander, 1894 (original combination).

1058 **Improved diagnosis:** Size 60-85 μm in diameter; shape discoidal; three spines: one frontal, one right
 1059 lateral, and one right posterior; pectinelles in dextro-frontal and left fragment >17 and 8-10,
 1060 respectively; left fragment with or without associated spine; posterior fringe ratio >1; without
 1061 precaudal dikinetids.

1062 **Voucher material:** Two slides, one from MOKOT and one from BOTAN populations, containing the
 1063 protargol-impregnated specimens are deposited in the National Museum of the Czech Republic,
 1064 Prague, Czech Republic (catalog number XXXXX).

1065 **GenBank sequence:** Six sequences were obtained: two from LAPLICE (PQ699748-9), and three
 1066 from BOTAN (PQ699731-3); one consensus sequence (PQ699753) was obtained from three
 1067 sequences derived from cloning from MOKOT.

Remarks: Based on morphology and molecular evidence, we consider *Discomorphella pectinata* to be conspecific with *Discomorphella pectinata*. Therefore, we synonymize *D. pectinata* with *D. pectinata*. The validity of the subspecies *Discomorphella pectinata bidenticulata* (Kahl, 1932) Corliss, 1960 requires further examination.

Incertae sedis within Odontostomatida: *Pelodinium reniforme* Lauterborn, 1908; *Tostonella* gen. nov.; *Limnomylestoma* gen. nov.

Genus *Pelodinium* Lauterborn, 1908

Improved diagnosis: Ellipsoidal shape, dorsal keel without frontal spine; posterior marginal heart-shape concavity present, bordered by few blunt spines; lapel-like structure unknown; inverse kinety present; two preoral kineties; two ventral kineties; 10 somatic kineties arranged in *Pelodinium* pattern, with long spine segments; one or two macronuclei, single micronucleus; freshwater habitat.

Type and only included species: *Pelodinium reniforme* Lauterborn, 1908

***Pelodinium reniforme* Lauterborn, 1908**

Synonyms: *Pelodinium rotundum* Kahl, 1926; *Epalxis penardi* Wetzel, 1928

Diagnosis: Size 20-50 µm; ellipsoidal shape; ventral margin spine present; posterior heart-shaped concavity surrounded by about six blunt spines; inverse kinety with two dikinetids; without caudal cirri.

Genus *Tostonella* gen. nov.

Zoobank registration: urn:lsid:zoobank.org:act:2A5185E1-12EE-4371-A1EA-66136A2683C5

Diagnosis: Discoidal shape, posterior spines present, hood-like structure present; epistomial fringe reduced to five ventrally located pectinelles; leftmost pectinelle displaced posteriorly; two preoral kineties, one ventral kinety, non-ciliated kineties present; two caudal cirri with long cilia; one macronucleus and one micronucleus.

Type and only included species: *Tostonella uncinata* comb. nov.

Etymology: Derived from the Mexican slang word "tostón" (a low-value coin), referring to the coin in shape and swimming pattern which mimics a flipping coin; feminine gender.

1098 ***Tostonella uncinata* comb. nov.**

1099 **Original combination:** *Epalxis uncinatum* Penard, 1922

1100 **Diagnosis:** Size 28-34 µm in diameter; discoidal shape; three posterior spines; five pectinelles, one
 1101 pectinelles displaced downwards; preoral kinety 1, 2, and ventral kinety with 5-8, 6-8, and 9-10
 1102 dikinetids, respectively; 10 membranelles; one and three non-ciliated kineties on the left and right
 1103 side, respectively; caudal cirri located on posterior spine I, each with seven kinetids; freshwater
 1104 species.

1105 **Type locality:** A freshwater pond in the Arkutino Reserve, Primorsko, Bulgaria. 42°19'53.0"N
 1106 27°43'33.3"E.

1107 **Voucher material:** One slide containing protargol-impregnated voucher specimens from the BUL7A
 1108 population, is deposited in the National Museum of the Czech Republic, Prague, Czech Republic
 1109 (catalog number XXXX).

1110 **GenBank sequence:** Two 18S rRNA gene sequences were obtained, one from BUL7A (PQ699734)
 1111 and one from CEDARKOR (PQ699737).

1113 **Genus *Limnomylestoma* gen. nov.**

1114 **Zoobank registration:** urn:lsid:zoobank.org:act:6C731B3B-67A3-4A22-B211-1E1FF21A5BCD

1115 **Diagnosis:** Shape semi-circular; hood-like structure present; posterior concavity absent; posterior
 1116 spines conspicuous; less than five pectinelles; two preoral kineties and one ventral kinety; seven non-
 1117 ciliated kineties; two caudal cirri arranged in amphikinetal pattern; lapel-like structure lamina-like,
 1118 i.e., without fold; inverse kinety present; one macronucleus and one micronucleus; freshwater habitat.
 1119 Phylogenetically, more closely related to *Tostonella* and *Discomorphella* than Mylestomatidae.

1120 **Type and only included species:** *Limnomylestoma shuriken* sp. nov.

1121 **Etymology:** A compound of Greek prefix "limno-" (lake, pond), referring to the freshwater nature of
 1122 the genus, and "mylestoma," referring to the morphological similarities to the genus *Mylestoma*;
 1123 neuter gender.

1125 ***Limnomylestoma shuriken* sp. nov.**

1126 **Zoobank registration:** urn:lsid:zoobank.org:act:1810DD96-B1E5-4140-95E6-B75FAB6431DF

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3 1127 **Diagnosis:** Size 24-33 × 18-26 μm, shape semi-circular in outline with two or three prominent spines:
4 1128 one short ventral spine, one posterior spine, and one fin-like structure; 3-4 epistomial pectinelles;
5 1129 preoral kinety 1, 2, and ventral kinety with 4-5, 2-4, and six dikinetids, respectively; seven non-
6 1130 ciliated somatic kineties; caudal cirri with 8 kinetids.
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8 1131 **Type locality:** Slatina pond, Dubeč, Czech Republic, 50°04'13.2"N 14°34'12.5"E, freshwater.
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10 1132 **Type material:** One slide, containing the protargol-impregnated holotype (marked with a black ink
11 1133 circle) and several paratype specimens, is deposited in the National Museum of the Czech Republic,
12 1134 Prague, Czech Republic (catalog number XXXX).
13
14 1135 **GenBank sequence:** Two partial 18S rRNA gene sequences with accession numbers PQ699759-60.
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16 1136 **Etymology:** Derived from the Japanese word "shuriken," referring to the cell shape that resembles the
17 1137 star-like shape of a shuriken, a traditional throwing star weapon; noun in apposition.
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26 1139 **Glossary**
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28 1140 Given the complexity of the ciliature and the general cell shape of odontostomateans, we explain here
29 1141 specific terms used in this paper. These terms are either adapted from previous works (Wetzel 1928;
30 1142 Klein 1930; Schrenk and Bardele 1991; Foissner et al. 1992; Tuffrau 1992; Paiva et al. 2017) or,
31 1143 where indicated, newly proposed in this work.
32
33 1144 **Auricles:** Conspicuous projections of the borders of *pectinelles* (Fig. 2O) (Paiva et al. 2017).
34
35 1145 **Ciliary sockets:** Small but conspicuous pits, delimited by the thick epiplasm layer, from which the
36 1146 cilia emerge. Also referred to as odontostomatid ciliary sockets (Paiva et al. 2017).
37
38 1147 **Cortical pits:** Small holes in the cortex, likely reduced *ciliary sockets*, immediately underneath the
39 1148 cell surface dilate into large parasomal sacs. Cortical pits are part of the *non-ciliated segments of*
40 1149 *kineties* (Schrenk and Bardele 1991).
41
42 1150 **Cortical slit:** Sharply demarcated, deep, more or less oval-shaped cortical depression occurring on the
43 1151 right and left sides of some Epalxellidae. Vary in number. Referred to by Kahl (1932a, b) as
44 1152 "Fensterbildungen" (window formations).
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46 1153 **Dorsal keel:** Flattened anterodorsal margin of the cortex. It is a dorsal continuation of the frontal
47 1154 spine or the hood-like structure (Fig. 2A-E) (Schrenk and Bardele 1991).
48
49 1155 **Epistomial fringe (also frontal band):** A conspicuous row of *pectinelles* running perpendicularly to
50 1156 the main body axis, starting from the left side of the cell, embracing the *ventral ridge*, and ending on
51 1157 the right side of the cell in a staggered manner. The most anterior frontal ciliary row is the longest,
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1158 while the most posterior is the shortest. The epistomial fringe can be long or short, continuous or
 1159 fragmented into two sections: Dextro-frontal fragment (F1) forming a frontal awning and left
 1160 fragment (F2); fragments linked by a conspicuous *epistomial fringe spacer*. (Schrenk and Bardele
 1161 1991; Tuffrau 1992; Paiva et al. 2017)

1162 ***Epistomial fringe spacer***: An elevation of the body surface separating the two fragments of the
 1163 *epistomial fringe* (Paiva et al. 2017).

1164 ***Frontal awning***: A protrusion of the region containing the epistomial fringe, as opposed to what is
 1165 present in *Saprodinium*, in which the fringe is etched in the cell surface, forming a frontal band (Paiva
 1166 et al. 2017).

1167 ***Frontal band***: see epistomial fringe.

1168 ***Inverse kinety***: Two or three dikinetids emerging from a cortical pit at the base of the *lapel-like*
 1169 *structure*. The inversion of the kinety is known from TEM observations (Schrenk and Bardele 1991).

1170 ***Lapel-like structure (new term)***: the outer left plate of the buccal cavity anteriorly folds outwards
 1171 forming a lapel-like structure, which in vivo resembles a spine and previously was referred as an oral
 1172 spine (Schrenk and Bardele 1991; Méndez-Sánchez et al. 2023), at the base of the fold, an *inverse*
 1173 *kinety* rises from a cortical pit (Fig. 2P).

1174 ***Papillary dikinetids***: Ciliated dikinetids at the end of the dextro-frontal fragment of the *epistomial*
 1175 *fringe* in which cilia arise from slightly elevated *ciliary sockets*, forming papillae on the cell surface
 1176 (Paiva et al. 2017).

1177 ***Pectinelle***: Longitudinal row of one to six, usually five, *ciliary sockets* from which the cilia of the
 1178 *epistomial fringe* arise. The pectinelles are separated by notched interkinetal ridges (Fig. 2N) or
 1179 *auricules*. Pectinelles might be homologous to the false kineties in metopids (Armophorea) (Lynn
 1180 2008).

1181 ***Subsurface basal bodies***: Non-ciliated basal bodies located below the epiplasm layer and interleaved
 1182 with parasomal sacs. Subsurface basal bodies are the components of the non-ciliated segments of
 1183 somatic kineties (Paiva et al. 2017). Note that this term was mistakenly used as a synonym for *cortical*
 1184 *pits*.

1185 ***Ventral flap***: Expansion of the left wall at the margin of ciliary sockets of the posterior part of ventral
 1186 kineties (Paiva et al. 2017).

1187 ***Ventral ridge***: Bulge of the cortex that supports the *epistomial fringe*, located above the cell equator
 1188 on the ventral side (Schrenk and Bardele 1991).

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Ventrocaudal spine (also spine 1): An extension of the cortex forming the most ventral spine on the posterior right side of the cell, which bears the ventral kineties (Schrenk and Bardele 1991).

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Author contributions DMS: conceptualization, methodology, investigation, data curation, formal analysis, writing-original draft, review and editing. OP: investigation, data curation, funding acquisition, writing-review and editing. MV: formal analysis, investigation, data curation, writing-review and editing. WAB: methodology, formal analysis, investigation, writing-review and editing. IC: conceptualization, methodology, supervision, funding acquisition, investigation, writing-review and editing.

Data availability 18S rRNA gene sequences obtained in this study have been submitted to GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>). Type and voucher material is available upon request from the National Museum of the Czech Republic, Prague, Czech Republic (Václavské nám. 68, 110 00 Nové Město, Czechia, Tel.: +420 224 497 111).

Conflict of Interest

The authors have no competing interests to declare that are relevant to the content of this article.

Animal and human rights statement This article does not contain data from human participants or animals.

Supplementary Data

Figure S1. Environmental phylogenetic tree of Odontostomatea.
Table 1. Origin of the studied strains.

- 1220 Table S1. Oligonucleotide primers to amplify the 18S rRNA gene sequence of Odontostomatean
 1221 ciliates.
- 1222 Table S2. Pebblescout summary of hits.
- 1223 Table S3. Biosamples used to extract the 18S rRNA sequences of environmental odontostomateans.
- 1224 Table S4. Genetic distance matrix of the 18S rRNA sequences from the studied populations and
 1225 published odontostomatean sequences.
- 1226 Table S5. Morphometric data of *Saprodinium mimeticum* IS7 and BOPAT strains.
- 1227 Table S6. Morphometric data of *Saprodinium dentatum* VLKOV strain.
- 1228 Table S7. Morphometric data of *Mircalla triangula* (MT) and *Mircalla polidori* (MP).
- 1229 Table S8. Morphometric data of *Epalxella exigua* LAUFENJ strain.
- 1230 Table S9. Morphometric data of *Mylestoma bipartitum* KLAGOS (K), *Mylestoma monodontum*
 1231 FRESHO (F) and FARO (FA), and *Limnomylestoma shuriken* SLATINA (S).
- 1232 Table S10. Morphometric data of *Tostonella uncinata* nov. sp. BUL7A strain.
- 1233 Table S11. Morphometric data of *Discomorphella pectinata* MOKOT and BOTAN strains.
- 1234 Video S1. *Saprodinium mimeticum*. <https://youtu.be/SeFwbKPey8Y>
- 1235 Video S2. *Saprodinium dentatum*. <https://youtu.be/oP-bELV-OU0>
- 1236 Video S3. *Mircalla polidorii*. <https://youtu.be/IkwTR66fdC8>
- 1237 Video S4. *Epalxella* cf. *antiquorum*. <https://youtu.be/bODB1p7d8Ik>
- 1238 Video S5. *Mylestoma monodontum*. https://youtu.be/qYj7T_Kdp0
- 1239 Video S6. *Limnomylestoma shuriken*. <https://youtu.be/CoDuqxbVeFo>
- 1240 Video S7. *Tostonella uncinata*. <https://youtu.be/QCvwr-y2qLo>
- 1241 Video S8. *Discomorphella pectinata*. <https://youtu.be/bZztOd2RD48>
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Figure legends

Fig. 1 Phylogeny of Odontostomatea based on 18S rRNA gene. **A** ML phylogenetic tree showing the newly sequenced strains and species (in bold) of odontostomeans. **B** ML tree showing the position of Odontostomatea within Ciliophora after adding the new sequences. **C** ML phylogenetic tree including the environmental odontostomean sequences from metatranscriptomes and metagenomes, plus the newly obtained sequences. For a full tree refer to Figure S1. Branch values represent bootstrap support of ML and Bayesian posterior probabilities of BI, values below 50 and 0.80, respectively, are not shown or are represented by an (*). Scale bars: 30 changes per 100 nucleotides.

Fig. 2 General morphology of Odontostomatea in vivo (**A–J**) and after impregnation (**K–R**). **A–C** Shape of the anterior region of the cell on the lateral view, ending as a frontal spine (**A**), as a hood-like structure (**B**) or blunt (**C**). **D–G** Shape of the posterior end, on lateral views, with prominent spines and broad posterior marginal concavity (**D**), wavy and with inconspicuous spines and broad posterior marginal concavity (**E**), smooth with reduced spines (**F**), or small heart-shaped concavity (**G**). **H–J** Posterior end in dorsal view ending smooth (**H**), with spines on both sides (**I**), or with concavity with inwardly folding spines (**J**). **K** General ventral view of the ciliature. **L, M** Organization of the pectinelles without (**L**) or with auricles (**M**). **N** Left lateral view of the buccal area showing the disposition of the lapel-like structure and the inverse kinety. **O–R** Different ciliature patterns: *Epalxella* pattern (**O**), *Pelodinium* pattern (**P**), *Mylestoma* pattern (**Q**) with caudal cirrus arranged as an amphikinetal pattern (**Qi**), and a semikinetal pattern (**Qii**), and *Discomorphella* pattern (**R**). *AM* adoral membranelles, *DK* dorsal keel, *EF* epistomial fringe, *IK* inverse kinety, *LAP* lapel-like structure, *PK* preoral kinety, *S-I* spine 1, *sVdK* supplemental ventral dikinetid, *VCS* ventrocaudal spine, *VK* ventral kinety.

Fig. 3 Morphology of *Saprodinium mimeticum* in vivo (**A–B, E–I**) and after impregnation (**C–D, J–Q**). **A–B** Representation of living specimens, **B** after Kahl 1932. **C, D** Representation of the ciliature. **E–H, J–K** Right (**E, H, J**) and left (**F–G, K**) lateral view of specimens showing the body shape, ciliature, and the spines (**G, H**), inset in **G** shows the frontal spine. **I** Two cells during conjugation. **L** Ventral kineties. **M** The beginning of the epistomial fringe and preoral kineties; the white double arrowhead shows the start of the fifth epistomial ciliary row. **N** Epistomial fringe and adoral membranelles. **O** Nuclear apparatus. **P, Q** Somatic kineties arranged in an *Epalxella* pattern. White arrowheads in **J, K, M–O** point to the inverse kinety. Black double arrowheads in **J, K, O** point to the micronucleus. Black arrowheads in **J, K, P, Q** point to the somatic kineties. *AM* adoral membranelles, *EF* epistomial fringe, *FS* frontal spine, *IK* inverse kinety, *Ma* macronucleus, *PK* preoral kinety, *S* posterior spine, *SK* somatic kinety, *VK* ventral kinety. Scale bars: 10 µm.

Fig. 4 Scanning electron micrographs of *Saprodinium mimeticum*. **A–C** Left (**A**) and right (**B–C**) lateral views show the cell shape and the spines. **D** Close-up view of **B** shows the epistomial fringe and the dorsal keel. **E** Left lateral view of the anterior cell half shows the anterior ciliated fragment of SK8. **F** Left lateral view of the mid-ventral edge showing the lapel-like structure, the inverse kinety (black arrowhead), the preoral kineties, and the ventral ridge bearing the epistomial fringe. **G** Left lateral view of the posterior cell half shows the thorn-like spines. **H** Close-up view of **B** showing the ventral kineties, dikinetids of VK1 are pointed by black arrowheads, the buccal lip (yellow arrowhead), and the membranelles (black double arrowhead). *DK* dorsal keel, *EF* epistomial fringe, *FS* frontal spine, *LAP* lapel-like structure, *PK* preoral kinety, *S* posterior spine, *SK* somatic kinety, *VK* ventral kinety. Scale bars: 10 µm (**A–C**), 5 µm (**D–H**).

Fig. 5 Morphology of *Saprodinium dentatum* after impregnation (**A–B**) and in electron scanning microscopy (**C–H**). **A–B** Representation of the ciliature. **C, D, H** left (**C, H**) and right (**D**) lateral views showing the general shape, spines, and spine segments of kineties, black double arrowhead in **C** shows the inverse kinety, while black arrowheads in **H** point to the spine segments of kineties. **E**

Epistomial ciliary sockets bearing dikinetids. **F** Left lateral view of the mid-ventral margin showing the lapel-like structure (yellow arrowhead) and preoral kineties. **G** Ventro-lateral view showing the posterior cavity surrounded by spines. *DK* dorsal keel, *EF* epistomial fringe, *FS* frontal spine, *IK* inverse kinety, *PK* preoral kinety, *S* posterior spine, *SK* somatic kinety, *sVdK* supplementary ventral dikinetids, *VK* ventral kinety. Scale bars: 10 μ m (C, D, F-H), 2 μ m (E).

Fig. 6 Morphology of *Mircalla polidorii* gen. nov., sp. nov. in vivo (**A, D-G**), after impregnation (**B, C, H-N**). **A** Representation of a living specimen. **B, C** Representation of the ciliature. **D, E, G, H, I** Right (**D, H**) and left (**E, G, I**) lateral views show the general shape and infraciliature; the white arrowhead in **I** shows the inverse kinety. **F** Left lateral view shows the ventral spine (black double arrowhead), the adoral membranelles, and two inner spines (black arrowheads). **J** Right lateral view showing the non-ciliated segments of the SK1-6. **K, L** Beginning (**K**) and continuation (**L**) of the epistomial fringe and preoral kineties. **M** Ventral kinety 1. **N** Posterior end showing the VK2 and the associated spine kineties of SK7-9. Black arrowheads in **D** and **F** point to two short posterior spines. A black double arrowhead in **E** and **F** points to the ventral spine. *AM* adoral membranelles, *EF* epistomial fringe, *IK* inverse kinety, *Ma* macronucleus, *Mi* micronucleus, *PK* preoral kinety, *SK* somatic kinety, *VCS* ventrocaudal spine (spine 1), *VK* ventral kinety. Scale bars: 10 μ m.

