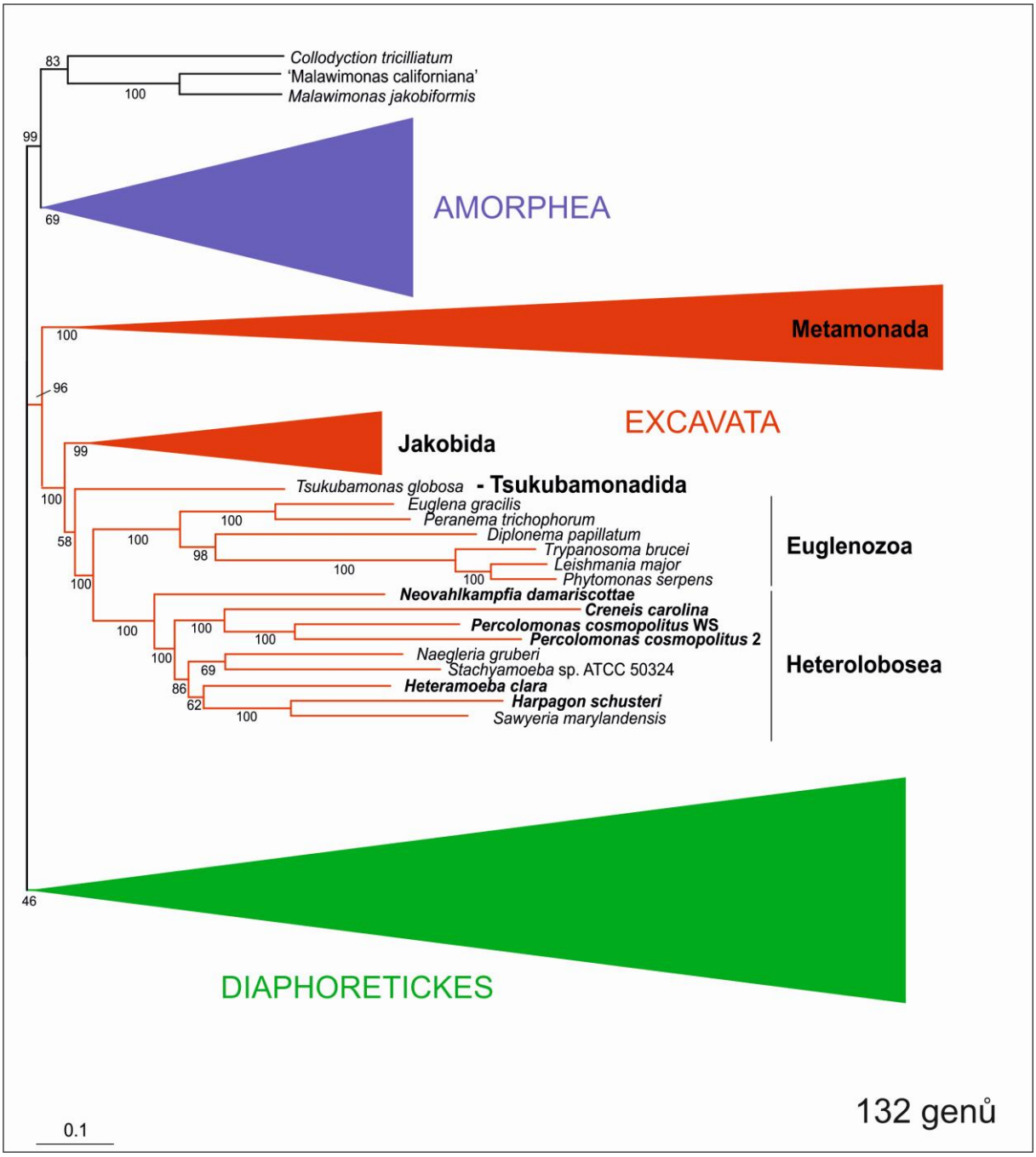
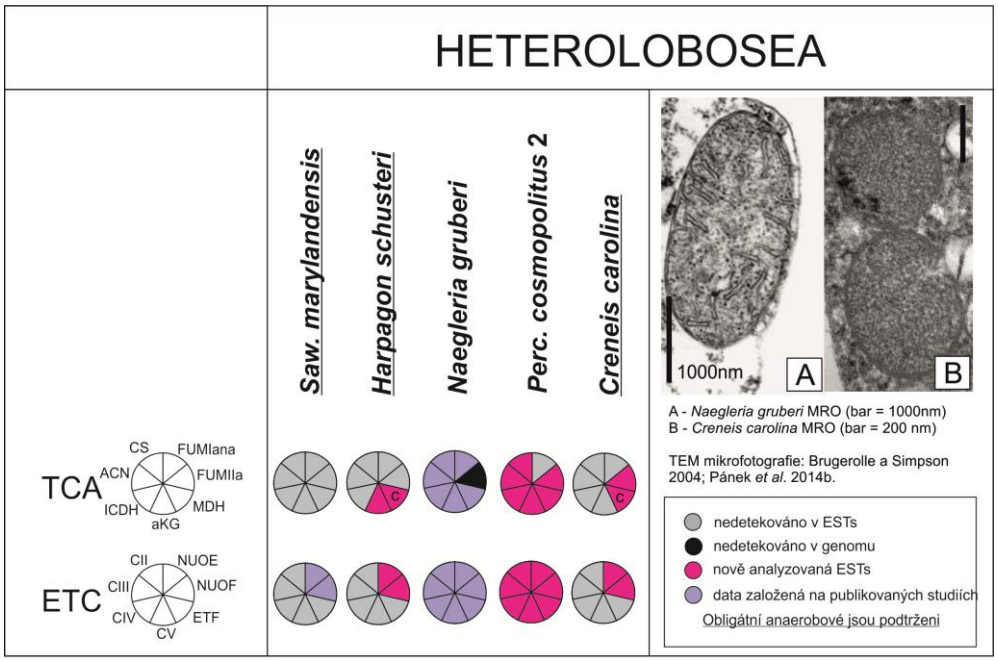
8. Přílohy

8.1. Obrázky S1 – S9

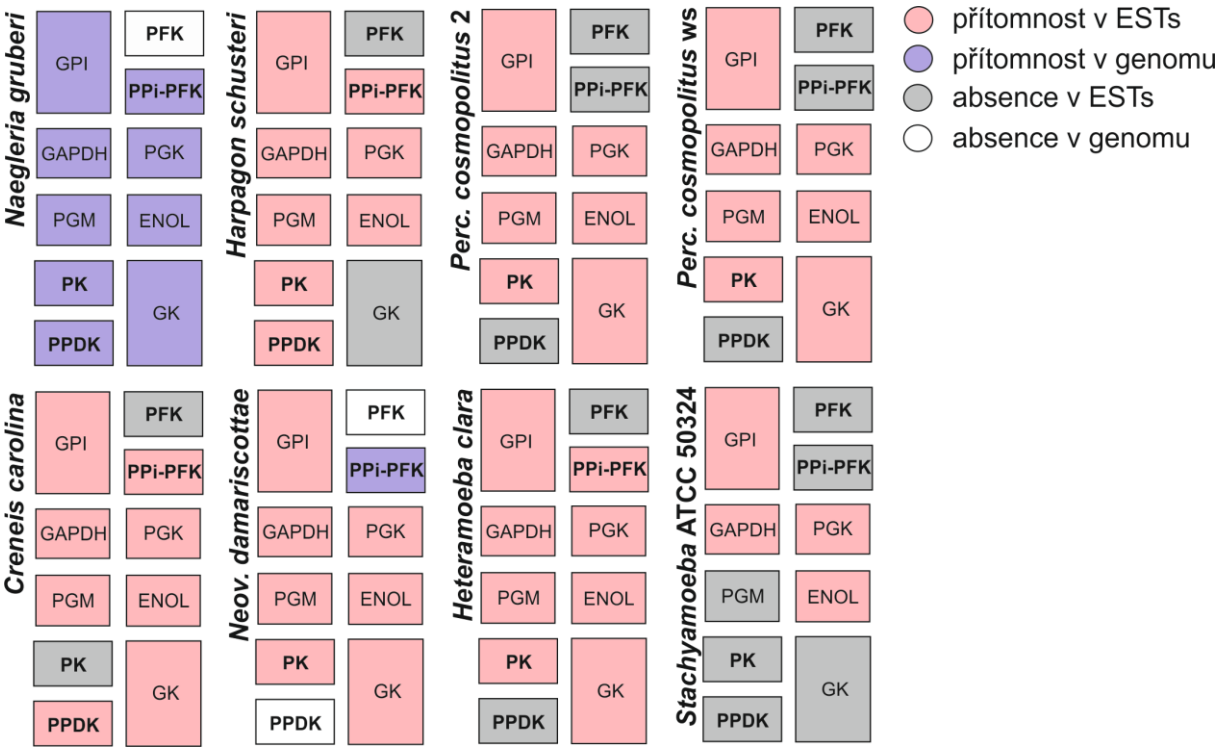


Obrázek S1. Fylogenomická analýza eukaryot založená na 132 genech. Strom byl konstruován v programu RAxML s použitím Maximum likelihood (PROTGAMMALGF model). Hodnoty v uzlech reprezentují bootstrapovou podporu, 300 bootstrapových pseudoreplikátů. Vyjma zástupců skupiny Heterolobosea a jí sesterské skupiny Euglenozoa byly názvy taxonů odstraněny, neboť v analýze byla použita i další naše doposud nepublikovaná data z jiných skupin eukaryot. Nově přidané OTU jsou označeny tučně. Analyzováno ve spolupráci s Dr. M. Kolískem.

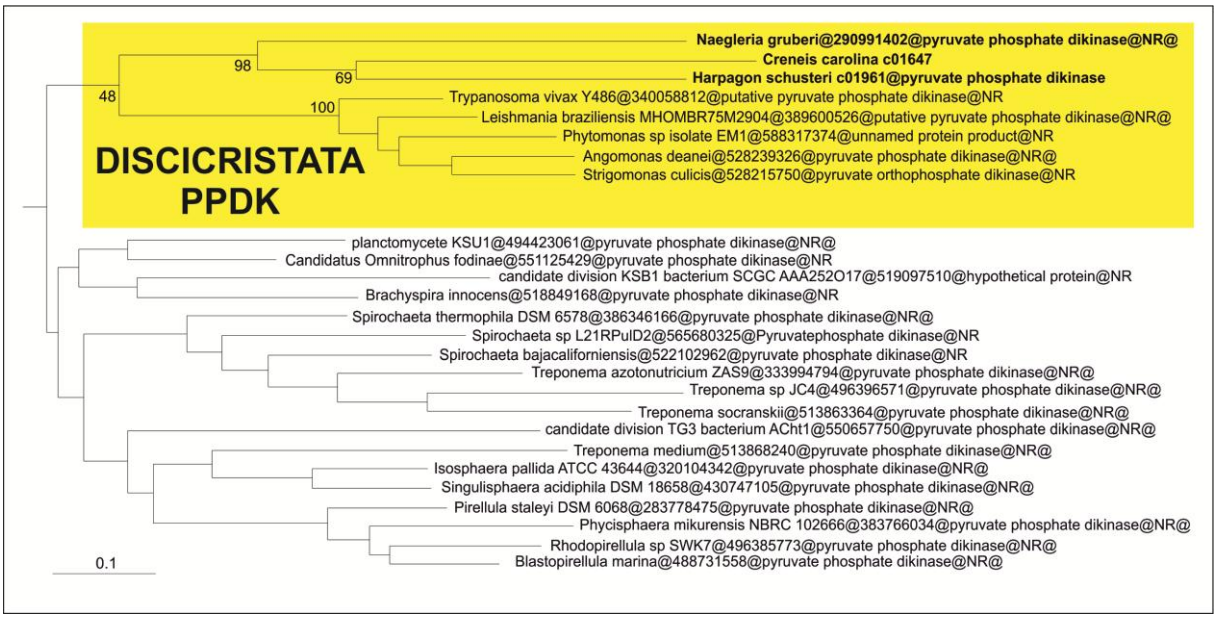


c - pouze cytoplasmatická isoforma MDH byla identifikována v EST datech

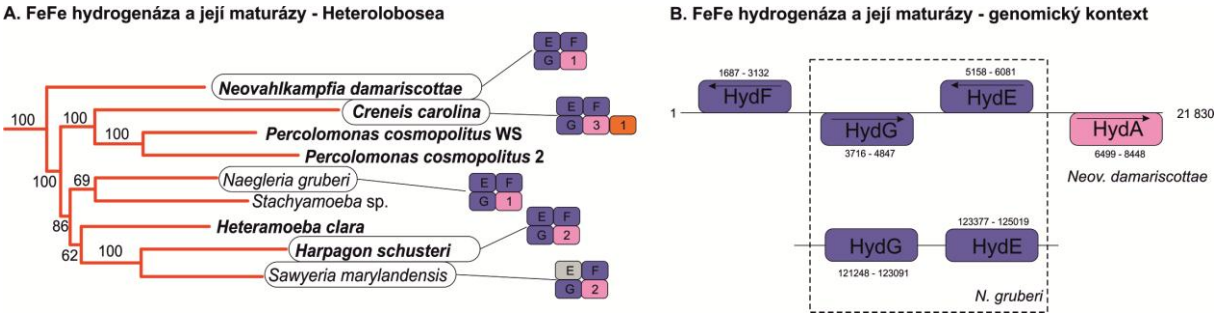
Obrázek S2. Enzymy Krebsova cyklu a dýchacího řetězce u heteroloboseí (*insilico* predikce). TCA: Krebsův cyklus: akonitáza (ACN); citrát syntáza (CS); fumaráza (FUMIana a FUMIIaer); isocitrát dehydrogenáza (ICDH); malát dehydrogenáza (MDH); α-ketoglutarát dehydrogenáza (aKG); ETC: Dýchací řetězec: komplexy I – V a flavoprotein přenášející elektrony (ETF); NuoE/F představují dvě podjednotky komplexu I. Sestaveno ve spolupráci s Dr. C. Stairs.



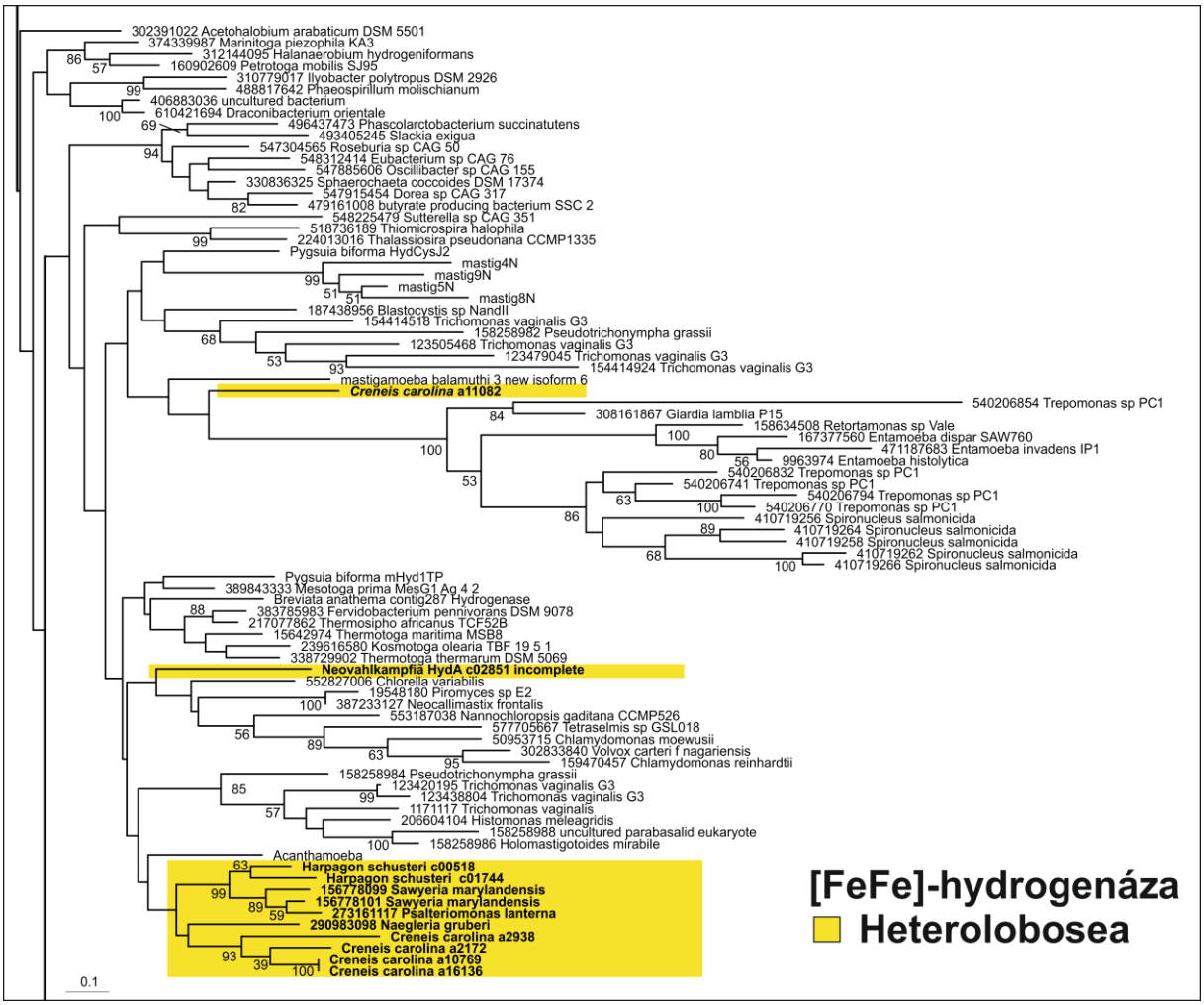
Obrázek S3: Glykolýza u heteroloboseí.GPI: glukóza-P-izomeráza; PFK: fosfofruktokináza; PPi-PFK: pyrofosfát-dependentní PFK; GAPDH:glyceraldehyd-3-P-dehydrogenáza; PGK: fosfoglycerát-kináza; PGM: fosfoglycerát-mutáza;ENOL: enoláza; PK: pyruvát-kináza; PPDK: pyruvát fosfát dikináza; GK: glycerol kináza.



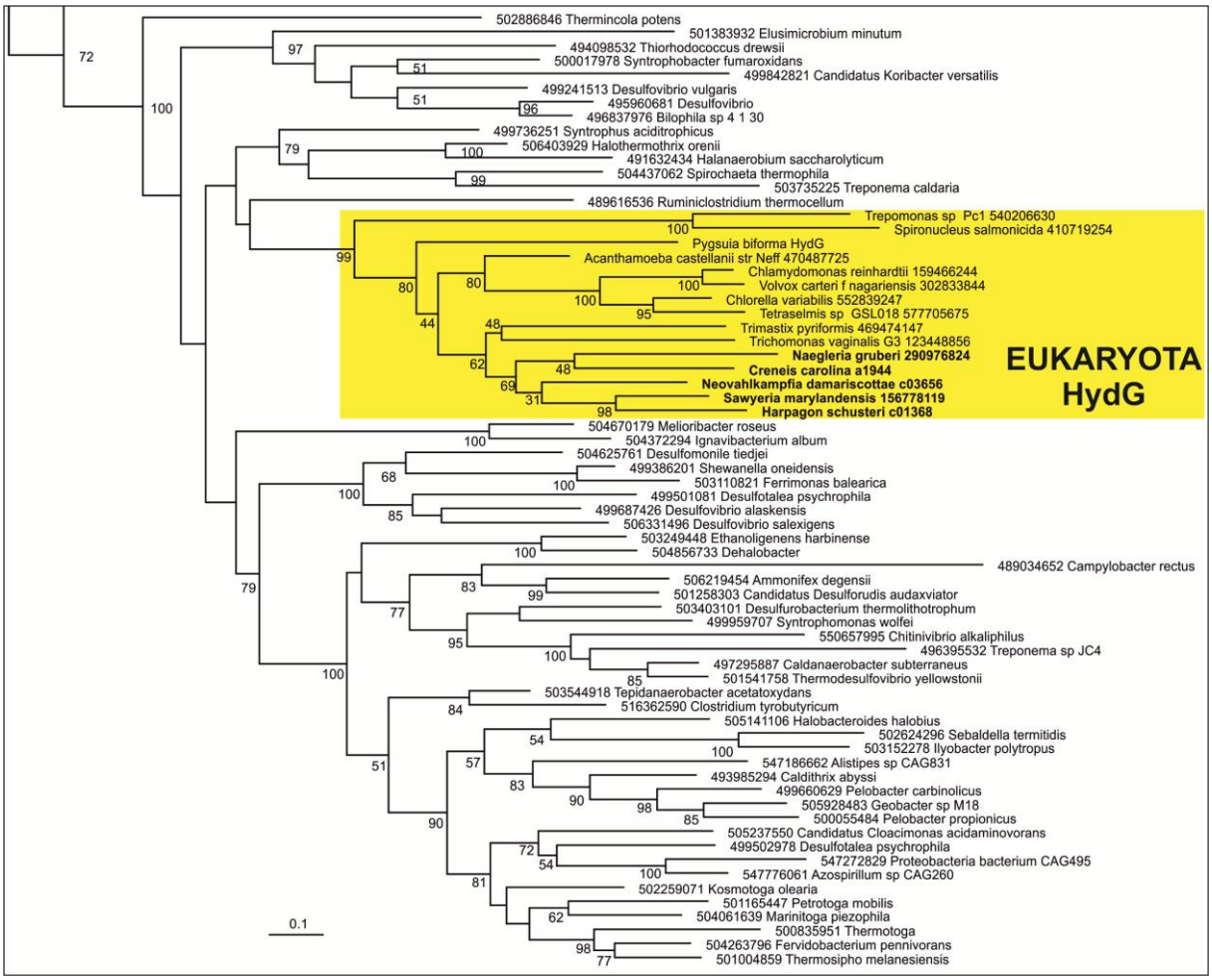
Obrázek S4. Fylogenetický strom pyruvát fosfát dikinázy (výřez). Barevně je vyznačena linie Euglenozoa + Heterolobosea (Discicristata), tučně pak sekvence heteroloboseí. Strom byl konstruován v programu RAxML (Maximum likelihood, LG4X model); hodnoty uvedené u jednotlivých větví reprezentují bootstrapovou podporu založenou na 500 pseudoreplikátech. Analyzováno ve spolupráci s Dr. C. Stairs.



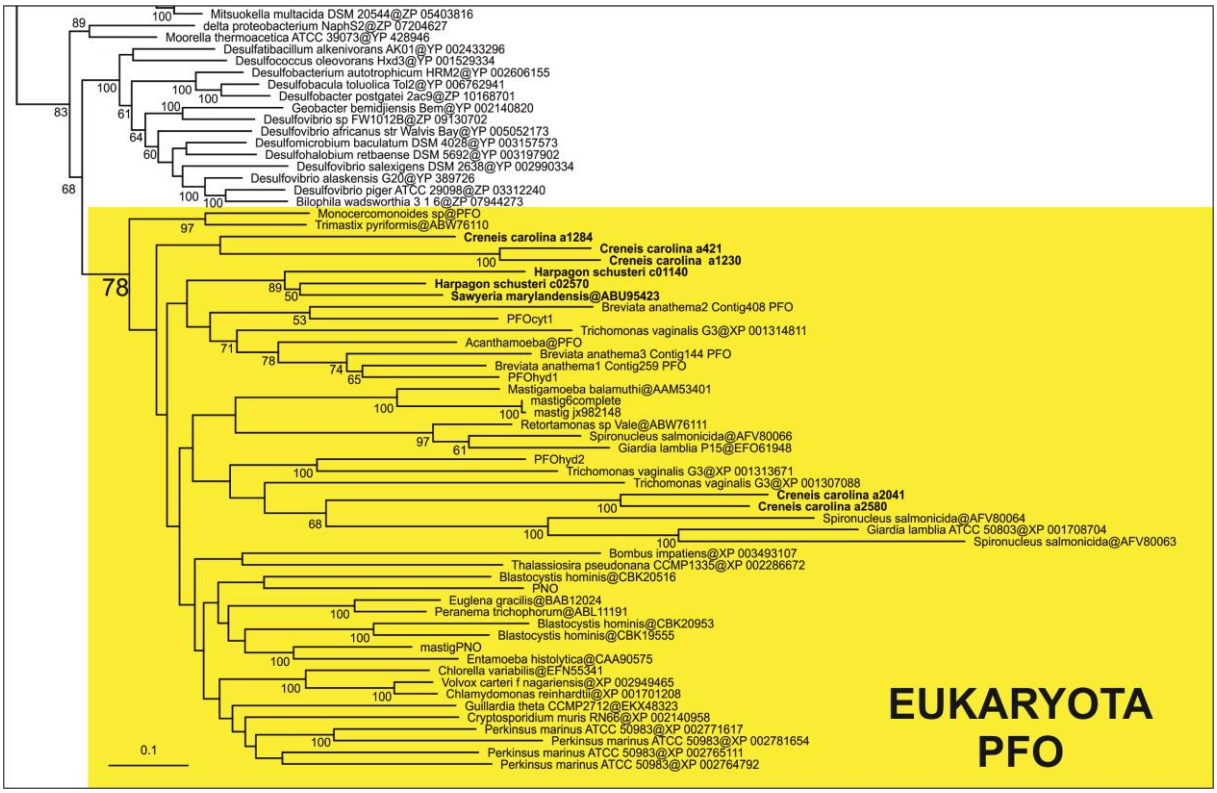
Obrázek S5. Výskyt [FeFe]-hydrogenázy a jejích maturáz u heteroloboseí. A. Výskyt těchto enzymů namapovaný na znalosti o fylogenezi heteroloboseí (tedy na naší předběžnou fylogenomickou analýzu). Modře je označena přítomnost maturáz, u S. marylandensis nebyla v transkriptomických datech nalezena maturázaHydE (naznačeno šedě). Růžově je označena přítomnost původní [FeFe]-hydrogenázy heteroloboseí (číslem uveden počet kopií). Oranžově označena přítomnost [FeFe]-hydrogenázy nejistého původu u *Creneiscarolina*. **B.**[FeFe]-hydrogenáza a její maturázy z pohledu umístění v genomu. U *N. damariscottae* jsou všechny čtyři geny velmi blízko sebe, u *N. gruberi* se vedle sebe nachází jen HydG a HydE. Analyzováno ve spolupráci s E. Zadrobílkovou.



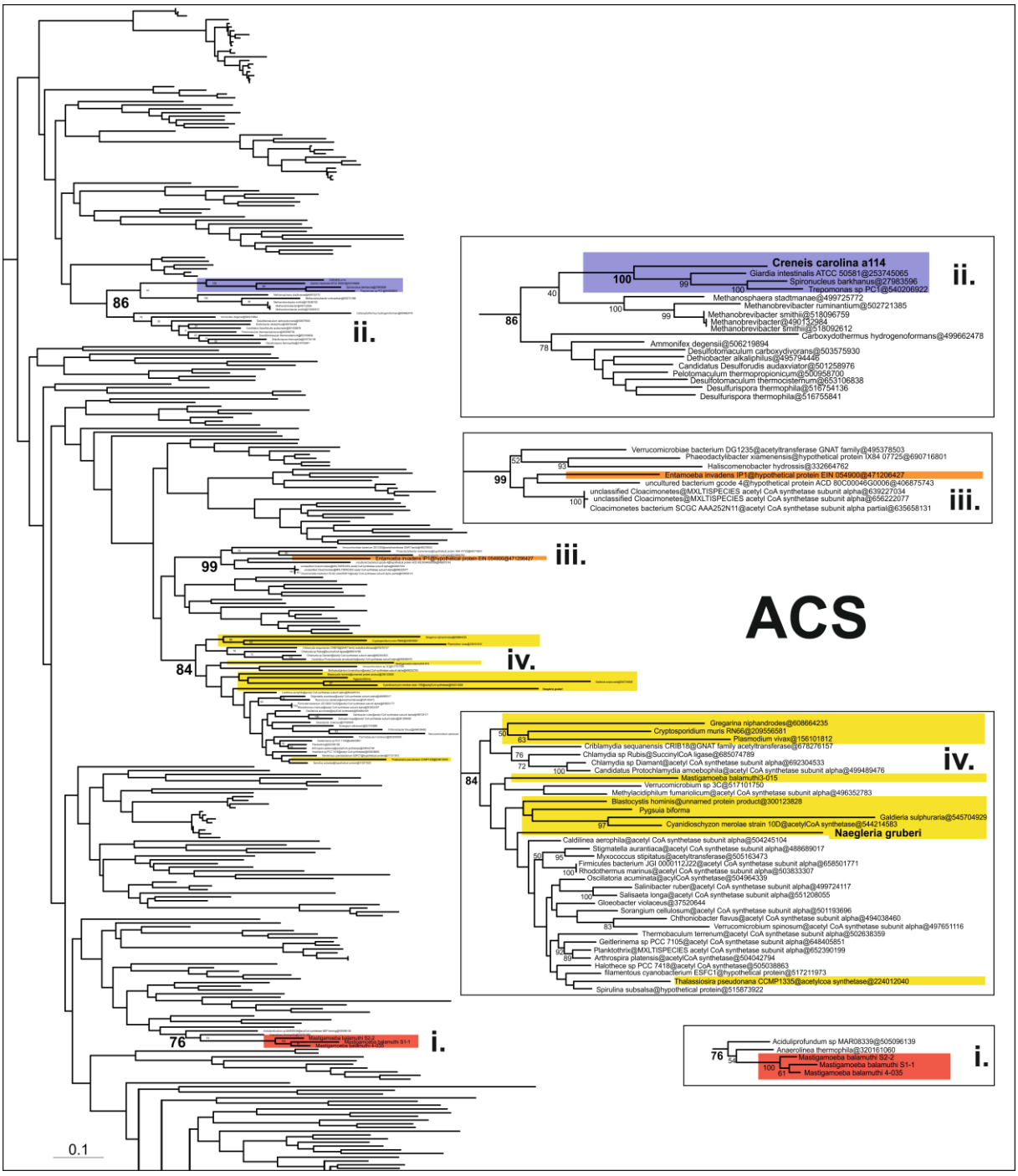
Obrázek S6. Fylogenetický strom [FeFe]-hydrogenázy (výřez). Barevně jsou vyznačeny sekvence patřící heteroloboseím. Strom byl konstruován v programu RAxML (Maximum likelihood, LG4X model); hodnoty uvedené u jednotlivých větví reprezentují bootstrapovou podporu založenou na 500 pseudoreplikátech. Analyzováno ve spolupráci s Dr. C. Stairs.



Obrázek S7. Fylogenetický strom maturázy HydG (výřez). Barevně jsou vyznačeny sekvence patřící eukaryotům, tučně pak sekvence heteroloboseí. Strom byl konstruován v programu RAxML (Maximum likelihood, LG4X model); hodnoty uvedené u jednotlivých větví reprezentují bootstrapovou podporu založenou na 500 pseudoreplikátech. Analyzováno ve spolupráci s Dr. C. Stairs.



Obrázek S8. Fylogenetický strom PFO (výřez). Barevně jsou vyznačeny sekvence patřící eukaryotům, tučně pak sekvence heteroloboseí. Strom byl konstruován v programu RAxML (Maximum likelihood, LG4X model); hodnoty uvedené u jednotlivých větví reprezentují bootstrapovou podporu založenou na 500 pseudoreplikátech. Analyzováno ve spolupráci s Dr. C. Stairs.



Obrázek S9. Fylogenetický strom ACS (výřez). Na levé straně je vyobrazen výřez fylogenetického stromu s barevně vyznačenými eukaryotickými sekvencemi. Různými barvami jsou označeny jednotlivé horizontální genové přenosy z prokaryot do eukaryot (podle Nývltová *etal*. 2015). Na pravé straně jsou zvětšeny jednotlivé výseky fylogenetického stomu ukazující detailněji bootstrapovou podporu a postavení jednotlivých eukaryotických linií.Strom byl konstruován v programu RAxML (Maximum likelihood, LG4X model); hodnoty uvedené u jednotlivých větví reprezentují bootstrapovou podporu založenou na 500 pseudoreplikátech. Analyzováno ve spolupráci s Dr. C. Stairs.

8.2. Ostatní publikace autora práce

Pánek, T., Čepička, I. (2012). Diversity of Heterolobosea in: Caliskan M. (ed.) Genetic diversity in microorganisms, InTech.

Yubuki, N., **Pánek, T.**, Yabuki, A., Čepička, I., Takishita, K., Inagaki, Y., Leander, B.S. (2015). Morphological identities of two different marine stramenopile environmental sequence clades: *Bicosoeca kenaiensis* (Hilliard, 1971) and *Cantina marsupialis* (Larsen and Patterson, 1990) gen. nov., comb. nov. Journal of Eukaryotic Microbiology 62: 532-542.

Céza, V., **Pánek, T.**, Smejkalová, P., Čepička, I. (2015). Molecular and morphological diversity of the genus *Hypotrichomonas* (Parabasalia: Hypotrichomonadida), with descriptions of six new species. European Journal of Protistology 51: 158-172.

Zhang, Q., Táborský, P., Silberman, J., **Pánek, T.**, Čepička, I., Simpson, A.G.B. (2015) Marine isolates of *Trimastix marina* form a plesiomorphic deep-branching lineage within Preaxostyla, separate from other known trimastigids (*Paratrimastix* n. gen.). Protist, in press.

8.2.1. Pánek, Čepička (2012)

Pánek, T., Čepička, I. (2012). Diversity of Heterolobosea in: Caliskan M. (ed.) Genetic diversity in microorganisms, InTech.

1

Diversity of Heterolobosea

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1. Introduction

Heterolobosea is a small group of amoebae, amoeboflagellates and flagellates (ca. 140 described species). Since heterolobosean amoebae are highly reminiscent of naked lobose amoebae of Amoebozoa, they were for a long time treated as members of Rhizopoda (Levine, 1980). The class Heterolobosea was established in 1985 by Page and Blanton (Page & Blanton, 1985) by uniting unicellular Schizopyrenida with Acrasida that form multicellular bodies. Later, it was suggested that Heterolobosea might be related to Euglenozoa (e.g., *Trypanosoma*, *Euglena*) instead of other amoebae (Cavalier-Smith, 1998; Patterson, 1988). This assumption based on the cell structure was supported also by early multigene phylogenetic analyses (Baldauf et al., 2000). Currently, the Heterolobosea is nested together with Euglenozoa, Jakobida, Parabasalia, Fornicata, Preaxostyla, *Malawimonas*, and *Tsukubamonas* within the eukaryotic supergroup Excavata (Hampl et al., 2009; Rodríguez-Ezpeleta et al., 2007; Simpson, 2003; Yabuki et al., 2011). The excavate organisms were originally defined on the basis of the structure of flagellar system and ventral feeding groove (Simpson & Patterson, 1999). However, Heterolobosea have lost some of these structures (Simpson, 2003).

The most important heterolobosean taxon is the genus *Naegleria* as *N. fowleri* is a deadly parasite of humans (Visvesvara et al., 2007) and *N. gruberi* is a model organism in the research of assembly of the flagellar apparatus (Lee, 2010). Both the species have been studied in detail for decades and genome sequence of *N. gruberi* was recently published (Fritz-Laylin et al. 2010). On the other hand, the other heteroloboseans are considerably understudied and undescribed despite their enormous ecological and morphological diversity. Many heteroloboseans have adapted to various extreme environments; halophilic, acidophilic, thermophilic, and anaerobic representatives have been described. Few heteroloboseans are facultative endobionts of both vertebrates and invertebrates. *Naegleria fowleri* and *Paravahlkampfia francinae* are even able to parasitize humans (Visvesvara et al., 2007, 2009). The genus *Stephanopogon*, whose members are multiflagellate, was once considered to be a primitive ciliate and was affiliated with Heterolobosea only on the basis of cell structure and phylogenetic position. Finally, acrasids have developed a simple form of aggregative multicellularity and represent the only known multicellular excavates.

2. Morphological diversity

Most heteroloboseans are unicellular and uninucleate, though several species are multinucleate at least in part of their life cycle, e.g., *Stephanopogon* spp., *Gruberella flavescens*,

Pseudovahlkampfia emersoni, Fumarolamoeba ceborucoi, Willaertia magna, and *Psalteriomonas lanterna* (Broers et al., 1990; De Jonckheere et al., 2011b; Page, 1983; Sawyer, 1980; Yubuki & Leander, 2008). All heteroloboseans lack a typical, "stacked" Golgi apparatus. Mitochondria of Heterolobosea are oval, elongated or cup-shaped and possess flattened, often discoidal cristae. Few species are anaerobic and their mitochondria lack cristae. The mitochondrion of Heterolobosea is often closely associated with rough endoplasmic reticulum.

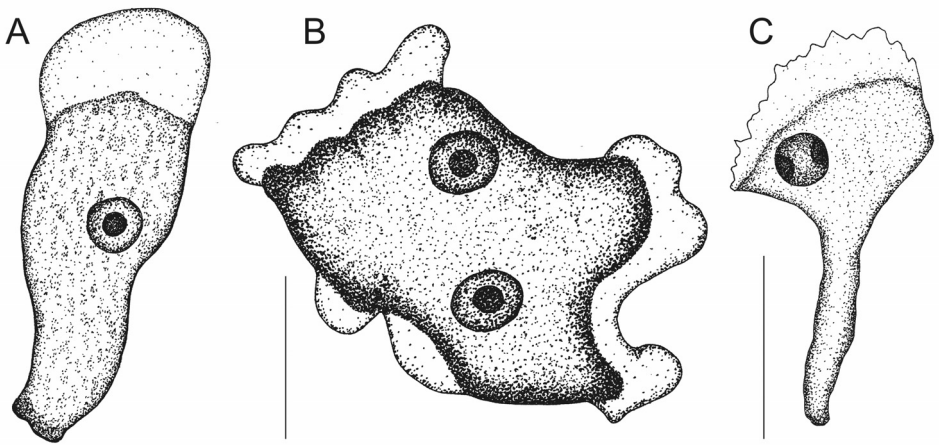

Fig. 1. Amoebae of Heterolobosea. **A**, *Acrasis rosea*; **B**, *Fumarolamoeba ceborucoi*; **C**, flabellate form of *Stachyamoeba lipophora*. Scale bars = 10 µm. After De Jonckheere et al., 2011b; Page, 1988; Olive & Stoianovitch, 1960).

Typical life-cycle of Heterolobosea consists of amoeboid, flagellate and resting stage (a cyst). However, one or two stages are unknown and presumably have been reduced in many taxa. Heterolobosean amoebae bear no flagella. Interestingly, the flagellar apparatus including basal bodies is assembled *de novo* during the transformation to the flagellate (see below). The amoebae are relatively uniform in shape and size. The locomotive forms are usually of the "limax" type (i.e. cylindrical monopodial amoebae, see fig. 1A) and move rapidly with eruptive lobopodia. Amoebae of some species, e.g., *Fumarolamoeba ceborucoi*, form subpseudopodia in all directions (De Jonckheere et al., 2011b; see fig. 1B). The locomotive form of *Stachyamoeba lipophora* is usually flattened ("flabellate") and its single pseudopodium bears many short subpseudopodia (Page, 1987; see fig. 1C). Many heterolobosean amoebae form a posterior uroid, sometimes with long uroidal filaments. *Vahlkampfia anaerobica* was reported to form a floating form (Smirnov & Fenchel, 1996). The heterolobosean amoebae do not possess any cytoskeleton-underlain cytostomes. However, the amoeba of *Naegleria fowleri* forms so-called amoebastomes, sucker-like surface structures that aid in phagocytosis (Sohn et al., 2010). The amoeboid stage is unknown (and possibly completely lost) in genera *Lyromonas*, *Pharyngomonas*, *Pleurostomum*, *Percolomonas*, and *Stephanopogon*.

Most heterolobosean flagellates have a groove-like cytostome that rises subapically (see fig. 2A-D). On the other hand, cytostomes of genera *Tetramitus*, *Heteramoeba*, and *Trimastigamoeba* open anteriorly (fig. 2E, G). Several heterolobosean flagellates, e.g., *Tetramitus* spp. and *Heteramoeba clara*, have a distinct collar or rim that circumscribes the

anterior end of the cell body (see fig. 2E). In *Tetramitus rostratus* and *Pleurostomum flabellatum* the collar is drawn out into a short rostrum (fig. 2F). The cytostome of *T. rostratus* and *P. flabellatum* has a broad opening and curves into the cell to form a microtubule-supported tubular feeding apparatus (fig. 2F). The cytostome of *Trimastigamoeba phillippinensis* is a gullet-like tube with flagella rising from its bottom (fig. 2G). *Pharyngomonas kirbyi*, the basal-most lineage of Heterolobosea, has a subtle ventral groove and sub-anterior curved cytopharynx (fig. 2A). The cytostome of genera *Naegleria*, *Willaertia* and *Euplaesiobystra* has been reduced (fig. 2H).

Heterolobosean flagellates typically possess two (Heteramoeba, Euplaesiobystra, Pleurostomum, Pocheina, most Naegleria and some Tetramitus species) or four (Lyromonas, Willaertia, Percolomonas, Pharyngomonas, Tetramastigamoeba, few Naegleria and most Tetramitus species) flagella which arise at the anterior end of the feeding apparatus. Only few heterolobosean species have a different number of flagella. However, the number of flagella may vary among individuals of a single species. For example, most Tetramitus jugosus and Oramoeba fumarolia flagellates are biflagellate, but cells with more flagella (up to 10 in O. fumarolia) were found as well (Darbyshire et al., 1976; De Jonckheere et al., 2011a). Psalteriomonas lanterna has four nuclei, four mastigonts, each with four flagella, and four ventral grooves (fig. 2D). Representatives of genus Stephanopogon have over one hundred flagella (fig. 2I).

The flagella are usually equal in length. Alternatively, some flagella may be longer than the other ones (*Percolomonas* spp., *Pharyngomonas kirbyi*). All four flagella of *Percolomonas descissus* beat synchronously and drive water with food particles into the cytostome. *P. cosmopolitus* often attaches to the substrate by the tip of the longest flagellum. Most unattached cells move with a skipping motion across the substrate, as the trailing flagellum repeatedly makes and breaks contact with the surface (Fenchel & Patterson, 1986). Two flagella of *Pharyngomonas kirbyi* are directed anteriorly and actively beat during swimming. The cells can attach to the substrate using these flagella. The remaining two flagella are directed posteriorly and beat slowly. They are used during feeding to drive the water into the cytostome (Park & Simpson, 2011).

In quadriflagellate heteroloboseans, the basal bodies of flagella are arranged into two linked similar dikinetids rather than a single tetrakinetid. Such an unusual organization of the mastigont is called "double bikont". The arrangement of the pairs between each other can be orthogonal (e.g., *Tetramitus rostratus*), in tandem (e.g., *Percolomonas descissus*) or side-by-side (*Pharyngomonas kirbyi*, *Percolomonas sulcatus*). The arrangement of basal bodies in a pair can be orthogonal (*Pharyngomonas kirbyi*), parallel or near parallel (other heteroloboseans) (Brugerolle & Simpson, 2004; Park & Simpson, 2011). The flagellar apparatus of most heterolobosean flagellates possesses only two structures characteristic for Excavata as defined by Simpson (2003). In contrast, the mastigont of *Pharyngomonas kirbyi*, the deepest-branching heterolobosean, is more plesiomorphic and displays additional two or three excavate features (for details see Park & Simpson, 2011). The arrangement of basal bodies within a pair of flagella also seems to be more plesiomorphic in *Pharyngomonas* than that of the other heteroloboseans. In addition, the flagellar apparatus of *Percolomonas sulcatus* seems to be more plesiomorphic as well and is the most obvious example of the double bikont organization (Brugerolle & Simpson, 2004; Park & Simpson, 2011). On the other hand, it lacks the additional excavate features observed in *Ph. kirbyi*.

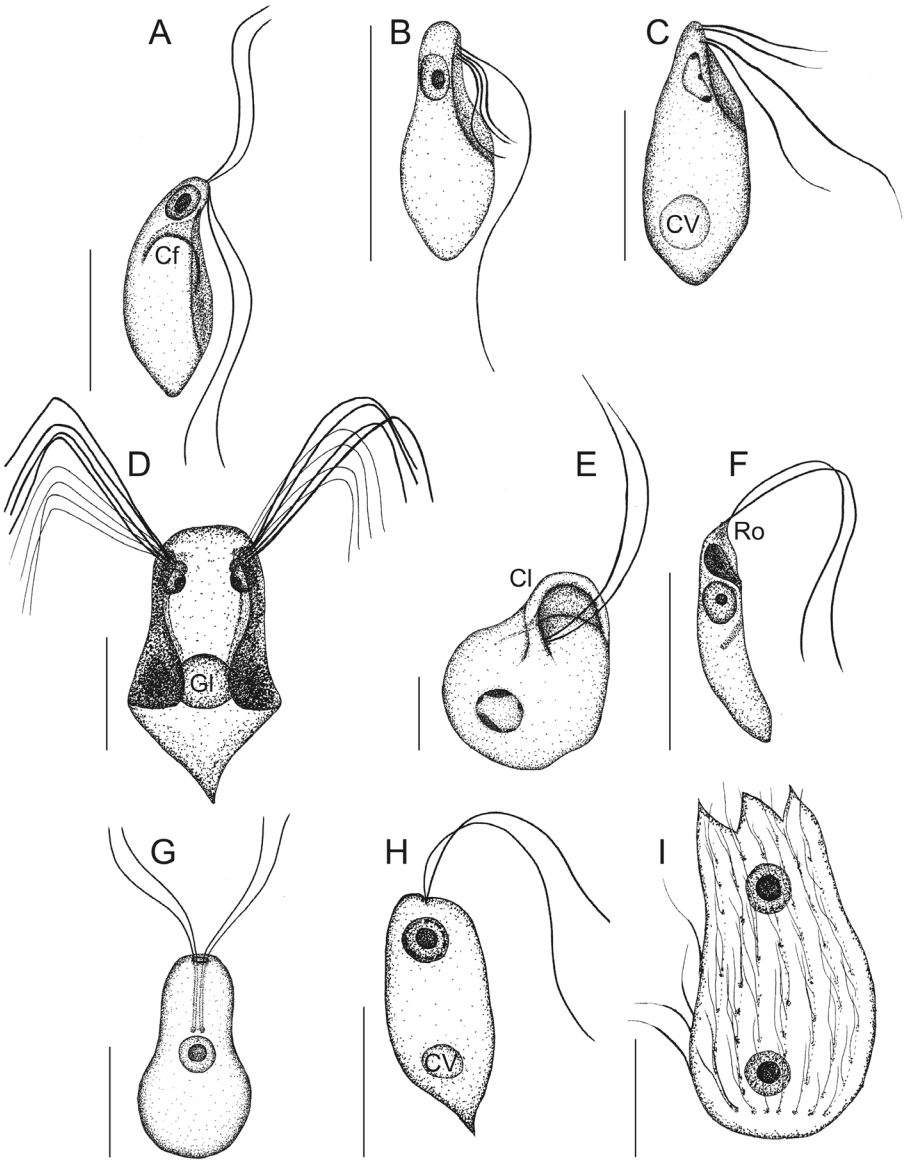

Fig. 2. Heterolobosean flagellates. **A**,*Pharyngomonas kirbyi*; **B**, *Percolomonas cosmopolitus*; **C**, *Percolomonas descissus*; **D**, *Psalteriomonas lanterna*; **E**, *Heteramoeba clara*; **F**, *Pleurostomum flabellatum*; **G**, *Trimastigamoeba philippinensis*; **H**, *Naegleria gruberi* **I**,. *Stephanopogon minuta*. Cf – cytopharynx; Cl – collar; CV – contractile vacuole; Gl – globule of hydrogenosomes; Ro – rostrum. Scale bars = 10 μm. After Broers et al., 1990; Bovee, 1959; Brugerolle & Simpson, 2004; Droop, 1962; Fenchel & Patterson, 1986; Page, 1967, 1988; Park et al., 2007; Park & Simpson, 2011; Yubuki & Leander, 2008.

Members of the eukaryovorous genus *Stephanopogon* are strikingly different from the other heterolobosean. Their vase-shaped and curved cell bodies possess several longitudinal rows of flagella and two isomorphic nuclei. The cytostome is slit-shaped, dorsally supported by a lip, and accompanied by ventral barbs in most species. The ventral side of the cell bears more than 100 flagella, while only ca. 13 flagella arise from the dorsal side (Yubuki & Leander, 2008).

The cyst is the third heterolobosean life stage. The cyst wall usually consists of two layers, ectocyst and endocyst. They are either closely associated to each other or can be separated and thus easily recognized by light microscope. The surface of the cyst is wrinkled, rough or smooth, and can be sticky (e.g., in *Paravahlkampfia*). Most heterolobosean cysts have no pores and presumably excyst by a wall rupture as in representatives of *Paravahlkampfia* (Visvesvara et al., 2009). The cyst of genera *Tulamoeba* and *Monopylocystis* has a single pore that penetrates the wall and is sealed with a mucoid plug (Park et al., 2009). Members of genera *Willaertia*, *Naegleria*, *Marinamoeba*, *Pernina*, and *Euplaesiobystra* have pores in the cyst wall. The cyst pores of *Naegleria*, *Willaertia* and *Pernina* are similar to each other in that the pores penetrate both the endocyst and ectocyst. In contrast, the pores of *Euplaesiobystra hypersalinica* do not penetrate the endocyst wall (Park et al., 2009). The cyst morphology of the genus *Tetramitus*, including the presence and number of pores, is highly variable. In many heterolobosean the cyst stage is unknown (*Pleurostomum*, *Neovahlkampfia*, *Sawyeria*, *Psalteriomonas*, *Lyromonas* etc.).

Members of the Acrasidae have developed an additional stage in their life cycle, a simple multicellular fruiting body (sorocarp) formed by an aggregation of amoebae. The Acrasidae is the only known multicellular lineage of Excavata. The cells are in the mature sorocarp differentiated into two types: basal stalk cells and distal spore cells. Unlike in *Dictyostelium* (Amoebozoa: Dictyosteliida), where the stalk-forming cells undergo programmed cell death, the stalk-forming cells of the Acrasidae do not lose their viability. The most studied species of multicellular heterolobosean is *Acrasis rosea*. Its sorocarps are complex with many branches ("arborescent"; fig. 4). Fruiting bodies of the recently described species *A. helenhemmesae* are simpler, uniseriate, and with only two or three bottle-shaped stalk cells (Brown M.W. et al., 2010). In contrast, the sorocarps of the putative acrasid *Pocheina flagellata* are globular (Olive et al., 1983).

3. Life cycles of Heterolobosea

Present knowledge on the life cycle of most heterolobosean is fragmentary and it has been studied in detail only in *Naegleria gruberi*. The main active stage of *N. gruberi* is the amoeba which relies on actin-based cytoskeleton (Walsh, 2007) and has no flagella, basal bodies or cytoplasmic microtubules. It normally feeds, moves, and divides. Under certain conditions the amoeba rapidly transforms to the flagellate or the cyst stage (fig. 3). The transformation to the flagellate stage is triggered by various stressors, such as changes in temperature, osmolarity or availability of nutrients. The flagellate of *N. gruberi* is a temporary stage persisting only for several hours. It does not divide and feed, and has no cytostome. This is a typical feature of most *Naegleria* species. In *Naegleria minor* and *Naegleria robinsoni*, however, the juvenile flagellates possess four flagella and divide once to form biflagellate cells similar to flagellates of the other *Naegleria* species (De Jonckheere, 2002). It was reported that the flagellate of *N. gruberi* plays a crucial role in shuttling from the benthos to the water surface

(Preston & King, 2003). Interestingly, the whole microtubule cytoskeleton of the flagellate, including flagella and their basal bodies, is formed *de novo* during the transformation from the amoeba (Fulton, 1977; Fulton & Dingle, 1971; Lee, 2010). The transformation is incredibly fast being completed within ca. two hours.

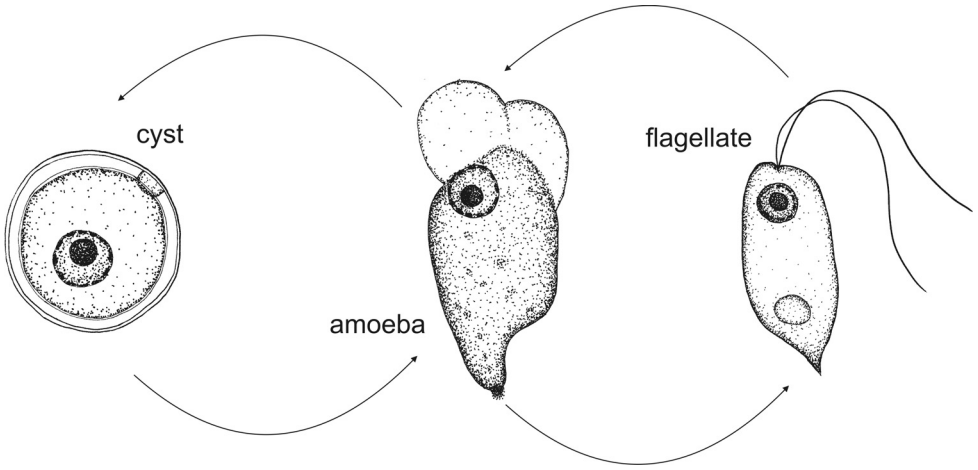

Fig. 3. The life cycle of *Naegleria gruberi*. After Page, 1967.

In contrast to *Naegleria*, flagellates of many other heteroloboseans are able to feed and divide, sometimes for long periods without reverting to the amoeba. In some heteroloboseans the flagellate is the only trophic stage and the ability to form amoebae has been presumably lost. On the other hand, the flagellate is unknown from even more heteroloboseans (see above). The ability to encyst is usually connected with the amoeba stage. The exceptions are *Percolomonas cosmopolitus* and *Stephanopogon* spp. which lack the amoeboid stage and encyst as flagellates (Fenchel & Patterson, 1986; Lwoff, 1936; Raikov 1969).

The amoeba-to-flagellate transformation of many heterolobosean species may be more or less successfully induced *in vitro* (see Page, 1988). In other species, the attempts were unsuccessful. The ability to form flagellates *in vitro* may vary also within the genus. For example, although most *Naegleria* and many *Tetramitus* species are known to produce flagellates, it seems that some of them have lost the ability (e.g., De Jonckheere, 2007; De Jonckheere et al., 2001). However, as culture requirements of almost no heteroloboseans have been studied in detail, some of them may be unable to transform *in vitro* and the observed inability to form certain life stages can be thus artificial. Indeed, strains of particular species known to produce flagellates (*Psalteriomonas lanterna*, *Heteramoeba clara*, *Willaertia*, some *Tetramitus* species) or cysts (*Percolomonas cosmopolitus*) were observed to lose the ability after a prolonged cultivation (Broers et al., 1990; Droop, 1962; Fenchel & Patterson, 1986; Page, 1988). In addition, De Jonckheere et al. (2011a) showed that the number of transformed flagellates of *Oramoeba fumarolia* depends on the type of bacterial prey.

The life cycle of acrasids is different from those described above and contains an additional multicellular stage, the sorocarp (see above, fig. 4). Individual amoebae of acrasids normally feed on bacteria. When starving, the amoebae aggregate to form sorocarps (Bonner, 2003).

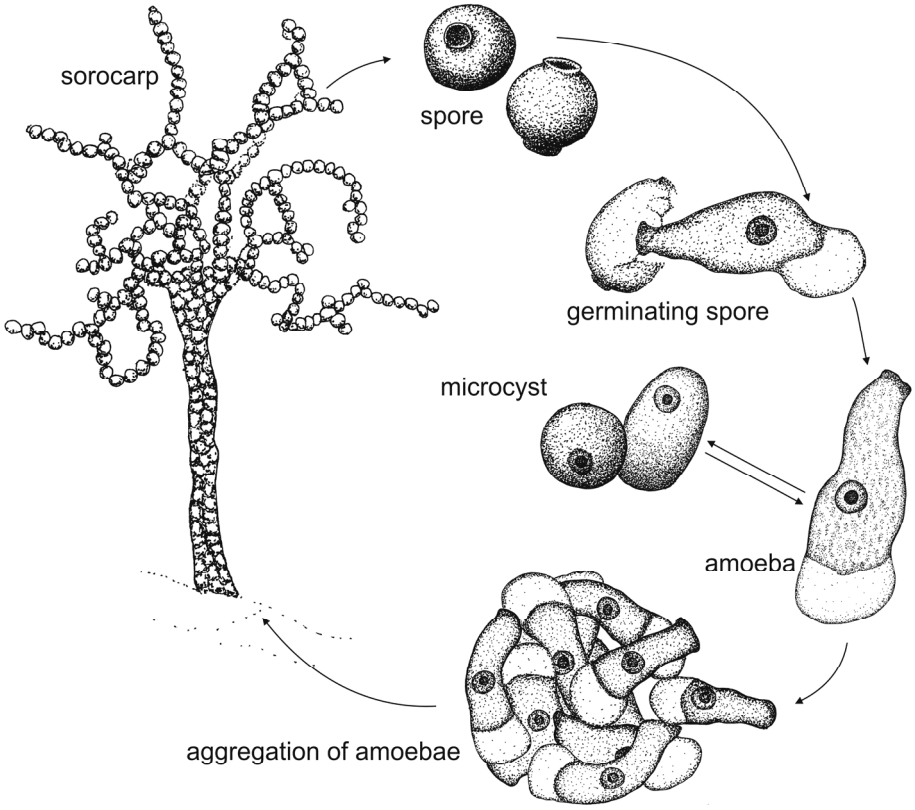

Fig. 4. Life cycle of *Acrasis rosea*. After Olive & Stoianovitch, 1960.

Both slightly different cell types of the fruiting body (basal stalk cells and distal spore cells) are capable of germination through excystment (Bonner, 2003; Brown M.W. et al., 2010). Both cell types of *Pocheina flagellata* are able to produce both amoebae and flagellates (Olive et al., 1983). The formation of multicellular bodies by the aggregation of individual cells is not unique feature of acrasids and has been well documented in several unrelated eukaryotic groups (see Brown M.W. et al., 2011) including the metazoan *Buddenbrockia plumatellae* (Morris & Adams, 2007). Unlike in *Dictyostelium*, the most-studied organism with the aggregative multicellularity, no motile ("slug") stage is formed during the ontogenesis of sorocarps of acrasids and their stalk-forming cells do not undergo the programmed cell death.

The question of the sexuality of Heterolobosea has not been elucidated yet. Although the sexual reproduction of several heterolobosean species has been discussed (e.g., Bunting, 1926; Droop, 1962; Fritz-Laylin et al. 2010; Olive et al., 1961; Olive, 1963; Pernin et al., 1992), no direct evidence has been found and the nature of the putative sexual processes remains unclear. Some authors hypothesize that the amoeba is the diploid stage whereas the flagellate is haploid and represents the gamete (Droop, 1962; Fulton, 1993). In fact, majority of the experiments and observations have to be revised and repeated using modern techniques.

The strongest evidence for sexuality of Heterolobosea has been brought by studies on *Naegleria*. Pernin *et al*. (1992) investigated the genetic structure of a natural population of *N. lovaniensis* by an isoenzyme analysis of 71 strains isolated in France. Analysis of single locus variation revealed that most strains were close to Hardy-Weinberg equilibrium. It indicated segregation and recombination between alleles. Recovery of relatively high number of distinct genotypic associations and the absence of linkage disequilibrium between genotypes at the different loci also supported the existence of recombination. In addition, the level of heterozygosyty in *N. gruberi* genome was reported as typical for sexual organism and most of meiotic genes defined by Ramesh (2005) were discovered (Fritz-Laylin et al., 2010). On the other hand, wild populations of *Naegleria gruberi* and *N. australiensis* showed large departures from Hardy–Weinberg equilibrium, low levels of heterozygosity, and strong linkage disequilibrium (Pernin & Cariou, 1997). These findings led to the conclusion that *N. gruberi* and *N. australiensis* have a predominantly clonal genetic structure in the wild. Different species of *Naegleria* thus could have different reproductive strategies.

4. Ecology of Heterolobosea

Heteroloboseans are heterotrophic prostists that inhabit a wide range of different habitats worldwide. Most heteroloboseans are bacteriovores, although cannibalism was reported in some species. Members of genus *Stephanopogon* are able to feed on diatoms and other eukaryotes. Most species of Heterolobosea live in soil and freshwater sediments. The number of marine species (30 – 50‰ salinity) is relatively low (e.g., *Neovahlkampfia damariscottae*, *Stephanopogon* spp., *Monopylocystis visvesvarai*, *Pseudovahlkampfia emersoni*). On the other hand, adaptations to various non-canonical enviroments occurred repeatedly in several heterolobosean lineages.

Heteroloboseans play a very important role in hypersaline habitats. About one third of species of heterotrophic protists recorded from this environment belong to Heterolobosea (e.g., *Pleurostomum* spp., *Pharyngomonas kirbyi*, *Euplaesiobystra hypersalinica*, and *Tulamoeba peronaphora*). However, the halophilic species do not form monophyletic group and differ in the response to various salinity levels. *Tulamoeba peronaphora* grows in the culture at 75‰ – 250‰ salinity, *Pharyngomonas kirbyi* up to 250‰, *Euplaesiobystra hypersalinica* and *Pleurostomum flabellatum* flourish in more than 300‰ salinity. The latter two species are true extremophiles, because they live in nearly salt-saturated solutions (Park et al. 2007, 2009; Park & Simpson, 2011). Some other heteroloboseans have adapted to extremely acidic habitats with pH < 3. Sheehan et al. (2003) detected DNA of *Naegleria* sp. from a thermal stream with pH 2.7. Amaral Zettler et al. (2002) discovered DNA of uncultured *Paravahlkampfia* sp. from the River of Fire (pH of 2.0). Another heterolobosean DNA sequences were reported in a recent study of the River of Fire (Amaral-Zettler et al., 2011). The only cultured acidophilic heterolobosean is *Tetramitus thermacidophilus* isolated from an acidic hot spring. This species flourishes at pH from 1.2 to 6 with the optimal pH of 3.0 (Baumgartner et al., 2009). Particular heterolobosean species differ in the range of temperature at which they are able to grow. Many of them are thermophilic. For example, *T. thermacidophilus* and *Oramoeba fumarolia* grow in temperature up to 54 °C (Baumgartner et al., 2009; De Jonckheere et al., 2011a). *Marinamoeba thermophila*, *Fumarolamoeba ceborucoi* and *Euplaesiobystra hypersalinica* grow up to 50 °C (De Jonckheere et al., 2009, 2011b; Park et al., 2009). Several heteroloboseans, importantly including pathogenic *Naegleria* strains, survive and divide in temperatures around 40 – 45 °C (De Jonckheere 2007; Guzmán-Fierros et al.,

2008; Park et al., 2007, 2009). In contrast to thermophilic heteroloboseans, there are also few reports on psychrophilic species adapted to cold environments. The growth optimum of *Vahlkampfia signyensis* is 10 °C and the cells die when the temperature exceeds 20 °C (Garstecki et al., 2005). *Tetramitus vestfoldii* isolated from microbial mat of a brackish Antarctic lake grows at 5 °C (Murtagh et al., 2002).

Representatives of at least two heterolobosean lineages have adapted to the life in anoxic/microoxic habitats (i.e. habitats without oxygen/with low concentration of oxygen). Mitochondria of most of them do not possess cristae. The first lineage is represented by the extreme halophile *Pleurostomum flabellatum*, the second one is more diversified and comprises *Psalteriomonas lanterna*, *Sawyeria marylandensis*, *Monopylocystis visvesvarai*, and most probably also *Percolomonas descissus*, *Lyromonas vulgaris* and *Vahlkampfia anaerobica* (Broers et al., 1990, 1993; O´Kelly et al., 2003; Smirnov & Fenchel, 1996). Mitochondrial derivates of *Psalteriomonas lanterna* and *Sawyeria marylandensis* were studied in detail and it was shown that they have been transformed to hydrogenosomes (Barberà et al., 2010; de Graaf et al., 2009). Interestingly, presumably aerobic *Naegleria gruberi* recently appeared to be a facultatively anaerobic protist. Its mitochondria possess cristae and a genome, and are probably equipped to function in both aerobic and anaerobic conditions (Fritz-Laylin et al., 2010; Ginger et al., 2010; Opperdoes et al., 2011).

Some heteroloboseans were reported to be endobionts or even pathogens of both invertebrates and vertebrates including humans. *Naegleria fowleri* causes primary amoebic meningoencephalitis (PAM, PAME, see Visvesvara et al., 2007), rare (235 reported cases worldwide, see De Jonckheere, 2011), but rapidly fatal disease of humans and other mammals. The total number of cases has been probably underestimated because *N. fowleri* lives in warm waters and it could be expected that most cases occur in tropical regions where the possibility of diagnosis is limited (De Jonckheere, 2011). Humans are typically infected while recreating in warm fresh water. In contrast to other CNS-infecting amoebae, *N. fowleri* infects primarily healthy individuals. The amoebae entry the central nervous system through the olfactory neuroepithelium and destroy host cells. Without prompt diagnosis and intervention, the patients die usually within two weeks of exposure; about 97 % of patients do not survive the infection. In addition to *N. fowleri*, pathogenicity was suggested also for *N. australiensis* and *N. italica* on the basis of tests on mice (De Jonckheere, 2002). There is a single report on PAM-like disease caused by *Paravahlkampfia francinae* (Visvesvara et al., 2009). In contrast to PAM caused by *N. fowleri*, the affected patient recovered within a few days. Several strains of *Vahlkampfia* sp., *Tetramitus ovis* and *Paravahlkampfia* sp. were isolated from keratitis patients (Aitken et al., 1996; Alexandrakis et al., 1998; De Jonckheere & Brown S., 2005a; Dua et al. 1998; Kinnear, 2003; Ozkoc et al., 2008; Walochnik et al., 2000). However, their importance in pathogenesis is unclear and no direct evidence of their pathogenity was indicated. Heteroloboseans were found also in the gut of animals (e.g., *Tetramitus* spp., *Paravahlkampfia ustiana*, *Percolomonas sulcatus*) and gills, skin, and internal organs of fish (*Naegleria* spp.) (e.g., Brugerolle & Simpson, 2004; Dyková et al., 2001, 2006; Schuster et al., 2003).

5. Taxonomy of Heterolobosea

The taxon Heterolobosea was created by Page & Blanton (1985) as a class unifying orders Schizopyrenida (limax-type amoebae, often with the flagellate stage) and Acrasida (aggregative amoebae forming multicellular sorocarps) on the basis of the common presence

of limax amoeba with eruptive lobopodia, discoidal mitochondrial cristae, and the absence of a stacked Golgi apparatus. However, it was later shown that several organisms with different morphology are closely related to the Heterolobosea (*Pharyngomonas*) or even form its internal branches (*Percolomonas*, *Stephanopogon*, *Lyromonas*, *Psalteriomonas*, *Pleurostomum*). Currently, two concepts of Heterolobosea, here called Heterolobosea *sensu lato* and Heterolobosea *sensu stricto*, respectively, exist. The concept of Heterolobosea *sensu lato* emphasizes monophyletic taxa and includes all aforementioned genera in Heterolobosea. It means, in fact, that Heterolobosea *sensu lato* is a group containing all descendants of the last common ancestor of *Pharyngomonas* and *Naegleria*. This concept is currently favored by most authors and we follow it as well.

In contrast, some authors emphasize the original definition of Heterolobosea *sensu* Page & Blanton (1985). The absence of mitochondrial cristae and microbodies in *Lyromonas*, *Sawyeria*, and *Monopylocystis*, the presumed absence of the amoeboid stage in *Percolomonas*, *Stephanopogon* and *Pharyngomonas* (but not in *Pleurostomum*), and the different arrangement of flagella of *Pharyngomonas* are considered so important that these genera cannot be members of Heterolobosea. Instead, they are classified in separate classes closely related to the Heterolobosea *sensu stricto*. The taxon corresponding to the Heterolobosea *sensu lato* was named Percolozoa (see Cavalier-Smith, 1991, 1993, 2003). The most recent version of this concept is represented by Cavalier-Smith & Nikolaev (2008). They divide the phylum Percolozoa into four classes: Pharyngomonadea (*Pharyngomonas*), Percolatea (*Percolomonas*, *Stephanopogon*), Lyromonadea (*Lyromonas*, *Psalteriomonas*, *Sawyeria*, *Monopylocystis*), and Heterolobosea (the rest of genera). The latter three classes are united within the subphylum Tetramitia whereas *Pharyngomonas* is the only member of the subphylum Pharyngomonada. Although we do not follow this concept because Heterolobosea *sensu stricto* is highly paraphyletic, we accept the division of Heterolobosea (*sensu lato*) into Pharyngomonada and Tetramitia as it is supported by phylogenetic analyses (see below).

The Pharyngomonada comprises a single family, Pharyngomonadidae, with two species of the genus, *Pharyngomonas*. Pharyngomonads are flagellates with four flagella; amoebae and cysts are unknown. In contrast to Tetramitia, basal bodies within a pair are arranged orthogonally. A large pharynx opens into the anterior end of the longitudinal ventral groove. The mastigont system of *Pharyngomonas* is more plesiomorphic than that of Tetramitia and shows more features of typical excavates (Park & Simpson, 2011).

Synapomoprhies of Tetramitia include parallel or nearly parallel basal bodies in a pair and a specific 17-1 helix in the secondary structure of SSU rRNA molecule (Cavalier-Smith & Nikolaev, 2008; Nikolaev et al., 2004). Tetramitia are currently classified into seven families though some authors recognize only three families of Heterolobosea (e.g., Patterson et al., 2002; Smirnov & Brown, 2004). In addition, several heterolobosean genera have not been assigned to any family (e.g., *Oramoeba*, *Fumarolamoeba*, *Pernina*, *Tulamoeba*, *Euplaesiobystra*). The paraphyletic family Vahlkampfiidae contains most heterolobosean genera (e.g., *Allovahlkampfia*, *Fumarolamoeba*, *Marinamoeba*, *Naegleria*, *Neovahlkampfia*, *Paravahlkampfia*, *Pseudovahlkampfia*, *Solumitrus, Tetramastigamoeba*, *Tetramitus*, *Vahlkampfia*, and *Willaertia*). It was defined by the presence of amoebae of the limax type and persistence of the nucleolus during mitosis. In addition, the genus *Pleurostomum*, whose amoeboid stage is unknown or has been lost, was placed within Vahlkampfiidae on the basis of its phylogenetic position (Park et al., 2007). In contrast to Vahlkampfiidae, the nucleolus of members of the family

Gruberellidae (genera *Gruberella* and *Stachymoeba*) disintegrates during mitosis. Members of two monotypic families, Lyromonadidae and Psalteriomonadidae, are anaerobic and possess acristate mitochondria. Park et al. (2007) suggested that Lyromonadidae might be a synonym of Psalteriomonadidae. *Lyromonas vulgaris* forms only flagellates with a single kinetid and longitudinal ventral groove, while *Psalteriomonas lanterna* is able to form also amoebae and its flagellates possess four mastigonts, nuclei and longitudinal ventral grooves. The anaerobic genera *Monopylocystis* and *Sawyeria* which were not placed into any family in the original description (O'Kelly et al., 2003) are sometimes placed into family Lyromonadidae or Psalteriomonadidae (Cavalier-Smith, 2003; Cavalier-Smith & Nikolaev, 2008). However, the authors did not specify in which family they classify the genera. The genus *Percolomonas* (family Percolomonadidae) comprises flagellates with four flagella (three in one species) and a longitudinal ventral groove. The amoeba stage is unknown or has been lost. The genus *Percolomonas* is most probably polyphyletic (see later). The peculiar eukaryovorous flagellates of the genus *Stephanopogon* with two nuclei and multiplicated flagella comprise the family Stephanopogonidae. Finally, heteroloboseans forming multicellular sorocarps have been accommodated within the family Acrasidae. Cells of their sorocarps are differentiated into morphologically distinct spores and stalk cells.

In addition to the aforementioned families, the family Guttulinopsidae comprising genera *Guttulinopsis* and *Rosculus* was sometimes affiliated with Heterolobosea (e.g., Smirnov & Brown, 2004), though many authors do not consider them as heteroloboseans (Page & Blanton, 1985).

The concept of the genus level of Heterolobosea was historically based on the cyst morphology and the presence/absence of the flagellate stage. All vahlkampfiids which do not form flagellates and whose cysts do not have pores were classified within the genus *Vahlkampfia* (see Brown S. & De Jonckheere, 1999). However, results of phylogenetic analyses showed that *Vahlkampfia* is polyphyletic. Consequently, genera *Paravahlkampfia*, *Neovahlkampfia*, *Allovahlkampfia*, *Fumarolamoeba*, and *Solumitrus* were created to accommodate species of *Vahlkampfia*-like morphology (Anderson et al., 2011; Brown S. & De Jonckheere, 1999; De Jonckheere et al., 2011b; Walochnik & Mulec, 2009). In addition, several former *Vahlkampfia* species were transferred to the genus *Tetramitus* (Brown & De Jonckheere, 1999). Moreover, *Vahlkampfia anaerobica* described by Smirnov & Fenchel (1996) is morphologically almost identical with *Monopylocystis visvesvarai* and is likely congeneric or conspecific with it. Phylogenetic position of several described *Vahlkampfia* species remains unknown. This applies also on the type species, *V. vahlkampfi*. Therefore, Brown & De Jonckheere (1999) nominated *V. avara* as a new type species of *Vahlkampfia*. However, such a change is not possible according to the International Code of Zoological Nomenclature and the current status of the genus *Vahlkampfia* is chaotic.

Although we agree with the classification of organisms of *Vahlkampfia*-like morphology into several independent genera based solely on molecular-phylogenetic analysis, we do not recognize the genus *Solumitrus* as it is closely related to *Allovahlkampfia* with identical morphology. The genetic distance between *S. palustris* and *A. spelaea* SSU rDNA is 93.7 % (Anderson et al., 2011) which is comparable to genetic distances within other heterolobosean genera. The situation is more complicated by the fact that *Allovahlkampfia* itself has been defined solely on the basis its phylogenetic position (Walochnik & Mulec, 2009). The authors did not include the genus *Acrasis* into their analysis and it was showed later that it is the

closest relative of *Allovahlkampfia* (Brown M.W. et al., 2010; present study). The morphology of *Acrasis* and *Allovahlkampfia* amoebae has not been compared and it cannot be ruled out that *Allovahlkampfia spelaea* and *Solumitrus palustris* are, in fact, members of the genus *Acrasis* with unknown or reduced multicellular stage.

The genus *Percolomonas* is even more problematic than *Vahlkampfia*. It was created by Fenchel & Patterson (1986) for the species *P. cosmopolitus* (originally described as *Tetramitus cosmopolitus*) and was later broadened to accommodate flagellates with one long and three shorter flagella inserting at the anterior end of a longitudinal feeding groove (Larsen & Patterson, 1990). In addition, the triflagellate *P. denhami* was described later (Tong, 1997). However, it was soon realized that most *Percolomonas* species were totally unrelated to the type species *P. cosmopolitus*. They were removed from *Percolomonas* and accommodated in genera *Trimastix* (Preaxostyla), *Carpediemonas* (Fornicata), which is, in fact, biflagellate, *Chilomastix* (Fornicata), and *Pharyngomonas* (Heterolobosea) (Bernard et al., 1997, 2000; Cavalier-Smith & Nikolaev, 2008; Ekebom et al., 1996). Moreover, the mastigont structure of *P. sulcatus* and *P. descissus* is so different from that of *P. cosmopolitus* (Brugerolle & Simpson, 2004) that they should be removed from the genus *Percolomonas* as well. The phylogenetic position of the remaining *Percolomonas* species (*P. denhami*, *P. similis* and *P. spinosus*) is uncertain and no sequence data are currently available. Finally, *P. cosmopolitus*, the only certain member of the genus *Percolomonas*, is possibly paraphyletic (see below) and its true identity is unknown.

6. Species concept of Heterolobosea

Ca. 140 species of Heterolobosea have been described so far. The species were originally distinguished on the basis of light-microscopic morphology. The cyst was the most important life stage as its morphological variability was sufficient to recognize most then-known species (see Page, 1988). Unlike the cysts, amoebae of particular heterolobosean species are usually indistinguishable and have been rarely used for the species identification. Finally, although Page (1988) stressed the importance of heterolobosean flagellates in taxonomy as their morphology is quite diverse (see above), their descriptions were often insufficient and cannot be considered in taxonomical studies. Moreover, the flagellates are unknown and presumably absent in many heterolobosean.

Since it is often impossible to differentiate between pathogenic and non-pathogenic *Naegleria* strains solely on the basis of their morphology, various biochemical and immunological methods have been applied (see Page, 1988). Later on, several new species of *Naegleria* were described on the basis of molecular markers (see De Jonckheere, 2002, 2011). This continuous process reached a peak in the explicit formulation of a molecular species concept of the genus *Naegleria* based on the ITS region (De Jonckheere, 2004). The concept was soon expanded to cover the whole Vahlkampfiidae (De Jonckheere & Brown S., 2005b). It was shown that vahlkampfiid ITS region is extremely variable. Interestingly, even strains with identical SSU rDNA sequences may slightly differ in the ITS region (Baumgartner et al., 2009; De Jonckheere & Brown S., 2005b). According to De Jonckheere & Brown S. (2005b), almost any two vahlkampfiid strains differing in ITS1, 5.8S rDNA or ITS2 sequences should be classified as different species even when their morphology and ecology are identical. The difference in the ITS region was often used as an accessory criterion in addition to morphological identification. However, several vahlkampfiid species were described solely

on the basis of minute differences in the ITS region without morphology being effectively involved. This was stated e.g. in the diagnosis of *Naegleria canariensis* (De Jonckheere, 2006): "Because of the morphological similarity of the cysts with those of other *Naegleria* spp. molecular identification is required. The species can be identified from the ITS2 sequence, which differs by 2 bp substitutions from that *N. gallica*. The ITS1 and 5.8S rDNA sequence is identical to that of *N. gallica*. ". Similarly, *Tetramitus anasazii* and *T. hohokami* were distinguished solely on the basis of few differences in the ITS region (De Jonckheere, 2007).