Fig. 7 Scanning electron micrographs of *Mircalla polidorii* gen. nov., sp. nov. **A, B** Right (**A**) and left (**B**) lateral views showing the general shape. **C, G** Left lateral views showing the arrangement of the ciliature; the double yellow arrowheads point to the ventral spine, and the black arrowhead the inverse kinety embraced by the lapel-like structure; inset in **C** shows the preoral kineties. **D-F** Left lateral view of the posterior cell half showing the arrangement of the posterior inconspicuous spines (yellow arrowheads), somatic kineties, and ventral kineties. *AM* adoral membranelles, *DK* dorsal keel, *EF* epistomial fringe, *PK* preoral kinety, *S* posterior spine, *SK* somatic kinety, *VK* ventral kinety. Scale bars: 10 μ m.

Fig. 8 Morphology of *Mircalla triangula* nov. comb. in vivo (**A, B, E-J**) and after impregnation (**C, D, K-P**). **A-B** Representation of living specimens, **B** after Kahl 1932. **C, D** Representation of the ciliature. **E-G** Right lateral views of living compressed specimens showing the epistomial fringe, the ridges of the non-ciliated kineties (black arrowheads), and two very short spines (white arrowheads). **H** Macronuclei. **I, J, O, P** left lateral views showing the general shape, the infraciliature, adoral membranelles, the conspicuous ventral spine (white arrow in **J**), and the potential lapel-like structure (white double arrowhead in **J**). The inset in **I** shows the short frontal spine (white arrowhead). **K, L, M** Right lateral view showing the ciliature. **N** Right lateral view showing the non-ciliated kineties. The black double arrowhead in **K, L**, and **P** points to the inverse kinety. *AM* adoral membranelles, *EF* epistomial fringe, *IK* inverse kinety, *Ma* macronucleus, *Mi* micronucleus, *PK* preoral kinety, *SK* somatic kinety, *sVdK* supplementary ventral dikinetids, *VK* ventral kinety, *VS* ventral spine. Scale bars: 10 μ m.

Fig. 9 Morphology of *Epalxella exigua* in vivo (**A, B, E-H**) and after impregnation (**C, D, I-P**). **A, B** Representation of living specimens, **B** after Kahl 1932, and Penard, 1922. **C, D** Representation of the ciliature. **E, I, J** Right lateral views showing the general shape, the epistomial fringe, and the SK6. The asterisk in **E** shows the vaulted lateral side. The black arrowhead points to the anterior end of the SK6, and the double black arrowhead to the fifth epistomial row in **J**. **F, G, L** Left lateral views showing the general shape and the ciliature. The double arrowhead in **G** points to the lapel-like structure; the inset in **L** shows the ventral kinety and the supplementary ventral dikinetids (dotted circle). **H** Left lateral view showing the window-like cortical slit (black arrowhead). **K** Right lateral view showing the non-ciliated kineties (black arrowheads). **M** Left lateral view showing the ciliature on the posterior cell half. **N** The beginning of the preoral kineties. **O** Adoral membranelles. **P** Nuclear apparatus. *EF* epistomial fringe, *IK* inverse kinety, *PK* preoral kinety, *SK* somatic kinety, *sVdK* supplementary ventral dikinetids, *VK* ventral kinety. Scale bars: 10 μ m.

Fig. 10 *Epalxella antiquorum* (A–D) and *Epalxella* cf. *antiquorum* (E–H). A–D Representation of living specimens after Penard, 1922 (A), Kahl 1932 (B, C), and Jankowski 1964 (D). E–H Living specimens showing the general morphology of *Epalxella* cf. *antiquorum*, the white arrowheads point to the posterior spines, the black arrowheads the window-like cortical slits. AM adoral membranelles, EF epistomial fringe, FS frontal spine, Ma macronucleus, PK preoral kinety, SK somatic kinety, VK ventral kinety. Scale bars: 20 µm.

Fig. 11 *Pelodinium reniforme* in vivo (A–C Idaho population, D–F MOKOT population) and after silver carbonate impregnation (G–I Idaho population). A Right lateral view of a slightly squashed cell showing the epistomial fringe (white arrow), the two preoral kineties (black arrowheads), the ciliated (black arrows) and unciliated (between white arrowheads) right somatic kineties, and the contractile vacuole (asterisk). B Left lateral view of same cell as (A) showing the very short left part of the epistomial fringe (white arrow), three of the five ciliated left somatic kineties (white arrowheads), and the blunt posterior spines (black arrowheads). C Right lateral view showing the epistomial fringe (white arrow), two right somatic kineties (white arrowheads), and a blunt posterior spine (black arrowhead). D Right lateral view showing the epistomial fringe (white arrow) and a right somatic kinety (black arrowhead). E Left lateral view showing prominent toothlike preoral spine (white arrowhead) and the adoral membranelles (black arrowhead). F Left lateral view showing cell with two macronuclear nodules, the epistomial fringe (white arrow), the preoral spine (white arrowhead), the adoral membranelles (black arrowhead) and the blunt posterior spines (black arrows). G Right lateral view of the infraciliature showing the epistomial fringe (white arrow), the ciliated (black arrows) and unciliated (between white arrowheads) right somatic kineties, and the preoral kineties (black arrowheads). H Left lateral view showing the adoral membranelles (white arrowhead), inverse kinety (black double arrowhead), epistomial fringe (white arrow), and the left somatic kineties (black arrowheads). I Left lateral view showing the adoral membranelles (black arrow), large prokaryotic endosymbionts (white arrowheads), and the left somatic kineties (black arrowheads). Ma macronucleus. Scale bars: 25 µm.

Fig. 12 *Odontostomatea* sp. from GROSERDU population in vivo (A–I) and after impregnation (J–M). A–I Living specimens in different focal points. J–M Ciliature after protargol impregnation. H, L Right lateral views showing the non-ciliated segments of SK1–5 (black arrowheads). AM adoral membranelles, EF epistomial fringe, PK preoral kinety, SK somatic kinety, VK ventral kinety. Scale bars: 10 µm.

Fig. 13 Morphology of *Mylestoma bipartitum* in vivo (A–C, G–M) and after impregnation (D–F, N–R). A–C Representation of living specimens; after Kahl 1928 (B), and Gourret and Roeser 1886 (C). D–F Representation of the ciliature, purple arrows point to the spines. G, H, I, O Left lateral views showing the general shape of the cell and the infraciliature; white arrowheads in H point to the ventral spines. J, K Right lateral view of the posterior cell half showing the posterior spines and the ventral kinety. L Ventral margin showing the lapel-like structure (white double arrowhead) and the inverse kinety (white arrowhead). M Ventrolateral view shows the furrows of the preoral kineties (white arrowheads) and the membranelles (black arrowhead). N Right lateral view shows the non-ciliated kineties and the inset the nuclear apparatus. P, Q Ventral margin showing the supplementary ventral dikinetid on a spine, and the ventral kinety. R Left lateral view of the posterior cell half showing the membranelles and the caudal kineties in a semikinetal pattern. The black arrow in J, P, and Q points to an additional dikinetid with long cilia. AM adoral membranelles, CC caudal cirri, EF epistomial fringe, IK inverse kinety, PK preoral kinety, SK somatic kinety, sVdK supplementary ventral dikinetid, VK ventral kinety. Scale bars: 10 µm.

Fig. 14 Morphology of *Mylestoma monodontum* sp. nov. after impregnation (A, B) and scanning electron microscopy (C, D). A, B Representation of the ciliature on the left side. C–D Left side showing the lapel-like structure, the inverse kinety (yellow double arrowhead), and preoral kineties.

The white arrowheads show the beginning of the SK6 and 7. **D** right posterior ventral margin showing the dikinetids of the ventral kinety (yellow arrowheads). *CC* caudal cirri, *EF* epistomial fringe, *IK* inverse kinety, *PK* preoral kinety, *SK* somatic kinety, *VK* ventral kinety. Scale bars: 10 μ m.

Fig. 15 Morphology of *Limnomylestoma shuriken* nov. sp. in vivo (**A, D-H**) and after impregnation (**B, C, I-L**). **A** Representation of a living specimen. **B, C** Representation of the ciliature and disposition of spines (black arrows). **D-G** Different focal points of the left (**D, E, F**) and right (**G**) lateral views show the general shape and the spines (black arrows). **H** Left lateral view showing the disposition of the caudal cirri (black double arrowheads) and their cilia. **I-L** Right (**I, K**) and left (**J, L**) lateral views show the infraciliature, and the inset in **J** shows the reduced epistomial fringe. The black arrowheads in **K** and **L** point to the non-ciliated kineties; the amphikinetal arrangement of the caudal kineties are shown in **L**. *AM* adoral membranelles, *CC* caudal cirri, *EF* epistomial fringe, *IK* inverse kinety, *Ma* macronucleus, *PK* preoral kinety, *SK* somatic kinety, *VK* ventral kinety. Scale bars: 10 μ m.

Fig. 16 Scanning electron micrographs of *Limnomylestoma shuriken* gen. nov., sp. nov. **A-D** Right (**A, B**) and left (**C, D**) lateral views showing the general shape, the ciliature, the spines (yellow arrows), and the posterior fin-like projection (double yellow arrowheads). **E-G** Left lateral views showing the opening of the buccal cavity, the membranelles, the inverse kinety, and the six dikinetids of ventral kinety (**E**); the lapel-like structure as a lamina (**F**); and the epistomial fringe pectinelles (asterisks) and preoral kineties (**G**). **H** Left lateral view of the posterior margin showing the two caudal cirri separated by a gap (amphikinetal pattern). The yellow arrowheads in **A, C**, and **E** point to the dikinetids of the ventral kinety. *AM* Adoral membranelles, *CC* caudal cirri, *EF* epistomial fringe, *HS* hood-like structure, *IK* inverse kinety, *PK* preoral kinety. Scale bars: 10 μ m.

Fig. 17 Morphology of *Tostonella uncinata* gen. nov., comb. nov. in vivo (**A, B, E-I**) and after impregnation (**C, D, J-N**). **A** Representation of living specimens, after Penard, 1922 (**B**). **C, D** Representation of the ciliature. **E, F, I-K** Left (**E, J, K**) and right (**F, I**) lateral views showing the general shape, infraciliature (**J, K**), and the spines (**E, F, I**). **G** Longitudinal crosssection in ventral view showing two posterior spines and the macronucleus. **H, M** Epistomial fringe and preoral kineties, black arrow points to the displaced pectinelle. **L** adoral Membranelles. **N** Nuclear apparatus. *AM* adoral membranelles, *BL* buccal lip, *CC* caudal cirri, *EF* epistomial fringe, *Ma* macronucleus, *PK* preoral kinety, *S* spine, *SK* somatic kinety, *VK* ventral kinety. Scale bars: 10 μ m.

Fig. 18 Scanning electron micrographs of *Tostonella uncinata* gen. nov., comb. nov. **A, D** Right lateral view showing the three posterior spines (**A**), and the folded dorsal keel. **B, E** Ventrolateral view showing the ventral ciliature (**B**), and the opening of the buccal cavity showing the membranelles, the buccal lip, and the dikinetids of the ventral kinety (yellow arrowheads in **E**). **C** Postero-right lateral view showing the disposition of the three spines. **F** Buccal cavity showing the membranelles. **G** Postero-right lateral margin showing two spines and the caudal kineties; the yellow arrowheads show the dikinetids of the ventral kinety. *AM* adoral membranelles, *BL* buccal lip, *CC* caudal cirri, *DK* dorsal keel, *EF* epistomial fringe, *PK* preoral kinety, *S* spine. Scale bars: 10 μ m.

Fig. 19 Morphology of *Discomorphella pectinata* in vivo (**A-G**) and after impregnation (**H-N**). **A, B, D** Right lateral view of two specimens in different focal points showing the frontal awning of the epistomial fringe and the posterior spine. **C** A specimen in ventral view showing the lateral spine. **E, I** Part of the epistomial fringe showing the pectinelles and the preoral kinety below it; the white arrowheads in **I** point to the non-ciliated kineties. **F** Left lateral view showing the fragmented epistomial fringe and caudal cirri (white arrowheads). **G** Left epistomial fringe fragment of two specimens showing the disposition of a fourth spine (white arrows); the arrowheads point to the pectinelles. **H, M** Right (**H**) and left (**M**) sides of two specimens showing the ciliature. **J** End of the dextro-frontal epistomial fringe fragment showing the papillary dikinetids (encircled). **K** Endoral kineties. **L** Nuclear apparatus, and adoral membranelles. **N** Disposition of the ventral kineties and

supplementary ventral dikinets. *AM* adoral membranelles, *CV* contractile vacuole, *DfF* dextro-frontal fragment of EF, *EF* epistomial fringe, *EFS* epistomial fringe spacer, *FS* frontal spine, *iEK* inner endoral kinety, *LF* left fragment of EF, *Ma* macronucleus, *Mi* micronucleus, *oEK* outer endoral kinety, *Pec* pectinelle, *PK* preoral kinety, *S* spine, *sVdK* supplementary ventral dikinets, *VK*: ventral kinety. Scale bars: 20 μ m (A-D, F-H, M, N), 10 μ m (E, I-L).

Fig. 20 Scanning electron micrographs of *Discomorphella pectinata*. **A, B** Right (**A**) and left (**B**) lateral view of two specimens showing the general morphology. **C** Preoral kinety with small pectinelles. **D** Papillary dikinets (yellow arrowheads) at the end of the dextro-frontal epistomial fragment. **E** Arrangement of pectinelles of the dextro-frontal epistomial fragment. **F** Beginning and disposition of pectinelles of the dextro-frontal epistomial fragment. **G** Posterior left ventral margin showing the ventral flap bearing the ventral kineties. **H** Caudal cirri. **I-K** Arrangement of the left epistomial fragment of three different specimens; yellow arrowhead points to the first pectinelle, white arrow points to the associated short spine. *DfF* dextro-frontal fragment of EF, *EF* epistomial fringe, *EFS* epistomial fringe spacer, *FS* frontal spine, *LF* left fragment of EF, *S* spine, *VK* ventral kinety. Scale bars: 10 μ m (A, B, G), 2 μ m (C-E, H), 5 μ m (F, I-K).

Supplementary figures

Fig. S1 Environmental phylogenetic tree of Odontostomatea. Labels in blue represent the obtained sequences in this study, and in green are the previously deposited sequences in GenBank.

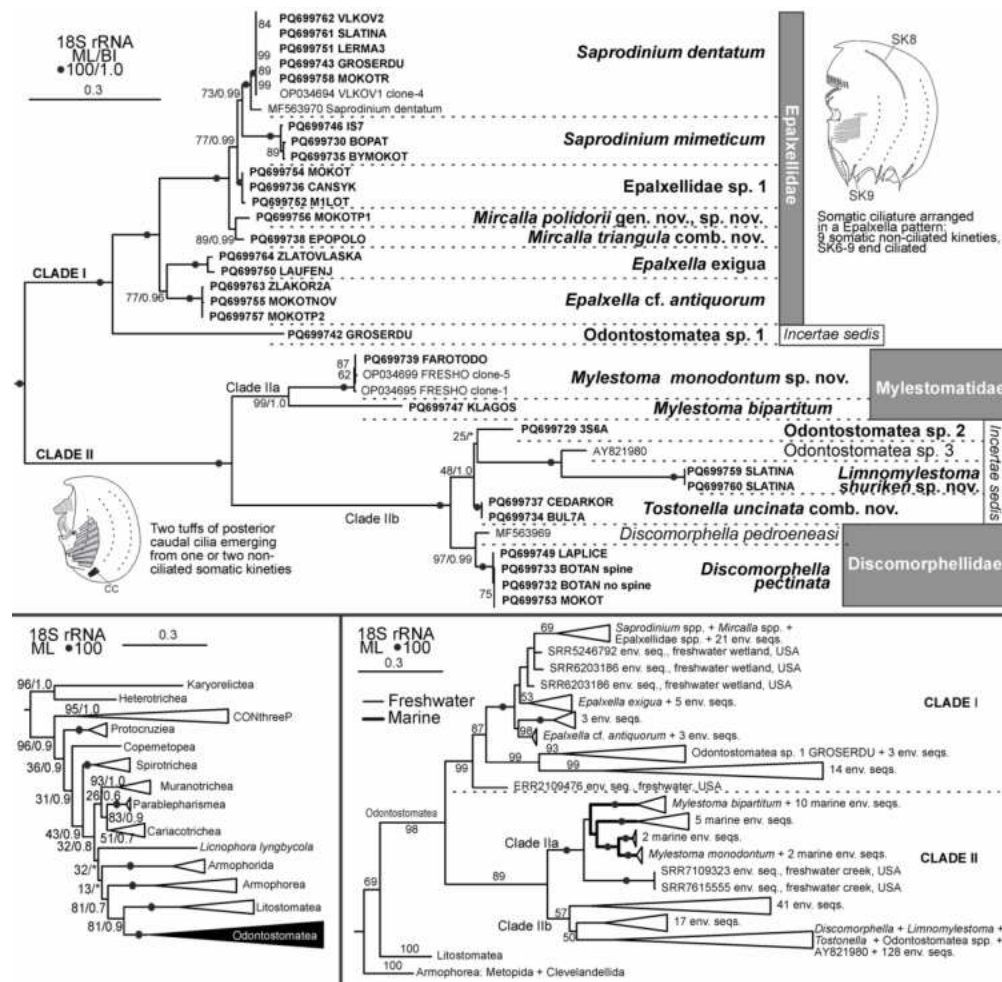


Fig. 1 Phylogeny of Odontostomatea based on 18S rRNA gene. A ML phylogenetic tree showing the newly sequenced strains and species (in bold) of odontostomateans. B ML tree showing the position of Odontostomatea within Ciliophora after adding the new sequences. C ML phylogenetic tree including the environmental odontostomatean sequences from metatranscriptomes and metagenomes, plus the newly obtained sequences. For a full tree refer to Figure S1. Branch values represent bootstrap support of ML and Bayesian posterior probabilities of BI, values below 50 and 0.80, respectively, are not shown or are represented by an (*). Scale bars: 30 changes per 100 nucleotides.

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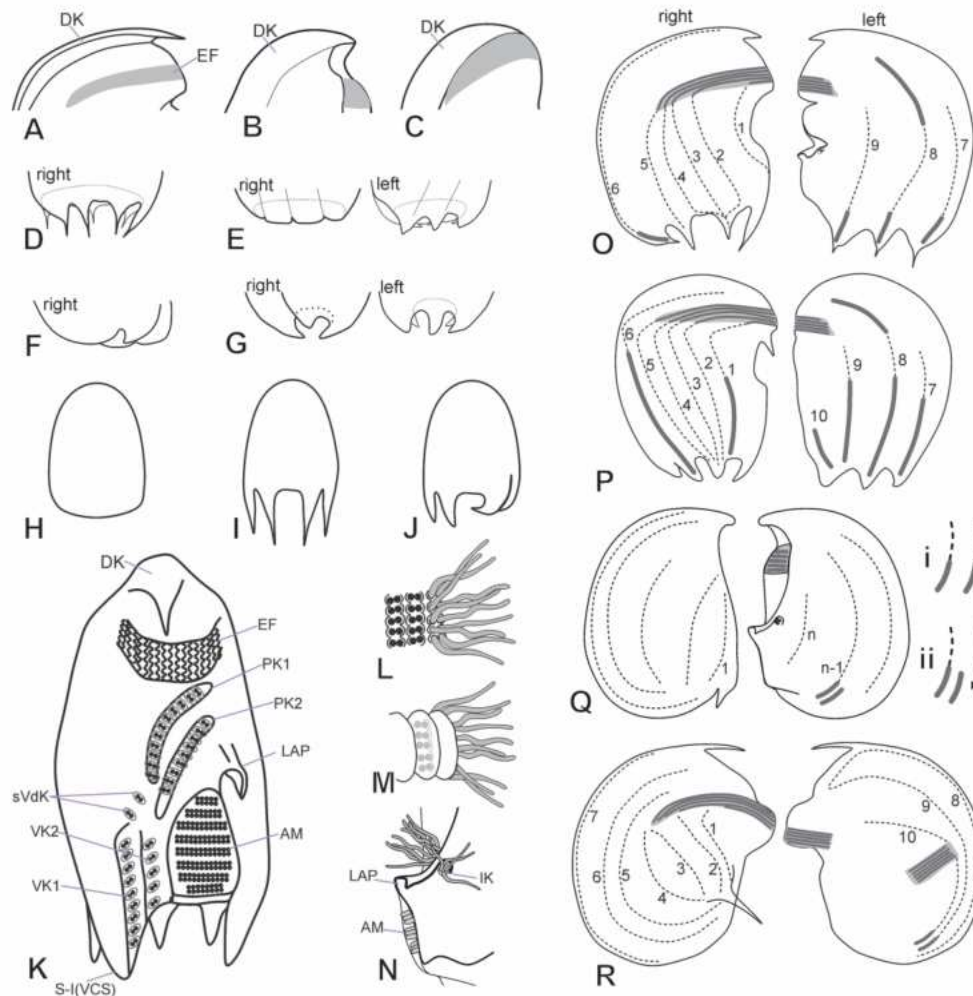
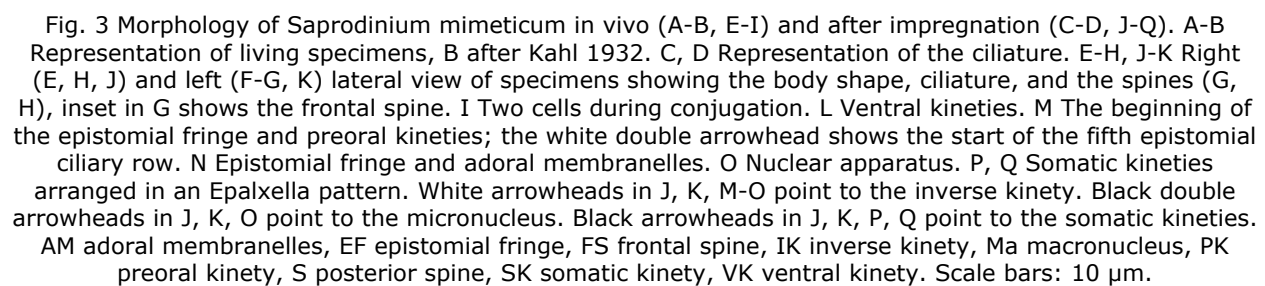


Fig. 2 General morphology of Odontostomatea in vivo (A–J) and after impregnation (K–R). A–C Shape of the anterior region of the cell on the lateral view, ending as a frontal spine (A), as a hood-like structure (B) or blunt (C). D–G Shape of the posterior end, on lateral views, with prominent spines and broad posterior marginal concavity (D), wavy and with inconspicuous spines and broad posterior marginal concavity (E), smooth with reduced spines (F), or small heart-shaped concavity (G). H–J Posterior end in dorsal view ending smooth (H), with spines on both sides (I), or with concavity with inwardly folding spines (J). K General ventral view of the ciliature. L, M Organization of the pectinellae without (L) or with auricles (M). N Left lateral view of the buccal area showing the disposition of the lapel-like structure and the inverse kinety. O–R Different ciliature patterns: Epalxella pattern (O), Pelodinium pattern (P), Mylestoma pattern (Q) with caudal cirrus arranged as an amphikinetal pattern (Q_i), and a semikinetal pattern (Q_{ii}), and Discomorphella pattern (R). AM adoral membranelles, DK dorsal keel, EF epistomial fringe, IK inverse kinety, LAP lapel-like structure, PK preoral kinety, S-1 spine 1, sVdK supplemental ventral dikinetid, VCS ventrocaudal spine, VK ventral kinety.

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173x166mm (300 x 300 DPI)

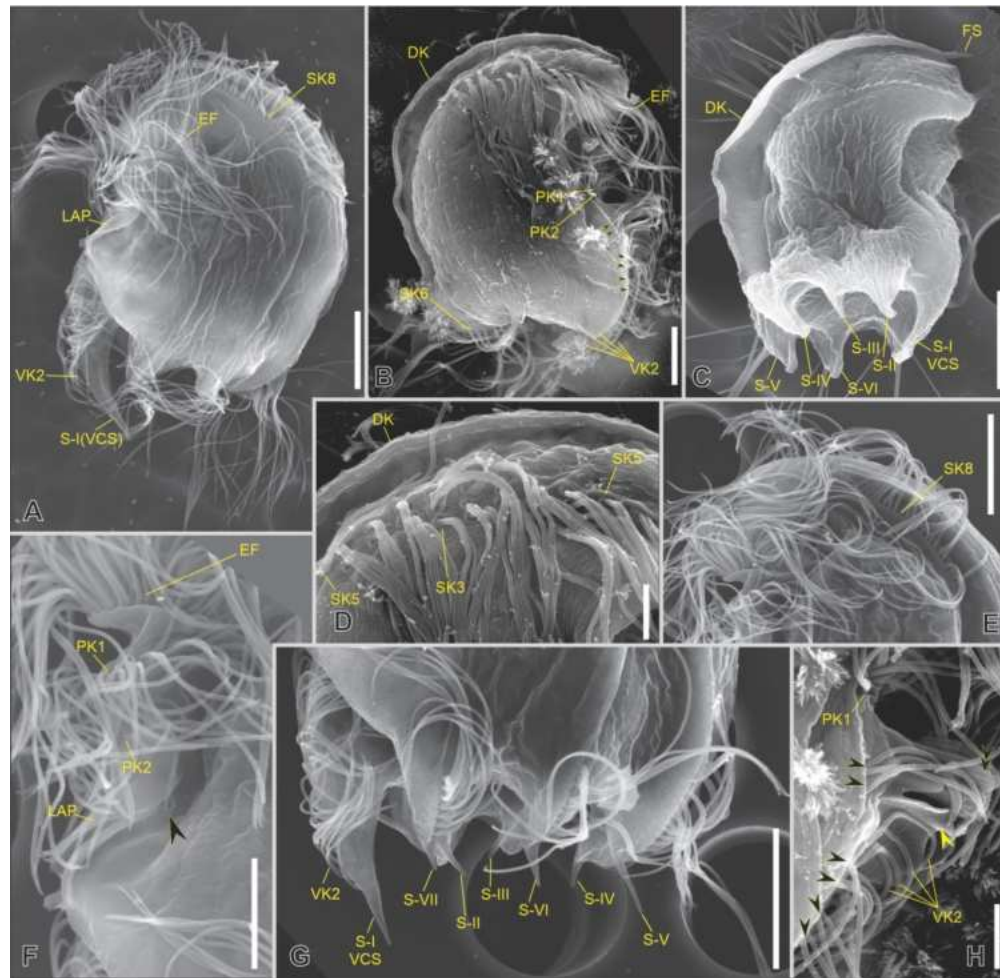


Fig. 4 Scanning electron micrographs of *Saprodinium mimeticum*. A-C Left (A) and right (B-C) lateral views show the cell shape and the spines. D Close-up view of B shows the epistomial fringe and the dorsal keel. E Left lateral view of the anterior cell half shows the anterior ciliated fragment of SK8. F Left lateral view of the mid-ventral edge showing the lapel-like structure, the inverse kinety (black arrowhead), the preoral kineties, and the ventral ridge bearing the epistomial fringe. G Left lateral view of the posterior cell half shows the thorn-like spines. H Close-up view of B showing the ventral kineties, dikinetids of VK1 are pointed by black arrowheads, the buccal lip (yellow arrowhead), and the membranelles (black double arrowhead). DK dorsal keel, EF epistomial fringe, FS frontal spine, LAP lapel-like structure, PK preoral kinety, S posterior spine, SK somatic kinety, VK ventral kinety. Scale bars: 10 μ m (A-C), 5 μ m (D-H).

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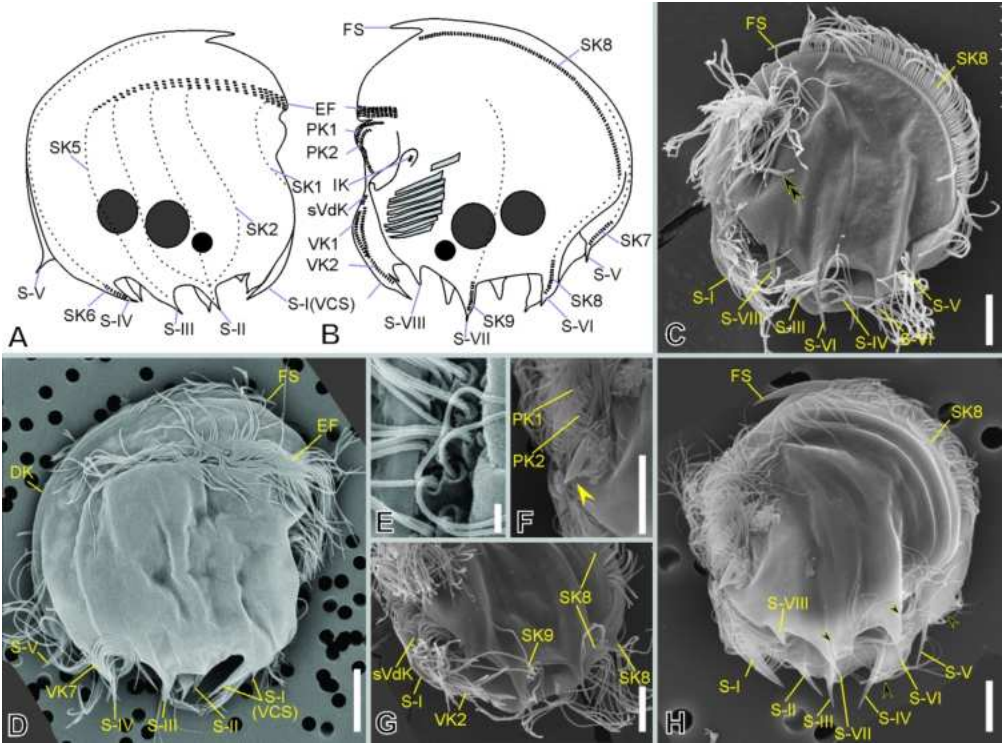


Fig. 5 Morphology of *Saprodinium dentatum* after impregnation (A-B) and in electron scanning microscopy (C-H). A-B Representation of the ciliature. C, D, H left (C, H) and right (D) lateral views showing the general shape, spines, and spine segments of kineties, black double arrowhead in C shows the inverse kinety, while black arrowheads in H point to the spine segments of kineties. E Epistomial ciliary sockets bearing dikinetids. F Left lateral view of the mid-ventral margin showing the lapel-like structure (yellow arrowhead) and preoral kineties. G Ventro-lateral view showing the posterior cavity surrounded by spines. DK dorsal keel, EF epistomial fringe, FS frontal spine, IK inverse kinety, PK preoral kinety, S posterior spine, SK somatic kinety, sVdK supplementary ventral dikinetids, VK ventral kinety. Scale bars: 10 μ m (C, D, F-H), 2 μ m (E).

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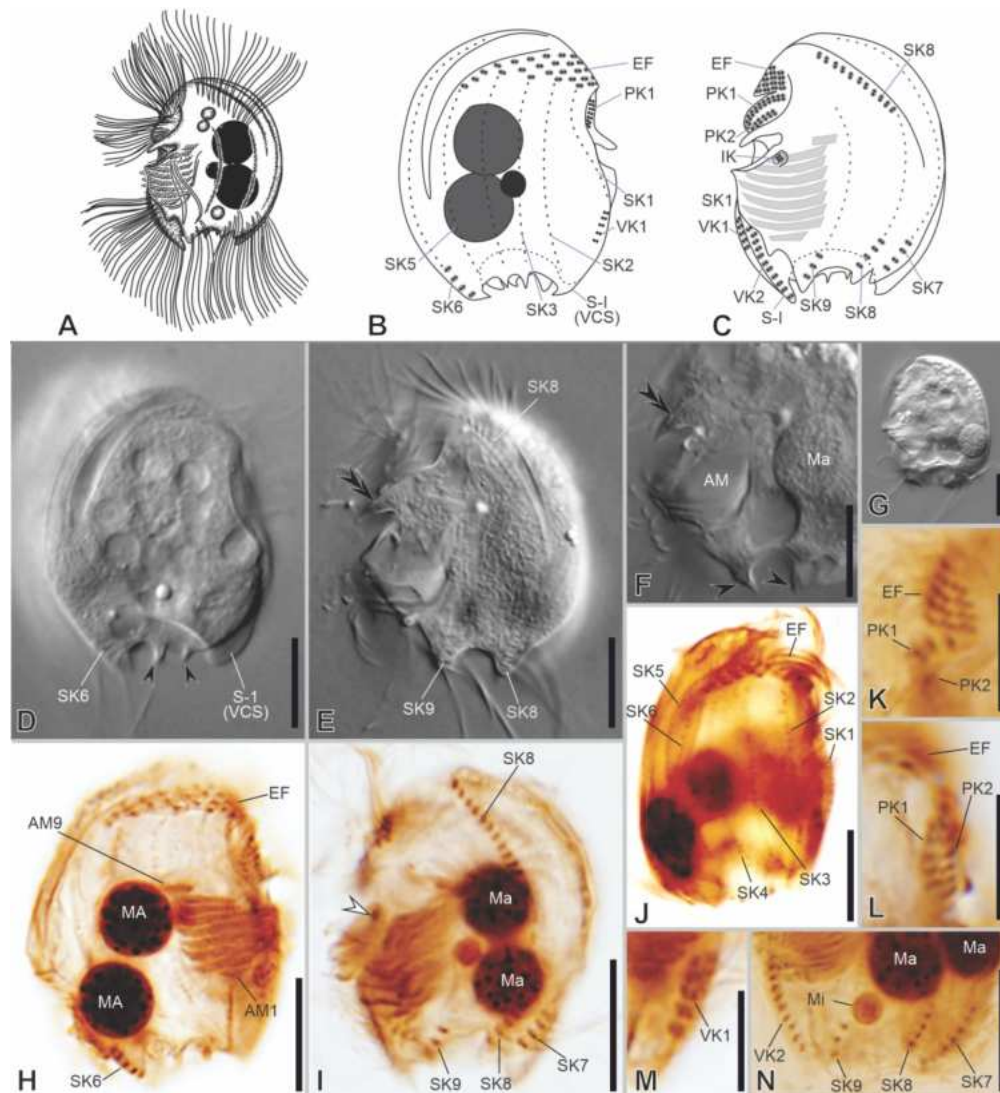


Fig. 6 Morphology of *Mircalla polidorii* gen. nov., sp. nov. in vivo (A, D-G), after impregnation (B, C, H-N). A Representation of a living specimen. B, C Representation of the ciliature. D, E, G, H, I Right (D, H) and left (E, G, I) lateral views show the general shape and infraciliature; the white arrowhead in I shows the inverse kinety. F Left lateral view shows the ventral spine (black double arrowhead), the adoral membranelles, and two inner spines (black arrowheads). J Right lateral view showing the non-ciliated segments of the SK1-6. K, L Beginning (K) and continuation (L) of the epistomial fringe and preoral kineties. M Ventral kinety 1. N Posterior end showing the VK2 and the associated spine kineties of SK7-9. Black arrowheads in D and F point to two short posterior spines. A black double arrowhead in E and F points to the ventral spine. AM adoral membranelles, EF epistomial fringe, IK inverse kinety, Ma macronucleus, Mi micronucleus, PK preoral kinety, SK somatic kinety, VCS ventrocaudal spine (spine 1), VK ventral kinety. Scale bars: 10 µm.

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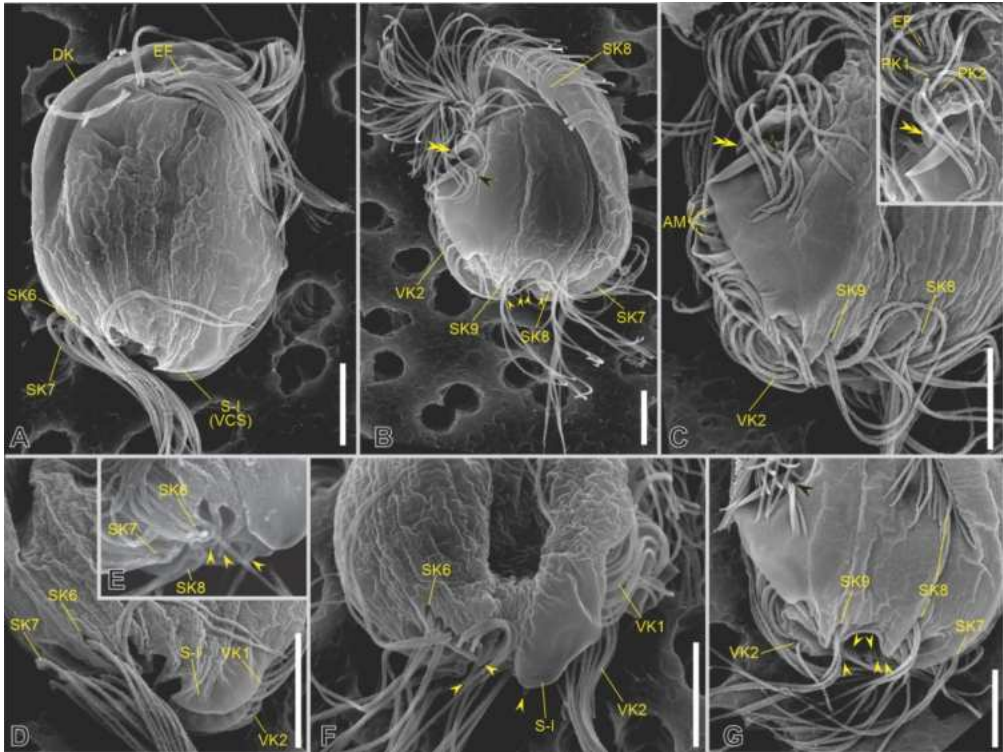


Fig. 7 Scanning electron micrographs of *Mircalla polidorii* gen. nov., sp. nov. A, B Right (A) and left (B) lateral views showing the general shape. C, G Left lateral views showing the arrangement of the ciliature; the double yellow arrowheads point to the ventral spine, and the black arrowhead the inverse kinety embraced by the lapel-like structure; inset in C shows the preoral kineties. D-F Left lateral view of the posterior cell half showing the arrangement of the posterior inconspicuous spines (yellow arrowheads), somatic kineties, and ventral kineties. AM adoral membranelles, DK dorsal keel, EF epistomial fringe, PK preoral kinety, S posterior spine, SK somatic kinety, VK ventral kinety. Scale bars: 10 μ m.

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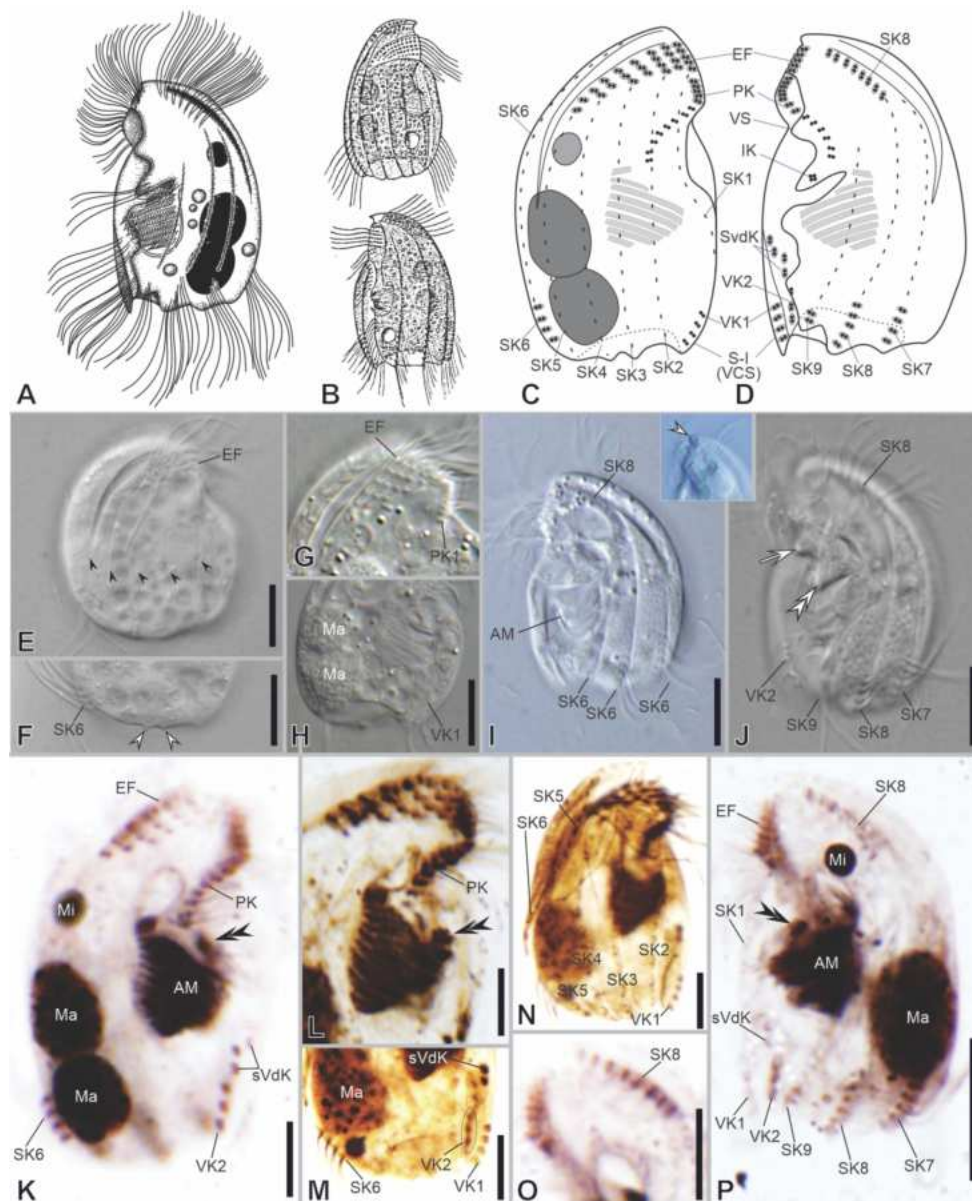


Fig. 8 Morphology of *Mircalla triangula* nov. comb. in vivo (A, B, E-J) and after impregnation (C, D, K-P). A-B Representation of living specimens, B after Kahl 1932. C, D Representation of the ciliate. E-G Right lateral views of living compressed specimens showing the epistomial fringe, the ridges of the non-ciliated kineties (black arrowheads), and two very short spines (white arrowheads). H Macronuclei. I, J, O, P left lateral views showing the general shape, the infraciliature, adoral membranelles, the conspicuous ventral spine (white arrow in J), and the potential lapel-like structure (white double arrowhead in J). The inset in I shows the short frontal spine (white arrowhead). K, L, M Right lateral view showing the ciliature. N Right lateral view showing the non-ciliated kineties. The black double arrowhead in K, L, and P points to the inverse kinety. AM adoral membranelles, EF epistomial fringe, IK inverse kinety, Ma macronucleus, Mi micronucleus, PK preoral kinety, SK somatic kinety, sVdK supplementary ventral dikinetids, VK ventral kinety, VS ventral spine. Scale bars: 10 µm.