Although we agree that the ITS region represents a quite effective DNA barcode of the Vahlkampfiidae and can be used for rapid determination of strains belonging to already described species, we are convinced that the concept is misleading when used for new species description. The biggest problem is that it assumes zero level of intraspecific polymorphism within the Vahlkampfiidae. However, the true variability of a vast majority of vahlkampfiid species is virtually unknown. It was convincingly shown that certain closely related *Naegleria* species differ in the ITS region sequence (De Jonckheere, 2004). There is, however, no reason to believe that it is true for the whole genus *Naegleria* or even for the whole Vahlkampfiidae, and, on the other hand, that the ITS region displays no intraspecific polymorphism. Indeed, it is a well-documented fact that there is some degree of ITS polymorphism within certain Vahlkampfiidae species. ITS regions of different strains of *Tetramitus jugosus* differ in a single nucleotide (De Jonckheere et al., 2005). Despite strains of *Naegleria fowleri* are even more variable, they are considered to be conspecific instead of belonging to several different species (De Jonckheere, 2004, 2011). Dyková et al. (2006) even showed that there exists an intragenomic polymorphism in *Naegleria clarki*. All these examples show that the ITS region should be no longer used for new species descriptions until the problem of species concept and intraspecific polymorphism within Heterolobosea is settled.

7. Phylogeny of Heterolobosea

Although many heterolobosean species have been successfully transferred into culture and many strains have been deposited into culture collections, the phylogeny of Heterolobosea has not yet been satisfactorily elucidated. Virtually all molecular-phylogenetic analyses with reasonable taxon sampling are based on a single locus, SSU rDNA (e.g., Brown M.W. et al., 2010; Cavalier-Smith & Nikolaev, 2008; De Jonckheere et al., 2011a, 2011b; Nikolaev et al., 2004; Park & Simpson, 2011; Park et al., 2007, 2009). To evaluate the resolving power of SSU rDNA, we performed a phylogenetic analysis which included members of all heterolobosean genera whose sequences are available, and important environmental sequences affiliated with Heterolobosea. Results of our analysis (fig. 5) were in agreement with previous studies.

Heterolobosea robustly split into two lineages, Pharyngomonada and Tetramitia. We divide here the Tetramitia into six clades whose interrelationships and internal phylogeny remain unresolved. Unfortunately, all the clades but E are currently indefinable on the basis of morphology. The current heterolobosean taxonomy is not consistent with delineation of these six monophyletic groups and has to be revised in the future. The family Vahlkampfiidae is highly paraphyletic.

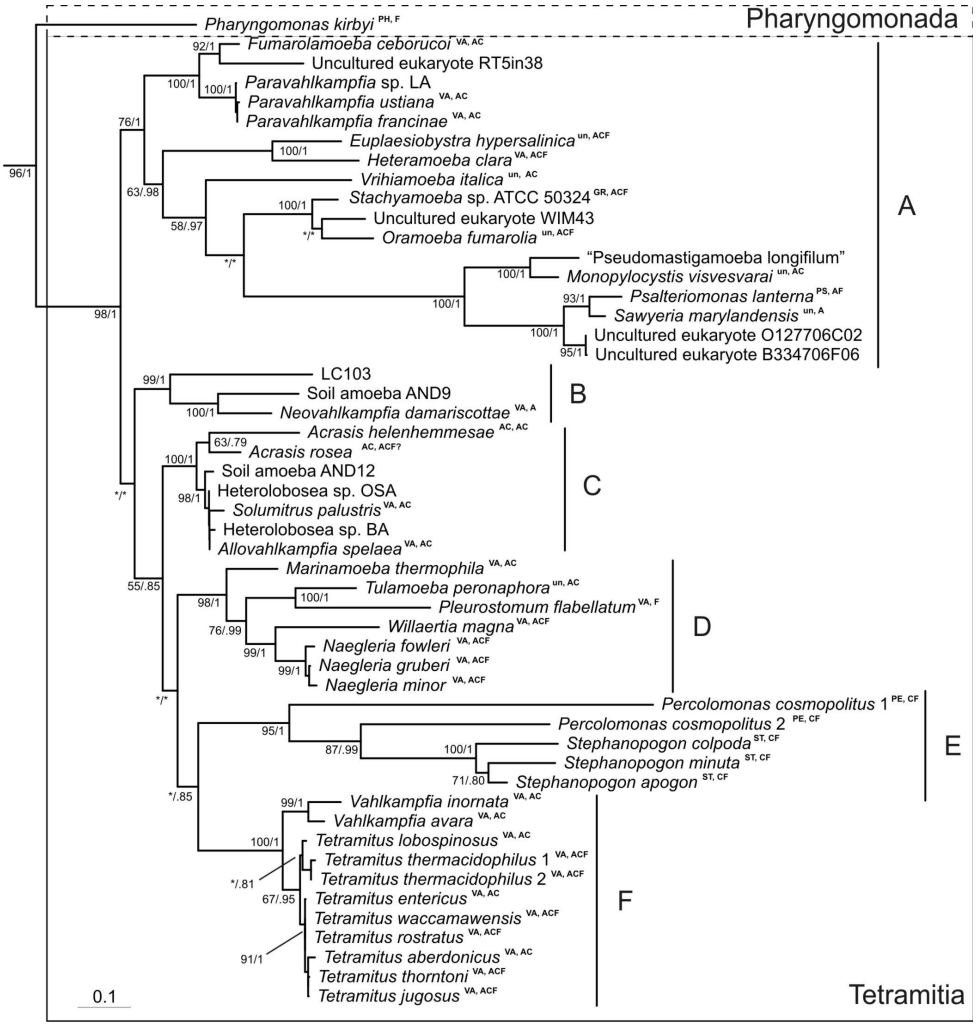

Fig. 5. Phylogenetic tree of Heterolobosea based on SSU rDNA sequences. The tree topology was constructed by the maximum likelihood method (ML) in RAxML 7.2.6 under the GTRGAMMAI model, and by the Bayesian method in MrBayes 3.1.2. under the GTR + Γ + I + covarion model. RAxML 7.2.6 was used for bootstrapping (1000 replicates). The tree was rooted with representatives of other Excavata lineages (outgroups were removed from the tree). The values at the nodes represent statistical support (ML bootstrap values/Bayesian posterior probabilities). Support values below 50%/.50 are represented by asterisks. Heterolobosea are divided into two branches (Pharyngomonada and Tetramitia). Six main clades of Tetramitia are labeled. The classification is indicated: AC – Acrasidae, GR – Gruberellidae, PE – Percolomonadidae, PH – Pharyngomonadidae, PS – Psalteriomonadidae, ST – Stephanopogonidae, un – unassigned, VA – Vahlkampfiidae, The occurrence of particular life stages is indicated: A – amoeba, C – cyst, F – flagellate.

Clade A consists of genera *Paravahlkampfia*, *Fumarolamoeba*, *Euplaesiobystra*, *Heteramoeba*, *Stachyamoeba*, *Vrihiamoeba*, *Oramoeba*, *Psalteriomonas*, *Sawyeria*, *Monopylocystis*, and several undetermined heteroloboseans (strains RT5in38, WIM43, and 'Pseudomastigamoeba longifillum', O127706C02, B334706F06). Moreover, morphological data suggest that *Vahlkampfia anaerobica*, *Lyromonas vulgaris*, and *Percolomonas descissus* belong to this clade as well (see above). Many members of the clade A display a unique morphology of the nucleolus. Typical heteroloboseans have nucleus with a single central nucleolus. In contrast, *Heteramoeba*, *Sawyeria*, *Monopylocystis*, *Stachyamoeba*, *Percolomonas descissus*, *Vahlkampfia anaerobica*, and at least some strains of *Psalteriomonas* possess parietal nucleoli or a thin ring of nucleolar material near the nuclear membrane. *Neovahlkampfia damariscottae* and undetermined heteroloboseans AND9 and LC103 constitute the clade B. Although it is quite robust (bootstrap support 99), its position within Tetramitia is uncertain. In some previous analyses the clade formed the basal branch of Tetramitia (Brown M.W. et al., 2010; Park & Simpson, 2011; Park et al. 2007, 2009) while it branched more terminally in the others (Cavalier-Smith & Nikolaev; 2008; De Jonckheere et al., 2011a, 2011b; Nikolaev et al., 2004; this study). *Acrasis*, *Allovahlkampfia*, *Solumitrus* and undetermined heteroloboseans BA, OSA, and AND12 formed tetramitian clade C. All representatives of the clade inhabit freshwater sediments and soil (however, the data are unavailable for strains BA and OSA). Members of the clade D (*Marinamoeba*, *Tulamoeba*, *Pleurostomum*, *Willaertia*, and *Naegleria*) live in wide range of habitats. Interestingly, at least some members of all the genera are able to grow at higher temperatures (40 – 50 °C). The Clade E comprises *Percolomonas cosmopolitus* and the genus *Stephanopogon* (i.e. Percolatea *sensu* Cavalier-Smith & Nikolaev, 2008). Both *Stephanopogon* and *P. cosmopolitus* form long branches in the phylogenetic trees and it cannot be ruled out that their grouping is, in fact, a result of long-branch attraction. However, Yubuki & Leander (2008) identified three morphological features shared by *Stephanopogon* and *P. cosmopolitus* suggesting that the clade E might be monophyletic. In addition, both *Stephanopogon* spp. and *P. cosmopolitus* have lost the amoeba stage and their ability to encyst as flagellates is unique among heteroloboseans. Tetramitian clade F is formed by the remaining genera *Vahlkampfia* and *Tetramitus*. Morphology and ecology of the genus *Tetramitus* is extremely diverse including characteristics used for generic determination (presence and number of cyst pores, number/presence of flagella, marine or freshwater lifestyle, etc.).

Our view on the evolution of Heterolobosea has completely changed after the application of methods of molecular phylogenetics. The analyses of sequence data are currently the only efficient tool for pinpointing relationships between species and genera although it is unable to resolve interrelationships between particular tetramitian clades. The analyses suggested that some morphologically well-defined genera (e.g., *Vahlkampfia* and *Percolomonas*) were polyphyletic. On the other hand, *Tetramitus* spp. is so diverse that it was impossible to group them into a single genus solely on the morphological base. Although it is currently impossible to define morphological synapomorphies of particular tetramitian clades, Heterolobosea itself and both its subphyla, Tetramitia and Pharyngomonada, seem to be well defined on both molecular and morphological level.

8. Conclusion

During the last decade, Heterolobosea have attracted considerable interest because of their extraordinary morphological, ecological and physiological diversity. Members of the most

studied genus *Naegleria* are medicinally important or became model organisms in cell biology. The insight obtained from the genome sequence of *N. gruberi* considerably improved understanding of the early eukaryotic evolution. However, our knowledge about Heterolobosea as a whole is still seriously limited. Since it is currently unclear whether Heterolobosea are sexual or asexual organisms, the biological species concept is not applicable. On the other hand, the current species concept of Heterolobosea based on the ITS region is misleading and most probably considerably overestimates the real number of extant species. Heterolobosean phylogeny is unclear as well. Although the monophyly of Heterolobosea and its split into Pharyngomonada and Tetramitia is strongly supported by both cell structure and molecular-phylogenetic analyses, the internal phylogeny of Tetramitia has not yet been satisfactorily elucidated. Since 18S rDNA has not sufficient resolving power, it is necessary to perform multigene phylogenetic analyses in order to improve the hetrolobosean phylogeny. In addition, it is important to obtain sequence data from so-far uncharacterized, potentially important taxa, such as *Percolomonas sulcatus* and *Gruberella flavescens*. There is also a strong possibility that some already-known enigmatic eukaryotes will be shown to belong to Heterolobosea as well. This has already happened in the case of the ciliate-resembling genus *Stephanopogon*. Finally, it is a well-known fact that the current taxonomy of Heterolobosea, particularly the family level, does not reflect the phylogeny and should be changed. This cannot be, however, achieved before the heterolobosean phylogeny is resolved.

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ORIGINAL ARTICLE

Morphological Identities of Two Different Marine Stramenopile Environmental Sequence Clades: *Bicosoeca kenaiensis* (Hilliard, 1971) and *Cantina marsupialis* (Larsen and Patterson, 1990) gen. nov., comb. nov.

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ABSTRACT

Although environmental DNA surveys improve our understanding of biodiversity, interpretation of unidentified lineages is limited by the absence of associated morphological traits and living cultures. Unidentified lineages of marine stramenopiles are called "MAST clades". Twenty-five MAST clades have been recognized: MAST-1 through MAST-25; seven of these have been subsequently discarded because the sequences representing those clades were found to either (1) be chimeric or (2) affiliate within previously described taxonomic groups. Eighteen MAST clades remain without a cellular identity. Moreover, the discarded "MAST-13" has been used in different studies to refer to two different environmental sequence clades. After establishing four cultures representing two different species of heterotrophic stramenopiles and then characterizing their morphology and molecular phylogenetic positions, we determined that the two different species represented the two different MAST-13 clades: (1) a lorica-bearing *Bicosoeca kenaiensis* and (2) a microaerophilic flagellate previously named "*Cafeteria marsupialis*". Both species were previously described with only light microscopy; no cultures, ultrastructural data or DNA sequences were available from these species prior to this study. The molecular phylogenetic position of three different "*C. marsupialis*" isolates was not closely related to the type species of *Cafeteria*; therefore, we established a new genus for these isolates, *Cantina* gen. nov.

ENVIRONMENTAL DNA sequence surveys combined with microscopic observations have greatly improved our knowledge of eukaryotic microbial diversity, especially from uncultured species (Edgcomb and Bernhard 2013; Kim et al. 2011; Kolisko et al. 2010; Takishita et al. 2010; Tarbe et al. 2011; Wylezich and Jürgens 2011). However, most of the sequences recovered from environmental DNA sequence surveys are unidentified at the cellular level and are deposited in GenBank with ambiguous labels, such as "uncultured eukaryotes". There are two types of sequences designated as uncultured eukaryotic lineages: (1) sequences from novel and undescribed organisms and (2) sequences from previously described species that lack any associated DNA sequence information. In the latter case, morphological information obtained before the routine use of molecular methods and more recently acquired DNA sequences from the same species remain unlinked.

Stramenopiles represent a huge group of photosynthetic eukaryotes that play an important ecological role as

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primary producers (e.g. kelps and diatoms); the group also comprises a diverse assortment of non-photosynthetic lineages that function as consumers (e.g. bicosoecids and heterotrophic and mixotrophic chrysophytes), decomposers (e.g. water molds and labyrinthulids) and parasites (e.g. *Blastocystis*) (Andersen 2004; Arndt et al. 2000; Patterson 1989; Zubkov et al. 2001). Environmental PCR surveys of small subunit ribosomal RNA (SSU rRNA) gene sequences from diverse environments have demonstrated a large number of marine stramenopile (MAST) clades without clear cellular identities (Massana et al. 2004). These clades represent cosmopolitan species and encompass a great deal of stramenopile diversity (Gómez et al. 2011; Kolodziej and Stoeck 2007; Massana et al. 2004; Rodríguez-Martínez et al. 2012; Takishita et al. 2010; Zuendorf et al. 2006). Because many stramenopiles are very small (< 10 μm) and lack easily recognizable features, identifying, and describing the organisms corresponding to the MAST clades is challenging. Twenty-five MAST clades have been identified and labeled MAST-1 through MAST-25; however, seven of these MAST clades have been discarded because (1) MAST-5 was found to be a chimeric sequences from a labyrinthulid and a dinoflagellate and (2) six of these clades were shown to affiliate with previously described taxonomic groups (e.g. MAST-14 was subsequently identified as members of the Bicosoecida). Therefore, there are currently 18 MAST clades without a clear cellular identity (Massana et al. 2014). Because they represent a diverse collection of small heterotrophic eukaryotes that diverged before the clade of photosynthetic stramenopiles, improved knowledge of these MAST clades is expected to play an important role in our understanding of the early evolutionary history of stramenopiles as a whole. For example, the anaerobic MAST-12 clade has been unexpectedly shown to be the sister clade to parasitic lineages like the human pathogen, *Blastocystis* (Gómez et al. 2011; Massana et al. 2014). The organisms representing MAST-12 are critical for understanding the evolution of parasites from free-living ancestors, the switch from oxic to anoxic lifestyles and the associated transformation of canonical mitochondria to hydrogenosomes. Nonetheless, the cellular identities of only two clades (MAST-3 and MAST-12) have been demonstrated with morphological data (Gómez et al. 2011; Kolodziej and Stoeck 2007), and a representative from only one of these clades (MAST-3) has been successfully brought into culture (Cavalier-Smith and Scoble 2013) since the first report of MAST clades by Massana et al. (2004).

One important point of confusion is that there are two different "MAST-13" clades that have been reported in the literature. One MAST-13 clade branches within the Bicosoecida (Zuendorf et al. 2006). Another "MAST-13" clade was described by Takishita et al. (2007) as a novel environmental sequence clade from an anaerobic environment, namely NAMAKO-31. Subsequent studies have propagated the double meaning for MAST-13. On one hand, Kolodziej and Stoeck (2007), Park and Simpson (2010), Orsi et al. (2011), Cavalier-Smith and Scoble (2013), and Massana et al. (2014) discussed only Zuendorf et al.'s version of MAST-13 and did not consider Takishita et al.'s version of MAST-13; however, Zuendorf et al.'s version of MAST-13 has been subsequently excluded from the MAST clades (Massana et al. 2014) because of its clear affiliation with the Bicosoecida. On the other hand, Takishita et al.'s (2010) analysis of environmental DNA sequences included only their version of MAST-13 (NAMAKO-31), but not Zuendorf et al.'s version of MAST-13 lineages. Only SSU rRNA gene sequences are available from these environmental sequence clades, and these sequences have never been studied together within the same phylogenetic analysis. Zuendorf et al.'s version of MAST-13 has been demonstrated to be a member of Bicosoecida; however, there is no detailed morphological data available from any of these particular organisms in question. In addition, the lack of cellular identities for these two lineages magnifies how poorly we understand the MAST clades in general.

The aims of this study were to (1) isolate cells that represent each of the two MAST-13 clades from environments that are similar to those where the two lineages had been reported, (2) establish the culture conditions necessary to maintain these isolates, (3) determine the molecular phylogenetic positions of these isolates on the basis of SSU rRNA gene sequences, and (4) describe the morphological traits of these isolates using light and transmission electron microscopy. The four isolates we studied here are very similar to two different flagellate species that have been reported from all over the world using light microscopy alone: *Bicosoeca kenaiensis* and "*Cafeteria*" *marsupialis* (Hilliard 1971; Larsen and Patterson 1990); no established cultures, ultrastructural data or DNA sequences were available from these flagellates prior to this study. The SSU rRNA gene sequences generated from our isolates branched separately, each within one of the two different "MAST-13" clades. These data allowed us to demonstrate the cellular identities for both clades and address taxonomic problems associated with the molecular phylogenetic position of "*Cafeteria*" *marsupialis*.

MATERIALS AND METHODS

Establishment, maintenance, and identification of cultures

An isolate with identical morphological traits as *B. kenaiensis* (NY0214) Hilliard 1971; was collected from surface water in the northwestern Pacific Ocean at Station 1 (29°34′38″ N, 144°34′20″E) on February 14, 2011 during a research cruise (MR 11-02) from February 11 to March 9, 2011 on the research vessel Mirai (Japan Agency for Marine-Earth Science and Technology—JAMSTEC). This culture is maintained in seawater with a grain of barley at 16 °C. Three different isolates with identical morphological traits of *Cafeteria marsupialis* Larsen and Patterson 1990 (=*Cantina marsupialis* gen. nov., comb. nov.) were isolated for this study. Isolate NY0206 was collected from soft mud samples in River Port, Nova Scotia, Canada

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(44°17′42″N, 64°19′7″W) on May 18, 2011. Isolate YPF1205 was collected from the sediment of the lagoon, Kai-ike (Satsumasendai, Kagoshima, Japan) (31°51′34″N, 129°52′31″E) on June 7, 2012. Isolate BUSSELTON was collected from a sandy beach in Busselton, Western Australia, Australia (33°38′38″S, 115°20′46″E) on April 24, 2010. NY0206 and YPF1205 are maintained in 3.3% Lysogeny Broth (LB) medium in anaerobic seawater at 16 °C, and BUSSELTON was maintained in seawater-based ATCC 1525 medium in microaerophilic conditions at room temperature. A monoeukaryotic culture of *C. marsupialis* (NY0206) has been deposited at the American Type Culture Collection (ATCC), Manasas VA. *Cantina marsupialis* (YPF1205) has been deposited at the National Institute for Environmental Studies (NIES), Tsukuba, Japan (accession number: NIES-3354). BUSSELTON died after 3 yr of continual (weekly) sterile transfers.

Light and transmission electron microscopies

Light micrographs of *B. kenaiensis* (NY0214) and *C. marsupialis* (NY0206) were taken with a Zeiss Axioplan 2 imaging microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with a Leica DC500 digital camera (Leica Microsystems Digital Imaging, Cambridge, UK). Light microscopy on *C. marsupialis* (YPF1205) was performed using an Eclipse E600 microscope (Nikon, Tokyo, Japan) equipped with a digital camera ORCA-ER (Hamamatsu Photo-nics KK, Hamamatsu, Japan). Light-microscopic observations of *C. marsupialis* (BUSSELTON) were performed using an Olympus Microscope BX51TF light microscope equipped with a DP70 color camera (Olympus Corporation, Tokyo, Japan) and Differential Interference Contrast. For transmission microscopy (TEM), cells of *B. kenaiensis* (NY0214) growing in a Lab-Tek II, eight well glass slide chamber (Nunc, Rochester, NY) were mixed with an equal volume of fixative containing 5% glutaraldehyde and 0.2 M sucrose in 0.2 M sodium cacodylate buffer (pH 7.2) at room temperature for 1 h. Cells were rinsed with 0.2 M sodium cacodylate buffer (pH 7.2) and then postfixed in 1% osmium tetroxide in 0.2 M sodium cacodylate buffer (pH 7.2) at room temperature for 1 h. The cells were then dehydrated through an ethanol series, which was then substituted with acetone. The cells were infiltrated with 1:1 mixture of JEMBED resin diluted with acetone and ultimately embedded in absolute resin.

Cells of *C. marsupialis* (NY0206) were collected in a 15-ml tube by centrifugation at 1,000 *g* for 5 min. The cell pellet was high-pressure frozen using the Leica EM HPM100 High Pressure Freezer. Cells were freeze-substituted in 1% osmium tetroxide and 0.1% uranyl acetate in HPLC grade acetone using the Leica Automatic Freeze Substitution chamber set to: −85 °C for 3 d, warming to −20 °C over 13 h and held at −20 °C for 8 h before warming to 4 °C over 12 h. The cells were washed in acetone, and then increasing concentrations of a 1:1 mixture of JEMBED resin diluted with acetone. The cells were infiltrated with 100% resin at room temperature for 8 h. The infiltrated samples were polymerized overnight at 65 °C.

Ultra-thin sections were cut on a Leica EM UC6 ultramicrotome and double stained with 2% (w/v) uranyl acetate and lead citrate (Reynolds 1963). The ultra-thin sections were observed using a Hitachi H7600 transmission electron microscope (Hitachi High Technologies Corporation, Tokyo, Japan).

DNA extraction, PCR amplification and phylogenetic analyses

Genomic DNA from *B. kenaiensis* (NY0214) and *C. marsupialis* (NY0206) were extracted using the MasterPure Complete DNA and RNA purification Kit (Epicentre, Maddison, WI). Polymerase chain reactions (PCR) were performed using PuRe Taq Ready-To-Go PCR beads kit (GE Healthcare, Buckinghamshire, UK). Nearly the complete SSU rRNA genes from the two strains were amplified using the following primers, PF1: 5′-GCGCTACCTGG TTGATCCTGCCAGT-3′ and R4: 5′-CCGTTAACGAACGAGACCTC-3′. The PCR conditions were 95 °C for 2 min; 35 cycles involving 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min; and 72 °C for 7 min. The amplified fragments were gel-purified using UltraClean 15 DNA Purification Kit (MO Bio, Carlsbad, CA) and sequenced directly. The extraction of DNA and the PCR amplification of an SSU rRNA gene sequence from the isolate YPF1205 followed the procedure described in Yabuki et al. (2010). Genomic DNA of *C. marsupialis* (BUSSELTON) was isolated using the ZR Genomic DNA II Kit™ (Zymo Research, Irvine, CA). Almost complete SSU rRNA gene was amplified using primers A (5′-CTGGTTGATCCTGCCAG-3′) and B (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) (Medlin et al. 1988) with an annealing temperature of 50 °C. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Venlo, the Netherlands) and sequenced directly. To increase the diversity of stramenopile SSU rRNA gene sequence in the molecular phylogenetic analyses, an SSU rRNA gene sequence of *Pendulomonas adriperis* (Placididea, Stramenopiles) was also obtained using the same methods described above for NY0214. This new monoeukaryotic strain (NY0181) was isolated from sediment sample taken from Sooke, British Columbia, Canada (48°22′16″ N, 123°43′3″W) on April 24, 2008 and identified by light microscopy based on previous report (Al-Quassab et al. 2002; Karpov et al. 2001; Lee 2006; Tong 1997). The sequences of *C. marsupialis* (NY0206, BUSSELTON, YPF1205), *B. kenaiensis* (NY0214) and *P. adriperis* (NY0181) were deposited into GenBank/EMBL/DDBJ (accession numbers: KM816645–KM816649).

The new SSU rRNA gene sequences were added to the alignment used in Yubuki et al. (2010). The alignment consisted of bicosoecids, placidids, slopalinids, *Blastocystis*, labyrinthulids, hyphochytridiomycetes, oomycetes, *Developayella*, ochrophytes, and at least two sequences from each of the 18 MAST clades; seven sequences representing the Alveolata and Rhizaria were used as the outgroup. All ambiguously aligned sites were manually excluded using Mesquite version 2.75 (Maddison and Maddison 2011) and the 128-taxon alignment contained 1,250

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unambiguously aligned nucleotide positions that were determined for the phylogenetic analyses. Maximum-likelihood (ML) analyses of the alignment were performed in Garli 2.0 under the general time reversible (GTR) model incorporating among-site rate variation approximated by a gamma distribution with the proportion of invariable sites (GTR + G+ I model) as suggested by the program jModelTest-2.1.6 (Darriba et al. 2012; Guindon and Gascuel 2003). ML bootstrap analyses were carried out for ML with 500 replicates to evaluate statistical reliability. Bayesian analyses were preformed with MrBayes v3.2.2 (Ronquist et al. 2011) using the same alignment. The program was set to operate with the GTR model incorporating among-site rate variation approximated by a gamma distribution and four Monte-Carlo-Markov chains starting from random trees. A total of 10,000,000 generations until the mean standard deviation of split frequencies based on last 75% of generations was lower than 0.01. The trees and log-likelihoods were sampled at 1,000 generation-intervals. The first 25% of the generations were discarded as burn-in using the sumt command. Posterior probabilities (PP) correspond to the frequency at which a given node was found in the postburn-in trees.

RESULTS

Comparative morphology of the new stramenopile isolates: *B. kenaiensis* (NY0214) and *C. marsupialis* gen. nov., comb. nov. (NY0206)

A single cell of *B. kenaiensis* was manually isolated with a micropipette to establish a mono-eukaryotic culture (NY0214) with environmental bacteria as a food source. The cells are ovoid, 2.42–6.21 μm long (average: 3.59 μm, $n = 30$), 2.25–3.67 μm wide (average: 3.00 μm, $n = 30$) and attach to the lorica with a posterior flagellum (Fig. 1). The anterior flagellum is about four times longer than the cell length and extends anteriorly when the cell is sitting in the lorica. The posterior flagellum contracts and the cell withdraws into the lorica when disturbed, and the anterior flagellum rolls up in to tight coil at the anterior end of the cell (Fig. 1C, 2B). The cells have a conspicuous lip for feeding on the anterior side (Fig 1A, B). The unpedicellate lorica is cylindrical, 8.61–9.65 μm long (average: 7.37 μm), and 3.70–4.48 μm wide (average: 3.31 μm) (Fig 1B). The margin of the lorica curves outward; the bottom of the lorica is rounded. The posterior flagellum is positioned within a groove located to the side of the cell (Fig. 1D, 2A). TEM data showed that the posterior flagellum is thickened by a dense rod that is adjacent to the axoneme (Fig. 2A, C, D). The anterior part of the cell contains a vacuole filled with premature mastigonemes and typical mitochondria with tubular cristae (Fig. 2D). Distinct clusters of ribosomes are distributed around the periphery of the cell (Fig. 2A). These observations are consistent with the original description of *B. kenaiensis* by Hilliard (1971). The morphology of our isolate also resembles the species, *Bicosoeca maris* and *B. szabadosi*, although the isolate described here differs from both of these species by the length of the flagella and the size of the lorica (Hamar 1979; Picken 1941). Moreover, unlike our isolate, *B. maris* lacks a dense rod in the posterior flagellum (Moestrup and Thomsen 1976). Although *B. kenaiensis* was originally described from freshwater habitats, our isolate, NY0214, was collected from seawater. Co-occurrence in seawater, brackish water and freshwater is relatively common in the genus *Bicosoeca*; for instance, mostly freshwater species (e.g. *B. kepneri* and *B. pulchra*) have also been recorded in seawater (Lee 2002; Vørs 1993).

A single cell of *C. marsupialis* was manually isolated with a micropipette to establish a mono-eukaryotic culture (NY0206) with environmental bacteria as a food source. *C. marsupialis* (NY0206) is a microaerophilic stramenopile with D-shaped cells that are 7.23–12.1 μm long (average: 8.55 μm, $n = 30$) and 5.03–9.13 μm wide (average: 6.24 μm, $n = 30$) (Fig. 3A, B). A lorica is not present. Cells swim with one flagellum oriented forward and one

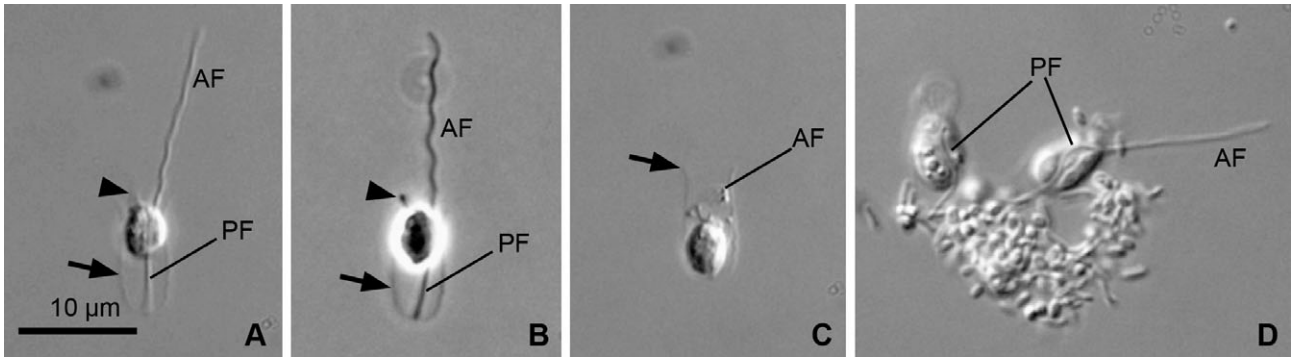

Figure 1 Light micrographs of *Bicosoeca kenaiensis* (NY0214). Arrows and arrowheads indicate the lorica and the feeding lip, respectively. AF = anterior flagellum; PF = posterior flagellum. All images are at the same scale. **A.** Differential interference contrast (DIC) light micrograph showing the lorica, anterior flagellum (AF) and posterior flagellum (PF). **B.** Phase contrast light micrograph showing the unpedicellate lorica. **C.** DIC light micrograph showing the cell withdrawing into the lorica and the anterior flagellum (AF) rolled into a tight coil. **D.** DIC light micrograph showing the posterior flagellum (PF) lying within the groove of the cell.

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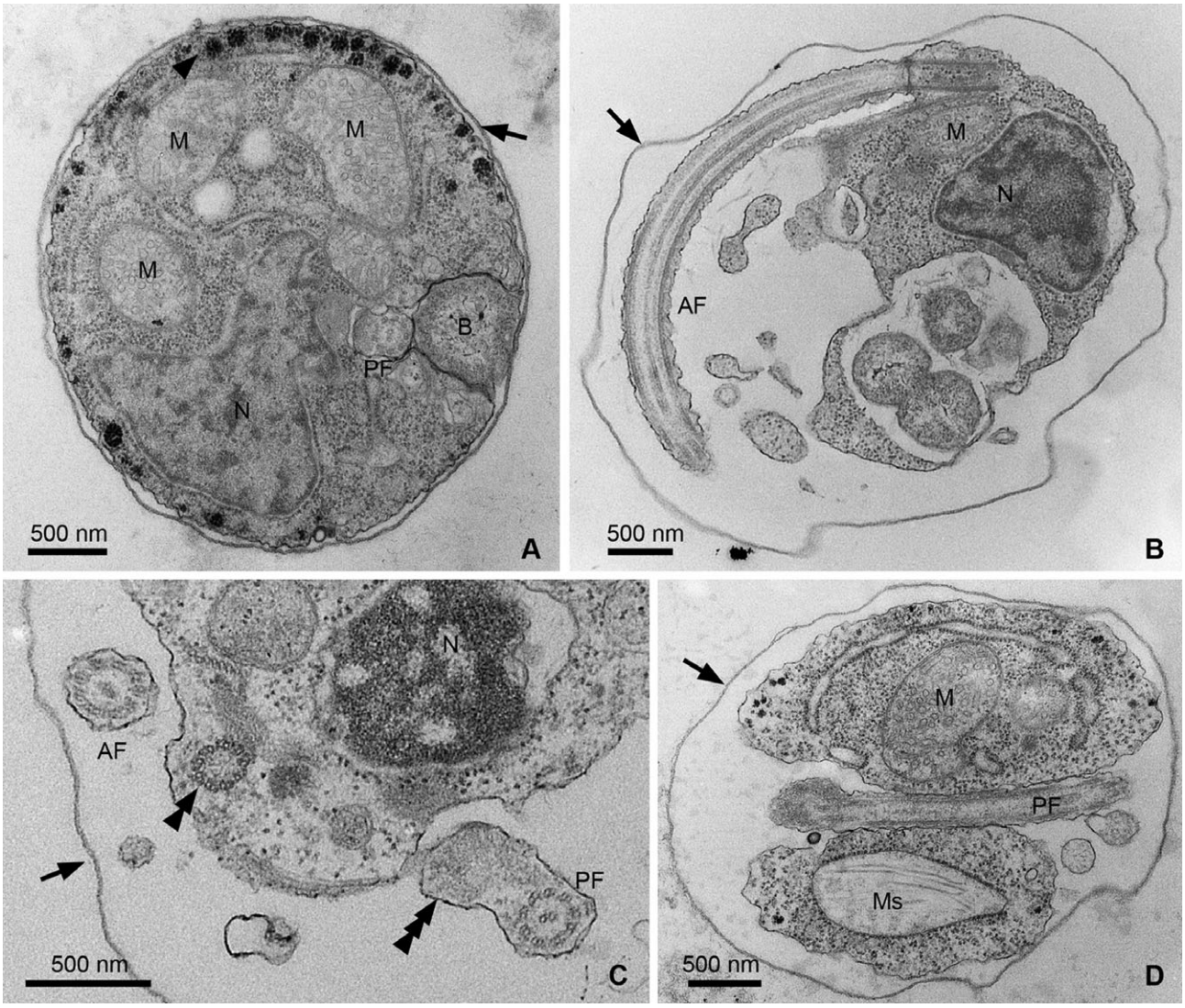

Figure 2 Transmission electron micrographs (TEM) of *Bicosoeca kenaiensis* (NY0214). Arrows indicate the lorica; AF = anterior flagellum; B = bacteria; M = mitochondria; N = nucleus; PF = posterior flagellum. **A.** Cross section of the cell showing the lorica, the PF and clusters of ribosomes (arrowhead) positioned in the periphery of the cell. **B.** Longitudinal section through the anterior flagellum (AF). **C.** Cross section showing a dense rod (triple arrowhead) adjacent to the axoneme of the posterior flagellum (PF) and the basal body (double arrowhead). **D.** TEM showing that the posterior flagellum (PF) sits within the feeding groove. Premature mastigonemes (Ms) are observed in the vacuole.

flagellum trailing behind. When attaching to the substrate with the posterior flagellum, the cells beat the anterior flagellum laterally. The anterior flagellum is about 1.5 times longer than the cell length; the posterior flagellum is about 1.2–1.5 times longer than the cell length. Bacteria are ingested within a deep ventral groove. The food vacuoles are located on the middle to posterior part of the cell (Fig. 3). Morphology of the two other isolates of *C. marsupialis* (YPF1205, BUSSELTON) is entirely consistent with that of the isolate NY0206 (Fig. 3C, D). These observations also agree with the original description of *C. marsupialis* by Larsen and Patterson (1990) and subsequent reports of this species (Ekebom et al. 1995/1996; Larsen and Patterson 1990; Lee 2006; Lee et al. 2003; Tong 1997). TEM data demonstrated that the cells have mitochondria (M) with a few tubular cristae (Fig. 4A, B). A striated rhizoplast is situated between the Golgi body and the nucleus and originated from the posterior side of the posterior basal body (Fig. 4C).

Phylogenetic positions of the new isolates as inferred from SSU rRNA gene sequences

We determined almost entire SSU rRNA gene sequences from *B. kenaiensis* (NY0214) and the three isolates of *C. marsupialis* (NY0206, YPF1205, BUSSELTON). The molecular phylogenetic tree inferred from an analysis of the 128-taxon alignment of previously described heterotro-

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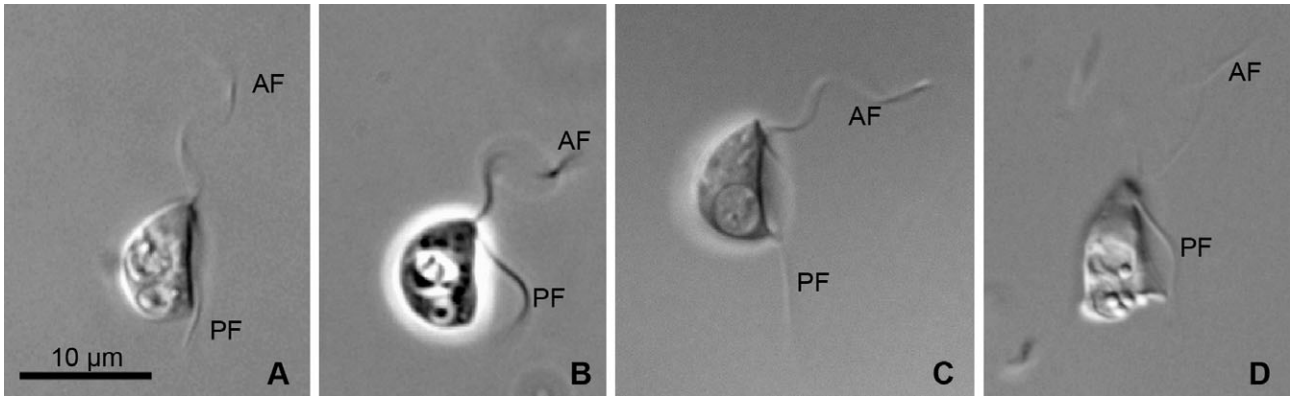

Figure 3 Light micrographs of three different strains of *Cantina* (formerly in *Cafeteria*) *marsupialis* gen. nov., comb. nov. AF = anterior flagellum; PF = posterior flagellum. All images are at the same scale. **A.** Differential interference contrast (DIC) light micrograph of *C. marsupialis* strain NY0206. **B.** Phase contrast light micrograph of *C. marsupialis* strain NY0206. **C.** DIC light micrograph of *C. marsupialis* strain YPF1205. **D.** DIC light micrograph of *C. marsupialis* strain BUSSELTON.

phic stramenopiles and representatives from all 18 MAST clades, is shown in Fig. 5. The monophyly of each MAST lineage is supported with moderate to high bootstrap values (BS) and Bayesian PP as demonstrated previously (Massana et al. 2004, 2014). Our isolate, *B. kenaiensis* (NY0206) nested within *Bicosoeca* (BS = 68, PP = 1.00). Moreover, it was more closely related to the aerobic marine environmental sequences associated with Zuendorf et al.'s (2006) version of MAST-13 (BS = 88, PP = 1.00) than *B. vacillans* and *B. petiolata*. The sequences from the three strains of *C. marsupialis* (NY0206, YPF1205, BUSSELTON) formed a monophyletic group with an environmental sequence NAMAKO-31 with high statistical support (BS = 100, PP = 1.00), which represents Takishita et al.'s (2007) version of MAST-13 from anaerobic environments. Our *Cantina marsupialis* (previously *Cafeteria marsupialis*) and MANAKO-31 do not group with the type species of *Cafeteria* (*C. roenbergensis*), Bicosoecida nor any stramenopile lineages with robust statistical support (Fig. 5).