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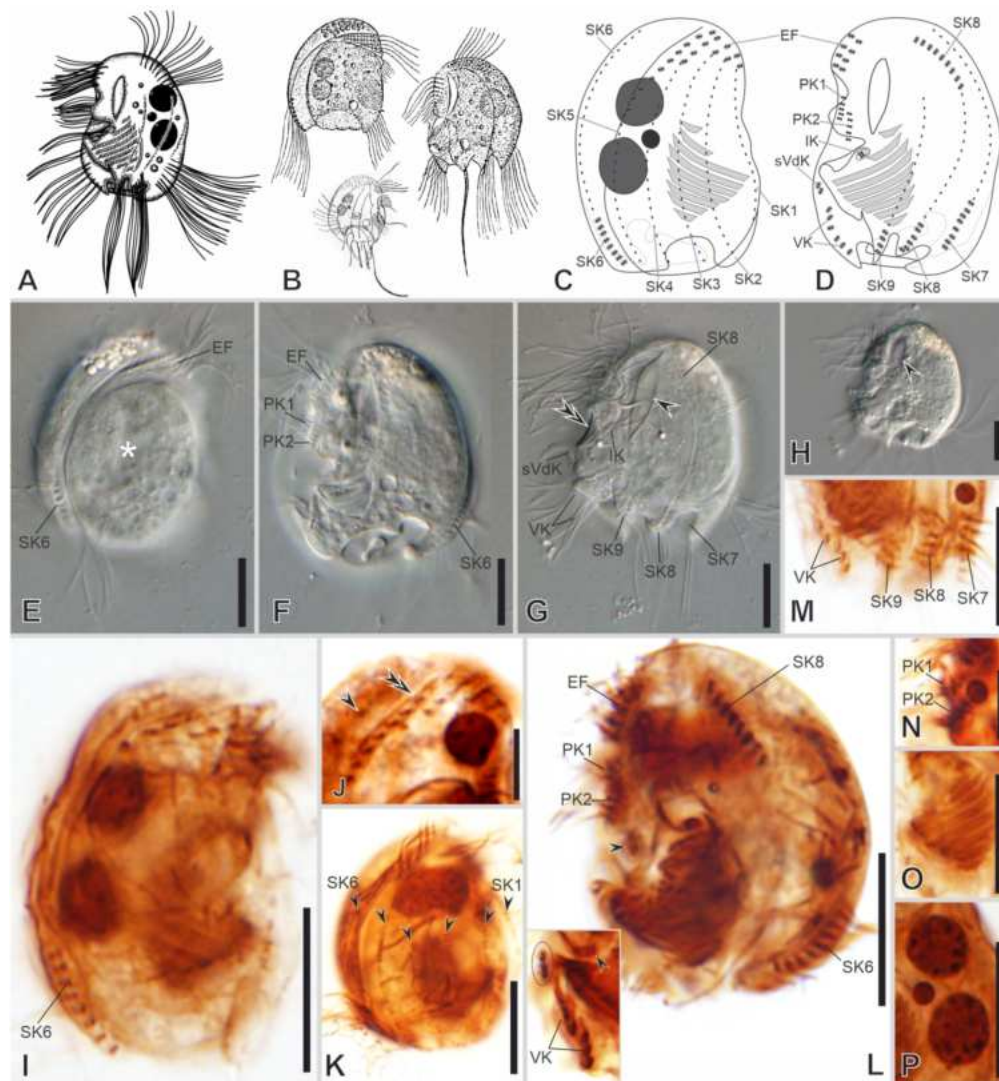


Fig. 9 Morphology of *Epalxella exigua* in vivo (A, B, E-H) and after impregnation (C, D, I-P). A, B Representation of living specimens, B after Kahl 1932, and Penard, 1922. C, D Representation of the ciliature. E, I, J Right lateral views showing the general shape, the epistomial fringe, and the SK6. The asterisk in E shows the vaulted lateral side. The black arrowhead points to the anterior end of the SK6, and the double black arrowhead to the fifth epistomial row in J. F, G, L Left lateral views showing the general shape and the ciliature. The double arrowhead in G points to the lapel-like structure; the inset in L shows the ventral kinety and the supplementary ventral dikinetids (dotted circle). H Left lateral view showing the window-like cortical slit (black arrowhead). K Right lateral view showing the non-ciliated kineties (black arrowheads). M Left lateral view showing the ciliature on the posterior cell half. N The beginning of the preoral kineties. O Adoral membranelles. P Nuclear apparatus. EF epistomial fringe, IK inverse kinety, PK preoral kinety, SK somatic kinety, sVdK supplementary ventral dikinetids, VK ventral kinety. Scale bars: 10 μ m.

173x187mm (300 x 300 DPI)

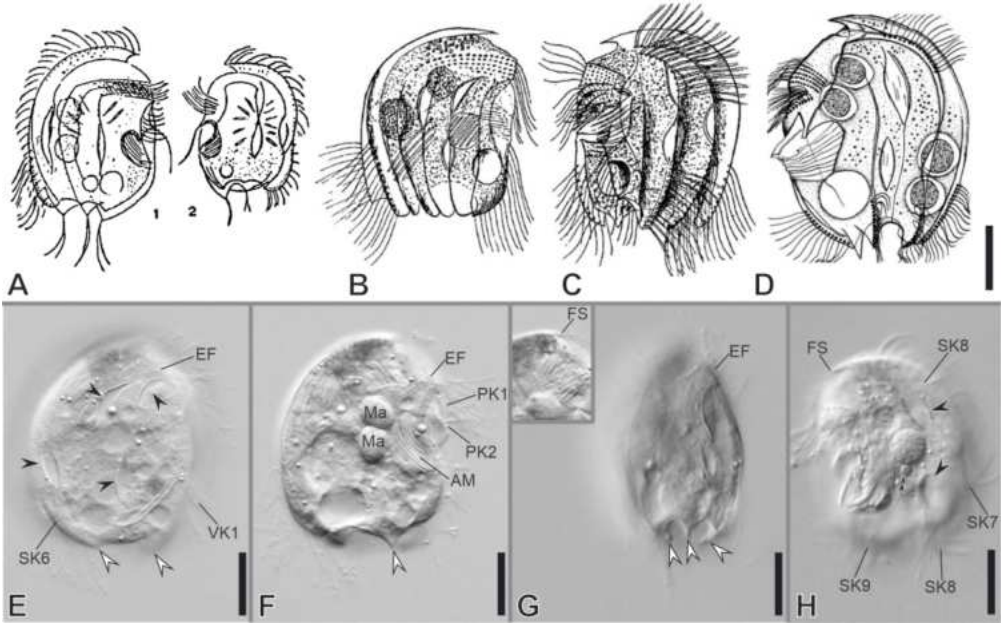


Fig. 10 *Epalxella antiquorum* (A-D) and *Epalxella* cf. *antiquorum* (E-H). A-D Representation of living specimens after Penard, 1922 (A), Kahl 1932 (B, C), and Jankowski 1964 (D). E-H Living specimens showing the general morphology of *Epalxella* cf. *antiquorum*, the white arrowheads point to the posterior spines, the black arrowheads the window-like cortical slits. AM adoral membranelles, EF epistomial fringe, FS frontal spine, Ma macronucleus, PK preoral kinety, SK somatic kinety, VK ventral kinety. Scale bars: 20 µm.

173x108mm (300 x 300 DPI)

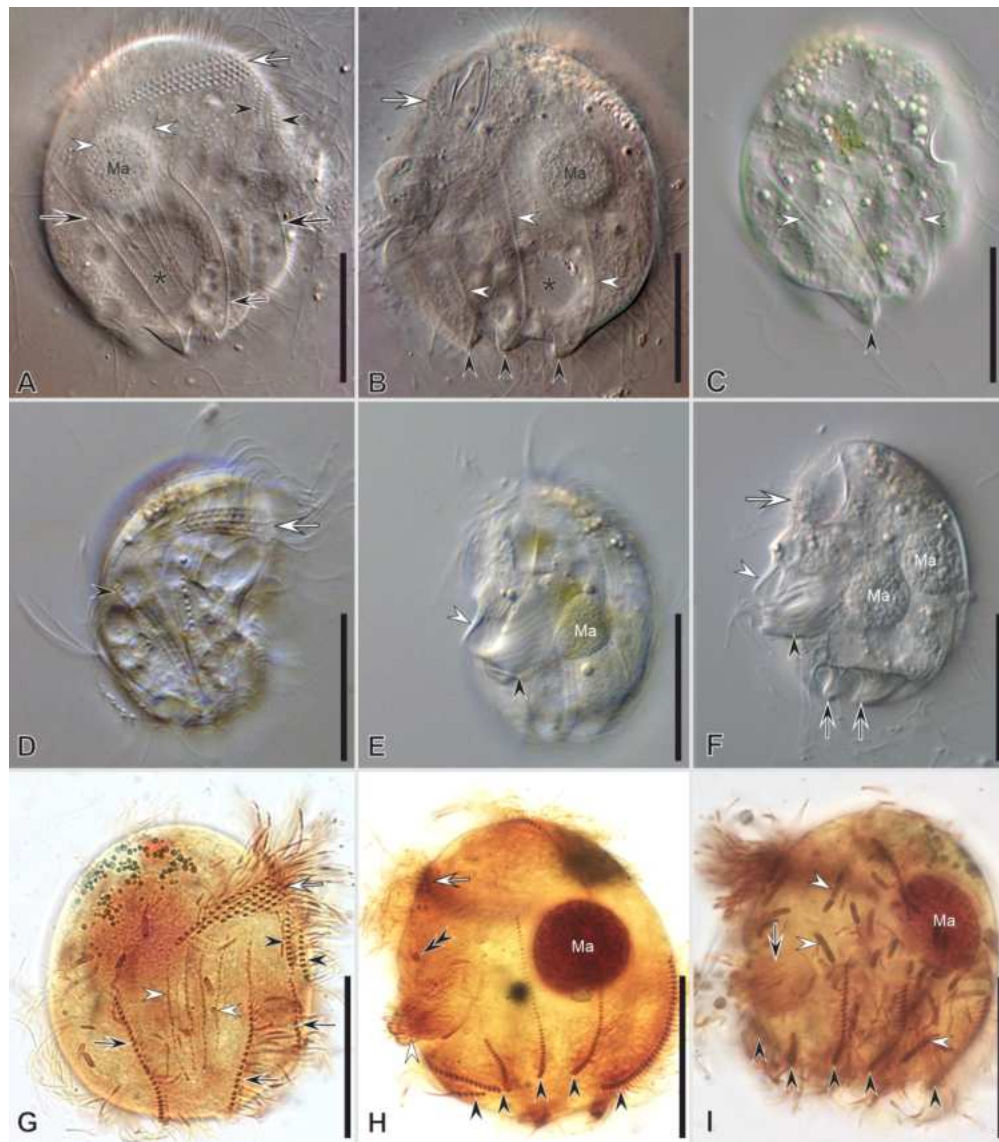


Fig. 11 *Pelodinium reniforme* in vivo (A–C Idaho population, D–F MOKOT population) and after silver carbonate impregnation (G–I Idaho population). A Right lateral view of a slightly squashed cell showing the epistomial fringe (white arrow), the two preoral kineties (black arrowheads), the ciliated (black arrows) and unciliated (between white arrowheads) right somatic kineties, and the contractile vacuole (asterisk). B Left lateral view of same cell as (A) showing the very short left part of the epistomial fringe (white arrow), three of the five ciliated left somatic kineties (white arrowheads), and the blunt posterior spines (black arrowheads). C Right lateral view showing the epistomial fringe (white arrow), two right somatic kineties (white arrowheads), and a blunt posterior spine (black arrowhead). D Right lateral view showing the epistomial fringe (white arrow) and a right somatic kinety (black arrowhead). E Left lateral view showing prominent toothlike preoral spine (white arrowhead) and the adoral membranelles (black arrowhead). F Left lateral view showing cell with two macronuclear nodules, the epistomial fringe (white arrow), the preoral spine (white arrowhead), the adoral membranelles (black arrowhead) and the blunt posterior spines (black arrows). G Right lateral view of the infraciliature showing the epistomial fringe (white arrow), the ciliated (black arrows) and unciliated (between white arrowheads) right somatic kineties, and the preoral kineties (black arrowheads). H Left lateral view showing the adoral membranelles (white arrowhead), inverse kinety (black double arrowhead), epistomial fringe (white arrow), and the left somatic kineties (black arrowheads). I Right lateral view showing the epistomial fringe (white arrow), the ciliated (black arrows) and unciliated (between white arrowheads) right somatic kineties, and the preoral kineties (black arrowheads).

I Left lateral view showing the adoral membranelles (black arrow), large prokaryotic endosymbionts (white arrowheads), and the left somatic kineties (black arrowheads). Ma macronucleus. Scale bars: 25 µm.

173x197mm (300 x 300 DPI)

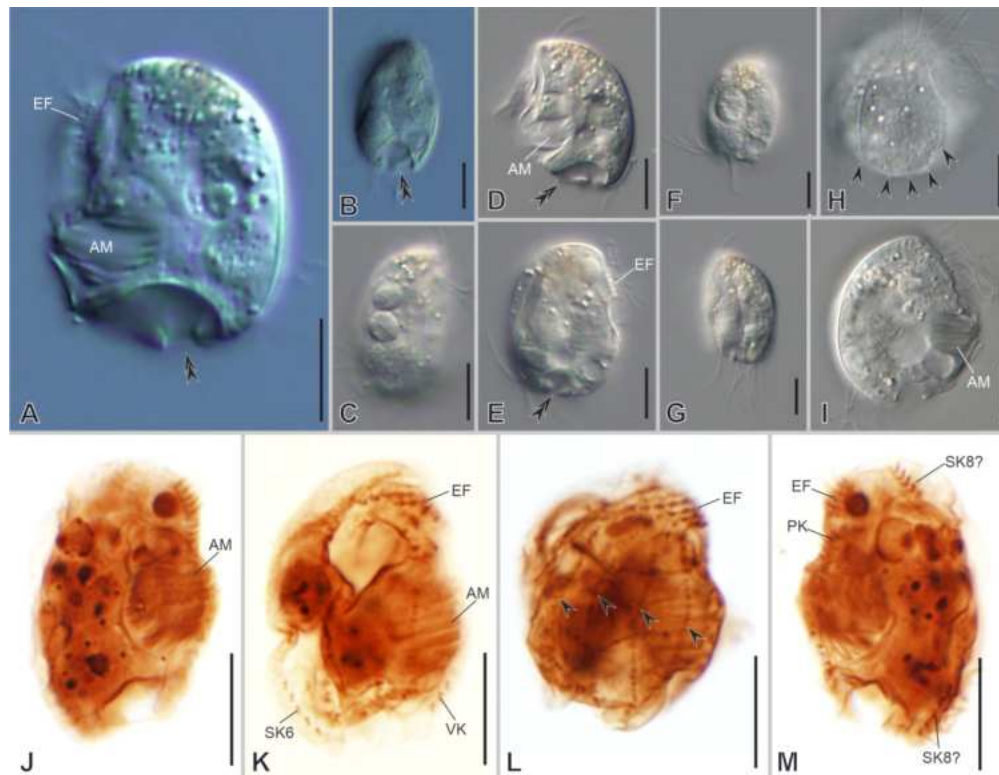


Fig. 12 *Odontostomatea* sp. from GROSERDU population in vivo (A-I) and after impregnation (J-M). A-I Living specimens in different focal points. J-M Ciliature after protargol impregnation. H, L Right lateral views showing the non-ciliated segments of SK1-5 (black arrowheads). AM adoral membranelles, EF epistomial fringe, PK preoral kinety, SK somatic kinety, VK ventral kinety. Scale bars: 10 μ m.

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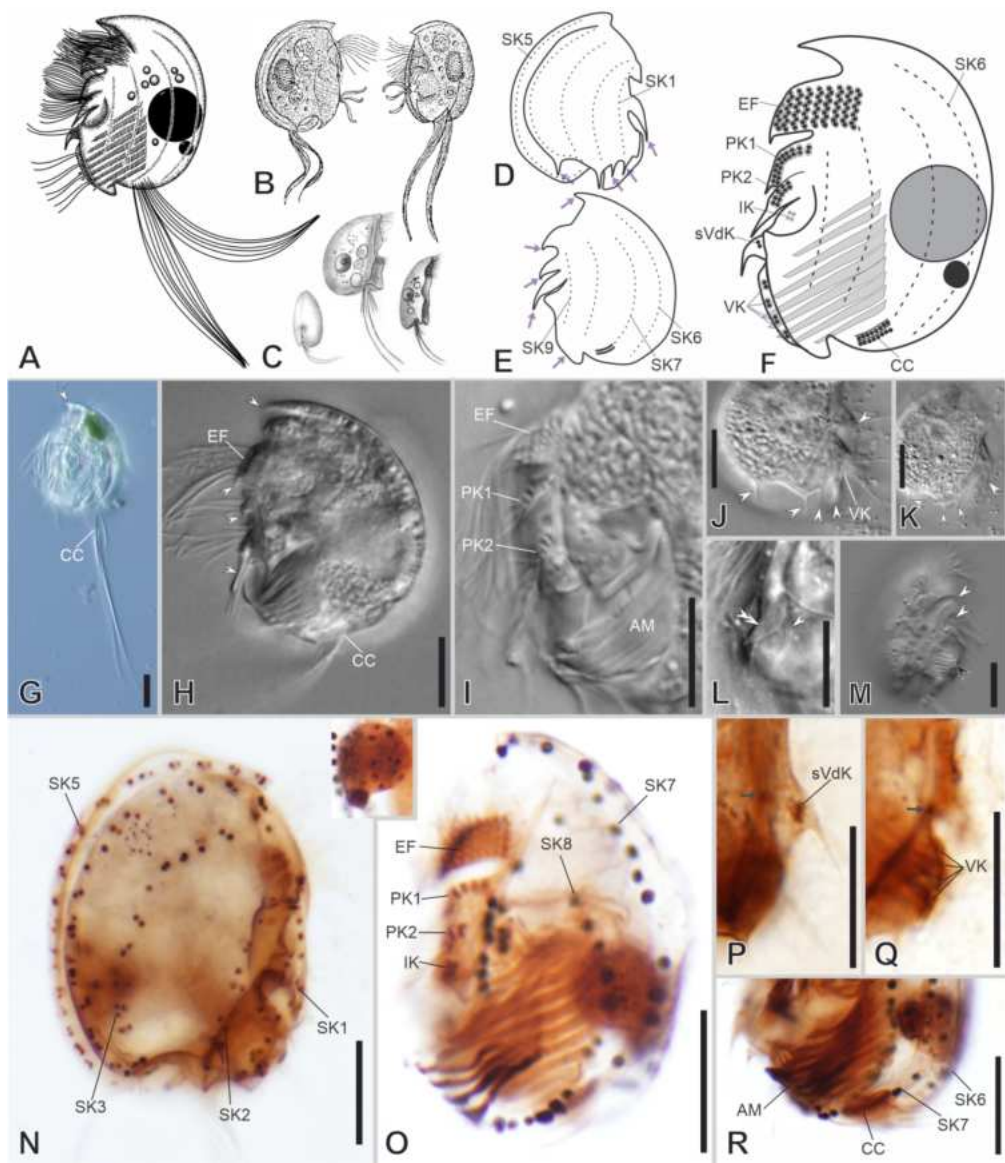


Fig. 13 Morphology of *Mylestoma bipartitum* in vivo (A-C, G-M) and after impregnation (D-F, N-R). A-C Representation of living specimens; after Kahl 1928 (B), and Gourret and Roeser 1886 (C). D-F Representation of the ciliature, purple arrows point to the spines. G, H, I, O Left lateral views showing the general shape of the cell and the infraciliature; white arrowheads in H point to the ventral spines. J, K Right lateral view of the posterior cell half showing the posterior spines and the ventral kinety. L Ventral margin showing the lapel-like structure (white double arrowhead) and the inverse kinety (white arrowhead). M Ventrolateral view shows the furrows of the preoral kineties (white arrowheads) and the membranelles (black arrowhead). N Right lateral view shows the non-ciliated kineties and the inset the nuclear apparatus. P, Q Ventral margin showing the supplementary ventral dikinetid on a spine, and the ventral kinety. R Left lateral view of the posterior cell half showing the membranelles and the caudal kineties in a semikinetal pattern. The black arrow in J, P, and Q points to an additional dikinetid with long cilia. AM adoral membranelles, CC caudal cirri, EF epistomial fringe, IK inverse kinety, PK preoral kinety, SK somatic kinety, sVdK supplementary ventral dikinetid, VK ventral kinety. Scale bars: 10 μ m.