DISCUSSION

Bicosoecids are abundant heterotrophic flagellates with a cosmopolitan distribution in freshwater, marine, brackish, and hypersaline environments (Arndt et al. 2000; Kim et al. 2010; Lee 2006; Lin et al. 2012; Park et al. 2006). Both *B. kenaiensis* and *C. marsupialis* gen. nov., comb. nov. (=*Cafeteria marsupialis*) have been previously described as bicosoecids. Molecular phylogenetic analyses in our present study demonstrated that these two species are members of different environmental sequence clades; however, both of them have been labeled "MAST-13" by different authors (Takishita et al. 2007; Zuendorf et al. 2006). Zuendorf et al.'s version of MAST-13 includes our isolate of *B. kenaiensis*, which is nested within the more inclusive *Bicosoeca* (Fig. 5). The sequences from our three microaerophilic strains of *C. marsupialis* formed a robust monophyletic group that branched closely with the environmental sequence "NAMAKO-31" (Takishita et al.'s MAST-13) which was also collected from a low-oxygen seawater environment (Takishita et al. 2007). Based on small divergence of the SSU rRNA gene sequence among NAMAKO-31 and our strains of *C. marsupialis*, it is reasonable to assume that NAMAKO-31 represents *C. marsupialis*. The environments from which the sequences in both environmental sequence clades were collected correspond to the environments where we collected our isolates of *B. kenaiensis* and *C. marsupialis*, respectively.

Park and Simpson (2010), Cavalier-Smith and Scoble (2013), and Massana et al. (2014) suggested that MAST-13 represents *Bicosoeca* based on molecular phylogenetic evidence, which is consistent with our results (Fig. 5). Small subunit rRNA gene sequences from only two other described species of *Bicosoeca* (*B. vacillans* and *B. petiolata*) have been reported prior to our study. No detailed morphological information other than from light micrographs and line drawings has been reported from *B. vacillans* and *B. petiolata* so far. Our strain of *B. kenaiensis* (NY0214) improved our understanding of morphological traits in the environmental sequence clade previously recognized as MAST-13. Of the 40 accepted species of *Bicosoeca* (Preisig et al. 1991), only several species (e.g. *B. lacustris*, *B. kepneri*, *B. maris*, *B. planctonica*) have been studied at the ultrastructural level prior our study (Belcher 1975; Mignot 1974; Moestrup and Thomsen 1976); the descriptions of most species are based only on light microscopy and line drawings. The morphological characters in some bicosoecid species descriptions, especially those from older literature, are difficult to interpret, so some of these descriptions could represent the same species with different names (synonyms). Indeed, some authors have already pointed out the need to revise the taxonomy of *Bicosoeca* (Al-Quassab et al. 2002; Aydin and Lee 2012; Lee 2007; Tong 1997; Tong et al. 1998). The current work offers a good example for how to improve the systematics of this genus by using a combination of detailed morphological traits and molecular phylogenetic

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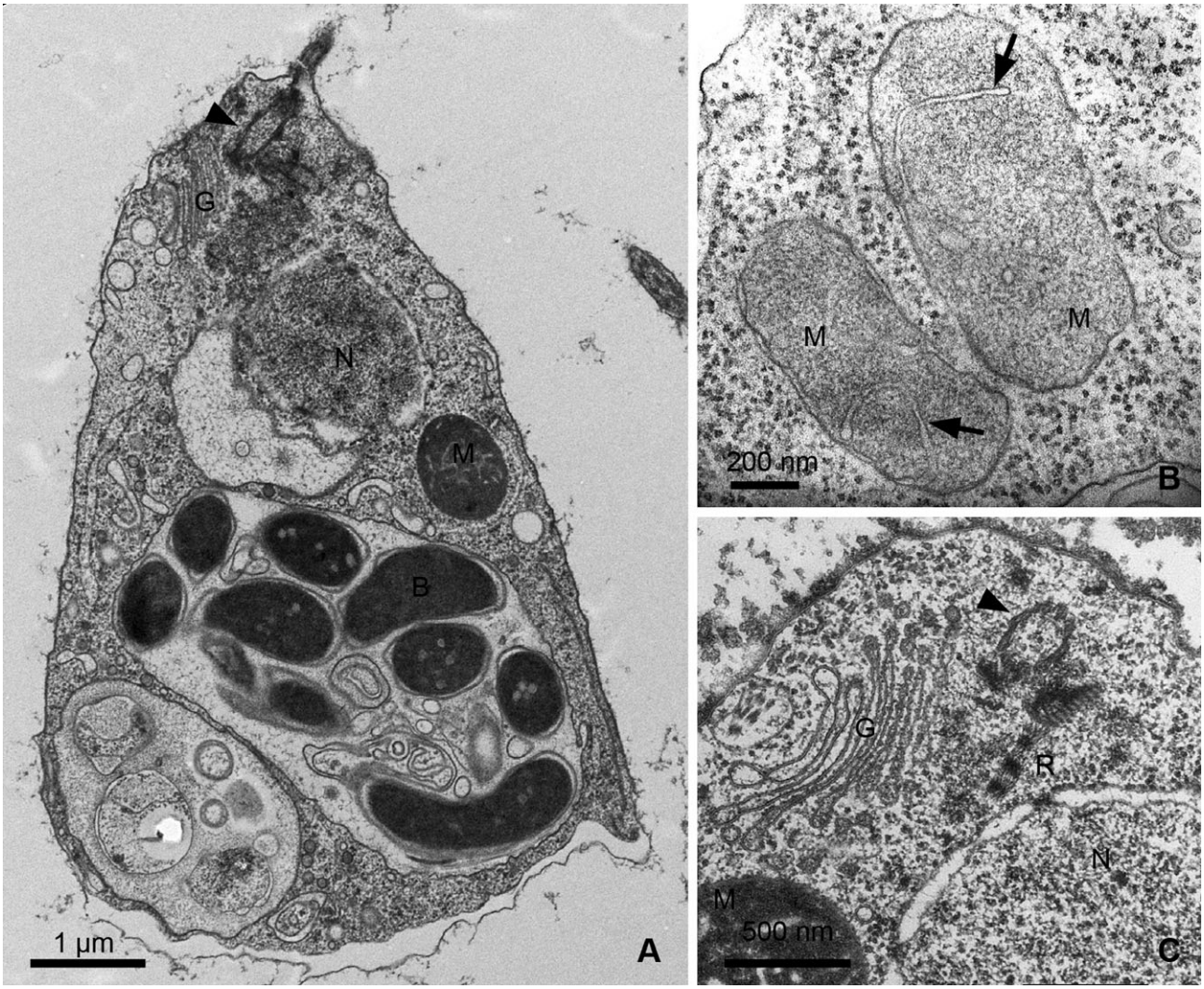

Figure 4 Transmission electron micrographs (TEM) of *Cantina* (formerly in *Cafeteria*) *marsupialis* gen. nov., comb. nov. (NY0206). B = bacteria; G = Golgi body; M = mitochondrion; N = nucleus. Arrowheads indicate the basal body. **A.** Longitudinal section of the cell. **B.** High magnification micrograph showing the small number of tubular mitochondrial cristae (arrows). **C**. High magnification micrograph showing a Golgi body and a rhizoplast (R) connecting a basal body and a microtubular root to the nucleus (N).

data. Nonetheless, *B. kenaiensis* (NY0214) represents the first species of *Bicosoeca* with enough associated data to infer the cellular identity of a previously unclear environmental sequence clade.

Although no DNA sequences were available prior to this study, data from light microscopy suggested that *Cantina marsupialis* (as *Cafeteria marsupialis*) occupies anaerobic sediments all over the world, such as Australia, Brazil, the United Kingdom, Japan and Canada (Ekebom et al. 1995/1996; Larsen and Patterson 1990; Lee and Patterson 1998, 2000; Lee 2006; Lee et al. 2003; Tong 1997; and this study). Our study demonstrated that an SSU rRNA sequence from *C. marsupialis* was previously reported as an environmental sequence (NAMAKO-31) from an anoxic environment (Takishita et al. 2007) and a few cristae in the mitochondria of *C. marsupialis* observed here reflect this organism's adaptations to low oxygen environments; for instance, *C. marsupialis* may retain a part of the mitochondrial electron transport chain as demonstrated in *Blastocystis* (Stechmann et al. 2008). Environmental DNA surveys are an efficient tool for elucidating broad patterns of species diversity in natural environments, but this approach has several limitations. For instance, some sequences from scarce organisms and divergent sequences from abundant organisms could fail to be recovered altogether. This issue has become evident in free-living fornicate clades (Excavata) from environmental PCR surveys. SSU rRNA gene sequences of one species, namely *Kipferlia bialata*, from these environmental PCR surveys are deposited in GenBank providing a misleading picture of the overall diversity of the Fornicata; culture-dependent methods have subsequently discovered five

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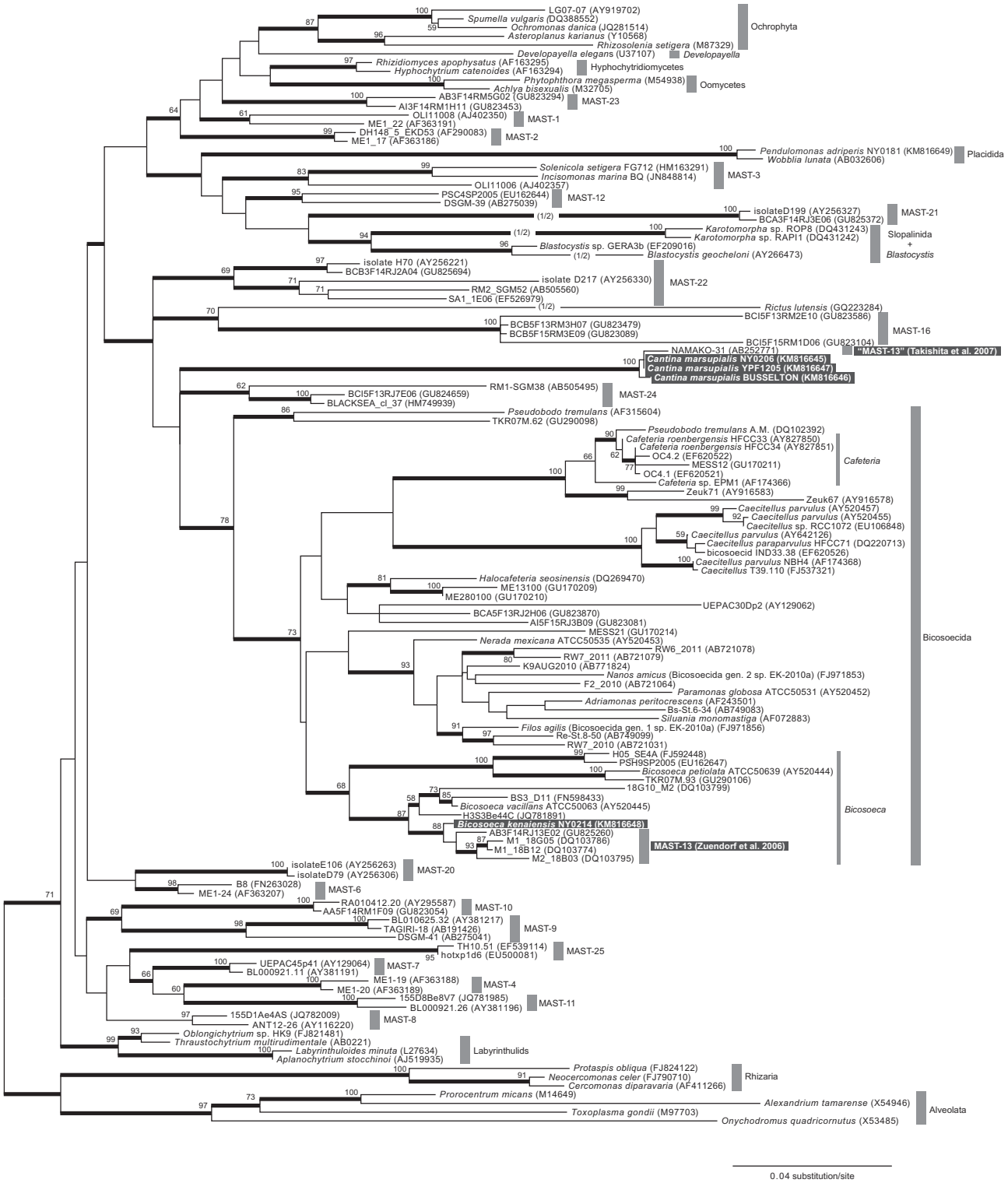

Figure 5 Phylogenetic tree inferred from a 128-taxon alignment of SSU rRNA gene sequences. The tree was inferred with Maximum Likelihood (ML) analyses using a GTR + G + I model. ML bootstrap values less than 50% are not shown. Nodes supported by Bayesian posterior probabilities ≥ 0.95 are shown by thick lines.

other free-living lineages of fornicates providing more comprehensive knowledge of the group (Kolisko et al. 2010; Takishita et al. 2012). The NAMAKO-31 lineage has only been reported once before in environmental DNA surveys, which implies that the distribution of *C. marsupialis* is restricted in nature. As Heger et al. (2014) pointed out,

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morphological data and molecular data are insufficient by themselves to understand the diversity, interactions and geographic distributions of different eukaryotic lineages. The integration of both kinds of information as studied in this work leads to much better understanding of these patterns.

Our molecular phylogenetic analyses showed that the anaerobic *Cantina* clade, including NAMAKO-31 in stramenopiles, is not resolved as was also reported in previous studies (Takishita et al. 2007, 2010). The phylogenetic affiliation of the *Cantina* clade with other anaerobic/microaerophilic lineages, such as *Rictus lutensis*, *Blastocystis*, slopalinids, MAST-9, MAST-12, MAST-16, and MAST-20 through MAST-24 is not confirmed in our analysis either, supporting the possibility that the anaerobic lineages within stramenopiles are either a paraphyletic or polyphyletic group (Massana et al. 2014). This would stand in contrast to the pattern observed in other major groups of eukaryotes where anaerobic lineages form only one or two clades, such as in euglenozoans, archamoebae, metamonads, and heteroloboseans (Hampl et al. 2005; Hampl et al. 2009; Pánek et al. 2012, 2014; Yubuki et al. 2013). Additional environmental sequences combined with culture-dependent studies of anaerobic stramenopiles are expected to resolve the phylogenetic position of *Cantina* and the overall phylogenetic pattern of anaerobic lineages within stramenopiles.

So far, marine environmental PCR surveys of SSU rRNA gene sequences have demonstrated that stramenopiles comprise a substantial portion of the total number of eukaryotic sequences recovered. For example, they represent over 30% of the total environmental eukaryotic lineages present in 13 different environmental gene libraries (Richards and Bass 2005) and 42% of total OTUs detected from the suboxic zone of the Black Sea (Wylezich and Jürgens 2011). Sequences from heterotrophic stramenopiles are also one of the most dominant lineages in RNA-derived clone library from an anoxic Danish fjord (Stoeck et al. 2007). Massana et al. (2006) demonstrated that marine stramenopiles occupied up to 35% of the abundance of heterotrophic flagellates in five different oceans, and MAST clades represent a significant fraction of the total number of cells present (e.g. MAST-4 alone represents 9%). Studies of community compositions in both marine and freshwater environments have demonstrated that stramenopile, mainly bicosoecids and chrysomonads, constitute 20–50% of the heterotrophic nanoflagellate biomass (Boenigk and Arndt 2002). Therefore, bicosoecids, including *B. kenaiensis*, contribute significantly to marine food webs, and our culturing-dependent and microscopical approaches help demonstrate the morphological identities of environmental sequences clades and improve our overall understanding of these ecologically important lineages.

TAXONOMIC SUMMARY

The morphological traits present in our three isolates of *C. marsupialis* gen. nov., comb. nov. are consistent with the original description of *C. marsupialis* (Larsen and Patterson 1990) and subsequent reports based on light microscopy (Ekebom et al. 1995/1996; Larsen and Patterson 1990; Lee 2006; Lee et al. 2003; Tong 1997). However, the molecular phylogenetic positions of our isolates were not closely affiliated with the type species of the genus, namely *Cafeteria roenbergensis*, or any other lineage of stramenopiles for that matter (Fig. 5). Therefore, we propose a new genus name and combination for this species based on molecular and ultrastructural data, namely *C. marsupialis* gen. nov., comb. nov.

Cantina gen. nov

Diagnosis. Stramenopiles with D-shaped cells possessing a large groove on the ventral side and two flagella; one oriented anteriorly and one oriented posteriorly. Mitochondria with a few tubular cristae. Microaerophilic, living in marine sediments.

Type species. *Cafeteria marsupialis* Larsen and Patterson 1990 (=*Cantina marsupialis* comb. nov.).

Etymology. "*Cantina*" refers to the English word "canteen", which has a similar meaning to the previous genus name *Cafeteria*. Gender feminine.

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Molecular and morphological diversity of the genus *Hypotrichomonas* (Parabasalia: Hypotrichomonadida), with descriptions of six new species

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Abstract

The genus *Hypotrichomonas* Lee, 1960 belongs to the small parabasalian class Hypotrichomonadea. Although five *Hypotrichomonas* species have been described from intestines of lizards and birds, some descriptions were brief and incomplete. Only the type species *H. acosta* has been observed repeatedly. We have established 23 strains of the genus *Hypotrichomonas* in culture. Phylogenetic and morphological analyses showed that these isolates represent eight distinct species, six of which are novel. Three of the species showed unusual morphology, such as a reduced undulating membrane, absence of the free part of the recurrent flagellum or a costa-like fiber. Our strains were isolated from a wide range of hosts including cockroaches, frogs, tortoises, lizards, snakes, marsupials, pigs, rodents, and primates. The genus *Hypotrichomonas* thus contains a relatively large number of species that differ in morphology, phylogenetic position and host range. It is remarkable that such diversity of hypotrichomonads was previously undetected, although a number of studies dealt with intestinal trichomonads of vertebrates and invertebrates. Our results indicate that the diversity of the genus *Hypotrichomonas* as well as of the whole Parabasalia is still only poorly understood, and the lineages described so far likely represent only a small fraction of the true diversity of parabasalids.

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Introduction

Parabasalia is a diverse lineage of flagellated protists belonging to the supergroup Excavata. It is defined morphologically by the presence of hydrogenosomes (anaerobic mitochondrion-related organelles), the arrangement of the flagellar apparatus, and closed mitosis with an extranuclear spindle (Cepicka et al. 2010). Most of the approximately 500 described species of Parabasalia (Adl et al. 2007) live as intestinal endobionts of various animals, particularly termites; only six free-living species have been described (see Yubuki et al. 2010). The Parabasalia have been traditionally divided into two groups according to the complexity of the cell (e.g., Levine et al. 1980): trichomonads, which usually possess smaller and less complex cells, with up to six flagella per mastigont; and the hypermastigotes, which are multiflagellate (possess multiple flagella per mastigont), with complex and often very large cells. The hypermastigotes live exclusively in the intestines of termites and wood-eating cockroaches, whereas the trichomonads are known from a wide range of vertebrates and invertebrates. However, it has been shown that the hypermastigote type of parabasalians has

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evolved many times independently by convergence, and that neither trichomonads nor hypermastigotes are monophyletic (e.g., Brugerolle and Patterson 2001; Hampl et al. 2004; Noda et al. 2009; Tai et al. 2015). The taxonomic division into the trichomonads (as order Trichomonadida) and hypermastigotes (as order Hypermastigida) has thus fallen into disuse. The taxonomic system of the Parabasalia currently identifies six classes: Trichomonadea, Hypotrichomonadea, Tritrichomonadea, Cristamonadea, Spirotrichonymphea, and Trichonymphea (Cepicka et al. 2010). In addition, it was recently shown that the genus *Lophomonas*, previously classified as a member of Cristamonadea, represents an independent evolutionary lineage of the Parabasalia (Gile and Slamovits 2012).

The diversity of Parabasalia is currently poorly understood, and numerous species await description. This can be demonstrated by the genus *Tetratrichomonas*. Several tetratrichomonad lineages have been detected in various host taxa, on the basis of SSU rDNA sequences, showing that the true diversity of this genus is far greater than the number of described species (Cepicka et al. 2006; Mantini et al. 2009; Smejkalová et al. 2012). Although the "environmental" diversity of other parabasalian genera is less explored, the hidden diversity within *Tetratrichomonas* is certainly not exceptional in this context.

On the other hand, many descriptions of new parabasalian species are too brief and do not contain sufficient morphological description. Although the importance of wet smear fixation for comparative morphology of the parabasalians was emphasized several decades ago (e.g., Honigberg 1951, 1963; Nie 1950), many authors employ dry smears stained with Giemsa, or even do not study the morphology of the organisms they are going to describe at all. Examples of this approach include the recent descriptions of *Tetratrichomonas empyemagena*, *Trichomonas gypaetinii*, *Trichomonas stableri*, and *Tritrichomonas blagburni* (Girard et al. 2014; Lopez-Escamilla et al., 2013; Martínez-Díaz et al., 2015; Walden et al. 2013), which are all based on sequence data, instead of careful morphological examination. Moreover, since no type material has been designated for any of these species, the descriptions are invalid under the International Code of Zoological Nomenclature.

Hypotrichomonas is one of the least-known trichomonad genera. Together with the genus *Trichomitus*, it belongs to the smallest parabasalian class, Hypotrichomonadea. Members of the Hypotrichomonadea are characterized by the presence of three anterior flagella, a lamelliform undulating membrane, a biramous parabasal body, and a cytoskeletal structure called comb-like structure in the mastigont (Brugerolle 1975/1976, 1991; Cepicka et al. 2010; Mattern et al. 1969). Members of the genus *Trichomitus* possess a costa, a striated fiber that underlies the undulating membrane; while members of *Hypotrichomonas* have lost this structure, leaving the undulating membrane unsupported (Honigberg 1963). The third evolutionary lineage of the Hypotrichomonadea, represented by strain KANEK, was identified on the basis of a SSU rDNA sequence, and its morphology remains unknown (Cepicka et al. 2010).

So far, five *Hypotrichomonas* species have been formally described. Four of them, *H. acosta*, *H. hemidactyli*, *H. osmaniae*, and *H. venkataramiahii*, have been obtained from the intestine of reptiles (Bhaskar Rao et al. 1976; Daykar et al. 1977; Krishnamurthy 1967; Moskowitz 1951), while *H. avium* was described from birds (Navarathnam 1970). Except for *H. acosta*, the descriptions are brief and incomplete, and the species have not been observed since the original description. Interestingly, recent molecular-phylogenetic studies based on SSU rDNA sequences revealed considerable diversity of organisms clustering with *H. acosta* in primates, beetles, reptiles, and rodents (Dimasuay and Rivera 2013; Kamaruddin et al. 2014; Smejkalová et al. 2012; Zhang et al. 2003).

To examine the diversity of this neglected parabasalian genus, we obtained 23 strains of *Hypotrichomonas* from various hosts, established them in culture, and examined their light-microscopic morphology and SSU rDNA phylogeny. Our data show that the genus *Hypotrichomonas* is considerably diverse from both morphological and molecular points of view. It confirms that the true diversity of trichomonads is still far from being satisfactorily described.

Material and Methods

Organisms

Information on the origin of strains included in this study is summarized in Table 1. *Hypotrichomonas* isolates were obtained between 2000 and 2012 from contents of the lower intestine (isolates from cockroaches and rodents) or feces. The hosts had been kept either by private keepers or in zoological gardens in the Czech Republic for a longer period or for several generations. An exception is the isolate GRAJC1 whose host, the striped field mouse (*Apodemus agrarius*), was captured from the wild in Grajcar (Slovakia) and was kept under laboratory conditions in Prague (Czech Republic) for two months. Trichomonads were isolated in Dobell and Laidlaw's biphasic medium (Dobell and Laidlaw 1926). Isolates from mammals were cultured at 37 °C and were subcultured every second or third day; isolates from frogs, reptiles, and cockroaches were cultured at room temperature and were subcultured once a week. Most cultures contained various endobiotic protists besides the trichomonads. A monoeukaryotic culture of the strain KOMBG1 was derived from the culture established by Smejkalová et al. (2012), which also contained *Blastocystis* sp. as well as *Hypotrichomonas mariae* sp. nov., by cultivation in modified TYSGM-9 medium (Diamond 1982), without mucin and Tween 80, and supplemented with rice starch. After the monoeukaryotic culture was created, the strain KOMBG1 was returned to the Dobell and Laidlaw's medium. The isolates except for CR are deposited in the culture collection

Table 1. List of strains included in the study.

Species	Isolate	Host species	Host common name	GenBank acc. numbers
H. acosta	CORAL1	*Corallus* sp.	Neotropical tree boa	KJ591561
	CR	*Calabaria reinhardtii*	Calabar ground boa	KJ591565
	EC	*Eryx conicus*	Rough-scaled sand boa	KJ591563
	LEP1	*Leptopelis* sp.	Forest tree frog	KJ591564
	VAR1H	Varanidae gen. sp.	Monitor lizard	KJ591562
H. carbonariae n. sp.	GECA1	*Geochelone carbonaria*	Red-footed tortoise	KJ591557
H. sanderai n. sp.	GRAJC1	*Apodemus agrarius*	Striped field mouse	KJ591566
H. mariae n. sp.	KOMBG1	*Otolemur garnettii*	Northern greater galago	HQ149966
H. osmaniae	CYCL2	*Cyclura nubila*	Cuban rock iguana	KJ591553
	EUMT	*Eumeces* sp.	Skink	KJ591554, KJ591556
	LASE1	*Laemanctus serratus*	Serrated casquehead iguana	KJ591555
H. imitans n. sp.	PRA	*Sus scrofa f. domestica*	Domestic pig	KJ591558
H. hampli n. sp.	MAR1	*Macropus rufus*	Red kangaroo	KJ591560
	KLRUD	*Wallabia rufogrisea*	Red-necked wallaby	KJ591559
H. blattarum n. sp.	ARCH1	*Archimandrita tessellata*	Giant leaf cockroach	KJ591544
	BAT1	*Blaberus atropos*	Cockroach	KJ591548
	BCR	*Blaberus craniifer*	Death's head cockroach	KJ591549
	BSP	*Blaberus* sp.	Cockroach	KJ591550
	BLAPT1	*Blaptica dubia*	Orange-spotted cockroach	KJ591545
	BLAPT2	*Blaptica dubia*	Orange-spotted cockroach	KJ591546
	EUBLAB	*Eublaberus* sp.	Cockroach	KJ591551
	GROM	*Gromphadorhina* sp.	Cockroach	KJ591552
	NANPA	*Nauphoeta cinerea*	Cinereous cockroach	KJ591547

of the Department of Parasitology of Charles University in Prague, Czech Republic.

Light microscopy

Light-microscopic observations were performed using a BX51 microscope equipped with a DP71 camera (Olympus, Tokyo, Japan). Cell morphology was examined in protargol-stained preparations. Moist films spread on coverslips were prepared from pelleted cultures obtained by centrifugation at 500 × *g* for 8 min. The films were fixed in Bouin-Hollande's fluid for 4 h, were washed with 70% ethanol, and stained with 1% protargol (Bayer, Leverkusen, Germany) following Nie's (1950) protocol. Morphology of some strains was also studied in living and formaldehyde-killed cells in order to examine the presence of knobs at the distal end of anterior flagella.

DNA extraction and PCR amplification

Genomic DNA was isolated using the High pure PCR template preparation kit (Roche). An approximately 1600 bp fragment of the SSU rDNA was amplified using trichomonad-specific primers 16Sl (5′-TACTTGGTTGATCCTGCC-3′; Tachezy et al. 2002) and 16SRR (5′-TCACCTACCGTTACCTTG-3′; Cepicka et al. 2005) with an annealing temperature of 58 °C. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and directly sequenced on the ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, USA). SSU rDNA of the strain EUMT of *Hypotrichomonas osmaniae* was cloned into the pGEM®-T EASY vector (Promega, Fitchburg, USA), and two clones originating from two separate PCR reactions were sequenced. Sequence data reported in this paper are available in GenBank under accession numbers KJ591544–KJ591566.

Phylogenetic analysis

A dataset containing SSU rDNA sequences of Hypotrichomonadea was constructed. It consisted of 24 new sequences, 30 sequences of the genus *Hypotrichomonas* retrieved from GenBank, and 8 sequences of the genus *Trichomitus* and Hypotrichomonadidae gen. sp. KANEK used as the outgroup. The sequence AB665801, labeled in GenBank as "uncultured rumen bacterium", was identified by BLAST to belong to the genus *Trichomitus*. The sequences were aligned using the MAFFT program (Katoh et al. 2002) with the help of the MAFFT 6 server (http://mafft.cbrc.jp/alignment/server/index.html) using the G-INS-i algorithm with default settings. The resulting alignment was manually edited in BioEdit 7.0.9.0 (Hall 1999). The length of the final alignment was 1467 characters, and

it is available upon request. Phylogenetic trees were constructed by the maximum likelihood method in RAxML 7.0.3 (Stamatakis 2006) under the GTRGAMMAI model. Bootstrap support values were generated using 1000 pseudoreplicates.

Results

Hypotrichomonas strains

In order to investigate the real diversity of the neglected genus *Hypotrichomonas* in detail, we examined protargol preparations of approximately 150 trichomonad strains deposited at the Department of Parasitology, Charles University in Prague, Czech Republic. 20 of them displayed typical features of *Hypotrichomonas*, such as three anterior flagella, undulating membrane, free distal portion of the recurrent flagellum, and the lack of a costa. We determined SSU rDNA sequences of all the strains; subsequent phylogenetic analysis placed all of them into this genus. We also included in the analysis three strains (PRA, GECA1, and KOMBG1), which showed morphology different from *Hypotrichomonas*, but were shown to be closely related to this genus by 18S rDNA analysis.

Phylogenetic analysis of SSU rDNA

The phylogenetic tree of Hypotrichomonadea as inferred from SSU rDNA sequences is shown in Fig. 1. The genus *Hypotrichomonas* was robustly monophyletic and split into eleven lineages, here considered separate species (see below): *H. acosta*, *H. osmaniae*, *H. imitans* n. sp., *H. carbonariae* n. sp., *H. hampli* n. sp., *H. blattarum* n. sp., *H. sanderai* n. sp., *H. mariae* n. sp., *Hypotrichomonas* sp. 1, *Hypotrichomonas* sp. 2, and *Hypotrichomonas* sp. 3. Monophyly of *H. acosta* was only poorly supported. The relationships between the particular lineages were generally unresolved. *H. imitans* n. sp., *Hypotrichomonas* sp. 1, *H. carbonariae* n. sp., *H. osmaniae*, *H. hampli* n. sp., and *H. blattarum* formed a robust clade without resolved interrelationships. *H. carbonariae* n. sp. was closely related to *H. osmaniae* with moderate support. *Hypotrichomonas* sp. 2 appeared closely related to *Hypotrichomonas* sp. 3, but the relationship was not highly supported. *H. sanderai* n. sp. and *H. mariae* n. sp. formed a robust clade.

The genetic distances (uncorrected p distances) between the lineages varied between 0.022 (*H. osmaniae* and *H. carbonariae* n. sp.) and 0.089 (*H. osmaniae* and *Hypotrichomonas* sp. 3). The maximum distance within particular lineages usually ranged between 0.003 and 0.007. The maximum distance within *Hypotrichomonas* sp. 3 reached 0.018. The maximum distance within *H. acosta* reached 0.036. However, when the divergent sequence JX565070 was not considered, the maximum distance lowered to 0.024. Further, when sequences JX565080 and JX565075 were removed that display unique nucleotides in certain otherwise highly conserved positions, the maximum distance between the remaining sequences of *H. acosta* was 0.009.

Light-microscopic morphology of protargol-stained cells

The light-microscopic morphology of trophozoites of the studied trichomonad strains is presented in Figs 2–4. The diameters of protargol-stained cells are summarized in Table 2. Cells of all strains were uninucleate and possessed three unequal anterior flagella ending with knobs, a single recurrent flagellum, an axostyle, and a pelta. The proximal part of the recurrent flagellum usually formed a well-developed undulating membrane (except for the strain PRA of *Hypotrichomonas imitans* n. sp.), and its posterior part did not attach to the cell body and continued by a free portion (except for the strain GECA1 of *H. carbonariae* n. sp.). The undulating membrane was not underlain by any fiber, with the exception of the strain KOMBG1 of *H. mariae* n. sp. The parabasal body was always biramous, though it was not clearly discernible in *H. blattarum* n. sp., *H. sanderai* n. sp., and *H. osmaniae*. Individual *Hypotrichomonas* species differed in cell diameter, relative length of the anterior flagella, and the character of the axostyle and undulating membrane.

Hypotrichomonas acosta (Moskowitz, 1951)

Morphology of the strains CORAL1 and LEP1 was closely examined (Fig. 2A–D). The cells were on average 8.2 μm long (excluding the protruding part of the axostyle) and 5.4 μm wide (including the undulating membrane). The longest anterior flagellum was approximately twice as long as the cell body. A well-developed undulating membrane reached approximately from one half to two thirds of the cell body and displayed several waves (Fig. 2B, C). The free distal part of the recurrent flagellum was longer than the cell body (Fig. 2C, D). The axostyle was of *Trichomonas*-type, and its trunk was relatively stout (Fig. 2B, D). Its protruding part was approximately 4.4 μm long. The pelta was thick and long (Fig. 2B–D).

Hypotrichomonas osmaniae Krishnamurthy, 1967

Cells of the strains CYCL2 and LASE1 were morphologically similar to each other (Fig. 2E–H). They were on average 8.5 μm long (excluding the protruding part of the axostyle) and 5.9 μm wide (including the undulating membrane). The longest anterior flagellum was approximately twice as long as the cell body. A well-developed undulating membrane often reached the posterior end of the cell body and displayed several waves (Fig. 2E–H). The free distal part of the recurrent flagellum was approximately as long as the cell body (Fig. 2F,

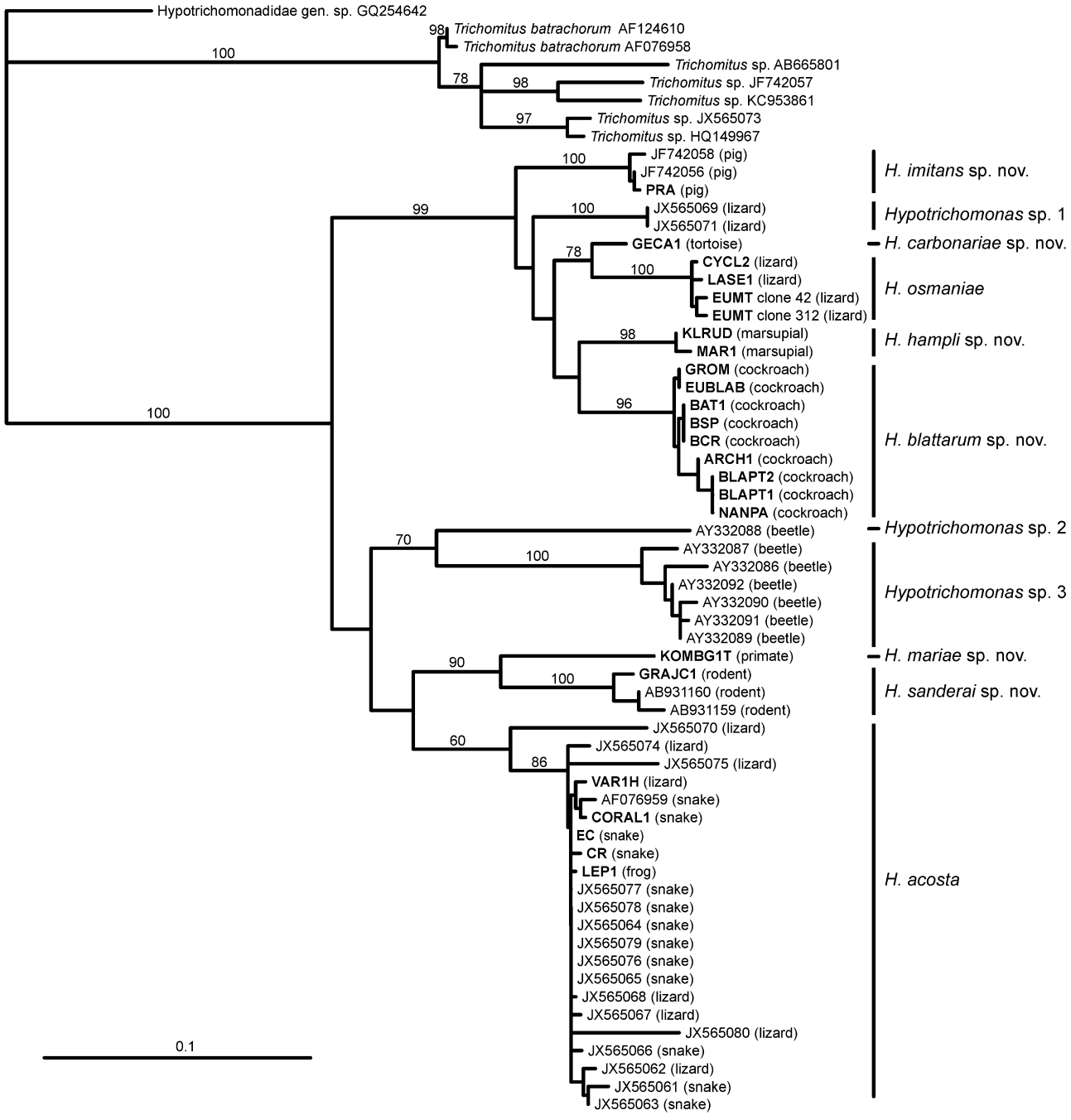

Fig. 1. Phylogenetic tree of Hypotrichomondea based on SSU rDNA sequences. The tree was constructed by the maximum likelihood method in RAxML under the GTRGAMMAI model; the values at the nodes represent statistical support in bootstrap values (RAxML). Support values below 50% not shown. The newly obtained sequences are in bold.

G). The axostyle was of *Trichomonas*-type, and its trunk was thinner than in *H. acosta* (Fig. 2E). The protruding part of the axostyle was approximately 5.6 μm long. The pelta was thick (Fig. 2E, G, H).

Hypotrichomonas hampli n. sp.

The strains MAR1 and KLRUD from marsupials were morphologically similar under the light microscope (Fig. 2I–L). The cells were on average 7.7 μm long (excluding the protruding part of the axostyle) and 6.2 μm wide (including the undulating membrane). The longest anterior flagellum was usually as long as the cell body (Fig. 2I, J, L). The well-developed undulating membrane reached approximately from one half to two thirds of the cell body and displayed several waves (Fig. 2I, J, L). The free distal part of the recurrent flagellum was approximately as long as the cell body (Fig. 2I, L). The axostyle was of *Trichomonas*-type, and

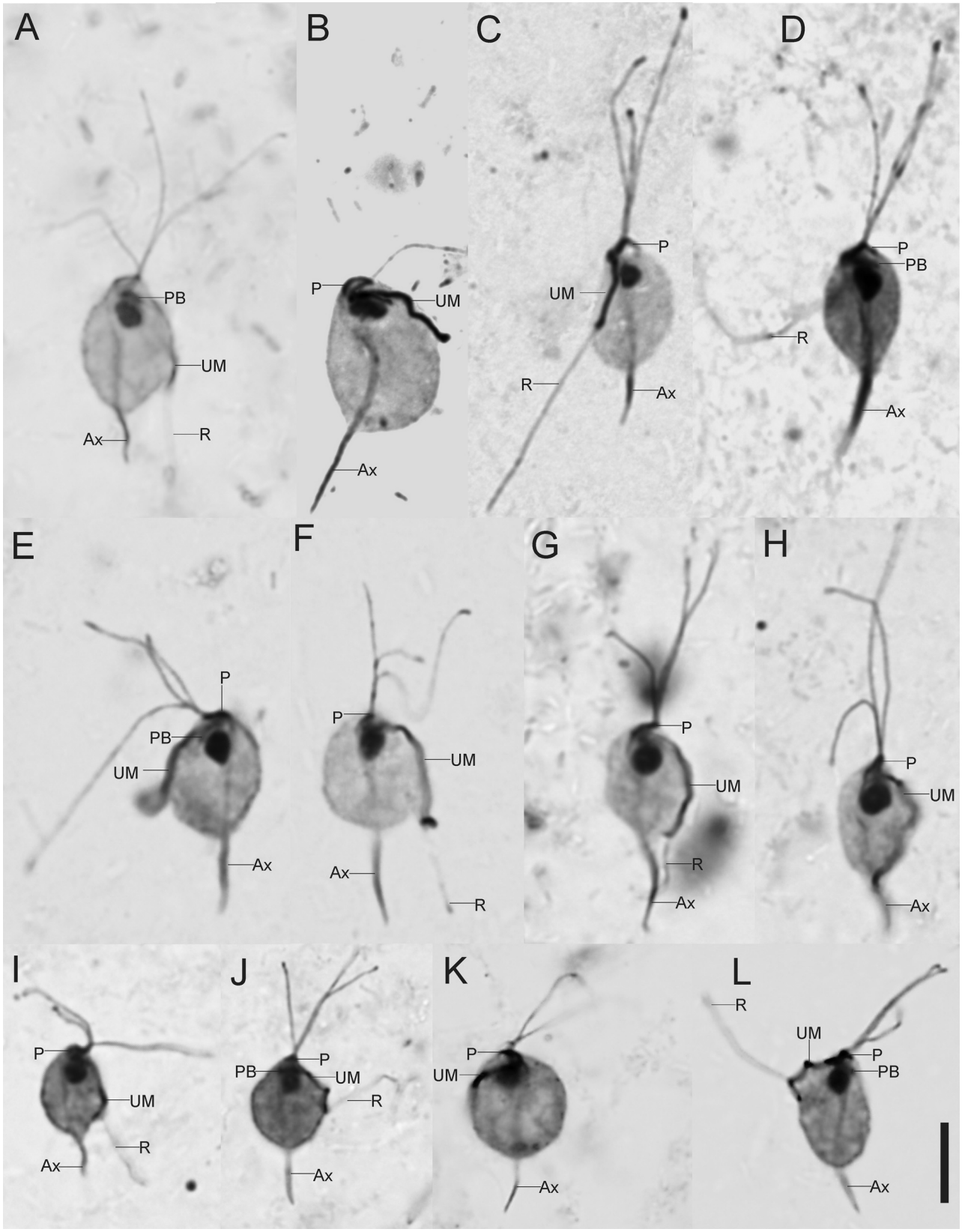

Fig. 2. Protargol-stained strains of *Hypotrichomonas* spp. (A–D): *Hypotrichomonas acosta*, A, B strain CORAL1, C, D strain LEP1; (E–H): *Hypotrichomonas osmaniae*, E, F strain CYCL2, G, H strain LASE1; (I–L): *Hypotrichomonas hampli* n. sp., I–K strain MAR1, L strain KLRUD. Ax – axostyle; P – pelta; PB – parabasal body; R – free part of recurrent flagellum; UM – undulating membrane. Bar = 5 μm.

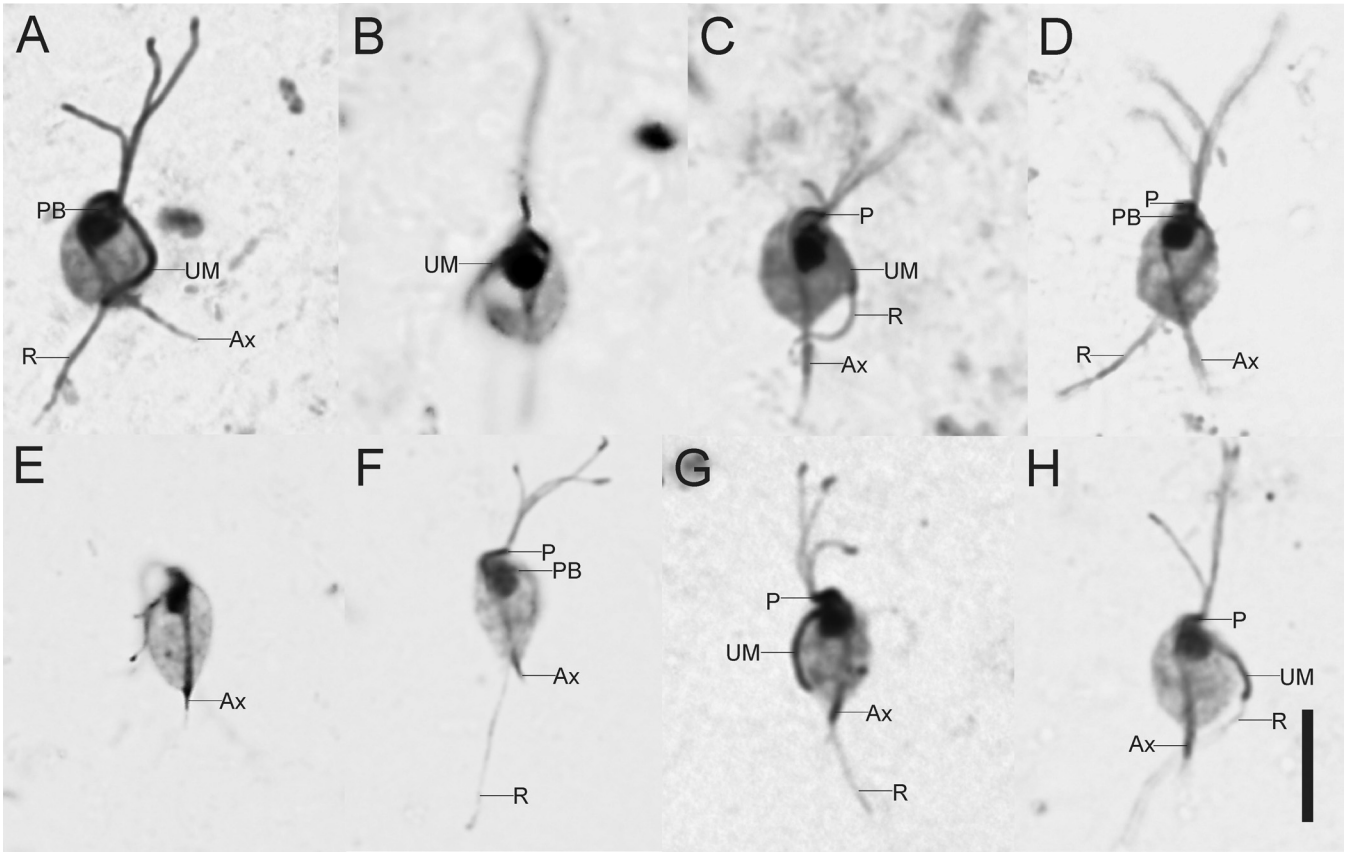

Fig. 3. Protargol-stained strains of *Hypotrichomonas* spp. (A–D): *Hypotrichomonas blattarum* n. sp., A–C strain BCR, D strain BSP; (E–H): *Hypotrichomonas sanderai* n. sp., strain GRAJC. Ax – axostyle; P – pelta; PB – parabasal body; R – free part of recurrent flagellum; UM – undulating membrane. Bar = 5 μm.

its trunk was slender (Fig. 2I, J, L). Its protruding part was approximately 2.5 μm long. The pelta was shorter than in *H. acosta* (Fig. 2I–L).

Hypotrichomonas blattarum n. sp.

The cells of *H. blattarum* n. sp. (Fig. 3A–D) were on average 5.4 μm long (excluding the protruding part of the axostyle) and 3.9 μm wide (including the undulating membrane). The longest anterior flagellum was approximately twice as long as the cell body (Fig. 3A, B, D). The well-developed undulating membrane reached approximately from one half almost to the posterior end of the cell and displayed a single large wave (Fig. 3A, C). The free distal part of the recurrent flagellum was somewhat longer than the cell body (Fig. 3A, D). The axostyle was of *Trichomonas*-type, and its trunk was thin (Fig. 3A, D). Its protruding part was approximately 2.6 μm long. The pelta was thick (Fig. 3A, C, D).

Hypotrichomonas sanderai n. sp.

The cells of strain GRAJC1 (Fig. 3E–H) were on average 5.0 μm long (excluding the protruding part of the axostyle) and 4.2 μm wide (including the undulating membrane). The longest anterior flagellum was slightly longer than the cell body (Fig. 3F–H). The well-developed undulating membrane reached approximately from halfway along the cell almost to the posterior end of the cell and displayed a single large wave (Fig. 3G, H). The free distal part of the recurrent flagellum was longer than the cell body (Fig. 3F, H). The axostyle was of *Tritrichomonas*-type (Fig. 3E–H). Its protruding part was approximately 1.5 μm long. The pelta was well developed (Fig. 3F–H).

Hypotrichomonas carbonariae n. sp.

The cells of the strain GECA1 (Fig. 4A–D) were on average 7.5 μm long (excluding the protruding part of the axostyle) and 5.8 μm wide (including the undulating membrane). The longest anterior flagellum was longer than the cell body, twice as long in some cells (Fig. 4A–D). The well-developed undulating membrane reached almost to the posterior end of the cell and displayed several waves (Fig. 4B–D). It was formed along the whole length of the recurrent flagellum (Fig. 4B–D); the flagellum lacked the free distal portion seen in other

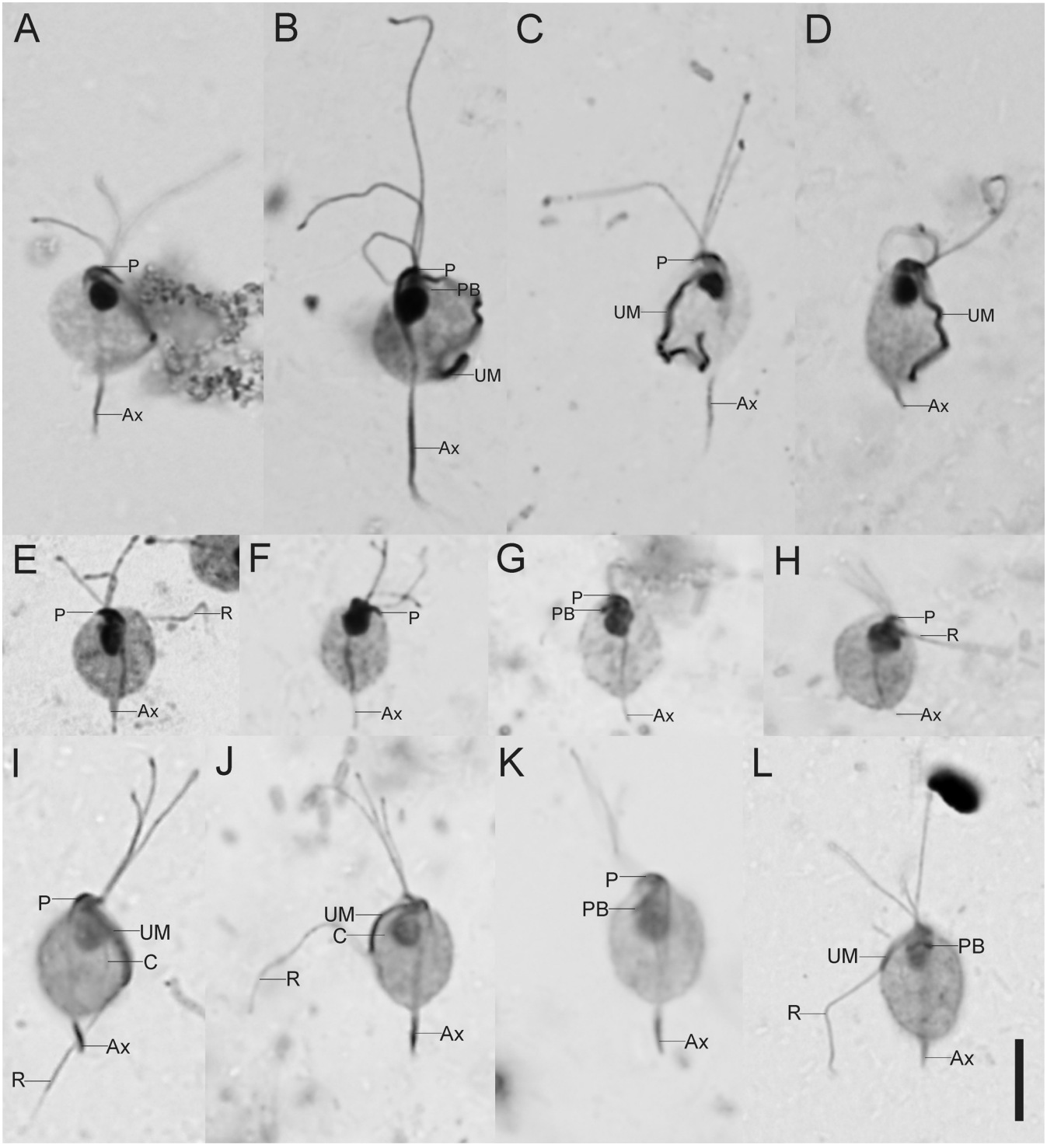

Fig. 4. Protargol-stained strains of *Hypotrichomonas* spp. (A–D): *Hypotrichomonas carbonariae* n. sp., strain GECA1; (E–H): *Hypotrichomonas imitans* n. sp., strain PRA; (I–L): *Hypotrichomonas mariae* n. sp., strain KOMBG1. Ax – axostyle; C – costa-like fiber; P – pelta; PB – parabasal body; R – free part of recurrent flagellum; UM – undulating membrane. Bar = 5 μm.

Hypotrichomonas species. The axostyle was of *Trichomonas*-type, and its trunk was slenderer than in *H. acosta* (Fig. 4A, B). The protruding part of the axostyle was approximately 4.7 μm long. The pelta was thick and long (Fig. 4A–D).

Hypotrichomonas imitans n. sp.

The cells of strain PRA (Fig. 4E–H) were on average 6.1 μm long (excluding the protruding part of the axostyle) and 4.9 μm wide. The anterior flagella were almost equal

Table 2. Dimensions (in μm) of protargol-stained specimens of *Hypotrichomonas* strains. Average of 50 specimens ± standard deviation (smallest–largest value).

Species	Isolate	Cell length	Cell width	Axostyle projection
H. acosta	LEP1	7.7 ± 0.9 (5.5–11.3)	5.4 ± 0.6 (4.4–7.8)	4.3 ± 1.6 (1.8–11.1)
	CORAL1	8.6 ± 1.6 (5.7–15.0)	5.4 ± 1.2 (3.7–10.7)	4.5 ± 1.7 (1.2–9.8)
H. carbonariae	GECA1	7.5 ± 1.1 (5.6–10.8)	5.8 ± 1.2 (3.6–10.3)	4.7 ± 1.7 (1.6–9.8)
H. osmaniae	CYCL2	8.2 ± 1.3 (5.6–12.1)	6.2 ± 1.2 (4.2–8.6)	4.7 ± 1.2 (2.1–7.0)
	EUMT	9.0 ± 1.2 (6.9–11.2)	5.9 ± 1.0 (4.4–8.9)	6.1 ± 2.1 (2.2–12.8)
	LASE1	8.2 ± 1.1 (6.0–11.5)	5.6 ± 0.7 (3.9–7.7)	6.0 ± 1.3 (2.4–11.7)
H. blattarum	BCR	5.3 ± 0.5 (4.0–6.5)	3.8 ± 0.5 (2.5–4.6)	2.9 ± 1.0 (0.7–6.8)
	BSP	5.4 ± 0.6 (4.1–6.4)	4.0 ± 0.4 (3.2–4.9)	2.3 ± 0.9 (0.4–4.5)
	ARCH1	5.3 ± 0.5 (4.2–7.0)	4.0 ± 0.4 (2.9–4.8)	2.5 ± 0.8 (1.0–5.1)
	BAT1	5.5 ± 0.6 (4.4–6.9)	3.9 ± 0.5 (2.7–5.0)	2.7 ± 0.7 (0.9–5.0)
H. hampli	KLRUD	7.9 ± 0.9 (5.9–10.2)	6.9 ± 0.8 (4.7–8.8)	2.2 ± 1.0 (0.8–5.0)
	MAR1	7.4 ± 0.9 (4.9–9.9)	5.5 ± 0.7 (3.9–6.8)	2.7 ± 0.7 (1.1–5.3)
H. imitans	PRA	6.1 ± 0.7 (4.7–8.0)	4.9 ± 0.5 (3.9–6.2)	1.5 ± 0.9 (0.4–3.5)
H. sanderai	GRAJC1	5.0 ± 0.5 (4.2–6.4)	4.2 ± 0.4 (3.3–5.0)	1.5 ± 0.6 (0.5–3.1)
H. mariae	KOMBG1T	7.5 ± 0.7 (6.4–9.7)	5.4 ± 0.5 (4.5–7.0)	3.0 ± 0.9 (1.5–5.3)

and shorter than the cell body (Fig. 4E, F, H). The undulating membrane was absent, and the recurrent flagellum attached to the cell body only by a very short proximal portion (Fig. 4E, H); the recurrent flagellum was usually shorter than the cell body. The axostyle was of *Trichomonas*-type, and its trunk was slender (Fig. 4E–H). The protruding part of the axostyle was approximately 1.5 μm long. The pelta was short (Fig. 4E, G, H).

Hypotrichomonas mariae n. sp.

The cells of the strain KOMBG1 (Fig. 4I–L) were on average 7.5 μm long (excluding the protruding part of the axostyle) and 5.4 μm wide (including the undulating membrane). The longest anterior flagellum was longer than the cell body (Fig. 4I, J); the anterior flagella were less unequal than in the other *Hypotrichomonas* species, with the exception of *H. imitans* n. sp. The well-developed undulating membrane reached approximately halfway along the cell body and did not display distinctive waves (Fig. 4I, J, L). The undulating membrane was underlain by a short fiber along its whole length (Fig. 4I, J). The axostyle was of *Trichomonas*-type, and its trunk was relatively thick (Fig. 4J, K). The protruding part of the axostyle was approximately 3.0 μm long. The pelta was well developed (Fig. 4I–K).

Morphology of living cells

In addition to protargol-stained preparations, cells of strains BCR, BSP (*Hypotrichomonas blattarum* n. sp.) and GRAJC1 (*H. sanderai* n. sp.) were observed alive. The anterior flagella of the living cells did not end in knob-like extensions, unlike in the protargol-stained cells. The motile cells, however, were too fast to be visualized. Therefore, the cells of the strain BCR were killed by formaldehyde and photographed. Similarly to the living cells, the formaldehyde-killed cells did not possess the conspicuous knobs at the distal end of the anterior flagella (Fig. 5).

Taxonomic summary

Parabasalia: Hypotrichomonadea: Hypotrichomonadida: Hypotrichomonadidae Honigberg, 1963: *Hypotrichomonas*, Lee 1960.

Hypotrichomonas acosta (Moskowitz, 1951)

Trichomonas acosta Moskowitz, 1951

New synonym. *Hypotrichomonas venkataramiahii* Daykar, Devi, Bhaskar Rao & Bhaskar Rao, 1977.

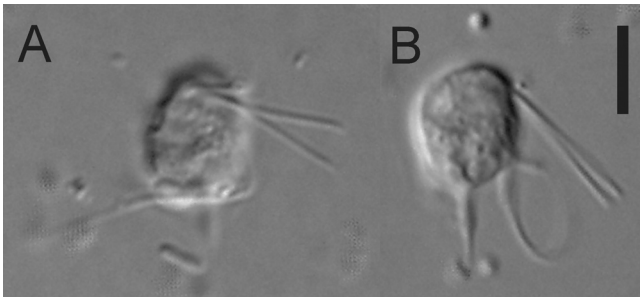

Fig. 5. Formaldehyde-killed cells of *Hypotrichomonas blattarum* n. sp., strain BCR (A, B). Bar = 5 μm.

Hypotrichomonas hampli n. sp.

Zoobank registration. urn:lsid:zoobank.org:act:7E3950E6-B4B6-4A1C-BE49-A4E2A824D32B.

Description. *Hypotrichomonas* with protargol-stained cells (without the axostyle protrusion and including the undulating membrane) 4.9–10.2 (mean 7.7) μm long and 3.9–8.8 (mean 6.2) μm wide, protruding part of axostyle 0.8–5.3 (mean 2.5) μm. Three unequal anterior flagella. Well-developed undulating membrane with several waves, usually does not reach the posterior end of cell. The recurrent flagellum continues distally by a free portion. Costa absent. Parabasal body biramous. *Trichomonas*-like axostyle slender with narrow capitulum. Pelta well developed.

Type material. Protargol preparations of the isolate MAR1, deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, catalog numbers 5/6–5/12.

Type host. *Macropus rufus* (Marsupialia: Diprotodontia: Macropodidae).

Other host. *Wallabia rufogrisea* (Marsupialia: Diprotodontia: Macropodidae).

Type locality. Isolated in Prague, Czech Republic. The type host species naturally occurs in Australia.

Habitat. Isolated from feces. Presumably lives in large intestine.

Etymology. Named after our friend and colleague, protistologist Dr. Vladimír Hampl.

Gene sequence. The SSU rDNA sequence from *H. hampli*, strain MAR1 has the GenBank Accession number KJ591560.

Remark. Differentiated from similar *H. osmaniae* by relatively shorter anterior flagella and shorter axostyle projection, from *H. blattarum* by larger cells, and from *H. avium* by termination of anterior flagella.

Hypotrichomonas imitans n. sp.

Zoobank registration. urn:lsid:zoobank.org:act:DB23C682-A138-4B8F-B1FB-ED5E1126C78F.

Description. *Hypotrichomonas* with protargol-stained cells (without the axostyle protrusion) 4.7–8.0 (mean 6.1) μm long and 3.9–6.2 μm (mean 4.9) wide, protruding part of the axostyle 0.5–3.5 (mean 1.5) μm. Three short, equal anterior flagella. Undulating membrane absent or rudimentary. Recurrent flagellum free along almost the whole length. Costa absent. Parabasal body biramous. *Trichomonas*-like axostyle slender with narrow capitulum. Pelta short.

Type material. Protargol preparations of the isolate PRA, deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, catalog numbers 2/37–2/39 and 3/1–3/3.

Type host. *Sus scrofa f. domestica* (Cetartiodactyla: Suina: Suidae).

Type locality. Prague, Czech Republic.

Habitat. Isolated from feces. Presumably lives in the lower intestine.

Etymology. *Imitans* [Latin adj.] – imitating, copying. This species is reminiscent of *Honigbergiella ruminantium*.

Gene sequence. The SSU rDNA sequence from *H. imitans*, strain PRA has the GenBank Accession number KJ591558.

Remark. The only *Hypotrichomonas* species without the undulating membrane.

Hypotrichomonas carbonariae n. sp.

Zoobank registration. urn:lsid:zoobank.org:act:F3DAEB27-4241-4C87-B6D4-713E08A0D92A.

Description. *Hypotrichomonas* with protargol-stained cells (without the axostyle protrusion and including the undulating membrane) 5.6–10.8 (mean 7.5) μm long and 3.6–10.3 (mean 5.8) μm wide, protruding part of axostyle 1.6–9.8 (mean 4.7) μm. Three unequal anterior flagella. Well-developed undulating membrane with several waves, extends up to the posterior end of the cell. No distal free portion of the recurrent flagellum. Parabasal body biramous. *Trichomonas*-like axostyle slender with narrow capitulum. Pelta well developed.

Type material. Protargol preparations of the isolate GECA1, deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, catalog numbers 2/30, 2/31 and 3/83–3/85.

Type host. *Geochelone carbonaria* (Testudines: Cryptodira: Testudinidae).

Type locality. Prague, Czech Republic. The type host species naturally occurs in South America.

Habitat. Isolated from feces. Presumably lives in large intestine.

Etymology. Named after the species epithet of the host, *Geochelone carbonaria*.

Gene sequence. The SSU rDNA sequence from *H. carbonariae*, strain GECA1 has the GenBank Accession number KJ591557.

Remark. The only *Hypotrichomonas* species without the free distal portion of the recurrent flagellum.

Hypotrichomonas blattarum n. sp.

Zoobank registration. urn:lsid:zoobank.org:act:AFD06105-5887-40C1-A74F-DDC08BD73E8A.

Description. *Hypotrichomonas* with protargol-stained cells (without the axostyle protrusion and including the undulating membrane) 4.0–7.0 (mean 5.4) μm long and 2.5–5.0 (mean 3.9) μm wide, protruding part of axostyle 0.4–6.8 (mean 2.6) μm. Three unequal anterior flagella. Undulating membrane with a single wave, usually does not reach

the posterior end of cell. The recurrent flagellum continues distally by a free portion. Costa absent. Parabasal body biramous. *Trichomonas*-like axostyle slender with narrow capitulum. Pelta well developed, relatively short.

Type material. Protargol preparations of the isolate BCR, deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, catalog numbers 3/31–3/35.

Type host. *Blaberus craniifer* (Blattodea: Blaberidae).

Additional hosts. *Blaberus atropos*, *Archimandrita tessellata* (Blattodea: Blaberidae).

Type locality: The type host species naturally occurs in Central America, Mexico, and Florida.

Habitat. Hindgut.

Etymology. *Blattarum* [Latin adj.] – of cockroaches. This species is found in cockroaches.

Gene sequence. The SSU rDNA sequence from *H. blattarum*, strain BCR has the GenBank Accession number KJ591549.

Remark. Differentiated from similar *H. osmaniae* and *H. hampli* by smaller cells, and from *H. avium* by termination of anterior flagella.

Hypotrichomonas mariae n. sp.

Zoobank registration. urn:lsid:zoobank.org:act:1EF056DA-979B-4860-9291-34ED9EA1FE67.

Description. *Hypotrichomonas* with protargol-stained cells (without the axostyle protrusion and including the undulating membrane) 6.4–9.7 (mean 7.5) μm long and 4.5–7.0 (mean 5.4) μm wide, protruding part of axostyle 1.5–5.3 (mean 3.0) μm. Three unequal anterior flagella. Undulating membrane without obvious waves, usually does not reach the posterior end of the cell. The recurrent flagellum continues distally by a free portion. Costa-like fiber underlying the undulating membrane present. Parabasal body biramous. *Trichomonas*-like axostyle relatively thick. Pelta well developed.

Type material. Protargol preparations of the isolate KOMBG1, deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, catalog numbers 3/86–3/90, 4/82 and 4/83.

Type host. *Otolemur garnettii* (Primates: Strepsirrhini: Galagidae).

Type locality. Prague, Czech Republic. The type host species naturally occurs in Somalia, Kenya, Tanzania, and on the islands of Zanzibar.

Habitat. Isolated from feces. Presumably lives in large intestine.

Etymology. Named in honor of Marie Čepičková, the wife of Ivan Čepička.

Gene sequence. The SSU rDNA sequence from *H. mariae*, strain KOMBG1 has the GenBank Accession number HQ149966.

Remark. The only *Hypotrichomonas* species with costa-like fiber.

Hypotrichomonas sanderai n. sp.

Zoobank registration. urn:lsid:zoobank.org:act:F91378EA-BCAC-4B8F-AC96-CBED86307D2A.

Description. *Hypotrichomonas* with protargol-stained cells (without the axostyle protrusion and including the undulating membrane) 4.2–6.4 (mean 5.0) μm long and 3.3–5.0 (mean 4.2) μm wide, protruding part of axostyle 0.5–3.1 (mean 1.5) μm. Three short, unequal anterior flagella. Undulating membrane without obvious waves, usually does not reach the posterior end of the cell. The recurrent flagellum continues distally by a free portion. Costa absent. Parabasal body biramous. *Tritrichomonas*-like axostyle. Pelta well developed.

Type material. Protargol preparations of the isolate GRAJC1, deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, catalog numbers 3/51–3/54.

Type host. *Apodemus agrarius* (Rodentia: Myomorpha: Muridae).

Type locality. Prague, Czech Republic. The host was captured in Grajcar, Slovakia.

Habitat. Large intestine.

Etymology. Named after the zoologist Dr. Martin Šandera, who provided the host of the type strain.

Gene sequence. The SSU rDNA sequence from *H. sanderai*, strain GRAJC1 has the GenBank Accession number KJ591566.

Remark. Shares *Tritrichomonas*-type axostyle with *H. hemidactyli*; differs from it by smaller cells.

Discussion

The genus *Hypotrichomonas* splits into eleven lineages with genetic distances between them being comparable to those between independent species of *Trichomonas*, *Tritrichomonas*, and *Tetratrichomonas* (see Cepicka et al. 2006). Eight lineages contained the newly-obtained strains. Trichomonads belonging to the distinct lineages were morphologically different and clearly represented separate species. The remaining three lineages of *Hypotrichomonas* consisted exclusively of uncultured organisms whose SSU rDNA sequences were retrieved from GenBank. Although their morphology could not be examined, they are genetically distinct from the other lineages as well as from each other and, therefore, also represent independent species. Our data confirm that the present knowledge of the diversity of intestinal trichomonads is far from being fully described.

The type species of the genus, ***Hypotrichomonas acosta***, has repeatedly been isolated from various snakes and lizards

(Dimasuay and Rivera 2013; Lee 1960; Moskowitz 1951; this study); its morphology was described in detail by Moskowitz (1951) and Lee (1960); Mattern et al. (1969) and Brugerolle (1975/1976) studied its ultrastructure. Typical features of *H. acosta* include relatively large cells (more than 7 μm) and a stout axostyle. Daykar et al. (1977) described ***Hypotrichomonas venkataramiahii*** from the intestinal contents of a single monitor lizard. The most significant difference between *H. venkataramiahii* and *H. acosta* reported by Daykar et al. (1977) was the presence of a pelta in the former species and its absence in the latter one. The authors apparently believed that *H. acosta* lacked a pelta, and they overlooked its clear demonstration by both Moskowitz (1951) and Lee (1960). When we compared the pelta of *H. venkataramiahii* with the descriptions *H. acosta*, we did not find any difference. Other features reported by Daykar et al. (1977) to distinguish between *H. venkataramiahii* and *H. acosta* were cell shape (elongated in the former species, ovoid or spherical in the latter one) and length of anterior flagella (slightly greater in the former species). However, we consider them both minute and insignificant. Therefore, we synonymize these two species, with *H. acosta* having priority.

Only five of our strains – one from a frog, three from snakes, and one from a lizard – were assigned to *H. acosta* based on their morphology and/or phylogenetic position. The morphology of strains LEP1 and CORAL1 closely corresponded to the descriptions of this species by Moskowitz (1951) and Lee (1960), particularly with respect to the relatively stout axostyle. Sixteen SSU rDNA sequences of uncultured trichomonads from snakes and lizards determined by Dimasuay and Rivera (2013) were affiliated with *H. acosta* in the phylogenetic analysis. The sequence JX565070 was closely related to the *H. acosta* clade; it is relatively divergent and may, in fact, represent a separate *Hypotrichomonas* species. However, in the absence of morphological data from this organism, we place it conservatively into *H. acosta*, as well.

Hypotrichomonas osmaniae is another species isolated from lizards (Krishnamurthy 1967). It is differentiated from *H. acosta* mainly because of a different cell shape and thinner axostyle. Although the cell shape is known to be variable in many parabasalian species, the axostyle thickness, in contrast, is a stable feature, often used to distinguish between different species of trichomonads (see Honigberg 1963). We classify three of our strains from lizards (EUMT, LASE1, and CYCL2) as *H. osmaniae* – although their cells morphologically resemble *H. acosta*, particularly with respect to size, they have a thinner axostyle. They are also phylogenetically distinct from *H. acosta*, which further supports the existence of *H. osmaniae* as a separate species.

The strains from marsupials, here considered to represent a separate species ***Hypotrichomonas hampli*** **n. sp.**, are morphologically similar to *H. osmaniae*. A slight difference between these two species lies in the length of anterior flagella, which are considerably shorter in *H. hampli*. In addition, cells of both strains of *H. hampli* n. sp. display much shorter axostyle projection than *H. osmaniae*. The distinctiveness of the two species is also justified by the fact that they are not specifically related to each other in the SSU rDNA tree.

Hypotrichomonas blattarum **n. sp.** is represented by multiple strains obtained from cockroaches and is distinguished from similar *H. osmaniae* and *H. hampli* n. sp. by having considerably smaller cells.

Hypotrichomonas hampli n. sp., *H. blattarum* n. sp., and *H. osmaniae* are morphologically similar to the poorly described and problematic species ***Hypotrichomonas avium***, the only hypotrichomonad known from birds (Navarathnam 1970). In the description of *H. avium*, Navarathnam differentiated it from *H. osmaniae* by the presence of an "endosome" (small, nucleolus-like body in the nucleus), cytostome, and the absence of pelta. However, it is obvious that the morphology of *H. avium* was misinterpreted. It is nowadays taken for granted that trichomonads do not possess a cytostome (Honigberg 1963). We also doubt that Navarathnam's species lacks a pelta, which is almost omnipresent among Trichomonadea, Tritrichomonadea, and Hypotrichomonadea. The manifestation of the "endosome" in the nucleus may differ between particular morphological methods (e.g. Honigberg 1951). Finally, the anterior flagella of *H. avium*, unlike the flagella of the three aforementioned species, were reported not to end in knobs. We consider this difference insignificant, because the knobs are most probably an artifact of fixation (compare Figs 2–4 with Fig. 5), and we cannot exclude the possibility that some fixations result in knobs whereas others do not. However, we conservatively retain *H. avium* as a separate species; the identity of hypotrichomonads from birds has to be clarified in the future.

Hypotrichomonas hemidactyli was obtained from a lizard by Bhaskar Rao et al. (1976). It clearly differs from the other species by possessing *Tritrichomonas*-type of axostyle (i.e. axostyle forming a tube which tapers abruptly to the point at the distal end, see Cepicka et al. 2010). This unusual feature is shared with the strain GRAJC1 which was isolated from a rodent and which we describe as a new species ***H. sanderai*** **n. sp**. Our observations are consistent with LVSEM pictures of a strain apparently belonging to *H. sanderai* recently published by Kamaruddin et al. (2014). *H. sanderai* can be clearly differentiated from *H. hemidactyli* by the cell size. The cells of *H. hemidactyli* were reported to measure 8.2 μm on average (Bhaskar Rao et al. 1976) whereas the mean length of the cells of the new species is only 5.0 μm. In addition, the undulating membrane of the strain GRAJC1 does not display multiple undulations reported from *H. hemidactyli*.

The remaining three species are morphologically less consistent with *Hypotrichomonas*, but belong into this genus on the basis of the phylogenetic analysis. ***Hypotrichomonas carbonariae*** **n. sp.** (strain GECA1) was isolated from a tortoise. It morphologically resembles the closely-related *H. osmaniae*, having relatively large cells and a thin axostyle. However, it differs from all other hypotrichomonads in the

absence of the free distal portion of the recurrent flagellum. This feature, relatively uncommon among trichomonads, is shared with unrelated genera *Trichomonas* and *Pseudotrichomonas* (Honigberg 1963; Yubuki et al. 2010). Geiman and Wichterman (1937) isolated a trichomonad from the intestine of a Galapagos tortoise and briefly described its morphology. The organism ("*Trichomonas* sp.") apparently belonged to the then-undescribed genus *Hypotrichomonas* – it possessed three anterior flagella and a relatively short undulating membrane that was not underlain by a costa. It displayed a relatively long free portion of the recurrent flagellum and, therefore, was distinct from *H. carbonariae* n. sp. With its large cells and a slender axostyle, the trichomonad was most similar to *H. osmaniae*. However, the report by Geiman and Wichterman (1937) was too brief to determine the species identity of the organism with certainty.

***Hypotrichomonas imitans* n. sp.** from pigs is unique among Hypotrichomonadida by the absence of an undulating membrane. Interestingly, its superficial morphology corresponds better to the genus *Honigbergiella* from the order Honigbergiellida (see Cepicka et al. 2010; Hampl et al. 2007). However, the biramous parabasal body and phylogenetic position support the inclusion of this species in the genus *Hypotrichomonas*. Because *H. imitans* n. sp. does not form a deep branch within the Hypotrichomonadida, and the last common ancestor of the Hypotrichomonadea most probably possessed a lamelliform undulating membrane (Cepicka et al. 2010), we conclude that the structure has been lost secondarily in this species.

The last strain, KOMBG1, from a primate, is described as ***Hypotrichomonas mariae* n. sp**. It is closely related to *H. sanderai* n. sp., and together they share a relatively thick axostyle. *H. mariae* n. sp. differs from all so-far known hypotrichomonads, including *H. sanderai* n. sp., by the presence of a fiber which is reminiscent of a costa.

The costa is a striated fiber which, although far from being omnipresent, is one of the hallmarks of trichomonads. When present, the costa underlies the undulating membrane. Two types of costae have been recognized that differ by the periodicity of the striation. The A-type costa is present in *Tritrichomonas*, some cristamonads, and *Trichomitus*, the close relative of *Hypotrichomonas*, whereas the B-type is characteristic for Trichomonadida (Brugerolle 1975/1976; Kulda et al. 1986). Although it is currently unclear whether the two types of the costa are homologous (Cepicka et al. 2010), it was proposed by Simpson and Patterson (2001) that the costa might be a homolog of the C fiber of the excavate taxa. Thus, it possibly represents the only remaining excavate feature of the Parabasalia (Simpson 2003). The presence of the undulating membrane with simultaneous absence of costa is not a unique feature of the genus *Hypotrichomonas* and is known also from genera *Monotrichomonas*, *Ditrichomonas* (Honigbergiellidae), *Lacusteria*, and *Pseudotrichomonas* (Trichomonadidae) (Bernard et al. 2000; Farmer 1993; Yubuki et al. 2010).

The presence of the thin fiber underlying the undulating membrane of *Hypotrichomonas mariae* n. sp. is surprising. If it is a homolog of a costa, the other *Hypotrichomonas* species must have lost this structure at least twice independently. However, the identity of the fiber in *H. mariae* has yet to be confirmed by electron microscopy.