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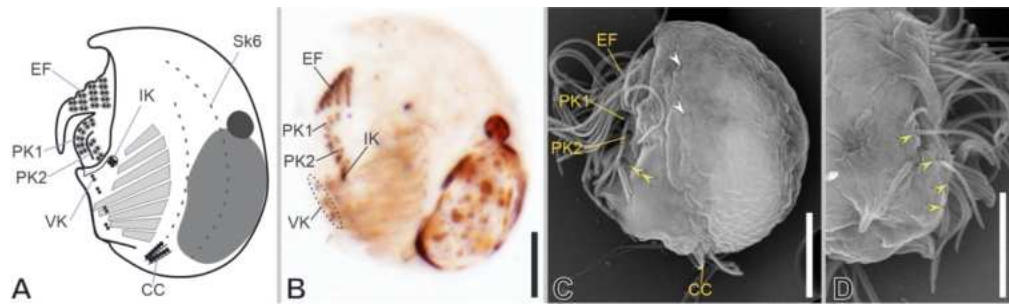


Fig. 14 Morphology of *Mylestoma monodontum* sp. nov. after impregnation (A, B) and scanning electron microscopy (C, D). A, B Representation of the ciliature on the left side. C-D Left side showing the lapel-like structure, the inverse kinety (yellow double arrowhead), and preoral kineties. The white arrowheads show the beginning of the SK6 and 7. D right posterior ventral margin showing the dikinetids of the ventral kinety (yellow arrowheads). CC caudal cirri, EF epistomial fringe, IK inverse kinety, PK preoral kinety, SK somatic kinety, VK ventral kinety. Scale bars: 10 µm.

173x51mm (300 x 300 DPI)

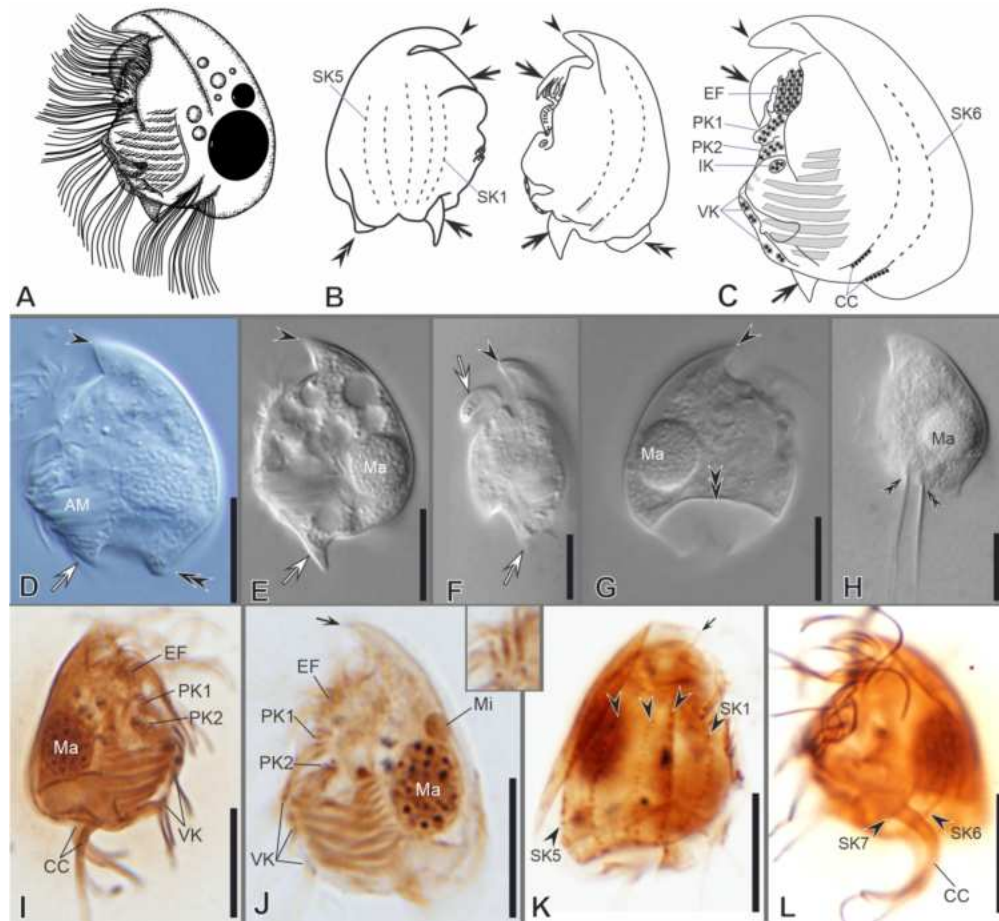


Fig. 15 Morphology of *Limnomylestoma shuriken* nov. sp. in vivo (A, D-H) and after impregnation (B, C, I-L). A Representation of a living specimen. B, C Representation of the ciliature and disposition of spines (black arrows). D-G Different focal points of the left (D, E, F) and right (G) lateral views show the general shape and the spines (black arrows). H Left lateral view showing the disposition of the caudal cirri (black double arrowheads) and their cilia. I-L Right (I, K) and left (J, L) lateral views show the infraciliature, and the inset in J shows the reduced epistomial fringe. The black arrowheads in K and L point to the non-ciliated kineties; the amphikinetal arrangement of the caudal kineties are shown in L. AM adoral membranelles, CC caudal cirri, EF epistomial fringe, IK inverse kinety, Ma macronucleus, PK preoral kinety, SK somatic kinety, VK ventral kinety. Scale bars: 10 µm.

173x160mm (300 x 300 DPI)

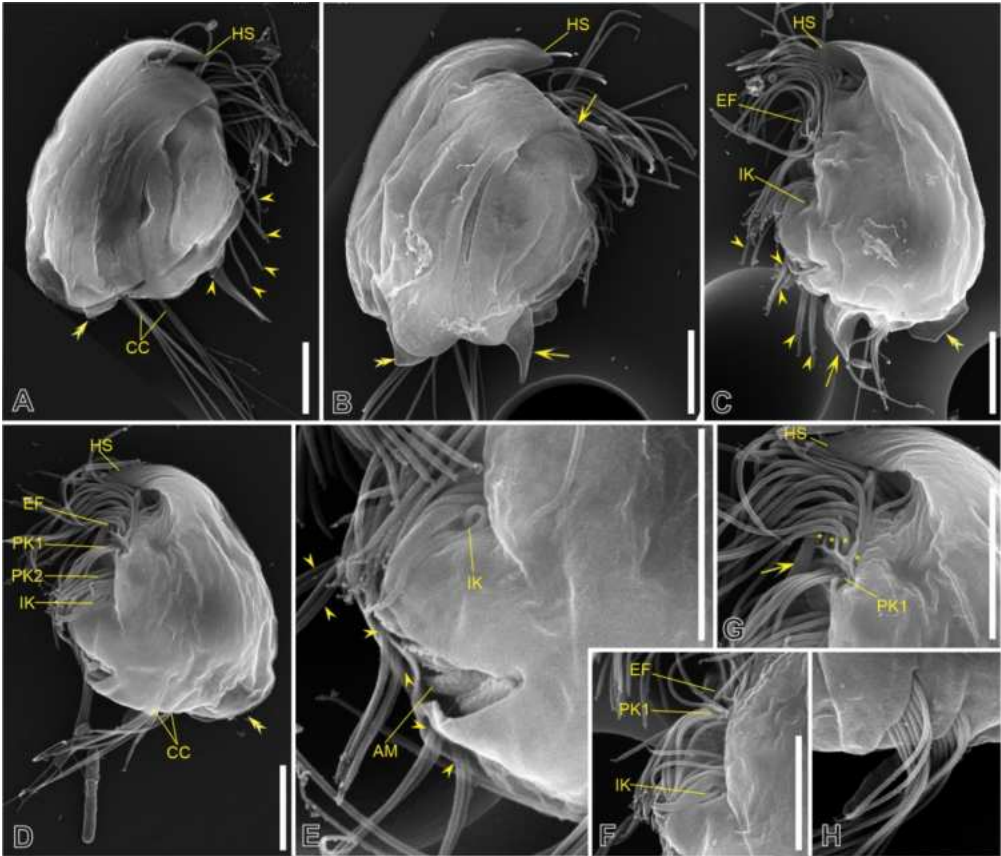


Fig. 16 Scanning electron micrographs of *Limnomylestoma shuriken* gen. nov., sp. nov. A-D Right (A, B) and left (C, D) lateral views showing the general shape, the ciliature, the spines (yellow arrows), and the posterior fin-like projection (double yellow arrowheads). E-G Left lateral views showing the opening of the buccal cavity, the membranelles, the inverse kinety, and the six dikinetids of ventral kinety (E); the lapel-like structure as a lamina (F); and the epistomial fringe pectinelles (asterisks) and preoral kineties (G). H Left lateral view of the posterior margin showing the two caudal cirri separated by a gap (amphikinetal pattern). The yellow arrowheads in A, C, and E point to the dikinetids of the ventral kinety. AM Adoral membranelles, CC caudal cirri, EF epistomial fringe, HS hood-like structure, IK inverse kinety, PK preoral kinety. Scale bars: 10 μ m.

173x148mm (300 x 300 DPI)

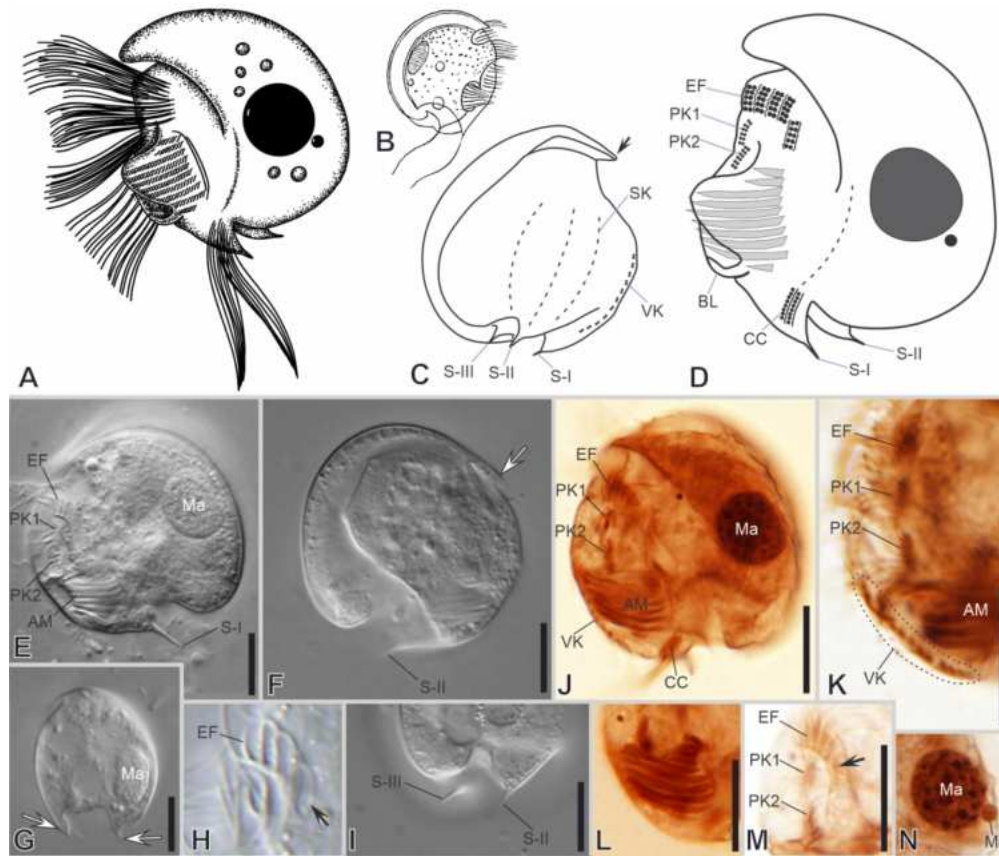


Fig. 17 Morphology of *Tostonella uncinata* gen. nov., comb. nov. in vivo (A, B, E-I) and after impregnation (C, D, J-N). A Representation of living specimens, after Penard, 1922 (B). C, D Representation of the ciliature. E, F, I-K Left (E, J, K) and right (F, I) lateral views showing the general shape, infraciliature (J, K), and the spines (E, F, I). G Longitudinal crosssection in ventral view showing two posterior spines and the macronucleus. H, M Epistomial fringe and preoral kineties, black arrow points to the displaced pectinelle. L adoral Membranelles. N Nuclear apparatus. AM adoral membranelles, BL buccal lip, CC caudal cirri, EF epistomial fringe, Ma macronucleus, PK preoral kinety, S spine, SK somatic kinety, VK ventral kinety. Scale bars: 10 μ m.

173x148mm (300 x 300 DPI)

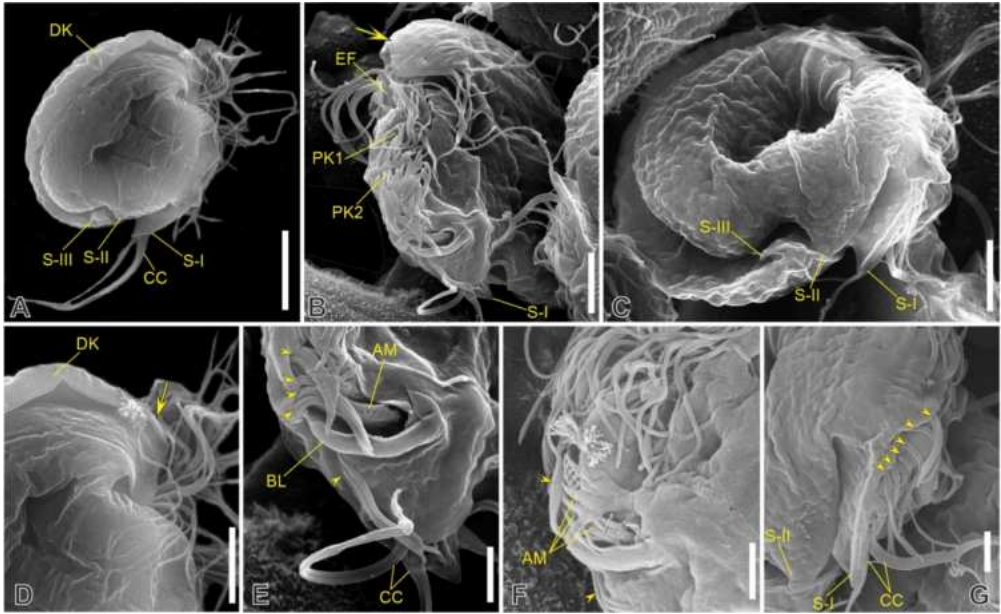


Fig. 18 Scanning electron micrographs of *Tostonella uncinata* gen. nov., comb. nov. A, D Right lateral view showing the three posterior spines (A), and the folded dorsal keel. B, E Ventrolateral view showing the ventral ciliature (B), and the opening of the buccal cavity showing the membranelles, the buccal lip, and the dikinetids of the ventral kinety (yellow arrowheads in E). C Postero-right lateral view showing the disposition of the three spines. F Buccal cavity showing the membranelles. G Postero-right lateral margin showing two spines and the caudal kineties; the yellow arrowheads show the dikinetids of the ventral kinety. AM adoral membranelles, BL buccal lip, CC caudal cirri, DK dorsal keel, EF epistomial fringe, PK preoral kinety, S spine. Scale bars: 10 μ m.

173x106mm (300 x 300 DPI)

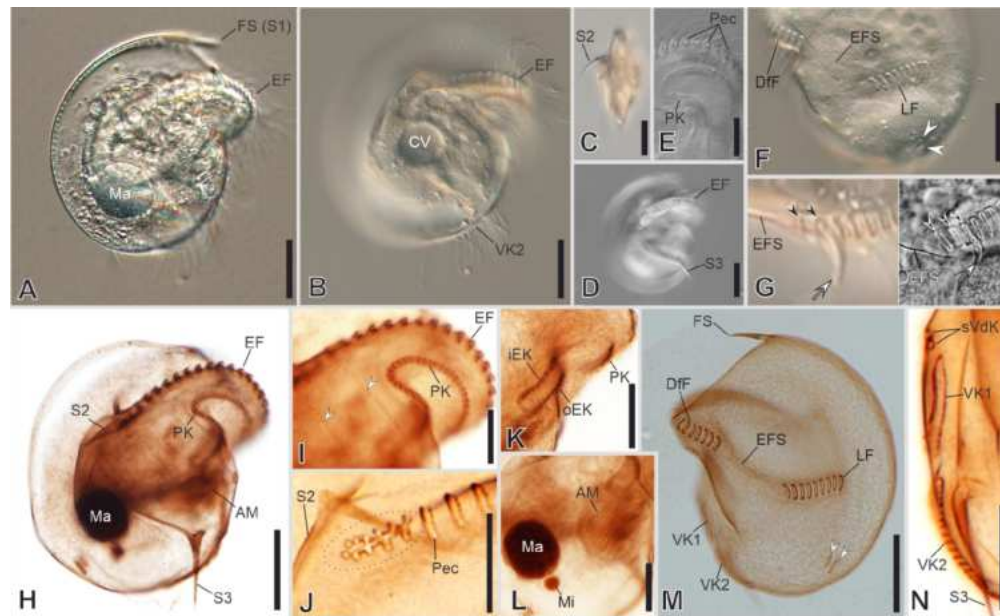


Fig. 19 Morphology of *Discomorphella pectinata* in vivo (A-G) and after impregnation (H-N). A, B, D Right lateral view of two specimens in different focal points showing the frontal awning of the epistomial fringe and the posterior spine. C A specimen in ventral view showing the lateral spine. E, I Part of the epistomial fringe showing the pectinelles and the preoral kinety below it; the white arrowheads in I point to the non-ciliated kineties. F Left lateral view showing the fragmented epistomial fringe and caudal cirri (white arrowheads). G Left epistomial fringe fragment of two specimens showing the disposition of a fourth spine (white arrows); the arrowheads point to the pectinelles. H, M Right (H) and left (M) sides of two specimens showing the ciliature. J End of the dextro-frontal epistomial fringe fragment showing the papillary dkinetids (encircled). K Endoral kineties. L Nuclear apparatus, and adoral membranelles. N Disposition of the ventral kineties and supplementary ventral dkinetids. AM adoral membranelles, CV contractile vacuole, DfF dextro-frontal fragment of EF, EF epistomial fringe, EFS epistomial fringe spacer, FS frontal spine, iEK inner endoral kinety, LF left fragment of EF, Ma macronucleus, Mi micronucleus, oEK outer endoral kinety, Pec pectinelle, PK preoral kinety, S spine, sVdK supplementary ventral dkinetids, VK: ventral kinety. Scale bars: 20 μ m (A-D, F-H, M, N), 10 μ m (E, I-L).

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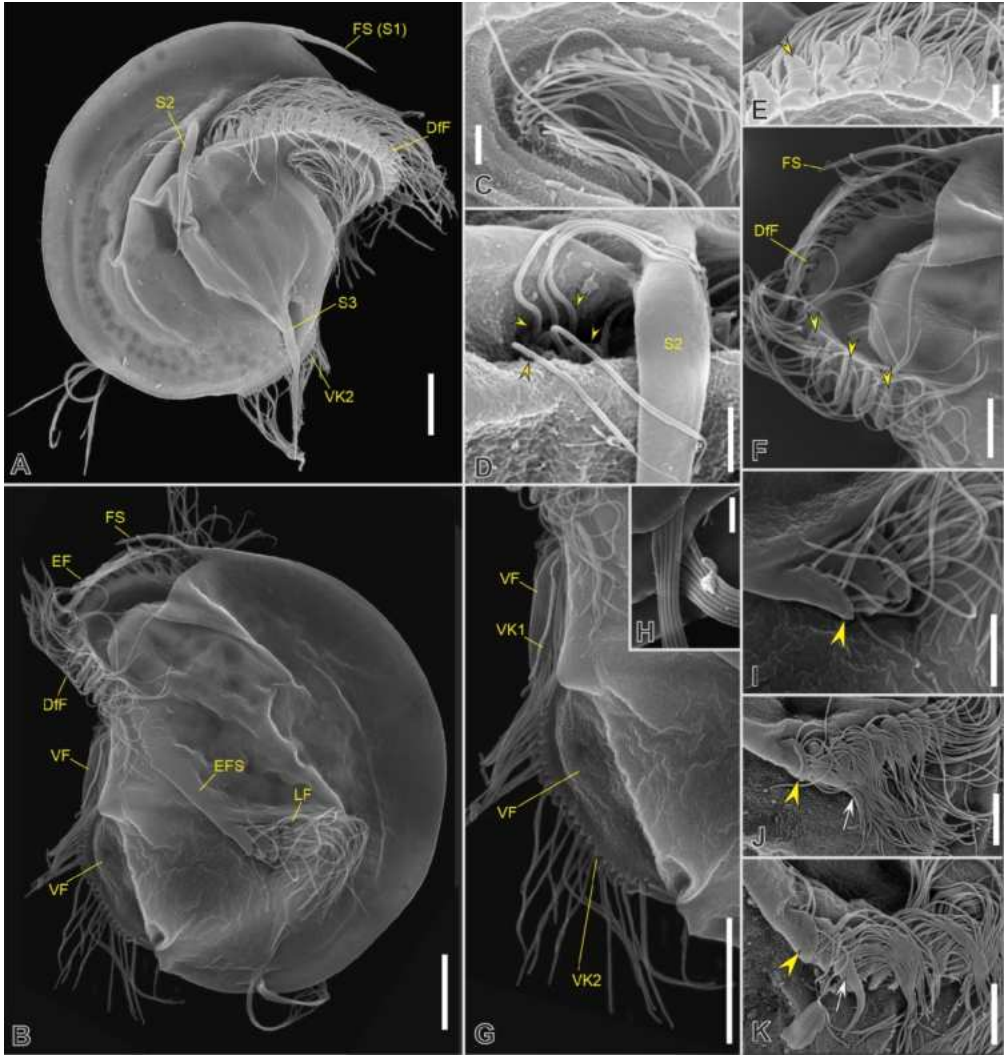


Fig. 20 Scanning electron micrographs of *Discomorphella pectinata*. A, B Right (A) and left (B) lateral view of two specimens showing the general morphology. C Preoral kinety with small pectinelles. D Papillary dikinetids (yellow arrowheads) at the end of the dextro-frontal epistomial fragment. E Arrangement of pectinelles of the dextro-frontal epistomial fragment. F Beginning and disposition of pectinelles of the dextro-frontal epistomial fragment. G Posterior left ventral margin showing the ventral flap bearing the ventral kineties. H Caudal cirri. I-K Arrangement of the left epistomial fragment of three different specimens; yellow arrowhead points to the first pectinelle, white arrow points to the associated short spine. DfF dextro-frontal fragment of EF, EF epistomial fringe, EFS epistomial fringe spacer, FS frontal spine, LF left fragment of EF, S spine, VK ventral kinety. Scale bars: 10 μ m (A, B, G), 2 μ m (C-E, H), 5 μ m (F, I-K).

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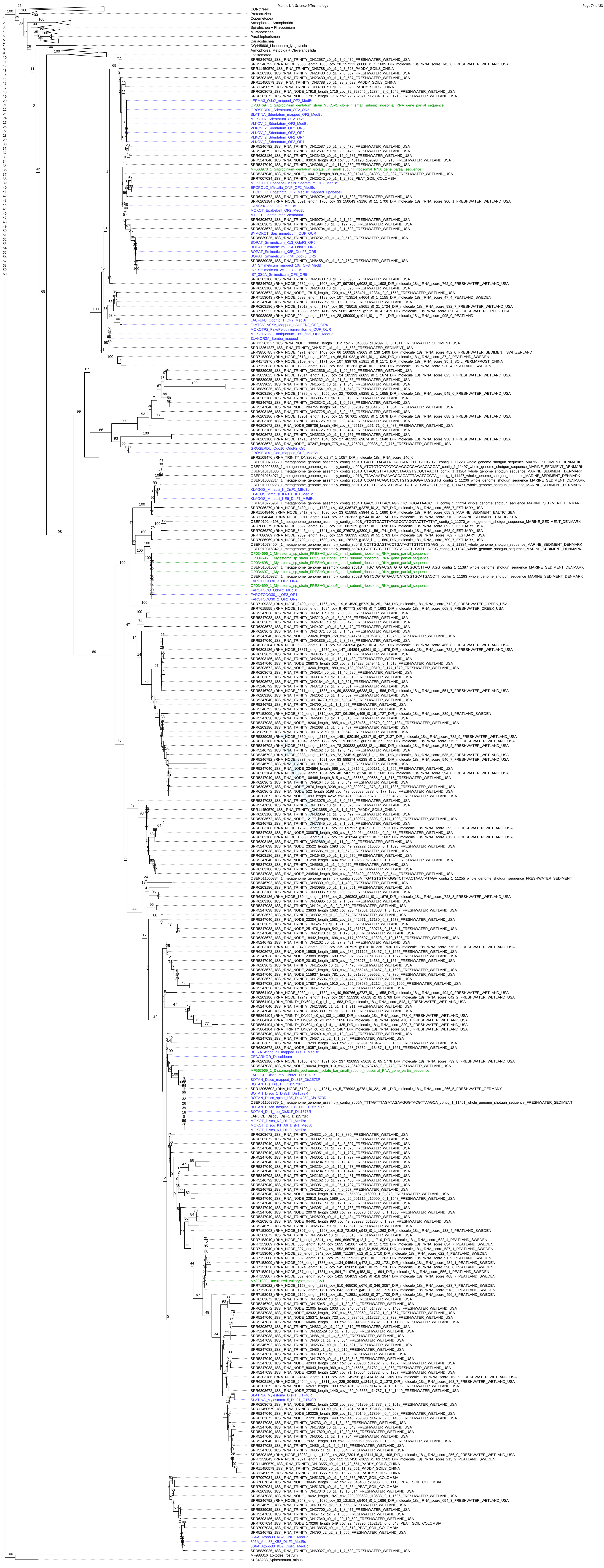


Table 1. Origin of the studied strains.

Strain	Species found	Sampling place	Coordinates
3S6A*	Odontostomatea sp.	A small pond, Croatia, freshwater	N.A.
BOPAT	<i>Saprodinium mimeticum</i>	A small pond in Smíchovský rybník, Karlovice, Czech Republic, freshwater	50°33'13.1" N, 15°10'59.8" E
BOTAN	<i>Discomorphella pectinata</i>	A small pond in the botanical Garden Faculty of Science, Charles University, Prague, Czech Republic, freshwater	50°04'20" N, 14°25'24" E
BUL7A*	<i>Tostonella uncinata</i>	A small pond within the Arkutino Reserve, Primorsko, Bulgaria, freshwater	42°19'53.0"N 27°43'33.3"E
BYMOKOT	<i>Saprodinium mimeticum</i>	Modřanské laguny, a small permanent pond adjacent to the Vltava River in Prague, Czech Republic, freshwater	49°59'44.5" N, 14°24'10.1" E
CANSYK	Epaxellidae sp.	A small pond, Gran Canaria, Canary Islands, freshwater	N.A.
CEDARKOR*	<i>Tostonella uncinata</i>	A small pond in dunes in the vicinity of Lake Korission, "Cedar forest", Korfu, Greece, freshwater	39°26'09.2"N 19°55'46.7"E
EPOPOLO*	<i>Mircalla triangula</i>	A small pond, Bayanga, Central African Republic, freshwater	2°50'32" N, 16°27'56" E
FAROTODO**	<i>Mylestoma monodontum</i>	A tidal wetland sediment in Olhão, Algarve, Portugal, brackish	37°01'49.4" N, 7°48'32.0" W
FRESHO**	<i>Mylestoma monodontum</i>	Fresh River, Falmouth, Massachusetts, USA, brackish	41°32'30.804" N, 70°37'19.92" W
GLENDWOODPEL	<i>Pelodinium reniforme</i>	A sulfidic sediments near the Boise River in Boise, Idaho Glenwood runoff, USA, freshwater	43°39'47.40" N, 116°16'57.12" W
GLENDWOODSA	<i>Saprodinium dentatum</i>		
GROSERDU	Odontostomatea sp. <i>Saprodinium dentatum</i>	Grosser Dutzendteich pond, in Nuremberg, Germany, freshwater	49°25'44.7" N, 11°07'03.7" E
IS7*	<i>Saprodinium mimeticum</i>	A small pond, Croatia, freshwater	N.A.
KLAGOS*	<i>Mylestoma bipartitum</i>	A shore in Porto Lagos, Greece, brackish	41°00'19.9" N, 25°06'00.0" E
LAPLICE	<i>Discomorphella pectinata</i>	A small pond, Łapalice, Poland, freshwater	54°19'48.7" N 18°08'21.3" E
LAUFENJ	<i>Epaxella exigua</i>	Ziegelweiher pond, Laufenburg, Switzerland, freshwater	47°33'35.5" N, 8°04'01.6" E
LERMA3	<i>Saprodinium dentatum</i>	Marsh, Atarascuillo, Lerma, State of Mexico, Mexico, freshwater	19°20'37" N, 99°30'09" W
MILOT	Epaxellidae sp.	A small puddle in Kaniera Lake Reed Trail, Latvia, freshwater	57°07" N, 23°28'41" E
MOKOT	<i>Discomorphella pectinata</i> Epaxellidae sp.	A small permanent pond adjacent to the Vltava River in Prague, Czech Republic, freshwater	49°59'24.9" N 14°24'04.1" E
MOKOTNOV	<i>Epaxella</i> cf. <i>antiquorum</i>		
MOKOTP1	<i>Mircalla polidorii</i>		
MOKOTP2	<i>Epaxella</i> cf. <i>antiquorum</i>		
MOKOTPEL	<i>Pelodinium reniforme</i>		
MOKOTR	<i>Saprodinium dentatum</i>		
SLATINA*	<i>Limnomylestoma</i> sp. <i>Saprodinium dentatum</i>	Slatina pond, Dubeč, Czech Republic, freshwater	50°04'13.2"N 14°34'12.5"E
VLKOV	<i>Saprodinium dentatum</i> <i>Epaxella</i> cf. <i>antiquorum</i>	A small permanent pond in the village of Vlkov, Czech Republic, freshwater	49°09'04.0" N 14°43'22.4" E
ZLAKOR2A	<i>Epaxella</i> cf. <i>antiquorum</i>	A small pool by the river Vltava River, Zlatá Koruna, Czech Republic, freshwater	48°51'19.0"N 14°22'18.8"E
ZLATOVLASKA	<i>Epaxella exigua</i>	A small pond, Pluhův Žďár, Czech Republic, freshwater	49°14'53.4"N 14°53'06.3"E

*Strains were cultured for short term. **Strains are maintained as long term cultures.

Table S1. Oligonucleotide primers used to capture the 18S rRNA gene sequence of Odontostomatean ciliates.

Primer	Sequence 5'-3'	Paired with primer/ Annealing temperature (°C)	Potential target
OdoF2	GTWGKGYTGTATTTATTAGATACT	Medlin Bc (52)	<i>Saprodinium dentatum</i> (SLATINA, VLKOV2, LERMA3) <i>Epaxellidae</i> (MOKOT, CANSYK, MILOT) <i>Mircalla polidorii</i> (MOKOTP1) <i>Mircalla triangula</i> (EPOPOLO) <i>Epaxella exigua</i> (LAUFENJ) <i>Epaxella</i> cf. <i>antiquorum</i> (ZLAKOR2A, MOKOTNOV) Odontostomatea (GROSERDU) <i>Mylestoma monodontum</i> (FARO)
		OdoR4 (52)	<i>Epaxella exigua</i> (ZLATOVASKA)
		OdoR5 (52)	<i>Saprodinium dentatum</i> (GROSERDU, MOKOTR) Odontostomatea (GROSERDU)
OdoF3	GTGCTGTATTTATTAGATACTAACCT	OdoR5 (52)	<i>Saprodinium mimeticum</i> (BOPAT)
		Medlin Bc (52)	<i>Saprodinium mimeticum</i> (IS7)
DisF1	GGTATGATAYCGAAAATCGAGC	Medlin Bc (52)	Odontostomatea (3S6A) <i>Tostonella uncinata</i> (BUL7A) <i>Mylestoma bipartitum</i> (KLAKOS)
		O1740R (52)	<i>Limnomylestoma shuriken</i> (SLATINA)
		Dis1573R (52)	<i>Discomorphella pectinata</i> (BOTAN, LAPLICE)
Dis425F	CAGGGARAWAGTGAAAARAC	Dis1573R (52)	<i>Discomorphella pectinata</i> (BOTAN)
Dis81F	AGACTGYGGATGGTTTAT	Dis1573R (52)	<i>Discomorphella pectinata</i> (BOTAN, LAPLICE)
OUF	ATCAGTTATAGTTTATATGATAGATT	OUR (45)	<i>Saprodinium mimeticum</i> (BYMOKOT) <i>Epaxella</i> cf. <i>antiquorum</i> (MOKOTP2)
OdoF3m	GGTTGTATTTATTAGATACTAACCG		
Dis1573R	GGCGGTGAGTACAAAAGA		
Odo1687R	ACCTTGTTACAACCTTYTNM		
Medlin Bc	TGATCCTTCTGCAGGTTACCT		
OdoR5	GAAACCTTGTTACAACCTCTRC		
OdoR1	CGATGGGSGGTGTCTACAAAKRRT	OdoF2 (52)	Works
OdoR2	TGGGSGGTGTCTACAAAKRRT	OdoF2 (52)	Works
OdoR3	CTATCCCATCACGATGCATA		
OdoR4	GATCTATCCCATCACGATGCATA		
O1740R	CTACWRAWACCTTGTTACAACCT		
O200F	TATTAGATACTAACCKWTGGTGA		
OdoF1	GKGYTGTATTTATTAGATACT		
OUR	AWACCTTGTTACAACCTTYT		
Sap836F	GGGCATTAGTATTTAATTATCAGAGG	Sequencing	
Sap836R	CCTCTGATAATTAAATACTAATGCCC	Sequencing	
Dis825F	GGARCRRTTGGRGRTGATAG	Sequencing	
Dis825R	CTATCAYCYCCAAYYGYTCC	Sequencing	
OintR1162	AGTTTTCSCGWGTTGAGTC	Sequencing	

Table S5. Morphometric data of *Saprodinium mimeticum* IS7 and BOPAT strains.

Character	Strain	Mean	M	SD	CV	Min	Max	N
Body length, in vivo	IS7	52,5	52,5	2,2	4,3	48,9	55,5	12
	BOPAT	54,9	54,9	2,5	4,6	50,1	59,9	12
Body width, in vivo	IS7	38,7	39,5	3,6	9,3	32,3	42,9	12
	BOPAT	37,4	37,5	3,8	10,2	29,2	43,8	12
Body length: width, ratio in vivo	IS7	1,4	1,3	0,1	7,5	1,2	1,6	12
	BOPAT	1,5	1,4	0,1	9,9	1,3	1,8	12
Body length	IS7	37,0	36,7	2,0	5,4	33,8	40,7	19
	BOPAT	48,0	46,9	4,0	8,3	41,7	56,0	19
Body width	IS7	30,1	30,5	2,5	8,4	25,2	34,8	19
	BOPAT	34,3	34,2	5,0	14,5	22,0	43,7	19
Body length: width, ratio	IS7	1,2	1,2	0,1	7,1	1,1	1,5	19
	BOPAT	1,4	1,4	0,2	10,9	1,2	1,9	19
Frontal spine, length	IS7	4,5	4,5	1,0	23,0	3,0	5,8	9
	BOPAT	6,1	5,9	0,7	12,1	4,9	7,1	9
Anterior cell end to anterior end of adoral zone, distance	IS7	19,0	19,4	1,3	6,8	16,5	21,0	19
	BOPAT	25,4	25,0	1,6	6,2	23,1	29,5	19
Anterior cell end to posterior end of adoral zone, distance	IS7	26,5	26,6	1,8	6,7	23,1	29,3	19
	BOPAT	33,5	33,1	2,2	6,5	30,4	38,9	19
Posterior cell end to anterior end of adoral zone, distance	IS7	19,9	19,9	1,3	6,4	18,1	22,5	11
	BOPAT	21,4	20,5	2,3	10,8	18,2	25,8	13
Posterior cell end to posterior end of adoral zone, distance	IS7	10,6	10,2	1,4	13,1	8,6	13,5	11
	BOPAT	10,6	10,0	1,8	17,0	8,8	14,8	13
Posterior cell end to adoral zone distance, ratio	IS7	0,5	0,5	0,1	10,2	0,4	0,6	11
	BOPAT	0,5	0,5	0,0	9,5	0,4	0,6	13
Anterior end to epistomial fringe, distance	IS7	7,9	7,7	1,5	19,1	4,3	10,3	19
	BOPAT	8,5	8,4	1,3	15,2	6,9	11,9	19
Epistomial fringe on left, length	IS7	7,1	7,0	1,3	18,5	4,8	9,7	19
	BOPAT	7,6	7,6	1,1	14,0	6,3	10,6	19
Epistomial fringe on right, length	IS7	22,3	22,3	1,9	8,4	18,2	25,8	17
	BOPAT	26,0	26,1	3,1	12,0	20,9	33,1	19
Macronucleus, number	IS7	2,6	3,0	0,8	28,9	1,0	4,0	19
	BOPAT	2,0	2,0	0,7	34,3	1,0	3,0	18
Macronucleus, length	IS7	6,3	6,2	1,0	15,8	4,6	8,8	49
	BOPAT	8,6	8,3	1,8	20,9	6,3	13,6	37
Macronucleus, width	IS7	4,8	4,8	0,9	19,5	3,0	7,7	49
	BOPAT	7,3	7,2	1,2	17,1	4,8	11,5	37
Micronucleus, diameter	IS7	2,6	2,7	0,4	14,3	2,0	3,0	5
	BOPAT	2,9	2,9	0,4	13,3	2,2	3,4	20
Epistomial fringe pectinelles, number	IS7	23,7	24,0	1,9	7,9	21,0	27,0	19
	BOPAT	23,9	24,0	1,6	6,7	21,0	27,0	19
Epistomial fringe pectinelles on the left, number	IS7	8,3	8,0	1,0	12,0	6,0	10,0	19
	BOPAT	8,2	8,0	0,6	7,4	7,0	9,0	19
Epistomial fringe pectinelles on the right, number	IS7	15,5	15,0	1,6	10,2	13,0	18,0	19
	BOPAT	15,7	16,0	1,2	7,9	13,0	18,0	19
Epistomial fringe ciliary rows, highest number	IS7	5,0	5,0	0,0	0,0	5,0	5,0	19
	BOPAT	5,0	5,0	0,0	0,0	5,0	5,0	19
Adoral membranelles, number	IS7	9,0	9,0	0,0	0,0	9,0	9,0	19
	BOPAT	9,0	9,0	0,0	0,0	9,0	9,0	19
Longest membranelle, length	IS7	8,8	8,6	1,1	12,4	7,5	11,5	19
	BOPAT	9,0	9,0	0,7	7,6	7,8	10,1	19
Posterior spines, including ventrocaudal spine, number	IS7	7,0	7,0	0,0	0,0	7,0	7,0	19
	BOPAT	7,0	7,0	0,0	0,0	7,0	7,0	19
Preoral kineties, number	IS7	2,0	2,0	0,0	0,0	2,0	2,0	19
	BOPAT	2,0	2,0	0,0	0,0	2,0	2,0	18
Dikinetids in preoral kinety 1, number	IS7	20,3	21,0	2,9	14,3	15,0	26,0	17
	BOPAT	22,4	23,0	1,9	8,4	19,0	25,0	17
Dikinetids in preoral kinety 2, number	IS7	13,6	13,0	2,3	17,3	9,0	18,0	17
	BOPAT	13,0	13,0	1,8	13,6	10,0	16,0	15
Dikinetids in ventral kinety 1, number	IS7	5,5	6,0	0,7	13,1	4,0	6,0	17
	BOPAT	5,6	6,0	0,5	8,9	5,0	6,0	16
Dikinetids in ventral kinety 2, number	IS7	15,8	16,0	1,4	8,7	13,0	17,0	19