We revealed members of the genus *Hypotrichomonas* in various host taxa. The hosts did not show any signs of disease. We, therefore, believe that the hypotrichomonads are harmless commensals. The route of infection between particular hosts has not yet been determined. Although no *Hypotrichomonas* species was reported to produce cysts, the oral-fecal route seems to be the most common pathway, though predation (including cannibalism) cannot be excluded.

Our data suggest that these organisms are common intestinal symbionts of various animal taxa, and that they must have been often overlooked by the previous authors who employed morphological methods. The majority of *Hypotrichomonas* species are known from vertebrates. Because members of the closely related genus *Trichomitus* as well as the undescribed lineage "KANEK" are known mostly from vertebrates as well (Cepicka et al. 2010; Honigberg 1963), we could hypothesize that the last common ancestor of all Hypotrichomonadea was an intestinal symbiont of a vertebrate. However, the diversity of trichomonads in insects, especially when termites are not considered, is only poorly understood (see Smejkalová et al. 2014), and we cannot reject the possibility that a number of still-undiscovered *Hypotrichomonas* species exist in these hosts.

From eleven *Hypotrichomonas* species with available molecular data, seven have been found in at least two host specimens and/or species. It is therefore possible to draw some conclusions about the **host specificity** within this genus. Most *Hypotrichomonas* species appear to have rather limited host range. For example, *H. imitans* n. sp. has been found only in domestic pigs (Mostegl et al., 2012; this study), but it has not been reported from cattle, although the cattle had been repeatedly examined for the presence of intestinal trichomonads employing cultivation, morphology, and molecular characterization (Cepicka et al. 2006; Jensen and Hammond 1964). Similarly, *H. sanderai* n. sp. has been isolated only from rodents, *H. hampli* n. sp. from marsupials, *H. blattarum* n. sp. from cockroaches, and *H. osmaniae* and *H.* sp. 1 from lizards. *H. acosta* lives predominantly in snakes (Moskowitz 1951; this study) but has also been isolated from lizards (Moskowitz 1951) and from a frog (this study). Although *H. acosta* seems to be the only *Hypotrichomonas* species that is not limited to a relatively narrow host taxon, it is possible that the frog acquired the infection accidentally and does not represent a natural host. Because the hypotrichomonads seem to be readily cultivable and host-specific, the genus *Hypotrichomonas* may become a valuable model

lineage for studying the host specificity of trichomonads in general in the future.

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8.2.4. Zhang *et al.* (*v tisku*)

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Marine isolates of *Trimastix marina* form a plesiomorphic deep-branching lineage within Preaxostyla, separate from other known trimastigids (*Paratrimastix* n. gen.)

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Running title: Ultrastructure and phylogeny of marine *Trimastix marina*

Abstract

Trimastigids are free-living, anaerobic protists that are closely related to the symbiotic oxymonads, forming together the taxon Preaxostyla (Excavata: Metamonada). We isolated fourteen new strains morphologically corresponding to two species assigned to *Trimastix* (until now the only genus of trimastigids), *Trimastix marina* and *Trimastix pyriformis*. Unexpectedly, marine strains of *Trimastix marina* branch separately from freshwater strains of this morphospecies in SSU rRNA gene trees, and instead form the sister group of all other Preaxostyla. This position is confirmed by three-gene phylogenies. Ultrastructural examination of a marine isolate of *Trimastix marina* demonstrates a combination of trimastigid-like features (e.g. preaxostyle-like I fibre) and ancestral characters (e.g. absence of thickened flagellar vane margins), consistent with inclusion of marine *T. marina* within Preaxostyla, but also supporting its distinctiveness from 'freshwater *T. marina*' and its deep-branching position within Preaxostyla. Since these results indicate paraphyly of *Trimastix* as currently understood, we transfer the other better-studied trimastigids to *Paratrimastix* n. gen. and Paratrimastigidae n. fam. The freshwater form previously identified as *T. marina* is described as *Paratrimastix eleionoma* n. sp., and *Trimastix pyriformis* becomes *Paratrimastix pyriformis* n. comb. Because of its phylogenetic position, 'true' *Trimastix* is potentially important for understanding the evolution of mitochondrion-related organelles in metamonads.

Key words: anaerobe; cytoskeleton; flagellar apparatus; oxymonads; phylogeny; ultrastructure.

Introduction

Trimastigids are a group of free-living heterotrophic flagellates with four flagella and a broad ventral feeding groove (Brugerolle and Patterson 1997; Grassé 1952; Saville Kent 1880-1882). Although little studied for most of the last 100 years, trimastigids have recently become important for researchers interested in eukaryotic cell evolution. Firstly, ultrastructural examinations have shown that trimastigids lack classical mitochondria, having instead small, double-membrane-bounded organelles without cristae (Brugerolle and Patterson 1997; O'Kelly et al. 1999; Simpson et al. 2000). Analyses of nuclear transcripts from '*Trimastix pyriformis'* (see below) have detected mRNAs for >20 putative 'mitochondrial proteins' (Hampl et al. 2008; Zubáčová et al. 2013). These include a complete glycine cleavage system, elements of which have been localized to the organelle, and hydrogenase maturases (Zubáčová et al. 2013), the presence of which suggests indirectly that the organelles might function as hydrogenosomes. Secondly, molecular phylogenetic studies of trimastigids demonstrated a close relationship to oxymonads, a better-known and more diverse group of anaerobic symbionts that had previously been of uncertain affinities (Dacks et al. 2001). Trimastigids and oxymonads figured prominently in systematic or evolutionary schemes for eukaryotes proposed near the turn of the 21st century, having been suggested as members of many of the various putatively deep-branching higher taxa within eukaryotes and not clearly related to well-established eukaryotic lineages (Cavalier-Smith 1998, 2002; Dacks et al. 2001). More recent phylogenetic analyses have shown that trimastigids and oxymonads are specifically related to other anaerobic excavates, including the mostly parasitic/commensal diplomonads, retortamonads and parabasalids, and the free-living *Carpediemonas*-like organisms, or 'CLOs' (Hampl et al. 2005, 2008, 2009; Kolisko et al. 2010; Simpson et al. 2006; Takishita et al. 2012). Trimastigids may therefore be useful organisms for investigations into the evolution of anaerobic mitochondrion-related organelles (Dacks et al. 2001; Hampl et al. 2008).

Despite this important evolutionary position, detailed data on the diversity and basic biology of trimastigids are limited. The only genus of trimastigids, *Trimastix,* was erected by Saville Kent in the late 19th century (Saville Kent 1880-1882), when he described *Trimastix marina* from a marine habitat. Later, Bernard et al. (2000) encountered organisms similar to Saville Kent's *T. marina* in both fresh water and marine-derived cultures. Adopting a broad species concept they regarded both types as belonging to the nomenclatural species *Trimastix marina*. Subsequent ultrastructural studies and molecular phylogenetic analyses of '*T. marina*' have used a freshwater isolate from Albury, Australia (Bernard et al. 2000; Dacks et al. 2001; Simpson et al. 2000, 2006). *Trimastix* currently includes at least two other described species, the marine *Trimastix inaequalis* and freshwater *Trimastix pyriformis* (Bernard et al. 2000), with a third entity, *Trimastix convexa* (Grassé 1952)*,* presently regarded as a synonym of *T. pyriformis* (see Bernard et al., 2000). Further, Dumas (1930) described the freshwater *Trimastix elaverinus*, which is of uncertain relationship to other trimastigids (see discussion). There are substantial data from isolates of *T. pyriformis sensu lato* (Brugerolle and Patterson 1997; O'Kelly et al. 1999; Simpson et al. 2000), however no marine isolates of *Trimastix*, especially of *T. marina,* have been characterized using ultrastructural or molecular techniques. Molecular phylogenetic studies have shown that several groups of small heterotrophic flagellates that were formerly treated as a single euryhaline species or genus have turned

out to be composed of distinct marine and freshwater clades - ancyromonads/planomonads and *Goniomonas* for example (Cavalier-Smith et al. 2008; Shalchian-Tabrizi et al. 2008; von der Heyden et al. 2004). It is possible that freshwater strains may only represent a portion of the major lineage diversity of trimastigids, therefore it is important to examine the similarities and phylogenetic relationships amongst trimastigids from marine and freshwater habitats.

Here we report SSU rRNA gene phylogenies that include numerous new trimastigid isolates, including marine isolates of *T. marina*. A three-gene phylogenetic analysis is also conducted. Surprisingly, marine and freshwater isolates identified as *T. marina* are not specifically related to each other, with marine *T. marina* forming the deepest known branch within Preaxostyla. An ultrastructural study of a marine *T. marina* isolate illustrates its morphological distinctiveness from the freshwater forms, including some potentially ancestral cytoskeletal features relative to other trimastigids. The classification of trimastigids is re-evaluated as a result of their division into at least two clades that are apparently not specifically related. Since *T. marina* is the type of *Trimastix*, the genus name *Trimastix* is retained for the clade containing 'true' (marine) *T. marina* isolates. However, *T. pyriformis* is moved to a new genus, *Paratrimastix* gen. nov., and a new species, *Paratrimastix eleionoma* sp. nov., is erected for freshwater isolates formerly assigned to the nomenclatural species *Trimastix marina*.

Results

New strains, and SSU rRNA gene phylogeny

A total of 14 new strains were established in culture. These were assignable by preliminary light microscopy examination to either *Trimastix pyriformis* (7 strains) or *Trimastix marina* sensu Bernard et al. 2000 (7 strains). Five of the latter were isolated from freshwater sites, and two (PCT & TINI) were isolated from marine sites (see Table 1). Strains PCT and TINI are the only extant isolates of *T. marina* from marine sites.

To examine the phylogenetic positions of these strains, an analysis of SSU rRNA genes with a broad sampling of eukaryotic lineages was performed using maximum likelihood (ML) and Bayesian inference (BI) approaches (Fig. 1). This analysis showed all the new isolates branching within a strongly supported Preaxostyla clade (ML bootstrap support – ML 89%, BI posterior probability – 1). The seven strains of *T. pyriformis* sensu Bernard et al. (2000) branched with the two previously cultured strains, ATCC 50598 and ATCC 50562, with very strong support (ML 94%, BI 1), with most having close affinities to ATCC 50598. The SSU rRNA gene sequences from the five new freshwater strains of *T. marina* sensu Bernard et al. (2000) were all extremely similar to the sole previously studied freshwater strain 'Albury' (see Bernard et al. 2000; Dacks et al. 2001). These two groups of freshwater, free-living Preaxostyla formed a moderately supported clade (ML 73%, BI 0.95), which we equate with the new genus *Paratrimastix* n. gen. (see taxonomic summary below). The SSU rRNA gene sequences of the two *T. marina* strains from marine habitats were very similar to one another, but they branched separately from all other trimastigids. Together with an environmental sequence (GenBank: AB505546) obtained from deep-sea sediment, marine *T. marina* strains instead formed the sister group to all other members of Preaxostyla. There was strong statistical support for the deep-branching position of this clade within Preaxostyla (i.e.

for all other Preaxostyla being a monophyletic group: ML 87%, BI 1). An additional analysis of SSU rRNA genes from Preaxostyla alone (i.e. without outgroups), but with more aligned positions, recovered an identical internal topology for Preaxostyla (not shown). The clan corresponding to *Paratrimastix* n. gen. and the *Trimastix marina +* AB505546*/'*other Preaxostyla' bipartition both received higher ML bootstrap support than in the original analysis - 87% (vs 73%) and 100% (vs 87%) respectively.

Three-gene phylogeny

The results of the SSU rRNA gene analysis were broadly confirmed by ML and BI analyses of a three-gene dataset (SSU rRNA, cytosolic Hsp90, beta tubulin) that included at least one isolate from each major subgrouping of trimastigid strains, as well as two oxymonads. The ML trees estimated from 43-taxon and 64-taxon datasets, with different outgroup samplings, showed similar topologies. Figure 2 shows the more robust results of the 43-taxon analysis, in which long-branching outgroup taxa were excluded (e.g., Fornicata and parabasalids). Marine *Trimastix marina* (isolate PCT) branched in a strongly supported Preaxostyla clade (ML 99-100% and BI 1 in both analyses) and diverged at the base of this clade, as in the SSU rRNA gene phylogenies. The support for this basal position was high in the 43-taxon analysis (ML 87%, BI 1; Figure 2). Support was lower in the 64-taxon analysis (i.e. with long-branching outgroup taxa included; ML 65%, BI 0.81; Supplementary Figure 1). The monophyly of *Paratrimastix* n. gen. was moderately supported by both datasets (ML 78%, BI 1; ML 85%, BI 1). AU tests were performed to test possible alternative placements of marine *T. marina*. The hypothesis that all trimastigids form a clade was not rejected by AU test at $\alpha = 0.05$ ($p = 0.468$), however a clade of marine *T. marina* and *Paratrimastix eleionoma* n. sp. (formerly freshwater *T. marina* sensu Bernard et al. 2000) was clearly rejected ($p = 0.005$).

Light microscopy comparison of *Trimastix marina*-like strains

The new freshwater isolates of *Paratrimastix eleionoma* n. sp. (formerly freshwater *Trimastix marina* sensu Bernard et al. 2000) are virtually identical to the Albury strain examined by Bernard et al. (2000) and Simpson et al. (2000). Strains WL and ELSUERO were documented in detail (Fig. 3). In both strains the cells are more-or-less broadly pyriform, with the apical end pointed, and with the ventral groove extending for most of the length of the cell (Fig. 3). WL cells were 14.7 – 26.3 µm long (av. 18.2 µm ± S.D. 2.3; n=50) and ELSUERO cells were 14.6 – 22.7 µm long (17.7 µm ± 1.8; n=50). The four flagella emerge in a cruciate pattern (anterior, posterior, left and right), with the anterior flagellum (F2) being markedly thickened (Fig. 3C, E). The right wall of the groove has a conspicuous bulged discontinuity at about the midpoint of the cell (Fig. 3F). The cells differ from those of marine *T. marina* (see below) in that the apex of the cell has a subtly bulbous end that is offset to the left of the anterior flagellum (Fig. 3A, G), the posterior end of the ventral groove is formed into a distinct lateral cytopharynx (Fig. 3A), and there is a posterior contractile vacuole. The cells are also larger on average than those of marine *T. marina* (see below), as well as considerably larger than *Paratrimastix pyriformis* comb. n.

The marine strains of *T. marina* are morphologically similar to each other. The cells are broadly pyriform, with the apical end pointed, with a conspicuous ventral groove and four flagella. The cell body

of strain PCT was 12-18 µm long (14.3 ± 1.3 µm; n=36), while that of strain TINI was 13–17.6 µm (14.9 ± 1.2 µm; n=50), so both are noticeably and significantly smaller on average than the freshwater strains reported above ($p < 0.0001$; 1-way ANOVA). Strain PCT was documented in detail (Figs 4, 5). The apex of the cell lacks a bulbous projection (see above), while the much thinner carinate process seen by TEM (see below) is difficult to see using light microscopy. The groove is typically about 3/4 cell length (Figs 4, 5H, I). The flagella originate at the head of the groove, extending to the anterior, posterior, left and right side of the cell body (Figs 4A, 5B, E – G). The anterior flagellum (F2) and posterior flagellum (F1) are 2 – 3.5 times the cell length (Fig. 5A, B, E). The anterior F2 flagellum inserts essentially apically, and its proximal portion is thickened (Fig. 5B – G). The proximal portion of the posterior F1 flagellum lies within the groove and beats with high-amplitude waves (Fig. 4A – C). The lateral flagella (F3, F4) are 1.5 times the cell length (Fig. 4A – C). The pyriform nucleus is located anteriorly, is 1/4 – 1/3 of the cell length, and has a large sub-posteriorly located nucleolus (Fig. 4B, D). Food vacuoles with prokaryotic prey are often present (Fig. 5F). No contractile vacuole was observed. The groove broadens to nearly the full width of the cell body at about 1/3 cell length from the anterior end (Fig. 5F). The left wall of the groove continuously curves from its origin to almost the posterior cell end. The right wall has a bulged discontinuity at about the midpoint of the cell (Fig. 5H, I), after which it is less conspicuous and angles left, making the groove narrower in the posterior part (Figs 4D, 5C, D, H). The groove eventually terminates in a narrow longitudinally oriented constriction (Figs 4C, 5H, arrowhead), and not a conspicuous, laterally oriented cytopharynx like that of *P. eleionoma* (see above and Simpson et al., 2000).

Ultrastructure of marine *Trimastix marina*, isolate PCT

Terms for general/excavate-specific ultrastructural components follow Yubuki et al. (2013), with the exception of the numbering of lateral basal bodies, which were reversed (see O'Kelly et al. 1999 for correct numbering for trimastigids). Terms for trimastigid-specific elements follow Simpson et al. (2000).

General appearance and organelles: The nucleus and flagellar apparatus are in the anterior part of the cell, with the flagellar apparatus located immediately subapically (Fig. 6A). The apical part of the cell is a carinate projection about 250 nm long that is supported by the dorsal fan microtubules and is attached to the anterior flagellum, F2 (Figs 6A; 7A – D). The nucleus normally lies more to the left side of the cell (Fig. 6G). There is one Golgi region represented by a stack of 7 – 10 membrane sacs, in the anterior left of the cell, near the R1 root – see below (Fig. 6B; see also Fig. 10A). The cytoplasm contains substantial numbers of small vesicles enclosing electron-dense granules, and numerous mitochondrion-related organelles (Fig. 6C). The granule-containing vesicles are typically 150 – 200 nm in diameter, with a single membrane that fits loosely around the rounded, homogenous granule (Fig. 6D). The mitochondrion-related organelles are ovoid-to-pill-shaped, typically 200 – 250 nm in diameter, and 500 – 900 nm in length (Fig. 6C). They are bounded by two closely adpressed membranes, lack cristae, and have a densely staining matrix (Fig. 6E). Cells typically contain several food vacuoles (Fig. 6A, C).

Flagellar structures: All flagella have a typical "9+2" arrangement of axonemal microtubules (Figs 6F, 7D). The posterior flagellum (F1) is extended by two very broad vanes (Fig. 6A, F, G). Transverse sections show a fine paraxonemal lamella supporting each vane (Fig. 6F, G). The ventral lamella and vane originates more proximally than the dorsal one (Fig. 6B). In any given section, the ventral vane is usually slightly broader than the dorsal vane (Fig. 6F, G). Each lamella connects to three axonemal doublets and extends to the vane edge as a narrow sheet, which lacks a thickened margin (Fig. 6F). The vanes are present for approximately the length of the groove.

The anterior flagellum (F2) is thickened on its dorsal-right side slightly distal to the point of emergence (Fig. 7A). The thickening lacks a regular paraxonemal structure, containing instead small granules and fine filamentous material (Figs 7A, C, D, 8F). Part of the base of this region is connected to the edge of the carinate process (Fig. 7A, D). This connection is made by fine filaments that extend from a thickened patch of the flagellar membrane and the similar patch of the cell membrane of the carinate process (Figs 7A, C, D, 8D – F). The gap maintained by the filaments of the connection structure is 80 – 105 nm wide **(**Fig. 7A, D), and spans approximately 300 nm along the conjoining structures (Fig. 8F).

Basal bodies: There are four basal bodies in the flagellar apparatus (Fig. 7B). The transition zone at the distal end of each basal body includes a dense axosome, which is associated with a fine transverse septum that includes the 'acorn' structure (Fig. 7C, F; see Geimer and Melkonian, 2004). Slightly below this the axonemal doublets become triplets (Fig. 7G). Cartwheels are present in the bases of all four basal bodies and are of typical substructure (Fig. 7C, E, H, 8G, 9A). The basal bodies are 400 – 500 nm long (Fig. 7C, E), with the shorter measurements generally from lateral basal bodies. The posterior and anterior basal bodies ('B1' and 'B2') are arranged at an obtuse angle (145°), and their proximal ends are separated by 50 nm at their closest (Figs 7B, 8A). The left and right basal bodies, 'B4' and 'B3' respectively, are both nearly orthogonal to both B1 and B2 and somewhat ventrally directed (Fig. 8B). B4 originates close to the ventral side of B2 and is directed leftwards (Figs 7B, 8B, G). The proximal end of B3 lies to the right of the proximal ends of both B1 and B2 (Figs 8G, 10A).

Anterior Roots: Two microtubular roots/protoroots are associated with B2. The major anterior microtubular root (R3) originates from the dorsal side of B2 (Fig. 8A, C – E) and has three microtubules (Fig. 8C). R3 extends anteriorly into the base of the carinate apical structure, on the immediate ventral side of the dorsal fan, as reported below (Fig. 8C – F). The second, minor root consists of 2 – 3 extremely short microtubules adjacent to the right ventral side of the proximal end of B2 (arrow in Fig. 8G).

The dorsal fan of approximately 30 individual microtubules originates at the apex of the cell, within the carinate structure (Fig. 7A – D). These microtubules are closely associated with R3, and with the base of the connection structure linking the carinate process and F2 (Figs 7D, 8C – F). The outer microtubules of the dorsal fan (outboard of R3) are linked to an electron-dense sheet that lies immediately under the cell membrane, including the thickened membrane of the connection structure (Figs 7D, 8D, E). The dorsal fan microtubules support the cell membrane outside the confines of the groove.

Structures associated with posterior basal body (B1): Basal body 1 is associated with two major microtubular roots (R1, R2), and a singlet root (S), which are all connected around their origins by an arc of homogenous dense material, the fibrous crescent (FC, Fig. 9A, B). The left arm of the FC connects to the C fibre, which is associated with R1 (Fig. 9A). Its right arm curves closely around the origin of the singlet root and is then continuous with the A fibre supporting R2 (Fig. 9A, B). The middle part of the FC connects to 3 triplets of B1 and is about 200 nm thick (Fig. 9A). The FC also extends to associate with B4 and the proximal portion of B2 (Fig. 8B).

R1 originates close to the left ventral side of B1. It has four microtubules near its origin (Fig. 9C), but expands to about 16 microtubules slightly distal to the level of insertion of the posterior flagellum 'F1' (Fig. 9E, F), though additional microtubules are added as others diverge off (see below). From its origin, R1 is closely linked on its dorsal side to the non-microtubular C fibre (Fig. 9D, E). The C fibre is about 200 nm thick and appears multilayered in transverse section (Fig. 9A, D – F). Longitudinal sections instead show lateral spoke-like fibres spaced at about 5 – 10 nm connected to the R1 microtubules, and backed by a denser region (Fig. 9G, H). The C fibre is present in the most anterior portion of the groove as it opens (Figs 8A, 9F), but then quickly reduces in breadth and ends (Fig. 9H). Shortly beforehand, individual microtubules begin to diverge from the right side of R1 (Fig. 9F). These 'R1-originating groove microtubules' (R1Mt) support the left part of the groove floor (see below).

R2 originates at the right ventral side of B1. As few as one microtubule is observed at its origin (Fig. 10A), but more distal sections show many microtubules (Fig. 10B, C), eventually >30 (e.g. Fig. 10G). The singlet root 'S' originates close to the right side of B1 and the dorsal side of R2 (Figs 9B, 10B). It is initially connected to the middle of the proximal part of R2 by a conspicuous, electron-dense singlet-associated fiber (SA; Fig. 9B, C). Thereafter, the A fibre forms, connecting to the dorsal side of the majority of the R1 microtubules (Figs 9B; 10C). The I fibre has a total thickness of 60 nm and is composed of a fine single-leaved sheet backed by a latticework structure that connects to the ventral face of R2 (Figs 9B, 10C, D). The B fibre originates close to the leftmost edge of R2, near its origin (Fig. 10B). In transverse section it appears as a single electron-dense sheet that is surrounded by more diffuse material, especially on its right side (Fig. 10B, C, E). Initially, the B fibre is straight (in transverse sections) and oriented dorso-ventrally, with its dorsal-most edge connected to the left-most edges of R2 and the I fibre, and aligned perpendicularly to these structures (Fig. 10C). Subsequently, the B fibre becomes displaced ventrally from R2, and follows the contour of the cell membrane on the right margin of the groove (Fig. 10D, E). This results in the dorsal edge of the B fibre lying closest to the middle-left of R2, where R2 splits (Fig. 10E, F – see below). Near the origin of the B fibre, indistinct fibrous material connects to its right side, and curves across the ventral face of the posterior basal body, to connect to B4 (short arrow in Figs 9B, C, E, 10A – C).

About 500 nm posterior to the opening of the groove R2 splits into an inner portion of seven microtubules (iR2) and an outer portion with the remaining microtubules (oR2, Fig. 10G – J). The A fibre and I fibre continue only with oR2 (Fig. 10E – J), while the dorsal side of iR2 is associated with a tubule of endoplasmic reticulum (Fig. 10F – I). The oR2 curves to form part of the support for the right wall of the groove (initially together with the B fibre), while iR2 remains associated with the floor of the groove (Fig.

10F – J). The curvature brings the oR2 and I fibre parallel to the B fibre, and eventually very closely associated with it (Fig. 10F – I). After the oR2-iR2 split the A fibre takes on the appearance of a series of short, linked bridges from each R2 microtubule (Fig. 10J).

The architecture of the groove: As the groove opens, its right margin is supported primarily by the B fibre, the center of the floor is supported by the singlet root (S), and the left part of the groove is supported by R1 and the R1Mt (Figs 9F; 11A). After the split of R2, the right margin of the groove is supported by the parallel and closely positioned B fibre and oR2 (Fig. 10H), while the right-most part of the groove floor is supported by iR2, and by a few microtubules that begin to diverge from the left side of oR2 (R2Mt in Fig. 10H, I). The B fibre ends before the midpoint of the cell. The left portion of the groove (more than half) is supported by S and by increasing numbers of microtubules derived from R1. R1 divides completely into individual microtubules (R1Mt) before the midpoint of the cell (Fig. 11A).

In the posterior portion of the groove the right margin is supported by just a few oR2-derived microtubules (Fig. 11B, C). These are not supported by either the I fibre or B fibre, instead a sheet-like composite fibre (CF) forms adjacent to the dorsal side of the rightmost 4 – 5 remaining microtubules (Fig. 11B, D). Transverse views of the CF shows it is formed by a sheet about 5 nm thick, with a dense rod structure supporting its outer margin (Fig. 11B; short arrow denotes the rod). Grazing sections show that the sheet is striated, with a periodicity of 25 nm (Fig. 11E). The iR2 is also associated with a dense rod in the posterior portion of the groove (Fig. 11D). This may be homologous to the 'Inner right root-associated Dense Rod' (IDR) reported by Simpson et al. (2000).

Discussion

The phylogenetic analyses indicate that marine *Trimastix marina* isolates are not specifically related to fresh water isolates corresponding to *T. marina sensu* Bernard et al. (2000) (i.e. *Paratrimastix eleionoma* n. sp.). Instead they form the deepest known branch within the Preaxostyla clade (trimastigids plus oxymonads). This topology implies the paraphyly (or polyphyly) of the taxon *Trimastix*, as it was previously understood. On the other hand, the deep position of marine *T. marina* in Preaxostyla also suggests that it may preserve some ancestral features of the Preaxostyla clade. Therefore it is important to compare the morphological features of marine *T. marina* (represented by isolate PCT) both with other Preaxostyla (other trimastigids, and where possible, oxymonads retaining plesiomorphic features) and with other 'typical excavates' (i.e. jakobids, some members of Fornicata, specifically *Carpediemonas*-like organisms and retortamonads, and now-controversially, *Malawimonas*), which are generally thought to represent broadly the ancestral state for excavates in general, including the Preaxostyla clade (Simpson 2003).

Ultrastructural similarities amongst Preaxostyla, especially trimastigids.

The flagellar apparatus cytoskeleton of marine *Trimastix marina* (isolate PCT) broadly conforms to the pattern shared by trimastigids and other 'typical excavates', with an R1 bearing a C fibre, a posterior singlet root, and an R2 root that splits into two parts (but see below) and that is associated with a B fibre

and I fibre (Simpson 2003; Simpson et al. 2000; Yubuki et al. 2013). The possession of a single permanent anterior R3 root associated with the 'dorsal fan' of cortical microtubules is shared with other trimastigids (i.e. *Paratrimastix* n. gen.), and most other typical excavates as well (Simpson 2003; Simpson and Patterson 1999). However, marine *T. marina* specifically resembles *Paratrimastix* spp. in the organization of the major root associated fibres, namely the I fibre, C fibre and the associated FC structure, and the B fibre.

The I fibre is one thin sheet connected to the R2 root by a lattice-like structure, like that found in *Paratrimastix eleionoma* n. sp. (freshwater *T. marina sensu* Bernard et al. 2000) and *Paratrimastix pyriformis* n. comb. (O'Kelly et al. 1999). This is different from the double-sheet I fibre found in most other excavates (Simpson et al. 2002b). Importantly, the paracrystalline part of the preaxostyle of oxymonads appears to be homologous to the I fibre of typical excavates, and has a similar substructure to that seen in trimastigids (Simpson et al. 2002b). Thus marine *T. marina* possesses the apomorphy proposed to define the taxon Preaxostyla (Simpson 2003), and is appropriately assigned to Preaxostyla, despite being the sister group to other Preaxostyla.

A markedly thickened fibrous crescent (FC) connecting the C fibre to the A fibre is another distinctive feature shared by marine *T. marina* and *Paratrimastix* spp. (Brugerolle and Patterson 1997; Simpson et al. 2000). There is a thick singlet-associated fibre in marine *T. marina*, as in *Paratrimastix* (O'Kelly et al. 1999; Simpson et al. 2000). These last two features have not been observed in other typical excavate groups (nor in the oxymonads). The B fibre of marine *T. marina* also most closely resembles that of *Paratrimastix* (and arguably the oxymonad *Monocercomonoides*) in being a very broad sheet-like structure that originates against the inner side of the R2 microtubular root (Brugerolle and Patterson 1997; Simpson et al. 2000). The B fibre in other typical excavates is less broad, and often originates in different positions within the flagellar apparatus – against R1 in fornicates (Simpson 2003; Simpson and Patterson 1999), or adjacent to basal body 1 in jakobids and malawimonads (O'Kelly and Nerad 1999; Simpson and Patterson 2001). The sheet-like appearance of the composite fibre and its substructure in grazing sections is consistent with the appearance in *Paratrimastix* spp. (Brugerolle and Patterson 1997; Simpson et al. 2000).

The substructure of the C fibre of marine *T. marina*, with long spoke-like fibres/vanes connecting the R1 microtubules and a body of dense material, is similar to that of *Paratrimastix* spp. and the oxymonad *Monocercomonoides* (Brugerolle and Patterson 1997; Simpson et al. 2000, 2002b). However, a similar C fibre substructure has also been reported recently in the fornicate *Kipferlia bialata* (Yubuki et al. 2013) and consequently it may not be an apomorphy for Preaxostyla.

Differences between marine *Trimastix marina* and *Paratrimastix* spp.

Despite previously being considered conspecific, marine *Trimastix marina* isolate PCT differs from *Paratrimastix eleionoma* n. sp. (formerly freshwater *T. marina*) in several ways, including the organization of the basal bodies, R3 root and iR2. In some cases *T. marina* differs from both *P. eleionoma* and *Paratrimastix pyriformis* n. comb. (see below).

The basal bodies of marine *T. marina* are of a typical size among eukaryotes (~500 nm), and all of

them have cartwheels. By contrast *P. eleionoma* has long basal bodies (~700 nm) and cartwheels are absent from B1 and B2 (Simpson et al. 2000). *P. pyriformis* has normal-length basal bodies (O'Kelly et al. 1999), but it is unclear if there are cartwheels in all four basal bodies.

The 3-microtubule R3 flagellar microtubular root of marine *T. marina* is much smaller than in *P. eleionoma*, where the R3 (AR) contains 15 or more microtubules (Simpson et al. 2000). An R3 of three or fewer microtubules was also found in *P. pyriformis* (Brugerolle and Patterson 1997; O'Kelly et al. 1999) and is common in other typical excavates, but not universal (Park et al. 2009; Simpson and Patterson 1999). Interestingly, the oxymonad *Monocercomonoides* has six microtubules in the homologous microtubular root (Simpson et al. 2002b).

Marine *T. marina* isolate PCT resembles most typical excavates in that R2 splits directly into inner and outer components (Simpson 2003). This is very different from *P. eleionoma*, where seven microtubules that diverged individually from the left edge of R2 'reassemble' into a single ribbon – the iR2 – in the middle of the groove floor (Simpson et al. 2000). Unfortunately, no published information resolves how the iR2 forms in *P. pyriformis* (Brugerolle and Patterson 1997; O'Kelly et al. 1999), though the spacing of the iR2 microtubules might indicate that it also forms by 'reassembly' (Simpson et al. 2000).

The paired opposed vanes of the posterior flagellum of marine *T. marina* are shared with other trimastigids, as well as many fornicates (Brugerolle 1973; Brugerolle and Patterson 1997; O'Kelly et al. 1999; Simpson 2003; Simpson et al. 2000; Yubuki et al. 2007). The margins to the vane lamellae are markedly thickened in both *P. eleionoma* and *P. pyriformis* (Brugerolle and Patterson 1997; O'Kelly et al. 1999; Simpson et al. 2000), and this was held to be a distinctive feature for the genus *Trimastix* when these two species were included (Simpson et al. 2000). The vanes of marine *T. marina* lack this thickened lamella margin, which is more similar to the vanes of other typical excavates.

One distinctive morphological feature that marine *T. marina* isolate PCT shares specifically with *P. eleionoma* is the pronounced basal thickening of the anterior flagellum (F2), its near-apical position, and its close association with an apical projection (carinate projection in marine *T. marina*; apical protrusion in *P. eleionoma*; though see below). The basal thickening is not present in *P. pyriformis* (or oxymonads), nor in other typical excavates (Bernard et al. 2000; Simpson et al. 2000). The paraxonemal material within the thickening is irregularly filamentous and granular in both *T. marina* and *P. eleionoma*, and there is a distinct region of thickened flagellar membrane (Simpson et al., 2000; see below). However, the paraxonemal material is on the left side of the axoneme in *P. eleionoma* (Simpson et al., 2000), rather than on the right as in marine *T. marina*. The apical carinate projection in marine *T. marina* is broadly similar to the 'apical protrusion' of *P. eleionoma* in that it is supported at least partly by the proximal ends of the dorsal fan microtubules, which originate substantially anterior to basal body 2. However, the projection in *P. eleionoma* is also supported by the very large R3 of this species (see above), and projects on the left dorsal side of basal body 2 (Simpson et al. 2000), not the right side as in marine *T. marina*. Interestingly *P. eleionoma* also has thickened membranes on one side of the flagellum, and on the nearest side of the apical projection (Simpson et al. 2000). Both thickenings are greater in area in *P. eleionoma* than in marine *T. marina*, however, and no filamentous connection between the flagellum and the apical projection was observed in *P. eleionoma* (Simpson et al. 2000).

Summary of cytoskeletal data

The ultrastructure of marine *Trimastix marina* isolate PCT shares substantial similarity with other trimastigids, including the apomorphy proposed to define the taxon Preaxostyla (preaxostyle-like I fibre substructure). Nonetheless it lacks features that are characteristic of trimastigids, including thickened vane margins, and a 'reassembled' inner R2 (if the latter is truly a feature of *Paratrimastix pyriformis n. comb.*). In these cases marine *T. marina* appears to have retained ancestral conditions of typical excavates, and the derived features are possible apomorphies for the new genus *Paratrimastix*. This combination of trimastigid-like and ancestral features supports the inclusion of marine *T. marina* within Preaxostyla, while also being consistent with its deep-branching position within Preaxostyla.

Consistent with its separation from other trimastigids as a whole, marine *T. marina* isolate PCT also lacks most or all of the distinctive features of *Paratrimastix eleionoma* n. sp. The one set of features they share – the thickened anterior flagellum and associated apical structures, shows substantial differences when compared in detail, especially a left-right inconsistency. For this reason it is questionable whether these features are homologous and therefore were present in the last common ancestor of Preaxostyla. We think it is more likely that the superficially similar anterior flagellum and apical structures represent convergent evolution.

Mitochondrion-related organelles of trimastigids

The mitochondrion-related organelles of marine *Trimastix marina* isolate PCT are broadly similar in size, shape and appearance (dense matrix; closely adpressed membranes) to those found in *Paratrimastix eleionoma* n. sp. and *Paratrimastix pyriformis* n. comb. (O'Kelly et al. 1999; Simpson et al. 2000). The tendency for the organelles to be associated with endoplasmic reticulum, as seen in *P. pyriformis* (O'Kelly et al. 1999), was not observed in marine *T. marina* isolate PCT. The organelles of *P. pyriformis* have been suggested to be ATP-generating hydrogenosomes, based on inferred mitochondrial-targeted proteins identified by EST surveys (Hampl et al. 2008). Because of its deep-branching position within Preaxostyla it would be especially interesting to examine the possible functions of mitochondrial organelles in *T. marina*, to give insight into the evolution of mitochondrial function in Preaxostyla, and all Metamonada.

Taxonomic issues

Based on molecular phylogenies and the ultrastructural comparison, marine *Trimastix marina* strains differ at the species level from multiple strains of the freshwater protist previously studied under the name '*Trimastix marina'*. The simplest hypothesis is that *T. marina* sensu Bernard et al. (2000) consists of two distinct clades that differ in habitat salinity, and each clade should be considered a separate nomenclatural species.

The original description of *T. marina* by Saville Kent (1880-1882) is more-or-less equally similar in morphology to the marine and freshwater forms of *T. marina* sensu Bernard *et al.* (2000). The size reported by Saville Kent (1880-1882) was ~18 µm, which overlaps with both the marine and freshwater strains reported here, though is more consistent with freshwater forms, if taken as an average (see

above). On the other hand, Saville Kent (1880-1882) specifically reports the absence of a 'distinct oral aperture', which accords better with marine forms. There are differences between Saville Kent's account and both forms, such as his reporting only three flagella, and a posterior location of the nucleus, but these are very plausibly inaccuracies in the original description (see Bernard et al. 2000). Crucially, however, Saville Kent's (1880-1882) observations were from marine material. Given the evidence in the current work for two salinity-demarcated clades of *T. marina* sensu Bernard et al. (2000), it is plausible that Saville Kent observed an organism belonging to the marine *T. marina* group identified here, and reasonable to assume that he did so. Therefore we propose retaining the name '*Trimastix marina*' for the marine clade, and erect a new species for the freshwater clade.

Molecular phylogenies indicate that marine *T. marina* is not specifically related to other trimastigids, and consistent with this, it lacks at least one previously proposed apomorphy of trimastigids – thickened flagellar vane margins (see above). This non-monophyly of trimastigids implies that marine *T. marina* should not be assigned to the same genus as other trimastigids. Unfortunately, *T. marina* is the type species of *Trimastix*, so it is the other better-studied *Trimastix* species, including 'freshwater *T. marina*', that require a new genus to house them. We propose the name *Paratrimastix* for this new genus, transferring to it *Trimastix pyriformis* (Klebs, 1893) Bernard et al. 2000 (basionym *Tetramitus pyriformis*), as *Paratrimastix pyriformis* n. comb. (here designated as the type species of the genus *Paratrimastix)*, and erect a new species, *Paratrimastix eleionoma* n. sp., for the 'freshwater *T. marina*' organisms. As a matter of expediency we also formally create the family Paratrimastigidae, currently housing *Paratrimastix* only (see taxonomic summary below). These nomenclatural acts are detailed in the taxonomic summary below.