	BOPAT	15,8	16,0	1,3	8,3	12,0	17,0	19
Somatic kineties, including epistomial fringe ciliary rows, number	IS7	9,0	9,0	0,0	0,0	9,0	9,0	19
	BOPAT	9,0	9,0	0,0	0,0	9,0	9,0	19
Associated spine kineties, number	IS7	5,0	5,0	0,0	0,0	5,0	5,0	19
	BOPAT	5,0	5,0	0,0	0,0	5,0	5,0	19
Non-ciliated kineties on left side, number	IS7	3,0	3,0	0,0	0,0	3,0	3,0	19
	BOPAT	3,0	3,0	0,0	0,0	3,0	3,0	12
Non-ciliated kineties on right side, number	IS7	6,0	6,0	0,0	0,0	6,0	6,0	19
	BOPAT	6,0	6,0	0,0	0,0	6,0	6,0	11
Dikinetids in associated spine kinety 6, number	IS7	8,5	9,0	0,6	7,2	7,0	9,0	19
	BOPAT	8,3	8,0	0,7	8,9	7,0	10,0	19
Dikinetids in non-ciliated kinety 7, number	IS7	26,6	27,0	2,6	9,7	23,0	30,0	10
Dikinetids in associated spine kinety 7, number	IS7	8,4	8,0	0,6	7,3	8,0	10,0	18
	BOPAT	8,7	9,0	0,7	8,6	8,0	10,0	19
Anterior fragment of kinety 8, length	IS7	13,1	13,2	2,0	14,9	10,1	16,2	19
	BOPAT	21,0	21,2	2,3	10,9	17,3	26,7	18
Dikinetids in anterior fragment of kinety 8, number	IS7	19,1	19,0	2,5	12,9	15,0	24,0	19
	BOPAT	25,8	26,0	3,1	12,0	22,0	32,0	17
Dikinetids in non-ciliated kinety 8, number	IS7	28,6	30,0	4,7	16,6	20,0	36,0	14
Dikinetids in associated spine kinety 8, number	IS7	7,5	7,5	0,9	12,3	6,0	10,0	18
	BOPAT	7,9	8,0	0,6	7,0	7,0	9,0	17
Dikinetids in non-ciliated kinety 9, number	IS7	26,8	26,5	6,1	22,7	18,0	37,0	12
Dikinetids in associated spine kinety 9, number	IS7	6,0	5,0	1,3	21,5	5,0	8,0	13
	BOPAT	6,3	6,0	1,0	15,3	5,0	8,0	18

Table S6. Morphometric data of *Saprodinium dentatum* VLKOV strains.

Character	Mean	M	SD	CV	Min	Max	N
Body length in vivo	73,9	75,3	4,5	6,1	65,4	78,4	10
Body width in vivo	67,3	67,1	6,1	9,0	56,6	75,2	10
Body length:width ratio in vivo	1,1	1,1	0,0	4,3	1,0	1,2	10
Body length	61,0	60,5	4,6	7,5	56,0	72,0	12
Body width	57,6	58,0	4,3	7,4	51,0	65,0	12
Body length:width ratio	1,1	1,1	0,0	4,6	1,0	1,1	12
Frontal spine, length	9,3	9,0	2,2	23,7	6,0	13,0	11
Anterior cell end to anterior end of adoral zone, distance	30,0	30,5	3,4	11,2	26,0	37,0	12
Anterior cell end to posterior end of adoral zone, distance	42,2	41,5	4,8	11,3	36,0	53,0	12
Posterior cell end to anterior end of adoral zone, distance	30,6	30,2	2,1	6,7	28,1	34,0	6
Posterior cell end to posterior end of adoral zone, distance	15,4	15,2	1,3	8,5	13,8	17,6	6
Posterior cell end to adoral zone distance, ratio	0,5	0,5	0,1	12,0	0,4	0,6	6
Anterior cell end to anterior end of epistomial fringe, distance	11,6	11,5	1,9	16,7	9,0	16,0	12
Epistomial fringe, right side, length	39,3	40,0	3,6	9,2	32,0	44,0	12
Macronuclear nodules, number	1,6	1,5	0,7	42,2	1,0	3,0	12
Length of Macronucleus	10,5	10,0	2,5	23,9	6,0	16,0	19
Width of macronucleus	10,0	9,0	2,6	26,5	7,0	16,0	19
Micronucleus, diameter	4,2	4,0	0,4	9,3	4,0	5,0	12
Epistomial fringe pectinelles, number	31,2	32,0	2,1	6,9	27,0	34,0	10
Epistomial fringe pectinelles on the left, number	10,5	10,0	1,7	16,1	8,0	14,0	11
Epistomial fringe pectinelles on the right, number	21,2	21,0	1,9	9,2	17,0	24,0	11
Epistomial fringe ciliary rows, highest number	5,0	5,0	0,0	0,0	5,0	5,0	11
Adoral membranelles, number	9,0	9,0	0,0	0,0	9,0	9,0	17
Longest membranelle, length	12,1	12,0	1,5	12,5	10,0	14,0	12
Posterior spines, including ventrocaudal spine, number	8,4	8,0	0,5	6,0	8,0	9,0	11
Preoral kineties, number	2,0	2,0	0,0	0,0	2,0	2,0	12
Dikinetids in preoral kinety 1, number	33,9	34,0	1,3	4,0	32,0	35,0	7
Dikinetids in preoral kinety 2, number	25,7	26,0	0,8	2,9	25,0	27,0	7
Dikinetids in ventral kinety 1, number	21,4	20,0	2,9	13,4	19,0	27,0	7
Dikinetids in ventral kinety 2, number	31,7	31,0	2,1	6,5	30,0	35,0	6
Supplementary ventral dikinetids, number	4,2	4,0	0,4	9,8	4,0	5,0	6
Somatic kineties, including epistomial fringe ciliary rows, number	9,0	9,0	0,0	0,0	9,0	9,0	12
Associated spine kineties, number	4,0	4,0	0,0	0,0	4,0	4,0	12
Non-ciliated kineties on left side, number	3,0	3,0	0,0	0,0	3,0	3,0	12
Non-ciliated kineties on right side, number	6,0	6,0	0,0	0,0	6,0	6,0	12
Dikinetids in associated spine kinety 6, number	13,4	13,0	1,1	8,4	12,0	15,0	7
Dikinetids in associated spine kinety 7, number	11,7	12,0	0,8	6,5	11,0	13,0	7
Dikinetids in anterior fragment of kinety 8, number	94,4	95,0	8,2	8,7	82,0	110,0	14
Dikinetids in associated spine kinety 8, number	12,1	12,0	1,3	11,1	10,0	14,0	7
Dikinetids in associated spine kinety 9, number	10,1	10,0	0,7	6,8	9,0	11,0	7

Table S7. Morphometric data of *Mircalla triangula* comb. nov. (MT) and *Mircalla polidorii* sp. nov. (MP).

Character	Species	Mean	M	SD	CV	Min	Max	N
Body length in vivo	MT	33,8	33,6	1,4	4,1	31,7	36,8	12
	MP	31,6	31,3	1,8	5,8	29,7	35,6	10
Body width in vivo	MT	23,6	23,6	1,7	7,2	20,3	27,1	12
	MP	25,2	25,3	1,5	6,1	22,9	27,6	10
Body length:width, ratio in vivo	MT	1,4	1,5	0,1	6,5	1,3	1,7	12
	MP	1,3	1,2	0,1	7,5	1,1	1,4	10
Body length	MT	25,4	25,0	1,9	7,4	22,2	28,9	19
	MP	26,5	26,3	2,2	8,3	23,6	29,5	10
Body width	MT	16,1	16,2	1,7	10,8	12,5	19,5	19
	MP	19,1	18,7	2,4	12,5	15,2	23,1	10
Body length:width, ratio	MT	1,6	1,6	0,1	6,7	1,4	1,9	19
	MP	1,4	1,4	0,1	8,8	1,3	1,6	10
Anterior end to anterior end of adoral zone, distance	MT	10,3	10,1	1,3	12,2	8,2	13,9	19
	MP	11,2	11,4	0,9	8,5	9,4	12,3	10
Anterior end to posterior end of adoral zone, distance	MT	17,8	17,4	1,6	9,2	16,0	22,8	19
	MP	19,9	20,0	1,5	7,6	17,6	21,7	10
Posterior cell end to anterior end of adoral zone, distance	MT	15,5	15,8	1,0	6,5	13,6	16,8	12
	MP	15,4	15,2	1,4	8,9	13,6	17,9	10,0
Posterior cell end to posterior end of adoral zone, distance	MT	6,7	6,1	1,3	18,8	5,3	8,6	10,0
	MP	7,5	7,7	0,8	10,1	6,2	8,5	12
Posterior adoral zone ratio	MT	0,5	0,5	0,0	6,4	0,4	0,5	12
	MP	0,4	0,4	0,1	11,8	0,4	0,5	10
Epistomial fringe on left, length	MT	NA	NA	NA	NA	NA	NA	NA
	MP	2,2	2,1	0,3	14,7	1,9	2,9	10
Epistomial fringe on right, length	MT	9,7	9,4	1,5	15,3	7,2	12,7	16
	MP	12,1	11,9	0,6	4,6	11,3	13,2	10
Macronucleus, number	MT	1,4	1,0	0,5	35,4	1,0	2,0	18
	MP	1,6	2,0	0,5	32,3	1,0	2,0	10
Macronucleus, length	MT	6,9	6,6	1,7	24,1	4,4	10,2	28
	MP	7,0	7,0	1,3	18,4	5,3	10,3	15
Macronucleus, width	MT	5,4	5,4	0,9	15,9	4,1	7,6	28
	MP	6,4	6,4	0,8	11,8	5,4	7,9	15
Micronucleus, diameter	MT	2,6	2,6	0,2	9,5	2,3	3,0	18
	MP	2,3	2,3	0,2	9,9	2,0	2,7	9
Epistomial fringe pectinelles, number	MT	7,1	7,0	0,7	10,1	6,0	8,0	16
	MP	12,1	12,0	1,3	10,6	11,0	15,0	10
Epistomial fringe pectinelles on the left, number	MT	NA	NA	NA	NA	NA	NA	NA
	MP	3,2	3,0	0,8	24,7	2,0	5,0	10
Epistomial fringe pectinelles on the right, number	MT	7,1	7,0	0,7	10,1	6,0	8,0	16
	MP	8,9	9,0	0,7	8,3	8,0	10,0	10
Epistomial fringe ciliary rows, highest number	MT	4,0	4,0	0,0	0,0	4,0	4,0	15
	MP	5,0	5,0	0,0	0,0	5,0	5,0	10
Adoral membranelles, number	MT	9,0	9,0	0,0	0,0	9,0	9,0	18
	MP	9,0	9,0	0,0	0,0	9,0	9,0	10
Longest membranelle, length	MT	6,0	6,2	0,6	10,6	4,5	7,0	19
	MP	8,2	8,3	0,9	10,7	6,9	9,5	10
Preoral kineties, number	MT	1,0	1,0	0,0	0,0	1,0	1,0	17
	MP	2,0	2,0	0,0	0,0	2,0	2,0	10
Dikinetids in preoral kinety 1, number	MT	13,4	13,0	1,5	11,5	10,0	15,0	17
	MP	8,9	9,0	0,9	9,8	8,0	10,0	10
Dikinetids in preoral kinety 2, number	MT	0,0	0,0	0,0	0,0	0,0	0,0	0
	MP	4,8	5,0	0,4	8,4	4,0	5,0	6
Dikinetids in ventral kinety 1, number	MT	3,9	4,0	0,3	8,8	3,0	4,0	16
	MP	4,4	4,0	0,5	11,9	4,0	5,0	9

Dikinetids in ventral kinety 2, number	MT	3,0	3,0	0,0	0,0	3,0	3,0	15
	MP	10,1	10,0	1,3	12,6	7,0	11,0	9
Supplementary ventral dikinetids, number	MT	3,7	4,0	0,5	13,0	3,0	4,0	13
	MP	0,0	0,0	0,0	0,0	0,0	0,0	0
Somatic kineties, including epistomial fringe ciliary rows, number	MT	9,0	9,0	0,0	0,0	9,0	9,0	9
	MP	9,0	9,0	0,0	0,0	9,0	9,0	8
Associated spine kineties, number	MT	5,0	5,0	0,0	0,0	5,0	5,0	13
	MP	4,0	4,0	0,0	0,0	4,0	4,0	7
Non-ciliated kineties on left side, number	MT	3,0	3,0	0,0	0,0	3,0	3,0	15
	MP	3,0	3,0	0,0	0,0	3,0	3,0	8
Non-ciliated kineties on right side, number	MT	6,0	6,0	0,0	0,0	6,0	6,0	15
	MP	6,0	6,0	0,0	0,0	6,0	6,0	7
Dikinetids in associated spine kinety 6, number	MT	4,2	4,0	0,5	13,0	4,0	6,0	18
	MP	4,9	5,0	0,4	7,8	4,0	5,0	7
Dikinetids in associated spine kinety 7, number	MT	4,9	5,0	1,0	20,9	3,0	6,0	18
	MP	4,7	5,0	0,5	10,7	4,0	5,0	9
Anterior fragment of kinety 8, length	MT	7,1	7,1	0,7	9,9	5,9	8,3	17
	MP	9,5	9,7	0,8	8,1	8,0	10,3	10
Dikinetids in anterior fragment of kinety 8, number	MT	7,4	7,0	0,6	8,2	6,0	8,0	17
	MP	12,6	12,5	0,7	5,5	12,0	14,0	10
Dikinetids in associated spine kinety 8, number	MT	4,4	4,5	0,6	14,2	3,0	5,0	16
	MP	4,1	4,0	0,8	19,0	3,0	5,0	9
Dikinetids in associated spine kinety 9, number	MT	2,0	2,0	0,0	0,0	2,0	2,0	14
	MP	2,4	2,0	0,5	21,6	2,0	3,0	9

Table S8. Morphometric data of *Epalxella exigua* LAUFENJ strain.

Character	Mean	M	SD	CV	Min	Max	N
Body length in vivo	33,1	33,1	2,4	7,3	30,2	36,8	5
Body width in vivo	25,2	24,6	2,2	8,7	23,1	28,8	5
Body length:width, ratio in vivo	1,3	1,3	0,1	8,0	1,1	1,4	5
Body length	24,6	24,8	0,7	3,0	23,7	25,4	6
Body width	17,3	17,7	1,1	6,6	15,0	18,0	6
Body length:width, ratio	1,4	1,4	0,1	5,9	1,3	1,6	6
Anterior end to anterior end of adoral zone, distance	11,7	11,7	0,8	7,0	10,7	13,0	6
Anterior end to posterior end of adoral zone, distance	20,2	20,2	0,6	2,8	19,5	21,2	6
Posterior cell end to anterior end of adoral zone, distance	13,4	13,4	0,9	6,8	12,2	14,7	6
Posterior cell end to posterior end of adoral zone, distance	4,2	4,0	0,7	16,2	3,4	5,1	6
Posterior cell end to adoral zone distance, ratio	0,3	0,3	0,0	10,6	0,3	0,4	6
Epistomial fringe on left, length	10,0	9,4	2,1	21,1	7,4	12,5	5
Epistomial fringe on right, length	2,7	2,5	0,7	26,1	2,1	3,9	6
Macronucleus, number	1,8	2,0	0,8	41,1	1,0	3,0	6
Macronucleus, length	5,7	5,2	1,7	29,3	3,9	9,1	11
Macronucleus, width	4,4	4,4	0,9	20,1	2,9	6,4	11
Micronucleus, diameter	2,0	2,0	0,2	7,9	1,8	2,2	6
Pectinelles of epistomial fringe, number	7,8	8,0	0,8	9,6	7,0	9,0	6
Epistomial fringe ciliary rows, highest number	5,0	5,0	0,0	0,0	5,0	5,0	6
Adoral membranelles, number	9,0	9,0	0,0	0,0	9,0	9,0	6
Longest membranelle, length	7,1	6,9	0,6	8,9	6,6	8,2	6
Preoral kineties, number	2,0	2,0	0,0	0,0	2,0	2,0	6
Dikinetids in preoral kinety 1, number	3,3	3,0	0,6	17,3	3,0	4,0	3
Dikinetids in preoral kinety 2, number	5,3	5,0	0,5	9,7	5,0	6,0	6
Dikinetids in ventral kinety 1, number	6,2	6,0	0,4	7,2	6,0	7,0	5
Supplementary ventral dikinetids, number	2,0	2,0	0,0	0,0	2,0	2,0	6
Somatic kineties, including epistomial fringe ciliary rows, number	9,0	9,0	0,0	0,0	9,0	9,0	6
Associated spine kineties, number	4,0	4,0	0,0	0,0	4,0	4,0	6
Non-ciliated kineties on left side, number	3,0	3,0	0,0	0,0	3,0	3,0	6
Non-ciliated kineties on right side, number	6,0	6,0	0,0	0,0	6,0	6,0	6
Dikinetids in associated spine kinety 6, number	7,8	8,0	0,4	5,2	7,0	8,0	6
Dikinetids in associated spine kinety 7, number	8,0	8,0	0,9	11,2	7,0	9,0	6
Anterior fragment of kinety 8, length	6,5	6,4	0,6	9,0	5,6	7,4	6
Dikinetids in anterior fragment of kinety 8, number	8,0	8,0	0,7	8,8	7,0	9,0	5
Dikinetids in associated spine kinety 8, number	6,2	6,0	0,4	6,6	6,0	7,0	6
Dikinetids in associated spine kinety 9, number	4,5	4,5	0,5	12,2	4,0	5,0	6

Table S9. Morphometric data of *Mylestoma bipartitum* KLAGOS (K), *Mylestoma monodontum* sp. nov. FRESHO (F) and FARO (FA), and *Limnomylestoma shuriken* sp. nov. SLATINA (S).