The identities of three other nomenclatural entities/species require brief comment. First, *Trimastix convexa* (Hollande, 1939) Grassé, 1952 was regarded as a junior subjective synonym of *P. pyriformis* (Klebs, 1893) by Bernard et al. (2000). If, in the future, it is proposed that *T. convexa* is a distinct species, it is likely that this will also require transfer to *Paratrimastix*. Second, *Trimastix inaequalis* Bernard et al. 2000 is an organism that was observed from marine material and documented only by photomicrography. It is superficially similar to *P. pyriformis* in most respects, suggesting that an assignment to *Paratrimastix* might ultimately be appropriate. However because of the habitat difference, and the very limited data available, the appropriate generic assignment of *T. inaequalis* is sufficiently doubtful that we prefer to not move this organism to *Paratrimastix* at present. This avoids adding a new name to the literature that has a substantial risk of being superfluous with the acquisition of new data.

Third, Dumas (1930) gave a brief account of a freshwater organism he described as *Trimastix elaverinus.* This was based on light microscopy, and illustrated by a small drawing of two cells, curiously listed in the figure legend as "*Trepomonas agilis*…(*Trimastix agilis*?)" (Dumas, 1930, plate XVII legend). The account is of an organism with a trimastigid-like cell profile and follows the original description of *Trimastix* (*marina*) by Saville Kent (1880-1882) in only documenting three flagella and in recording a sub-anteriorly positioned contractile vacuole (again, presumably actually the nucleus). The sparseness and indifferent quality of the report mean that the identity of the organism is very uncertain. The account resembles *T. marina* and *P. eleionoma* in the length and anterior direction of the anterior

flagellum, however, the small cell size (~10 µm) and report that it is a notably fast-moving organism ("Animal très agile."; Dumas, 1930, p. 136), are inconsistent with *P. eleionoma* in particular, but are consistent with (small cells of) *P. pyriformis.* It also shows some similarity to *Dallingeria*, a triflagellated organism of uncertain affinity (see Saville Kent, 1880-1882; Patterson and Zölffel, 1991). In summary, it is possible that Dumas (1930) observed a *P. pyriformis*-like organism, or a genuinely novel trimastigid morphospecies perhaps similar to a smaller, faster *P. eleionoma*, or a cell that was not a trimastigid at all. Given this uncertainty we feel it would be premature to move *T. elaverinus* to *Paratrimastix,* and thereby generate a new name that may well prove superfluous when more data are available.

Taxonomic Summary

1) New nomenclatural acts (all authored by Zhang, Táborsky, Silberman, Pánek, Čepička and Simpson)

Paratrimastigidae n. fam.
Definition. The most inclusive clade containing *Paratrimastix pyriformis* (Klebs, 1893) Zhang et al., 2015 [i.e. n. comb. in this work], but not *Trimastix marina* Saville Kent 1880-1882 or *Pyrsonympha vertens* Leidy, 1877 or *Monocercomonoides melolonthae* (Grassi, 1879) Travis, 1932. This is a branch-based definition. Abbreviated definition: > *Paratrimastix pyriformis* (Klebs, 1893) Zhang et al., 2015 ~ *Trimastix marina* Saville Kent 1880-1882 ∨ *Pyrsonympha vertens* Leidy, 1877 ∨ *Monocercomonoides melolonthae* (Grassi, 1879) Travis, 1932.

Type (and sole) genus. *Paratrimastix* n. gen.

Etymology. Paratrimastig- (considered as the stem of the name) + -idae.

***Paratrimastix* n. gen.**
Diagnosis. Free-living Preaxostyla with four flagella, one directed anteriorly (or anterio-laterally), one posteriorly, and others laterally; with suspension feeding groove. Posterior flagellum (F1) with two vanes with thickened vane margins.

Type species. *Paratrimastix pyriformis* n. comb. (basionym *Tetramitus pyriformis* Klebs, 1893) by original designation.

Included species. *Paratrimastix pyriformis* (Klebs, 1893) n. comb.; *Paratrimastix eleionoma* n. sp.

Etymology. Gr. prep. *para-*, beside, next to; *Trimastix*, existing genus of protists. Considered as feminine gender.

Paratrimastix pyriformis (Klebs, 1893) n. comb.

basionym Tetramitus pyriformis Klebs, 1893
= *Percolomonas pyriformis* (Klebs, 1893) Larsen and Patterson, 1990
= *Trimastix pyriformis* (Klebs, 1893) Bernard, Simpson and Patterson, 2000.

Subjective synonym (see Bernard et al. 2000): *Trimastix convexa* (Hollande, 1939) Grassé, 1952 (basionym *Coelotrichomastix convexa* Hollande, 1939)

***Paratrimastix eleionoma* n. sp.**
Diagnosis. *Paratrimastix* cells with near-apical anterior flagellum with thickened base, a small, bulbous apical protrusion, and conspicuous, laterally directed cytopharynx at the posterior end of groove.

Type material. The name-bearing type, a holotype, is the protargol-stained cell of the strain WL depicted in Fig. 3F. The permanent slide preparation containing the cell is deposited in the collection at the Department of Parasitology, Charles University in Prague, catalogue number 10/57.

Type Locality. A small pond adjacent to the south eastern end of Williams Lake, Halifax, Nova Scotia, Canada (44°37'18"N 63°35'20"W)

Habitat. Fresh-water hypoxic sediments.

Etymology. *eleionoma*. In Greek legend the Eleionomae were a type of female water spirit (naiad) that lived in swamps, and deceived travellers. The name refers to the freshwater anaerobic habitats of this organism, and also the deceptive nature of its similarity to *Trimastix marina*.

Gene sequence. The SSU rRNA gene sequence of *Paratrimastix eleionoma* strain WL has the GenBank accession number XXXX

2) Emended diagnoses.

Trimastigidae Saville Kent, 1880-1882.
Emended diagnosis (definition). The most inclusive clade containing *Trimastix marina* Saville Kent 1880-1882 but not *Paratrimastix pyriformis* (Klebs, 1893) Zhang et al., 2015 [i.e. n. comb. in this work], or *Pyrsonympha vertens* Leidy, 1877 or *Monocercomonoides melolonthae* (Grassi, 1879) Travis, 1932. This is a branch-based definition. Abbreviated definition: > *Trimastix marina* Saville Kent 1880-1882 ~ *Paratrimastix pyriformis* (Klebs, 1893) Zhang et al., 2015 ∨ *Pyrsonympha vertens* Leidy, 1877 ∨ *Monocercomonoides melolonthae* (Grassi, 1879) Travis, 1932.

Type (and sole) genus. *Trimastix* Saville Kent, 1880-1882.

***Trimastix* Saville Kent, 1880-1882.**

Emended Diagnosis: Free-living Preaxostyla with four flagella directly more-or-less anteriorly, posteriorly, left and right, and suspension feeding groove. Posterior flagellum (F1) with two vanes without thickened vane margins. No conspicuous lateral cytopharynx.

Type species: *Trimastix marina* Saville Kent, 1880-1882.

Other nominal species (though see above):

Trimastix elaverinus Dumas, 1930

Trimastix inaequalis Bernard, Simpson and Patterson, 2000

Methods

Isolation and culture: Strains of *Paratrimastix eleionoma* and *P. pyriformis* were isolated from freshwater hypoxic sediments worldwide (Table 1). *Trimastix marina* isolates PCT and TINI were obtained from samples collected from tidal estuarine/marine sediments from Atlantic USA and Oman, respectively (Table 1). The isolates were cultured in 15 ml conical tubes containing 10 ml of media: ATCC medium 802 for freshwater isolates, ATCC 1525 medium for TINI and ATCC 1525 medium supplemented by 1 ml of simplified ATCC 1034 medium (without folic acid and yeast nucleic acid added) for PCT. The cultures were maintained at room temperature and were subcultured once a week.

DNA extraction and gene sequencing: *Trimastix marina* PCT cells were cultured at 21 ^{o}C for 7 days before DNA extraction. About 2.5 x 10^{6} cells were pelleted by centrifugation at 3,000 g for 8 min, and genomic DNA was extracted using a DNAeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Genomic DNA of other strains was isolated from 0.2 – 0.5 ml of densely grown cultures, also using the DNAeasy Blood & Tissue Kit.

SSU rRNA genes were amplified by PCR using the universal eukaryotic primers “EukA” and “EukB” (Medlin et al. 1988). For freshwater strains, PCR-amplification was performed in 50 µl of a cocktail containing 0.05 U/µl Taq polymerase (Fermentas: ThermoFisher Scientific, Waltham, USA), 1.9 mM Mg^{2+}, 0.1 mM dNTPs, 250 nM primers, all in 1× Taq buffer (Fermentas), with an annealing temperature of 50 ^{o}C. The SSU rRNA gene of strain TINI was amplified in 50 µl of a cocktail containing 2.25 mM LA PCR reaction buffer with $MgCl_2$ (Top-Bio, Vestec, Czech Republic), 0.3 mM dNTPs (Top-Bio), 0.2 µM DMSO, 1.25 µM primers, and 0.05 U/µl LA polymerase (Top-Bio), with an annealing temperature of 55 ^{o}C. In the case of strain PCT PCR-amplification was performed in 20 µl of a cocktail containing 0.05 U/µl Taq polymerase (Invitrogen; Themofisher Scientfic), 1.5 mM Mg^{2+}, 0.2 mM dNTPs, 1 µM primers, all in 1× PCR buffer (Invitrogen), with an annealing temperature of 53 ^{o}C. The amplification products were either purified using the MinElute PCR Purification Kit (Qiagen) and directly sequenced (most strains), or were gel-purified with the Zymoclean™ GEL DNA Recovery Kit (Zymo Research, Irvine, USA; strains CIZOV2, COROMOTO, KIZILLAR, PCT, and TINI), or Qiaquick® gel extraction kit (Qiagen; strain PCT), then cloned into a pGEM vector (Invitrogen) using the pGEM®-T EASY VECTOR SYSTEM I (Promega, Fitchburg, USA). In

the latter cases, several individual clones were partially sequenced with one or two positive clones selected for final sequencing. For strains BALI, CIZOV2, IND7, and TUN4 only partial sequences were determined.

DNA sequences encoding cytosolic Hsp90 and beta tubulin proteins were determined for *T. marina* strain PCT. A mostly complete Hsp90 coding region sequence was PCR-amplified using degenerate primers 100XF and 970XR (Simpson et al. 2002a), and a near-complete beta-tubulin gene was amplified using degenerate primers Btub A and Btub B (Edgcomb et al. 2001). The PCR conditions were the same as described above (for PCT) except that 5% v/v acetamide (final concentration) was added to the cocktail, and the annealing temperature was 52 ^{o}C. The amplification products were gel-purified, cloned into a pGEM vector (as described above), and two and four individual clones were partially sequenced for Hsp90 and beta tubulin genes respectively, with one clone of each selected for complete bi-directional sequencing. No introns were detected in the beta tubulin gene sequence. Two introns were predicted in the Hsp90 gene.

The GenBank numbers of the new sequences reported in this study are XXXX-YYYYY.

Phylogenetic analysis: Two datasets of aligned SSU rRNA gene sequences were created. The 'main' dataset contained 14 newly determined sequences, 16 Preaxostyla sequences retrieved from GenBank, 4 environmental SSU rRNA gene sequences identified by BLAST as close relatives of trimastigids, and 43 sequences representing broad eukaryotic diversity, used as outgroups. The second 'Preaxostyla-only' dataset contained 34 SSU rRNA gene sequences exclusively from Preaxostyla (including newly determined sequences), and longer than 650 bp. The sequences of both datasets were aligned using MAFFT (Katoh et al. 2002) on the MAFFT 6 server (http://mafft.cbrc.jp/alignment/server/) with the G-INS-i algorithm at default settings, followed by manual correction and exclusion of poorly aligned sites in BioEdit 7.0.4.1 (Hall 1999). The final datasets contained 1165 and 1617 well-aligned sites, respectively, and are available from the corresponding author upon request. Phylogenetic trees were constructed by maximum likelihood (ML) and Bayesian Inference (BI) methods. The ML analysis was performed using RAxML 7.0.3 (Stamatakis 2006) under the GTRGAMMAI model, with 10 random taxon additions to generate starting trees. Bootstrap support was inferred from 1000 pseudoreplicate datasets. Bayesian inference was performed using MrBayes 3.2 (Ronquist et al. 2013) under the GTR + I + Γ + covarion model. MCMCs with four chains (default temperature parameter, sampling frequency 0.002) were run for 3 x 10^6 generations (main dataset) or 2 x 10^6 generations (Preaxostyla-only dataset) at which point the mean standard deviation of split frequencies based on the last 75 % of generations was < 0.01. The first 25 % of generations were then removed as burn-in.

For three-gene phylogenies inferred SSU rRNA nucleotide, Hsp90 amino acid, and beta tubulin amino acid sequences from 64 diverse eukaryotes were examined, including *Trimastix marina* PCT, *Paratrimastix eleionoma* Albury, *Paratrimastix pyriformis* ATCC 50562 and two oxymonad species. An alignment for each gene was constructed using CLUSTALX 2 (Thompson et al. 1994) in the program SEAVIEW (Gouy et al. 2010), followed by manual editing and exclusion of ambiguous regions in SEAVIEW. The final SSU rRNA gene alignment retained 1166 nucleotide sites, while the Hsp90 and beta tubulin

alignments retained 596 and 409 amino acid sites respectively.

Two three-gene datasets were analysed. The first included all 64 species, while the second had all the long-branch groups excluded (i.e. diplomonads, *Carpediemonas*-like organisms, parabasalids, Euglenozoa and Amoebozoa), and retained 43 species. Maximum Likelihood analyses were performed using RAxML 7.2.6. Models were set separately for DNA and amino acids, with an LGGAMMA model for each protein and GTRGAMMA model for the SSU rRNA genes. Branch lengths were estimated separately for the different partitions in the alignment. The ML tree searches used 20 random taxon additions to generate starting trees, while bootstrap analyses were performed on 1000 pseudoreplicate datasets. Bayesian analyses were performed using MrBayes 3.1.2 with a GTR + gamma model for SSU rRNA and the estimated amino acid model from MrBayes for each protein, with among-site rate variation modeled on gamma distributions. Branch lengths were estimated separately for each gene. Two duplicate runs with four chains were run for 10^7 generations (64-taxon analysis) and 4×10^6 generations (43-taxon analysis), with default temperature parameter and sampling frequency 0.001 in both cases. The average standard deviation of split frequencies for the last 75% of generations was 0.04 for the 64-taxon analysis, and the first 50% of generations were discarded as burn-in. The average standard deviations of split frequencies for the last 75% of generations was 0.01 for the 43-taxon analysis, so the first 25% of generations were discarded as burn-in.

Approximately unbiased (AU) tests were performed using CONSEL 0.1 for the 43-taxon three-gene dataset, to test two alternative hypotheses: the monophyly of all trimastigids and the grouping of freshwater and marine *Trimastix marina*. For each hypothesis, RAxML was used to search for the best constrained ML trees in which the related taxa consistently formed a monophyletic group. The site-wise likelihoods were calculated for each tree topology with RAxML, using the same models for different partitions as mentioned above.

Light Microscopy: Live cells of *Trimastix marina* isolate TINI and *Paratrimastix eleionoma* isolates WL and ELSUERO were observed with an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP71 camera. The lengths of 50 cells of each strain were measured from digital micrographs using the camera software (QuickPHOTO CAMERA 2.3). *Paratrimastix eleionoma* strain WL was also stained with protargol in order to create type material. Moist films spread on cover slips were prepared from pelleting cells by centrifugation at 500 g for 8 minutes. For better adherence to the cover slip, 1 µl of the sample was mixed with 1 µl of egg white diluted to 1:5 with ATCC medium 802 prior to the wet smear preparation. The films were then fixed in Bouin-Hollande's fluid for ca. 5 hours, were washed with 70% ethanol, and were stained with 1% protargol (Bayer, Germany) following the Nie's (1950) protocol.

Live *T. marina* isolate PCT cells were observed with differential interference and phase contrast microscopy using a Zeiss Axiovert 200M microscope (Zeiss, Oberkochen, Germany) equipped with a Zeiss Axiocam HR digital camera. Lengths of live cells (n=36) were determined from digital micrographs using the camera software (Axiovision 4.6).

Transmission electron microscopy of *Trimastix marina* strain PCT: For transmission electron microscopy

(TEM), cells were centrifuged at 1500 *g* for 3 min. After discarding the supernatant, the pellet was suspended in 500 µl of a cocktail containing 140 µl 25% glutaraldehyde and 360 µl culture medium. The solution was placed on ice for 15 seconds, then quickly combined with 500 µl of an ice-cold cocktail of 250 µl 4% OsO_4 and 250 µl culture medium, such that the final concentrations of glutaraldehyde and OsO_4 were 3.5%, and 1% respectively. The material was held on ice for 30 minutes and then centrifuged (2000 *g* for 1 min). After rinsing three times with culture medium, cells were suspended in 200 µl distilled water, then trapped in 2% (w/v) agarose. Agarose blocks were dehydrated by applying a graded ethanol series, and embedded in Spi-pon 812 resin (SPI-CHEM^{TM}). Serial sections (~70 nm) were cut with a diamond knife on a Leica UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany). Sections were then stained with saturated uranyl acetate in 50% ethanol and with lead citrate. Sections were observed using a Tecnai 12 electron microscope (FEI, Hillsboro, USA) with a goniometer stage. Micrographs were taken using a Gatan 14-megapixel digital camera (Gatan, Warrendale, USA).

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Figure Legends

Figure 1. Maximum likelihood (ML) tree of eukaryotes, based on SSU rRNA gene sequences. The new sequences are shown in bold. Numbers at nodes show ML bootstrap values and Bayesian Inference (BI) posterior probabilities; values <50/0.9 not shown. The scale bar corresponds to 0.1 expected substitutions per site.

Figure 2. Maximum Likelihood (ML) tree of 43 eukaryotes, estimated for a dataset of beta tubulin and cytosolic HSP90 inferred amino acid sequences, and SSU rRNA genes. The sequences for marine *Trimastix marina* isolate PCT (shown in bold) are newly reported here. Numbers at nodes show ML bootstrap values and Bayesian Inference (BI) posterior probabilities; Asterisk (*) means the node was not present in the BI tree. The scale bar corresponds to 0.05 expected substitutions per site.

Figure 3. Light micrographs of *Paratrimastix eleionoma*, gen. nov., sp. nov., isolates ELSUERO (A - C) and WL (D - G). Images A-F are Differential Interference Contrast (DIC) micrographs of live cells. Image G is a bright-field micrograph of the protargol-stained cell that is the holotype of *Paratrimastix eleionoma* sp. nov. **A.** Ventral view (seen from dorsal side) showing groove and anterior flagellum. **B.** Ventro-lateral view showing broad groove and posterior flagellum. **C.** Lateral view showing shape of the cell and thickened base of anterior flagellum. **D.** Ventro-lateral view of the cell. **E.** Lateral view showing thickened base of anterior flagellum. **F.** Lateral view showing shape of the cell and right wall of groove (note bulge; double arrowhead). **G.** Lateral view showing four flagella, groove and nucleus with central nucleolus. Scale bar in F represents 10 µm for A-F. Scale bar in G represents 10 µm.

Abbreviations for figures 3-5: **Ap:** apical projection (*P. eleionoma* only); **Cy**: (lateral) cytopharynx (*P. eleionoma* only); **F1**: Flagellum 1 (posterior flagellum); **F2**: Flagellum 2 (anterior flagellum); **F3:** Flagellum 3 (right flagellum); **F4:** Flagellum 4 (left flagellum); **G:** Groove; **N**: Nucleus; **Arrow:** thickened portion of flagellum 1; **Arrowhead:** posterior narrowing of groove (*T. marina* only); **Double arrowhead:** Bulge/discontinuity in right groove wall.

Figure 4. Light micrographs of a single live cell of marine *Trimastix marina*, strain PCT, phase contrast. **A – D.** lateral views showing the flagella, nucleus and groove. Note the narrow posterior end of groove in C and D (arrowhead in C). Scale bar represents 10 µm.

Figure 5. Light micrographs of live marine *Trimastix marina*, isolate PCT. Micrographs A and B are phase contrast images; C – I are Differential Interference Contrast (DIC) images. **A, B.** Lateral views showing length of anterior and posterior flagella (F2 and F1 respectively, in B). **C, D.** Lateral views showing the thickened base of anterior flagellum (arrow in D), and absence of separate, bulbous apical projection. **E – G.** dorsal (E, G) and ventral (F) views showing the broad groove and flagellar insertion. Note asymmetrically thickened base of anterior flagellum (compare with B), and food vacuole in E and F. **H, I.** Lateral views showing right wall of the groove with bulge (double arrowhead), and narrow posterior end of groove (arrowhead in H). Scale bar (bottom right) represents 10 µm for all micrographs.

Figure 6. *Trimastix marina*, isolate PCT, transmission electron micrographs, general cell features. **A.** Cell in longitudinal section, lateral view (ventral side to left of image). **B.** Longitudinal section through anterior-left portion of the groove, showing position of Golgi region and posterior flagellum (F1; near point of insertion). **C.** Section showing abundance and relative size of mitochondrion-related organelles and dense membrane-bounded granules. **D.** Dense membrane-bounded granule. **E.** Mitochondrion-related organelle, showing double membrane (arrow). **F.** Transverse section of posterior flagellum (F1), showing dorsal and ventral vanes, without thickened margins to the lamellae (d, v). **G.** Transverse section through middle portion of the groove, showing the supporting structures for the wall and floor of groove and the position of nucleus. Scale bar in A represents 2 µm. Scale bars in B and F represent 200 nm. Scale bars in C and G represent 500 nm. Scale bar in D represents 100 nm for both D and E.

Abbreviations for figures 6-11: **A**: A fibre; **B**: B fibre; **B1**: Basal body of the posterior flagellum (F1); **B2**: Basal body of the anterior flagellum (F2); **B3**: Basal body of the right flagellum (F3); **B4**: Basal body of the left flagellum (F4); **C**: C fibre; **CF**: Composite Fibre; **Co**: Connection structure; **Cp**: Carinate projection; **d**: dorsal vane; **ER**: Endoplasmic Reticulum; **F**: dorsal Fan of microtubules; **FA: (**proximal) Flagellar Apparatus**; FC**: Fibrous Crescent; **FV**: Food Vacuole; **F1**: Flagellum 1; **F2**: Flagellum 2; **GR**: Golgi Region; **I**: I fibre; **IDR**: Inner R2-associated Dense Rod; **iR2**: inner portion of the Root 2; **MRO:** Mitochondrion-Related Organelles; **N**: Nucleus; **oR2**: outer portion of Root 2; **oR2Mt**: oR2-originating groove Microtubules; **R1**: Root 1; **R2:** Root 2; **R3**: Root 3; **R1Mt**: R1-originating groove Microtubules; **R2Mt:** R2-originating groove Microtubules; **S**: Singlet microtubular 'root'; **SA**: Singlet root-Associated fibre. **v**: ventral vane. **Arrowhead:** granule-containing vesicles.

Figure 7. *Trimastix marina*, isolate PCT, transmission electron micrographs, general views of the flagellar apparatus. **A.** Longitudinal section showing the anterior flagellum (F2) and connection to the apical carinate process supported by the dorsal fan (F). **B.** Flagellar apparatus showing the four basal bodies. **C.** Longitudinal section of anterior basal body (B2), showing cartwheel at base. **D.** Transverse view of the anterior flagellum (F2) and carinate process. **E.** Longitudinal section of posterior basal body (B1), showing cartwheel. **F-H.** Non-consecutive serial sections of anterior basal body (B2). Note the transition zone including axosome and 'acorn' in F, and cartwheel in H. Scale bar in A represents 200 nm for A. Scale bar in E represents 200 nm for B, C, E. Scale bar in D represents 200 nm for D, F-H.

Figure 8. *Trimastix marina*, isolate PCT, transmission electron micrographs, flagellar apparatus elements focused around anterior flagellum/basal body (B2). Cell's ventral is to the bottom of all images, with cells' left to the right of the image, and vice versa. **A.** Approximately coronal section of the proximal flagellar apparatus showing the relationships between the major anterior cytoskeletal elements. **B.** Transverse section of the anterior basal body (B2) showing origin of microtubular root 3 (R3). **C-E.** Consecutive sections of the carinate process and emerging anterior flagellum (F2) showing the direction of root 3 (R3) and dorsal fan (F). **F.** Section through apex of cell, including carinate process, showing the dorsal fan (F) near its origin, and root 3. **G.** Transverse section of basal body 2. Arrow shows the minor root. Scale bar in A represents 200 nm. Scale bar in C represent 200 nm for B-E and G. Scale bar in F represents 200 nm.

Figure 9. *Trimastix marina*, isolate PCT, transmission electron micrographs, proximal flagellar apparatus elements focused around posterior flagellum/basal body (B1). Cell's ventral is to the bottom of images A-F, with cells' left to the right of the image, and vice versa. **A.** Transverse section through the posterior basal body (B1) showing origins of the main groove-supporting microtubular roots (R1, R2). **B.** Transverse section of the proximal part of R2 showing the Singlet Associated fiber and Singlet root (SA + S), and the A and B fibres (A, B). **C.** Origin of microtubular root 1 (R1). **D.** Immediately distal section after the origin of R1. Note large C fibre (C) with multilayered appearance. **E.** Transverse section near the distal end of B1. **F.** Transverse section of the opening of groove, showing the support of the left wall of the groove by R1 backed by the C fibre. Note also the B fibre (B) supporting the right wall, and the central position of the singlet root (S). **G, H.** More longitudinal sections of R1, showing the substructure of the C fibre and its abrupt termination in the anterior portion of the groove. Short arrows in B, C, E denote the indistinct fibrous material that runs between B and the left basal body (B4). Scale bar in A represents 200 nm for all images.

Figure 10. *Trimastix marina*, isolate PCT, transmission electron micrographs, anterior portion of right wall of groove. Cell's ventral is to the bottom of all images, with cells' left to the right of the image, and vice versa. **A-C.** Transverse sections aligned to root 2 (R2), showing its origin. Short arrows denote the indistinct fibrous material connected to the B fibre. **D.** Proximal transverse section of R2 showing the I fibre. **E-I.** Images from non-consecutive serial sections of the right wall of the anterior portion of the

groove showing the splitting of R2 into inner and outer sections (iR2, oR2), and change in alignment of oR2 and the B fibre. **J.** Section at a similar level to H showing appearance of the A fibre (A). Scale bar in A represents 200 nm for A – C. Scale bar in D represents 200 nm for D – J.

Figure 11. *Trimastix marina*, isolate PCT, transmission electron micrographs, middle and posterior portions of groove. **A.** Transverse view of microtubules supporting middle and left portion of the groove. **B.** Transverse view of the right portion of the groove near the distal part. Note the Composite Fibre (CF; short arrow indicates the dense element of CF). **C.** Oblique/lateral section near posterior end of cell showing grazing sections of oR2, iR2 and the microtubules supporting the groove floor. **D.** Section from same series as C, passing through right wall of groove, showing two profiles of the CF close to oR2Mt, and the probable IDR (Inner R2-associated Dense Rod) close to iR2. **E.** Grazing view of CF showing striations of sheet-like region, and dense element (short arrow). Scale bars in A, B, E represent 200 nm. Scale bars in C and D represent 500 nm.

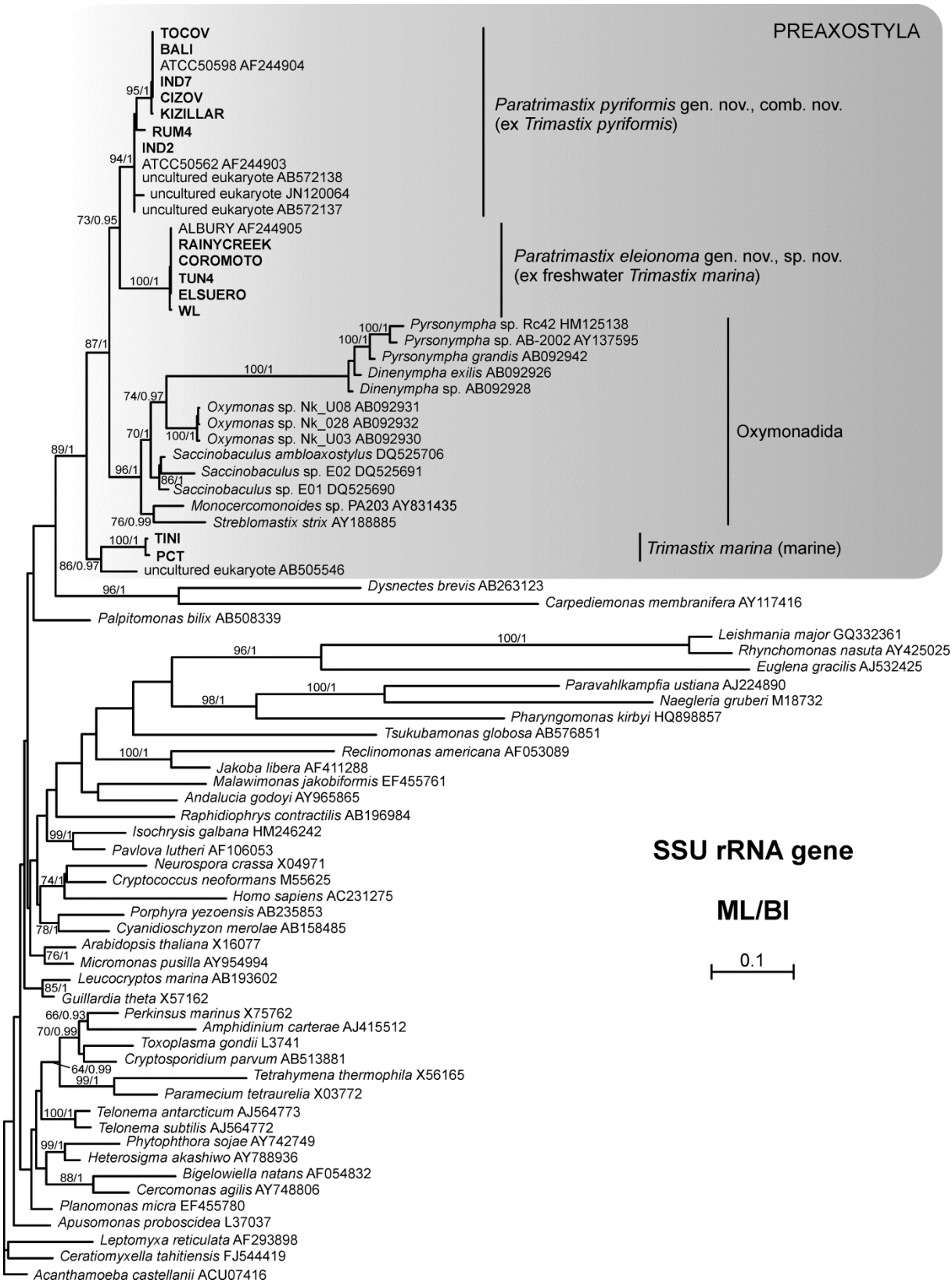
Supplementary Figure 1. Maximum Likelihood (ML) tree of 64 eukaryotes (including long branch outgroups), estimated for a dataset of beta tubulin and cytosolic HSP90 inferred amino acid sequences, and SSU rRNA genes. Numbers at nodes show ML bootstrap values. The scale bar corresponds to 0.05 expected substitutions per site.

Supplementary Figure 2. Bayesian Inference (BI) tree of 64 eukaryotes (including long branch outgroups), estimated for a dataset of beta tubulin and cytosolic HSP90 inferred amino acid sequences, and SSU rRNA genes. Numbers at nodes show posterior probabilities. The scale bar corresponds to 0.05 expected substitutions per site.

Table 1. List of isolates reported in this study, with sample locations and habitat

Species	Strain	Locality	Coordinates	Elevation	Habitat
Trimastix marina	PCT	Marstons Mills, MA, USA	41°38'N, 70°24'W	0 m	Marine sediment
	TINI	Tiwi, Oman	22 °49'N, 59°15'E	0 m	Marine sediment
Paratrimastix pyriformis comb. n.	BALI	Bali island, Indonesia	n.a.	n.a.	Fresh-water sediment
	CIZOV2[a]	Čížov, Czech Republic	48 °52'N, 15°52'E	420 m	Fresh-water brook
	IND2[a]	Bhangarh, India	27 °05'N, 76°17'E	360 m	Fresh-water sediment
	IND7[a]	Bhangarh, India	27 °05'N, 76°17'E	360 m	Fresh-water brook
	KIZILLAR[a]	Kizillar, Turkey	37 °31'N, 35°42'E	330 m	Fresh-water sediment
	RUM4	Sfântu Gheorghe, Romania	44 °53'N, 29°34'E	1 m	Fresh-water sediment
	TOCOV[a]	Former village Tocov, Czech Republic	50 °18'N, 13°05'E	590 m	Fresh-water sediment
Paratrimastix eleionoma sp. n.	COROMOTO	Coromoto lagoon, Venezuela	8°35'N, 71 °01'W	3300 m	Fresh-water sediment
	ELSUERO	El Suero lagoon, Venezuela	8°32'N, 71 °00'W	4200 m	Fresh-water sediment
	RAINYCREEK	Bon Echo Provincial Park, ON, Canada	44°54'N, 77°17'W	315 m	Fresh-water sediment
	TUN4	Chomutov, Czech Republic	50 °27'N, 13°21'E	420 m	Fresh-water sediment
	WL	Halifax, NS, Canada	44°37'N, 63°35'W	25 m	Fresh-water sediment

[a]same isolate as in Pánek et al. (2012).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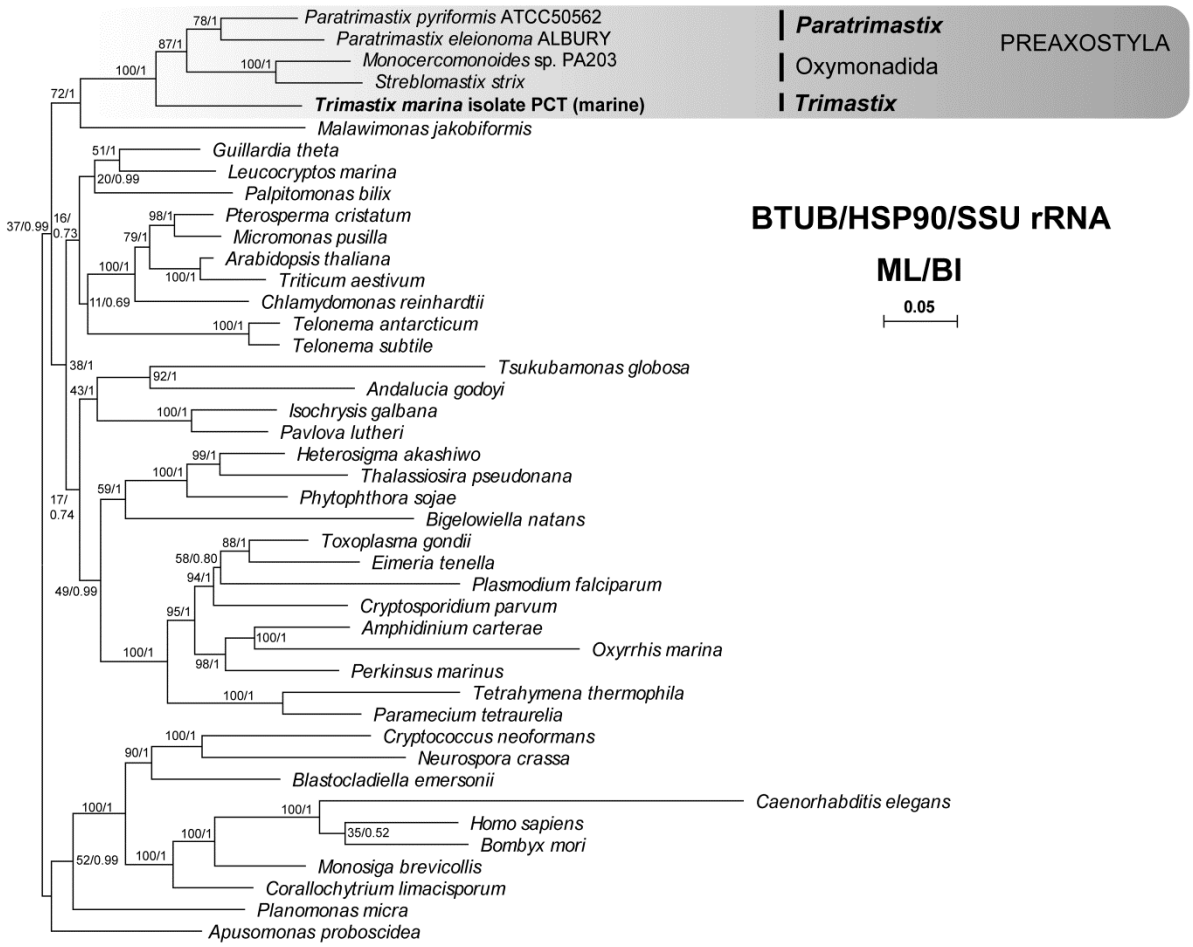
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Guillardia theta
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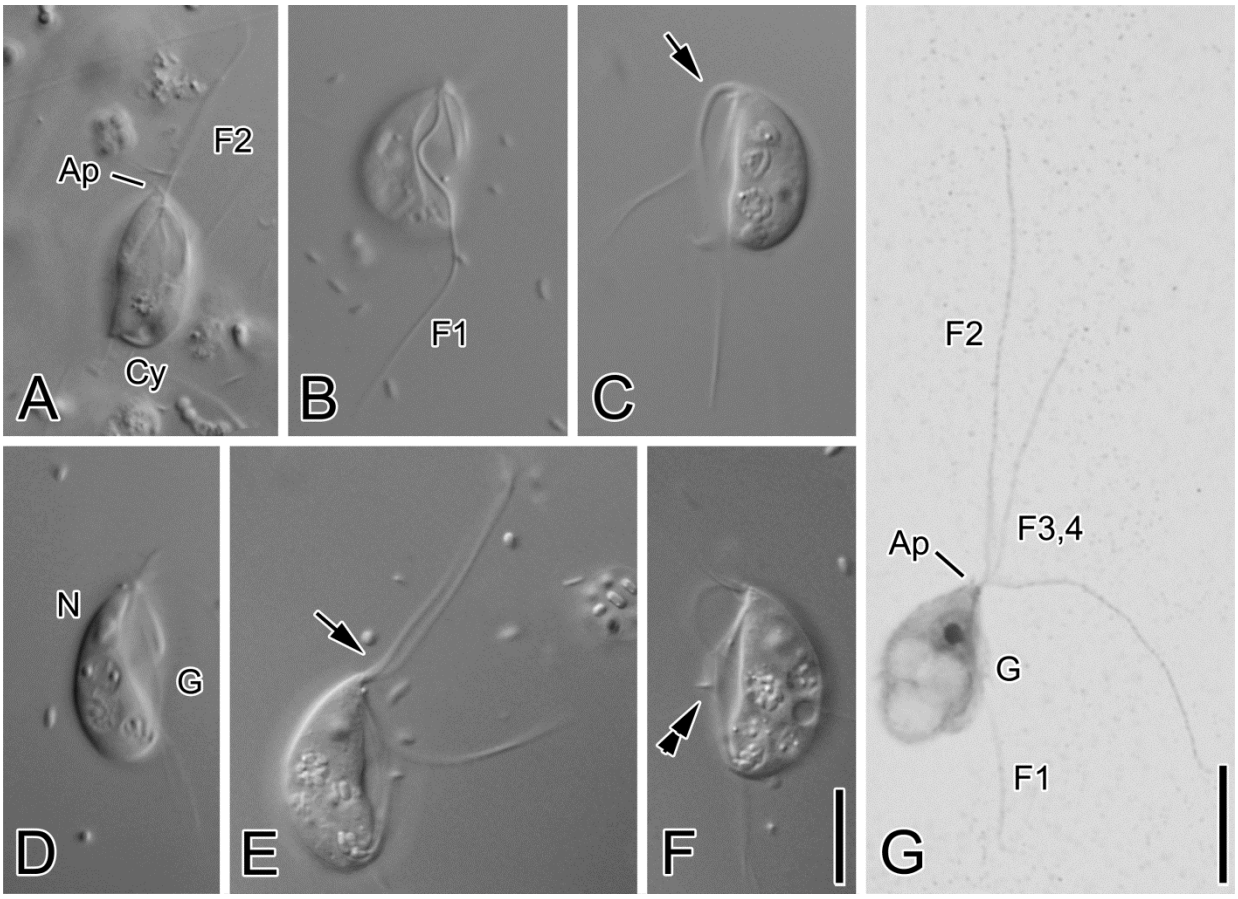