Character	Species	Strain	Mean	M	SD	CV	Min	Max	N
Body length in vivo	<i>M. bipartitum</i>	K	36,7	36,9	2,3	6,2	33,4	41,8	11
	<i>M. monodontum</i>	F	21,7	21,3	2,0	9,2	18,9	25,0	17
		FA	20,7	21,3	1,4	6,8	18,3	22,5	12
	<i>L. shuriken</i>	S	28,0	27,2	2,9	10,5	24,2	33,3	10
Body width in vivov. v.	<i>M. bipartitum</i>	K	27,5	27,8	2,5	9,1	24,2	31,2	11
	<i>M. monodontum</i>	F	18,0	18,0	2,3	13,0	13,6	21,2	17
		FA	16,8	16,7	1,7	10,0	14,3	19,8	12
	<i>L. shuriken</i>	S	21,9	22,4	2,2	10,0	18,3	25,9	10
Body length:width ratio in vivo	<i>M. bipartitum</i>	K	1,3	1,3	0,1	6,3	1,2	1,5	11
	<i>M. monodontum</i>	F	1,2	1,2	0,1	6,4	1,1	1,4	17
		FA	1,2	1,2	0,1	4,5	1,1	1,3	12
	<i>L. shuriken</i>	S	1,3	1,3	0,1	5,7	1,2	1,4	10
Body length	<i>M. bipartitum</i>	K	29,5	29,6	2,7	9,1	22,8	34,1	25
	<i>M. monodontum</i>	F	19,8	19,7	2,2	10,9	15,9	25,4	17
		FA	19,8	20,0	2,2	11,2	16,0	24,0	11
	<i>L. shuriken</i>	S	19,2	19,2	1,9	10,0	15,8	23,5	42
Body width	<i>M. bipartitum</i>	K	21,3	21,4	2,2	10,3	16,9	25,4	24
	<i>M. monodontum</i>	F	16,4	17,1	2,2	13,7	13,2	21,1	17
		FA	16,7	17,0	1,3	8,1	14,0	19,0	11
	<i>L. shuriken</i>	S	15,1	15,4	1,5	9,9	12,2	18,6	41
Body length:width ratio	<i>M. bipartitum</i>	K	1,4	1,4	0,1	7,4	1,2	1,6	24
	<i>M. monodontum</i>	F	1,2	1,2	0,1	6,7	1,1	1,4	17
		FA	1,2	1,2	0,1	5,6	1,1	1,3	11
	<i>L. shuriken</i>	S	1,3	1,3	0,1	9,1	1,0	1,5	41
Anterior end to anterior end of adoral zone, distance	<i>M. bipartitum</i>	K	15,7	15,8	1,7	10,9	12,9	20,5	25
	<i>M. monodontum</i>	F	9,0	8,6	1,4	15,7	6,3	11,8	17
		FA	9,8	10,0	1,5	15,7	6,0	12,0	11
	<i>L. shuriken</i>	S	10,4	10,3	1,4	13,3	8,1	13,2	42
Anterior end to posterior end of adoral zone, distance	<i>M. bipartitum</i>	K	25,3	25,4	2,3	9,1	19,6	30,5	25
	<i>M. monodontum</i>	F	17,5	17,8	1,7	9,6	14,1	20,9	17
		FA	17,4	17,0	1,7	10,1	14,0	20	11
	<i>L. shuriken</i>	S	16,9	17,0	1,6	9,6	13,4	19,7	42
Posterior cell end to anterior end of adoral zone, distance	<i>M. bipartitum</i>	K	13,9	13,6	1,7	12,1	9,1	16,5	25,0
	<i>M. monodontum</i>	F	10,8	10,6	1,2	10,8	9,5	13,6	17
		FA	10,0	10,0	1,3	13,4	8,0	12	11
	<i>L. shuriken</i>	S	8,8	8,9	1,0	11,3	6,9	11,6	42,0
Posterior cell end to posterior end of adoral zone, distance	<i>M. bipartitum</i>	K	4,2	4,2	1,1	26,4	1,8	6,3	25,0
	<i>M. monodontum</i>	F	2,3	2,0	0,8	37,1	1,3	5	17
		FA	2,5	2,0	1,2	49,4	1,0	5	11
	<i>L. shuriken</i>	S	2,3	2,3	0,7	29,3	1,2	4,2	42,0
Posterior cell end to adoral zone distance, ratio	<i>M. bipartitum</i>	K	0,3	0,3	0,1	22,2	0,1	0,4	25,0
	<i>M. monodontum</i>	F	0,2	0,2	0,1	28,4	0,1	0,3	17
		FA	0,2	0,2	0,1	37,8	0,1	0,4	11
	<i>L. shuriken</i>	S	0,3	0,3	0,1	23,4	0,1	0,4	42,0
Macronucleus, number	<i>M. bipartitum</i>	K	1,0	1,0	0,0	0,0	1,0	1,0	25
	<i>L. shuriken</i>	S	1,0	1,0	0,0	0,0	1,0	1,0	43
Macronucleus, length	<i>M. bipartitum</i>	K	8,5	8,4	0,9	10,8	6,9	10,0	23
	<i>M. monodontum</i>	F	10,1	10,1	1,7	16,7	7,7	12,7	17
		FA	9,7	9,0	1,8	18,4	8,0	14,0	11
	<i>L. shuriken</i>	S	7,3	7,2	1,2	16,1	4,4	10,4	43
Macronucleus, width	<i>M. bipartitum</i>	K	7,0	6,6	1,3	18,8	4,8	9,8	23
	<i>M. monodontum</i>	F	5,4	5,3	1,2	21,4	4,2	7,4	17
		FA	4,7	5,0	1,0	21,3	3,0	6,0	11
	<i>L. shuriken</i>	S	5,4	5,3	0,7	13,1	4,4	7,6	43
Micronucleus, diameter	<i>M. bipartitum</i>	K	2,8	2,9	0,5	18,2	2,0	3,6	20
	<i>M. monodontum</i>	F	2,1	2,1	0,4	18,8	1,7	2,8	13
		FA	2,1	2,0	0,6	27,0	1,0	3,0	10
	<i>L. shuriken</i>	S	2,2	2,1	0,3	15,0	1,7	3,1	36
Pectineles in epistomial fringe, number	<i>M. bipartitum</i>	K	8,4	8,5	0,6	7,7	7,0	9,0	14
	<i>M. monodontum</i>	F	4,0	4,0	0,0	0,0	4,0	4,0	10
		FA	4,0	4,0	0,0	0,0	4,0	4,0	9
	<i>L. shuriken</i>	S	3,8	4,0	0,4	10,7	3,0	4,0	30
Epistomial fringe ciliary rows, highest number	<i>M. bipartitum</i>	K	5,0	5,0	0,0	0,0	5,0	5,0	13
	<i>M. monodontum</i>	F	5,0	5,0	0,0	0,0	5,0	5,0	10
		FA	5,0	5,0	0,0	0,0	5,0	5,0	9
	<i>L. shuriken</i>	S	5,0	5,0	0,0	0,0	5,0	5,0	12
Adoral membranelles, number	<i>M. bipartitum</i>	K	8,9	9,0	0,3	3,2	8,0	9,0	23

	<i>M. monodontum</i>	F	7,9	8,0	0,3	4,3	7,0	8,0	16
		FA	7,5	7,0	0,5	7,0	7,0	8,0	11
	<i>L. shuriken</i>	S	9,0	9,0	0,0	0,0	9,0	9,0	37
Longest membranelle, length	<i>M. bipartitum</i>	K	10,7	10,6	1,4	12,8	8,6	13,2	24
	<i>M. monodontum</i>	F	6,6	6,4	0,9	13,4	5,3	8,8	17
		FA	6,2	6,0	0,8	12,1	5,0	7,0	11
	<i>L. shuriken</i>	S	6,3	6,4	0,6	9,3	4,9	7,6	41
Shortest membranelle, length	<i>L. shuriken</i>	S	4,0	4,0	0,0	0,0	4,0	4,0	38
	<i>M. monodontum</i>	F	2,0	1,9	0,3	15,3	1,5	2,5	17
		FA	2,6	3,0	0,5	19,1	2,0	3,0	11
Adoral zone, length	<i>M. monodontum</i>	F	8,5	8,4	0,8	9,7	6,9	9,6	17
		FA	7,5	7,0	0,7	9,1	7,0	9,0	11
Preoral kineties, number	<i>M. bipartitum</i>	K	2,0	2,0	0,0	0,0	2,0	2,0	10
	<i>M. monodontum</i>	F	2,0	2,0	0,0	0,0	2,0	2,0	5
		FA	2,0	2,0	0,0	0,0	2,0	2,0	2
	<i>L. shuriken</i>	S	2,0	2,0	0,0	0,0	2,0	2,0	22
Dikinetids in preoral kinety 1, number	<i>M. bipartitum</i>	K	9,7	10,0	0,8	8,1	8,0	11,0	11
	<i>M. monodontum</i>	F	6,0	6,0	0,0	0,0	6,0	6,0	5
		FA	5,5	5,5	0,6	10,5	5,0	6,0	4
	<i>L. shuriken</i>	S	4,9	5,0	0,4	7,3	4,0	5,0	28
Dikinetids in preoral kinety 2, number	<i>M. bipartitum</i>	K	7,2	7,0	1,3	17,4	6,0	9,0	11
	<i>M. monodontum</i>	F	3,0	3,0	0,0	0,0	3,0	3,0	6
		FA	3,0	3,0	0,0	0,0	3,0	3,0	2
	<i>L. shuriken</i>	S	3,3	3,0	0,6	16,7	2,0	4,0	24
Dikinetids in ventral kinety 1, number	<i>M. bipartitum</i>	K	2,7	3,0	0,6	21,7	2,0	3,0	3
	<i>M. monodontum</i>	F	3,9	4,0	0,4	9,8	3,0	4,0	7
		FA	4,0	4,0	0,0	0,0	4,0	4,0	6
	<i>L. shuriken</i>	S	6,0	6,0	0,0	0,0	6,0	6,0	25
Supplementary ventral dikinetids, number	<i>M. bipartitum</i>	K	1,0	1,0	0,0	0,0	1,0	1,0	13
Somatic kineties, number	<i>M. bipartitum</i>	K	9,0	9,0	0,0	0,0	9,0	9,0	15
	<i>M. monodontum</i>	F	7,0	7,0	0,0	0,0	7,0	7,0	6
		FA	7,0	7,0	0,0	0,0	7,0	7,0	6
	<i>L. shuriken</i>	S	7,0	7,0	0,0	0,0	7,0	7,0	9
Non-ciliated kineties on left side, number	<i>M. bipartitum</i>	K	5,0	5,0	0,0	0,0	5,0	5,0	12
	<i>M. monodontum</i>	F	2,0	2,0	0,0	0,0	2,0	2,0	6
		FA	2,0	2,0	0,0	0,0	2,0	2,0	6
	<i>L. shuriken</i>	S	5,0	5,0	0,0	0,0	5,0	5,0	11
Non-ciliated kineties on right side, number	<i>M. bipartitum</i>	K	4,0	4,0	0,0	0,0	4,0	4,0	11
	<i>M. monodontum</i>	F	5,0	5,0	0,0	0,0	5,0	5,0	6
		FA	5,0	5,0	0,0	0,0	5,0	5,0	6
	<i>L. shuriken</i>	S	2,0	2,0	0,0	0,0	2,0	2,0	9
Rows of caudal cirri, number	<i>M. bipartitum</i>	K	2,0	0,0	0,0	2,0	2,0	17,0	18
	<i>M. monodontum</i>	F	2,0	2,0	0,0	0,0	2,0	2,0	17
		FA	2,0	2,0	0,0	0,0	2,0	2,0	11
	<i>L. shuriken</i>	S	2,0	2,0	0,0	0,0	2,0	2,0	35
Caudal cirri, length, in vivo	<i>M. bipartitum</i>	K	44,3	43,6	6,0	13,5	37,6	56,0	10
	<i>M. monodontum</i>	F	24,4	25,1	4,1	16,8	17,2	31,0	16
		FA	24,6	25,3	3,0	12,2	19,8	28,4	10
	<i>L. shuriken</i>	S	19,3	19,3	3,1	16,2	15,2	24,1	6
Kinetids in each caudal cirri, number	<i>M. bipartitum</i>	K	9,6	10,0	0,7	7,6	8,0	10,0	9
	<i>M. monodontum</i>	F	6,0	6,0	0,0	0,0	6,0	6,0	8
		FA	6,0	6,0	0,0	0,0	6,0	6,0	8
	<i>L. shuriken</i>	S	8,0	8,0	0,0	0,0	8,0	8,0	17

Table S10. Morphometric data of *Tostonella uncinata* sp. nov. BUL7A strain.

Character	Mean	M	SD	CV	Min	Max	N
Body length in vivo	31,3	31,5	1,6	0,1	28,1	33,6	17
Body width in vivo	30,8	31,1	1,7	0,1	28,1	33,1	17
Body length:width, ratio in vivo	1,0	1,0	0,0	0,0	1,0	1,1	17
Body length	28,0	28,3	1,3	0,0	25,3	29,5	12
Body width	27,3	27,1	1,9	0,1	24,8	29,7	11
Body length:width, ratio	1,0	1,0	0,1	0,0	0,9	1,1	11
Anterior cell end to posterior end of frontal band, distance	6,9	6,3	1,6	0,2	4,9	9,9	11
Anterior end to anterior end of adoral zone, distance	16,4	16,9	1,4	0,1	14,1	18,1	12
Anterior end to posterior end of adoral zone, distance	24,9	25,1	1,4	0,1	22,2	26,7	12
Posterior cell end to anterior end of adoral zone, distance	11,6	11,4	1,4	0,1	10,1	15,4	12,0
Posterior cell end to posterior end of adoral zone, distance	3,1	2,6	1,5	0,5	1,7	7,3	12,0
Posterior cell end to adoral zone distance, ratio	0,3	0,2	0,1	0,3	0,2	0,5	12,0
Macronucleus, number	1,0	1,0	0,0	0,0	1,0	1,0	12
Macronucleus, diameter	8,8	9,0	1,0	0,1	6,8	10,6	12
Micronucleus, diameter	2,0	2,0	0,1	0,1	1,9	2,2	10
Pectinelles of epistomial fringe, number	5,0	5,0	0,0	0,0	5,0	5,0	8
Epistomial fringe ciliary rows, highest number	5,0	5,0	0,0	0,0	5,0	5,0	8
Adoral membranelles, number	10,0	10,0	0,0	0,0	10,0	10,0	12
Longest membranelle, length	8,8	9,1	1,0	0,1	7,1	10,0	12
Preoral kineties, number	2,0	2,0	0,0	0,0	2,0	2,0	12
Dikinetids in perioral kinety 1, number	6,9	7,0	1,2	0,2	5,0	8,0	7
Dikinetids in perioral kinety 2, number	7,3	7,5	1,0	0,1	6,0	8,0	4
Dikinetids in ventral kinety 1, number	9,8	10,0	0,4	0,0	9,0	10,0	5
Somatic kineties, number	4,0	4,0	0,0	0,0	4,0	4,0	4
Non-ciliated kineties on left side, number	1,0	1,0	0,0	0,0	1,0	1,0	4
Non-ciliated kineties on right side, number	3,0	3,0	0,0	0,0	3,0	3,0	4
Rows of caudal cilia, number	2,0	2,0	0,0	0,0	2,0	2,0	11
Caudal cirri, length, in vivo	25,5	23,7	4,7	0,2	21,6	38,2	11
Kinetids in each caudal cirri, number	6,7	6,0	1,2	0,2	6,0	8,0	3

Table S11. Morphometric data of *Discomorphella pectinata* MOKOT and BOTAN strains.

Character	Strain	Mean	M	SD	CV	Min	Max	N
Body length, in vivo	MOKOT	72,7	71,2	4,7	6,4	66,9	78,7	9,0
	BOTAN	74,2	74,5	4,4	5,9	67,0	80,0	12,0
Body width, in vivo	MOKOT	64,6	63,7	5,9	9,1	56,4	71,0	9,0
	BOTAN	72,0	72,5	4,6	6,4	63,0	79,0	12,0
Body length:width, ratio in vivo	MOKOT	1,2	1,2	0,1	5,0	1,1	1,2	5,0
	BOTAN	1,0	1,0	0,1	5,1	0,9	1,1	12,0
Body spines, number	MOKOT	3,0	3,0	0,0	0,0	3,0	3,0	13,0
	BOTAN	3,0	3,0	0,0	0,0	3,0	3,0	12,0
Frontal spine, length	MOKOT	11,5	11,5	1,3	11,2	10,0	13,0	4,0
	BOTAN	15,1	15,0	1,2	7,7	13,0	17,0	12,0
Lateral spine, length	MOKOT	16,7	16,0	2,1	12,5	15,0	19,0	3,0
	BOTAN	22,0	21,0	1,7	7,9	21,0	24,0	3,0
Posterior spine, length	MOKOT	21,0	21,0	2,0	9,5	19,0	23,0	3,0
	BOTAN	26,0	25,5	3,3	12,8	21,0	30,0	10,0
Body length	MOKOT	62,5	62,3	4,9	7,8	53,1	69,8	13,0
	BOTAN	60,6	61,7	3,8	6,3	54,2	64,3	5,0
Body width	MOKOT	63,2	64,1	4,9	7,7	52,7	71,6	12,0
	BOTAN	61,1	60,0	7,8	12,7	55,4	74,4	5,0
Body length:width, ratio	MOKOT	1,0	1,0	0,1	6,2	0,9	1,1	12,0
	BOTAN	1,0	1,0	0,1	11,9	0,8	1,2	5,0
Anterior end to anterior end of adoral zone, distance	MOKOT	38,0	36,2	5,2	13,6	31,4	45,5	8,0
Anterior end to posterior end of adoral zone, distance	MOKOT	48,6	48,3	4,2	8,7	43,1	54,1	8,0
Posterior cell end to anterior end of adoral zone, distance	MOKOT	39,2	27,2	19,4	49,5	19,5	65,8	13,0
Posterior cell end to posterior end of adoral zone, distance	MOKOT	32,7	15,7	24,6	75,2	11,7	65,8	13,0
Posterior cell end to adoral zone distance, ratio	MOKOT	0,7	0,6	0,2	29,7	0,5	1,0	13,0
Anterior end to fragment 1 of epistomial fringe, distance	MOKOT	12,6	12,8	4,5	35,2	5,4	20,2	13,0
	BOTAN	18,0	18,0	2,6	14,3	15,1	21,5	5,0
Longest membranelle, length	MOKOT	12,1	12,1	1,5	12,4	9,6	13,8	8,0
Macronucleus, number	MOKOT	1,0	1,0	0,0	0,0	1,0	1,0	13,0
	BOTAN	1,0	1,0	0,0	0,0	1,0	1,0	4,0
Macronucleus, length	MOKOT	17,8	18,0	2,6	14,5	11,8	21,5	13,0
	BOTAN	15,8	14,8	2,5	16,0	14,0	19,5	4,0
Macronucleus, width	MOKOT	13,6	14,0	1,6	11,8	10,4	16,2	13,0
	BOTAN	15,5	14,5	2,0	13,2	14,4	18,5	4,0
Micronucleus, number	MOKOT	1,0	1,0	0,0	0,0	1,0	1,0	11,0
	BOTAN	1,0	1,0	0,0	0,0	1,0	1,0	3,0
Micronucleus, diameter	MOKOT	4,3	4,2	1,1	24,5	2,0	6,4	11,0
	BOTAN	2,8	2,8	0,1	3,6	2,7	2,9	3,0
Epistomial fringe spacer, distance	MOKOT	24,6	24,9	4,0	16,3	16,9	30,3	13,0
	BOTAN	16,6	16,7	3,6	21,9	12,6	20,2	4,0

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dFS: anterior end to the anterior end of the front-most pectinelle of left fragment of epistomial fringe, distance	MOKOT	36,3	38,0	4,3	11,9	28,7	42,3	13,0
	BOTAN	33,0	33,9	4,2	12,7	27,6	36,5	4,0
Fringe spacer, ratio	MOKOT	0,6	0,6	0,0	7,5	0,5	0,6	13,0
	BOTAN	0,5	0,5	0,0	7,8	0,5	0,6	4,0
dF2a: posterior end of the front-most pectinelle in left fragment of epistomial fringe to the posterior end, distance	MOKOT	28,0	27,9	4,1	14,5	20,1	35,6	13,0
	BOTAN	27,5	27,3	1,8	6,4	25,7	29,5	4,0
dF2b: posterior end of the hind-most pectinelle in left fragment of epistomial fringe to the posterior end, distance	MOKOT	20,7	20,2	2,5	12,0	16,6	25,5	13,0
	BOTAN	20,2	19,7	2,0	9,9	18,5	23,1	4,0
Posterior fringe ratio	MOKOT	1,4	1,4	0,1	10,6	1,1	1,7	13,0
	BOTAN	1,4	1,4	0,2	13,3	1,1	1,5	4,0
Pectinelles of dextro frontal fragment of epistomial fringe, number	MOKOT	21,2	21,0	0,4	2,1	21,0	22,0	13,0
	BOTAN	21,2	21,0	0,4	1,9	21,0	22,0	6,0
Pectinelles of left fragment of epistomial fringe, number	MOKOT	9,0	9,0	0,6	6,4	8,0	10,0	13,0
	BOTAN	8,6	9,0	0,5	6,4	8,0	9,0	5,0
Adoral membranelles, number	MOKOT	9,0	9,0	0,0	0,0	9,0	9,0	8,0
	BOTAN	9,0	9,0	0,0	0,0	9,0	9,0	4,0
Ciliary sockets of the perioral kinety, number	MOKOT	24,7	25,0	3,8	15,5	20,0	34,0	11,0
	BOTAN	24,4	25,0	1,9	8,0	21,0	26,0	5,0
Dikinetids in inner endoral kinety, number	MOKOT	11,5	12,0	2,3	20,3	8,0	15,0	11,0
	BOTAN	8,8	8,5	1,0	11,1	8,0	10,0	6,0
Dikinetids in outer endoral kinety, number	MOKOT	13,4	14,0	1,9	14,2	10,0	15,0	10,0
	BOTAN	10,8	10,0	1,1	10,1	10,0	12,0	5,0
Supplementary ventral dikinetids, number	MOKOT	2,0	2,0	0,0	0,0	2,0	2,0	3,0
	BOTAN	2,0	2,0	0,0	0,0	2,0	2,0	4,0
Dikinetids in ventral kinety 1, number	MOKOT	16,0	16,0	1,9	12,0	13,0	19,0	7,0
	BOTAN	14,2	14,5	1,0	6,9	13,0	15,0	6,0
Dikinetids in ventral kinety 2, number	MOKOT	20,0	20,0	1,2	6,0	18,0	22,0	12,0
	BOTAN	16,8	17,0	0,4	2,4	16,0	17,0	6,0
Rows of caudal cirri, number	MOKOT	2,0	2,0	0,0	0,0	2,0	2,0	12,0
	BOTAN	2,0	2,0	0,0	0,0	2,0	2,0	6,0