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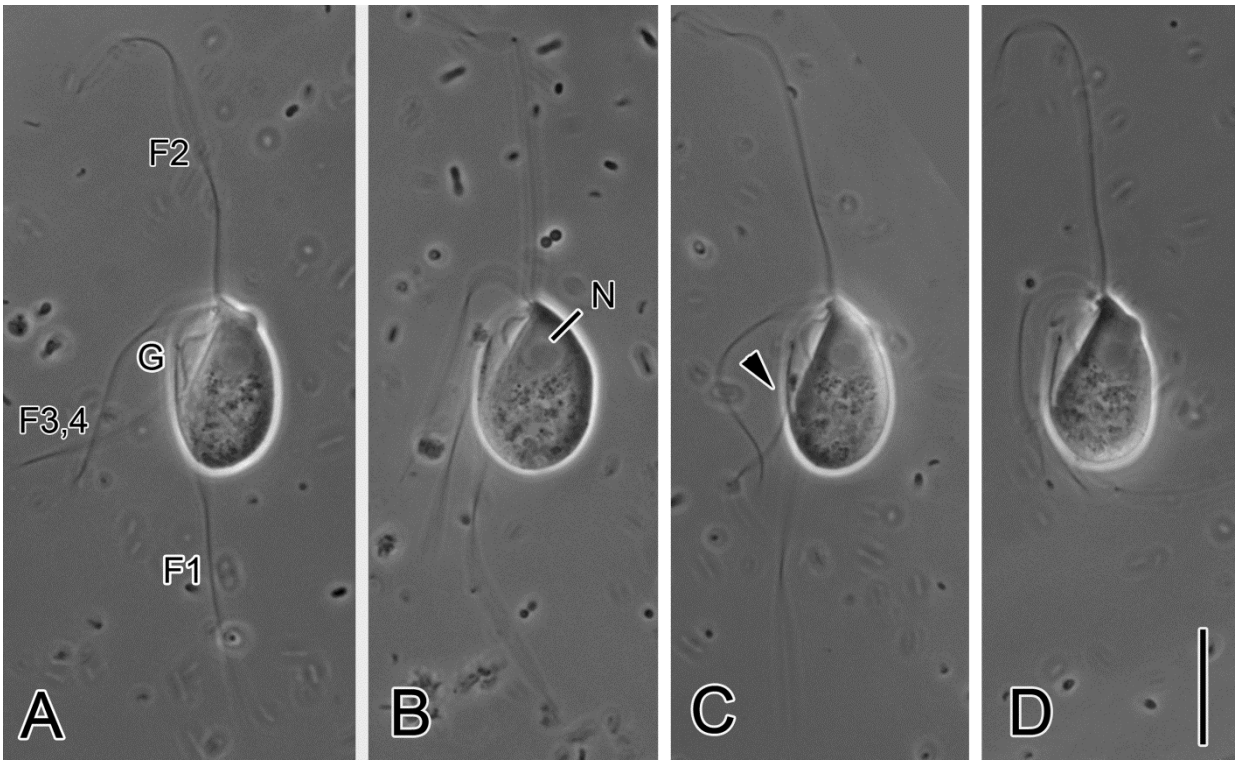

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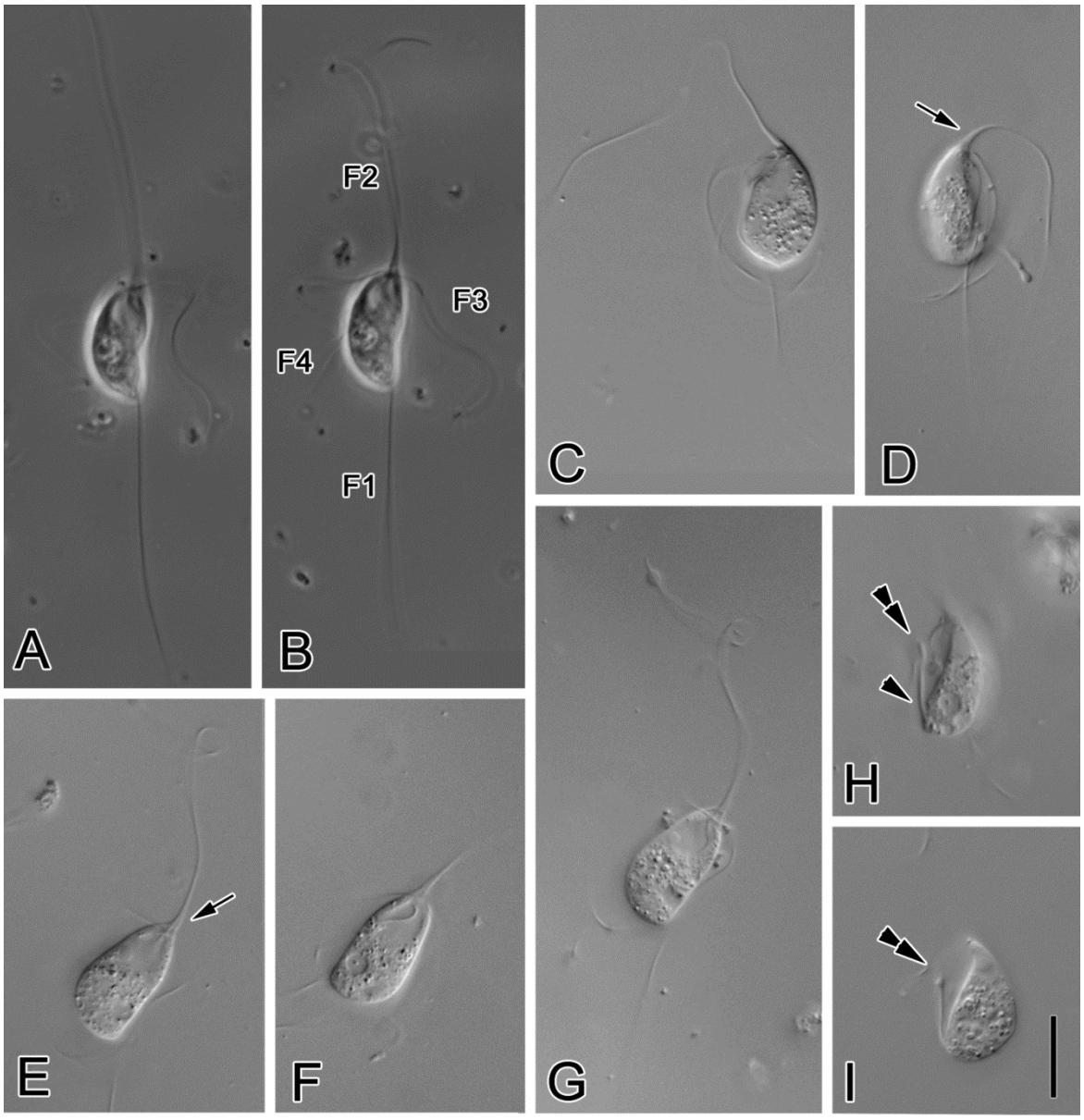

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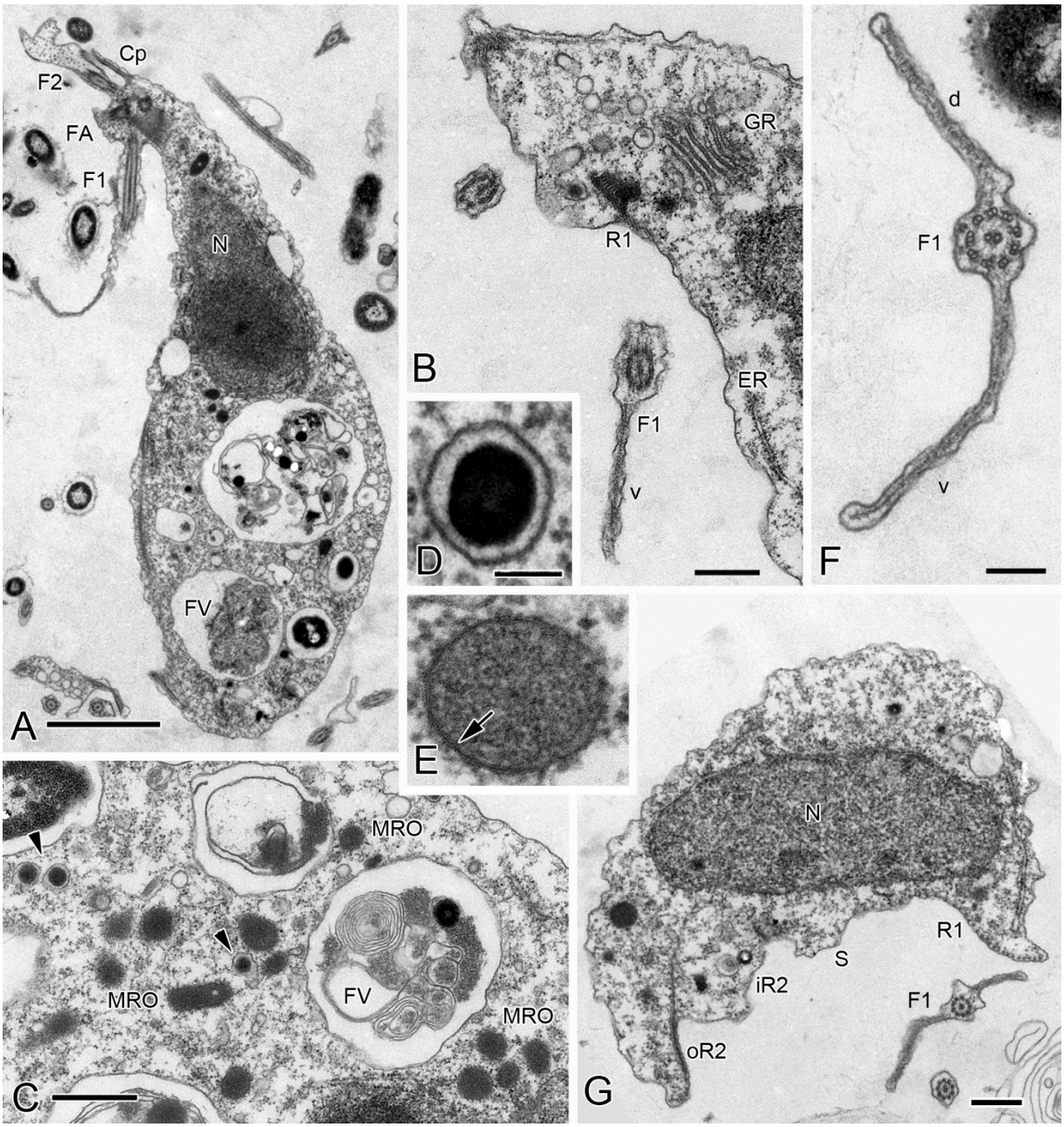
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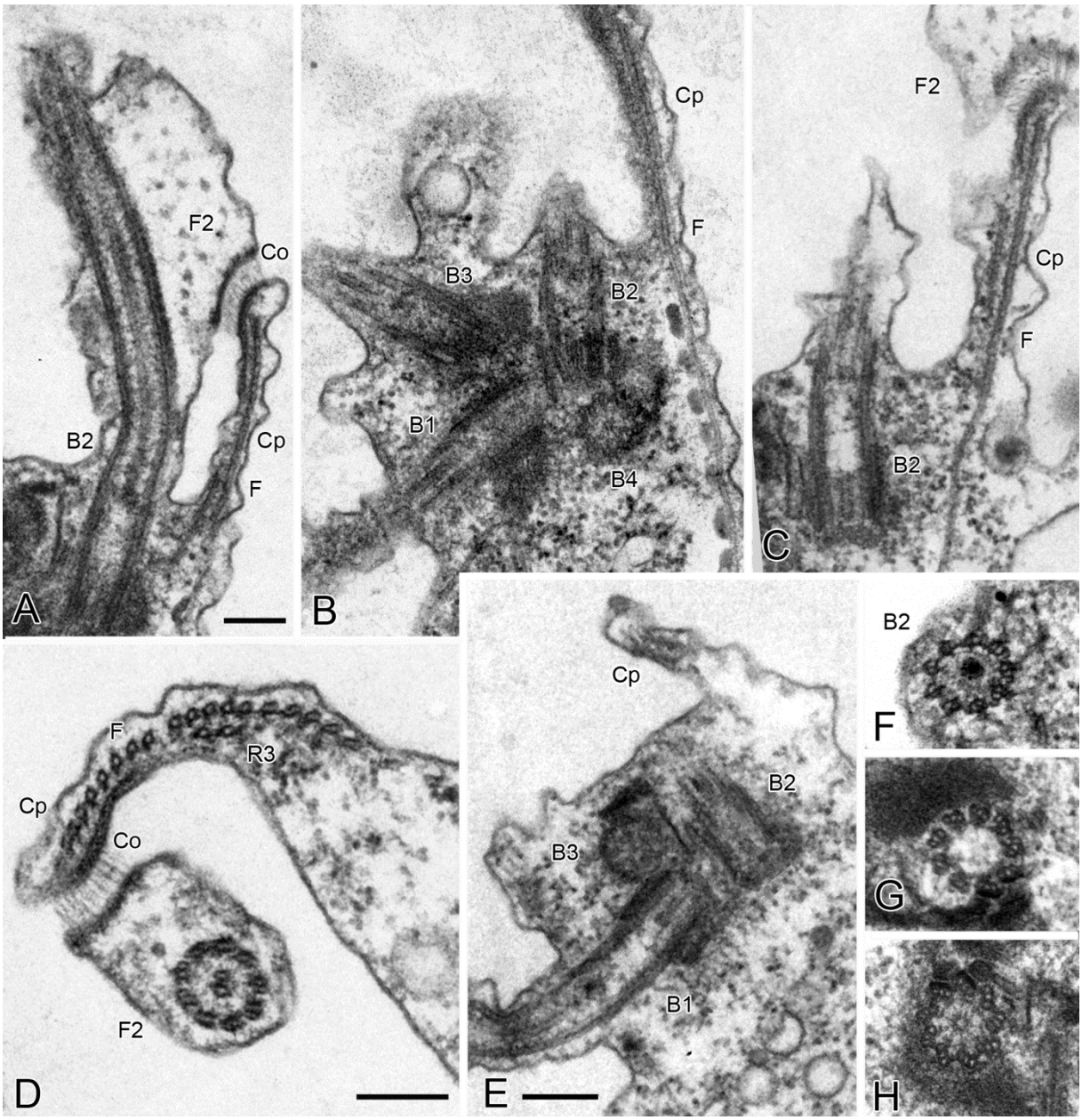
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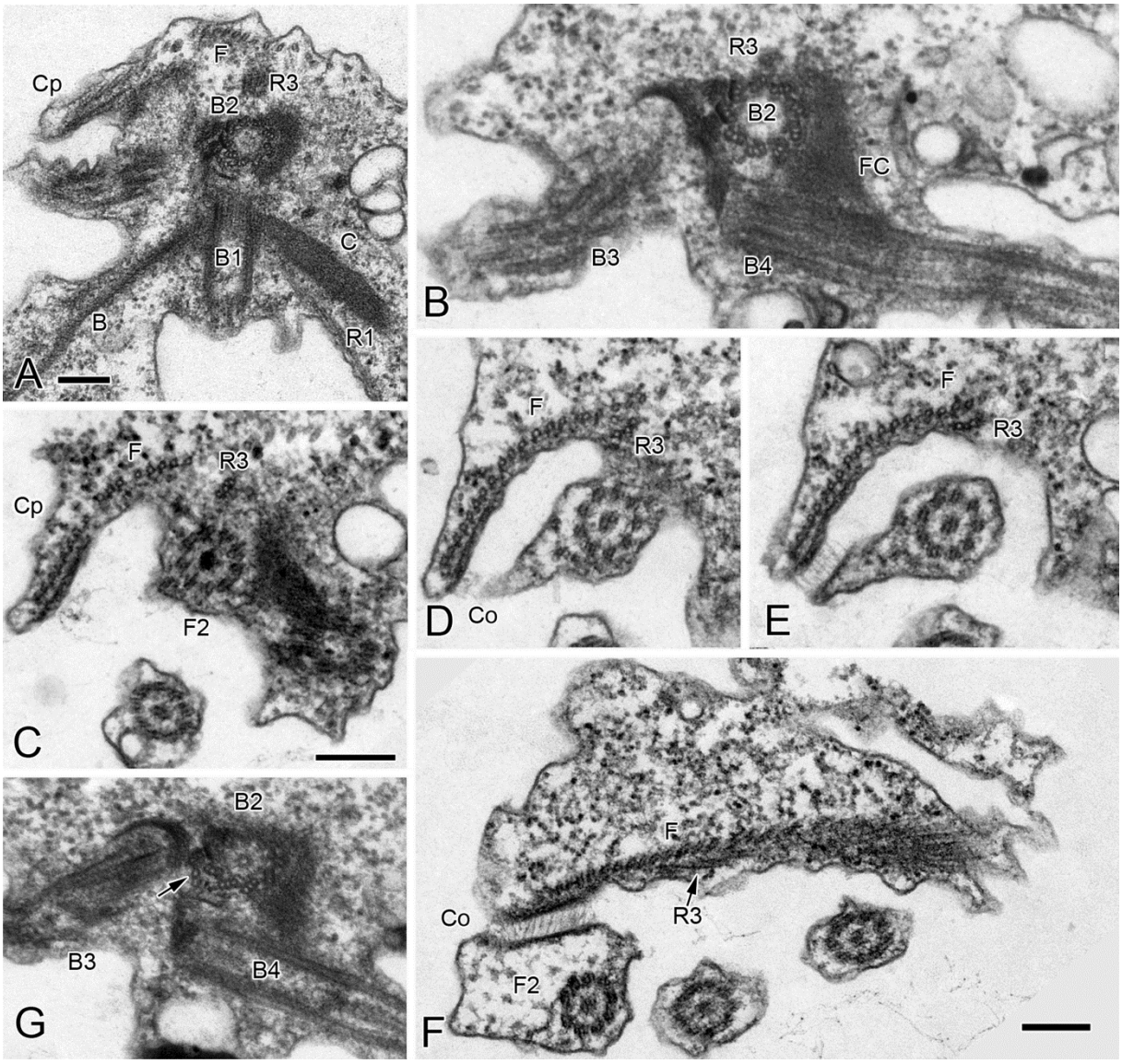
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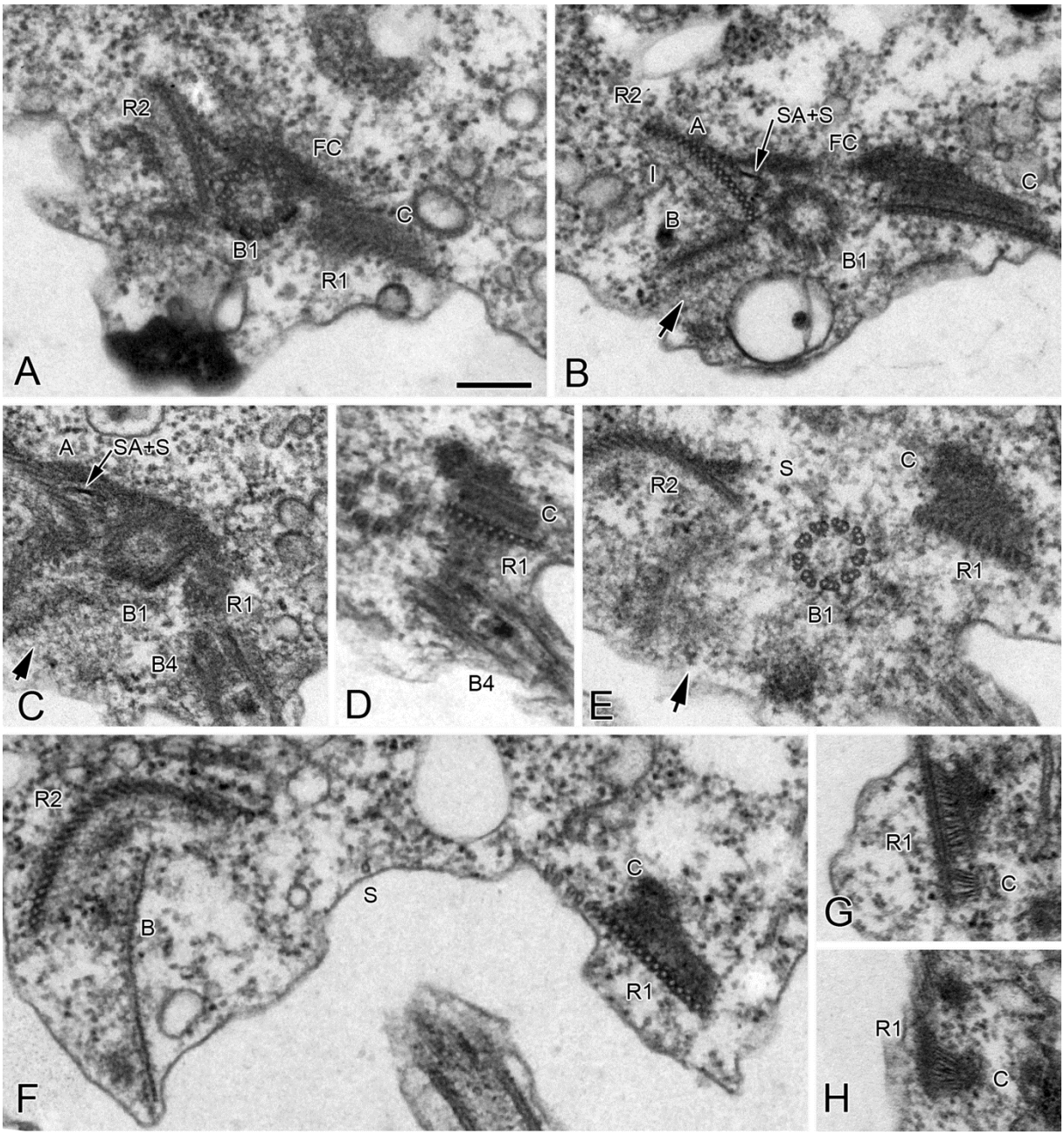
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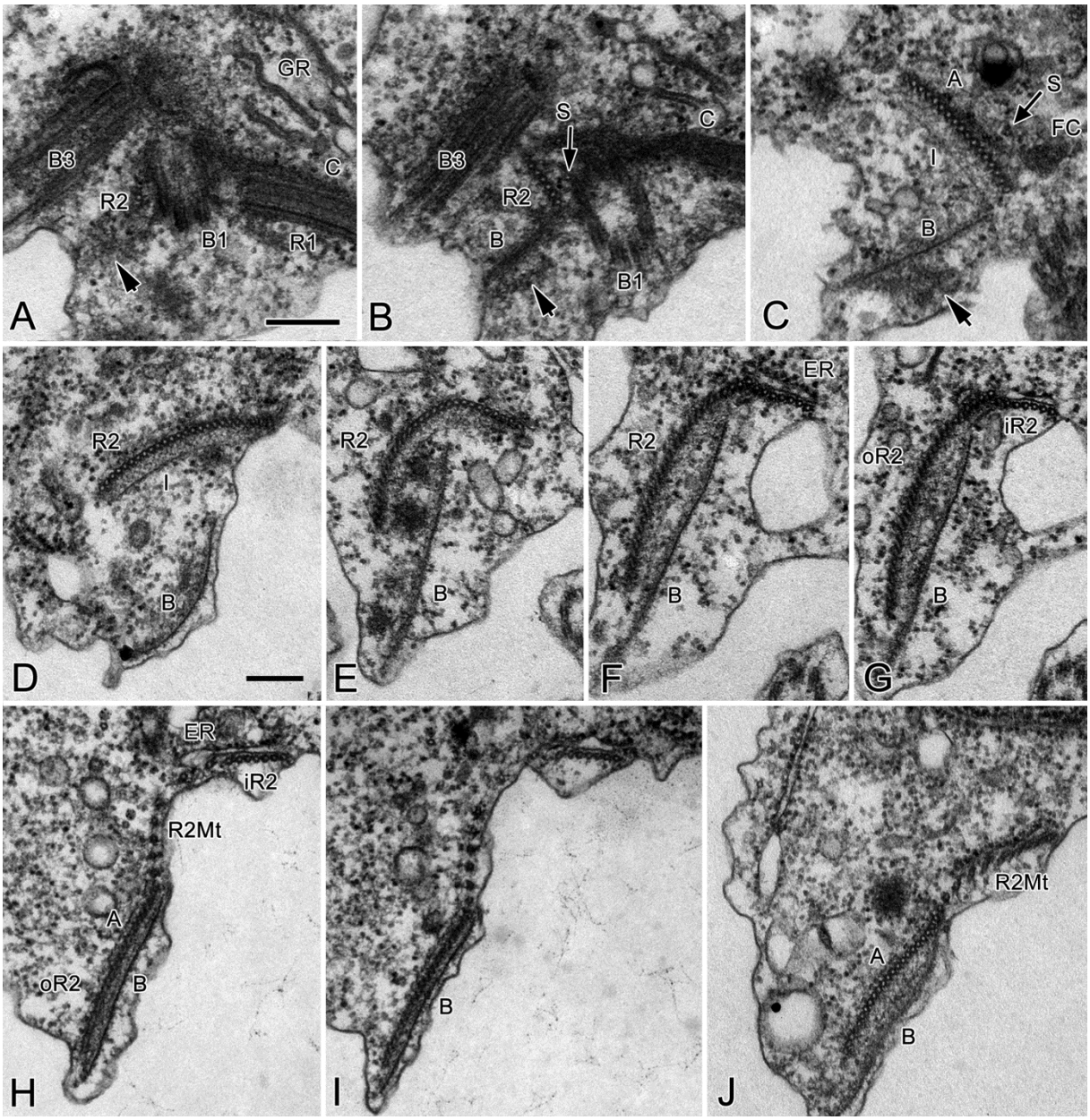
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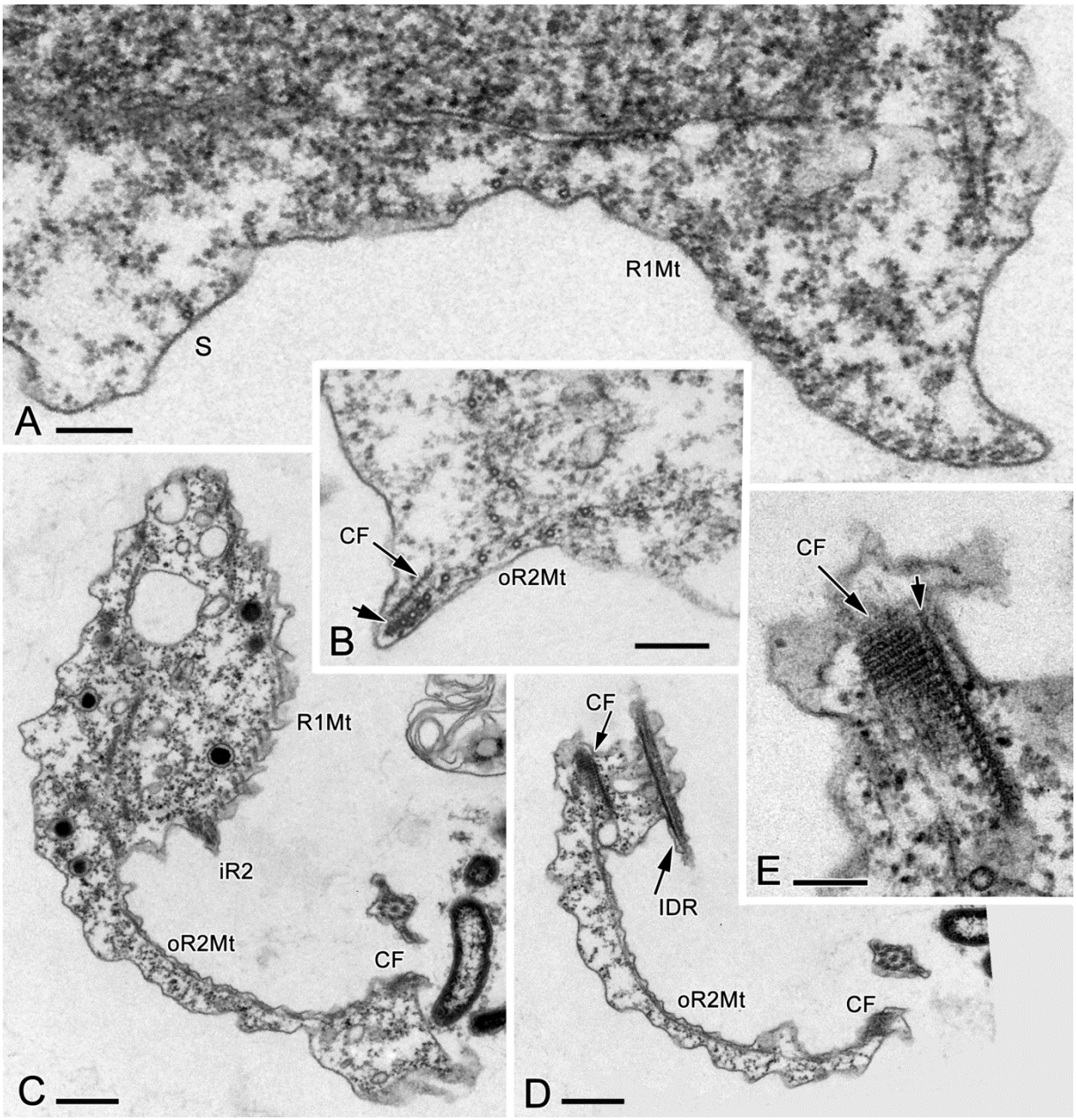

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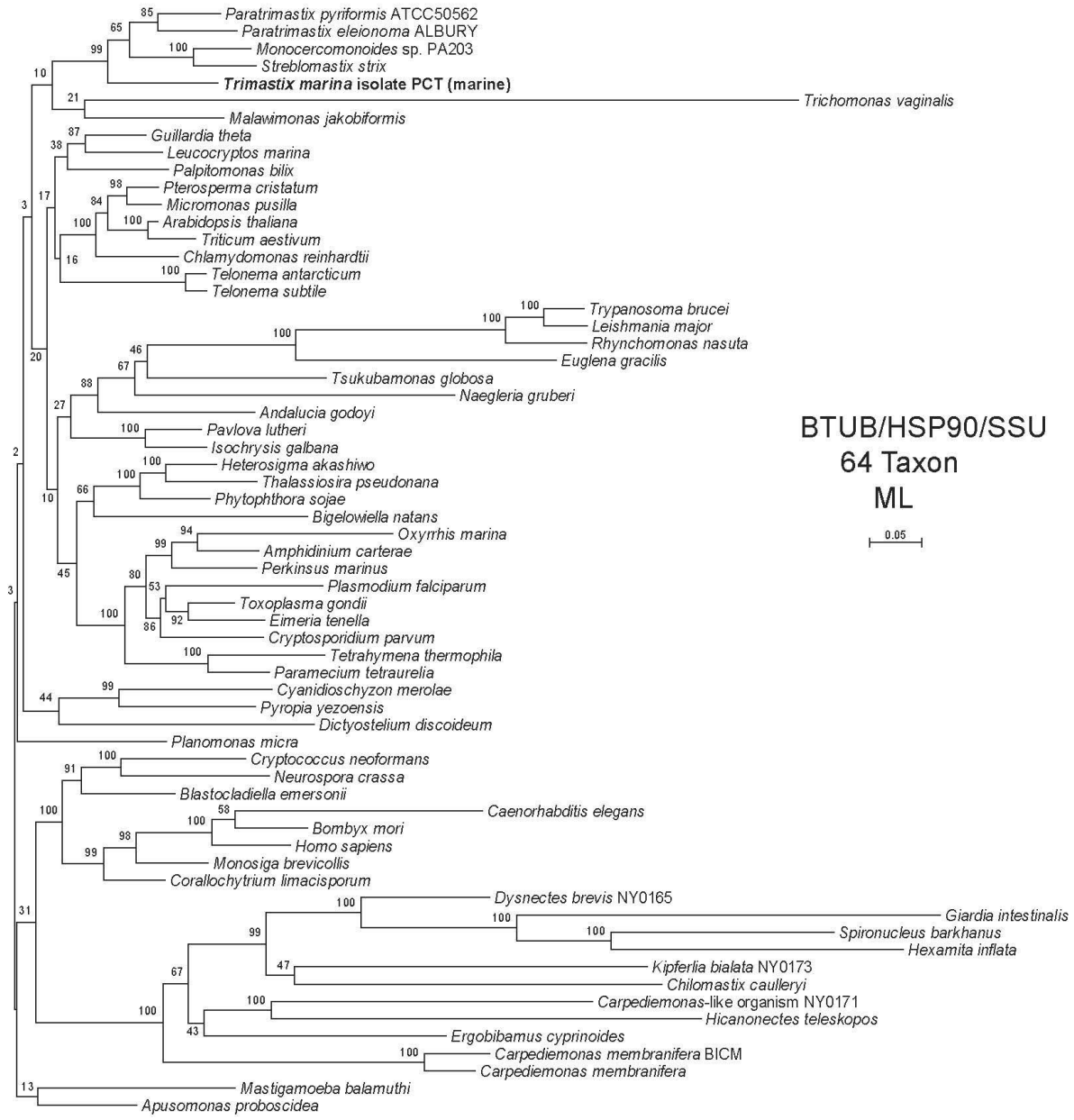
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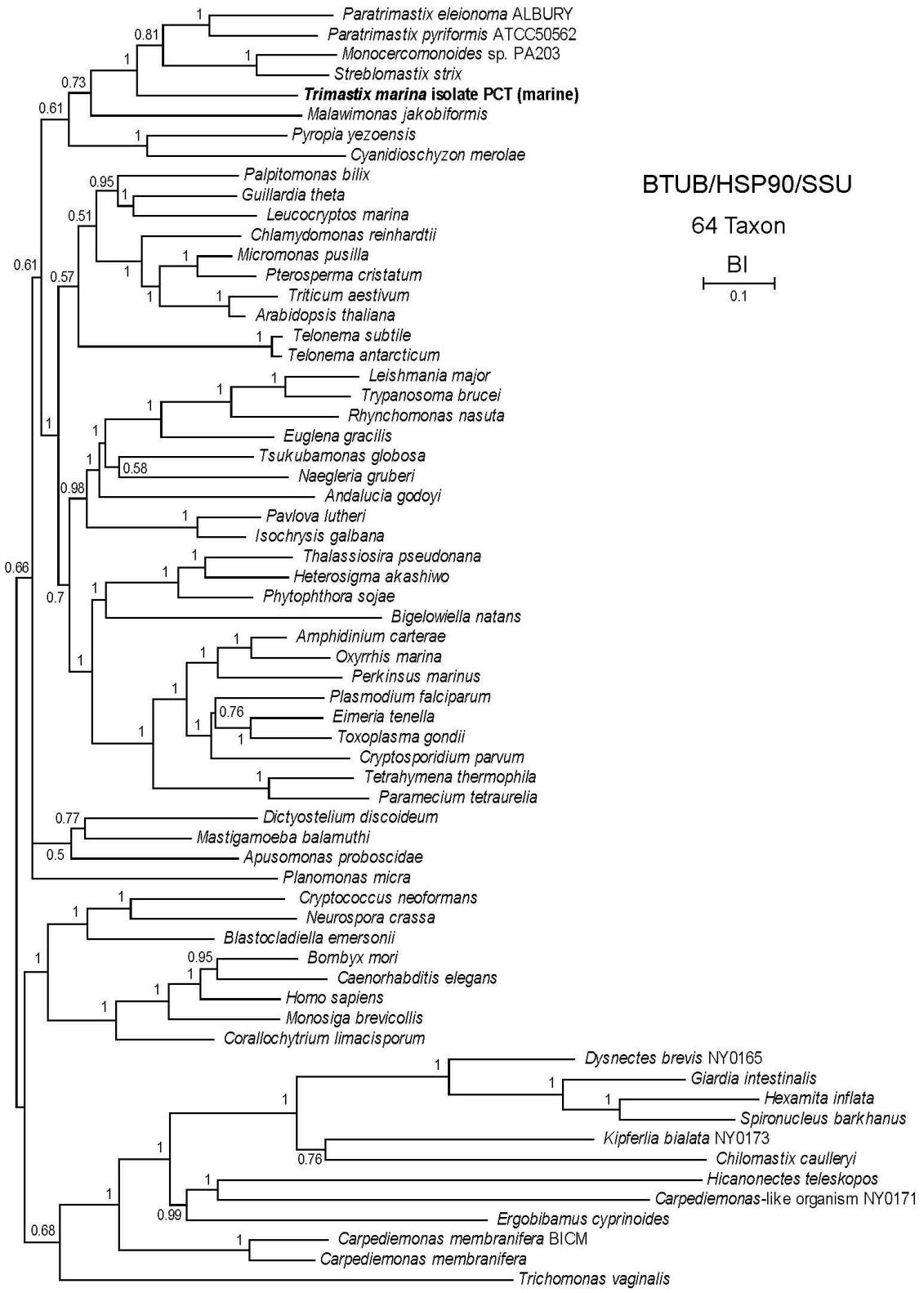
Figure S1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Palpitomonas bilix
Guillardia theta
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Micromonas pusilla
Pterosperma cristatum
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Heterosigma akashiwo
Phytophthora sojae
Bigelowiella natans
Amphidinium carterae
Oxyrrhis marina
Perkinsus marinus
Plasmodium falciparum
Eimeria tenella
Toxoplasma gondii
Cryptosporidium parvum
Tetrahymena thermophila
Paramecium tetraurelia
Dictyostelium discoideum
Mastigamoeba balamuthi
Apusomonas proboscidae
Planomonas micra
Cryptococcus neoformans
Neurospora crassa
Blastocladiella emersonii
Bombyx mori
Caenorhabditis elegans
Homo sapiens
Monosiga brevicollis
Corallochytrium limacisporum
Dysnectes brevis NY0165
Giardia intestinalis
Hexamita inflata
Spironucleus barkhanus
Kipferlia bialata NY0173
Chilomastix caulleryi
Hicanonectes teleskopos
Carpediemonas-like organism NY0171
Ergobibamus cyprinoides
Carpediemonas membranifera BICM
Carpediemonas membranifera
Trichomonas vaginalis
BTUB/HSP90/SSU
64 Taxon
BI
0.1

Figure S2