Charles University

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Elucidating the evolution and diversity of colonial chrysophytes

Po stopách evoluční historie a diverzity koloniálních zlativek

Ph.D. thesis

Supervisor: doc. Mgr. Pavel Škaloud, Ph.D.

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Author's declaration

I hereby declare that I have submitted neither this thesis nor its parts to acquire any other academic degree. I have written thesis independently using listed references.

Prohlášení autora

Čestně prohlašuji, že jsem nepředložil práci ani její podstatnou část k získání jiného nebo stejného akademického titulu, že jsem práci zpracoval samostatně, a že jsem uvedl všechny použité informační zdroje a literaturu.

In Prague / V Praze 29. 7. 2021 Martin Pusztai

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ORIGINAL PAPERS

This thesis is based on the following four papers:

I. **Pusztai, M.**, Čertnerová, D., Škaloudová, M., & Škaloud, P. (2016). Elucidating the phylogeny and taxonomic position of the genus *Chrysodidymus* Prowse (Chrysophyceae, Synurales). *Cryptogamie, Algologie*, 37(4), 297-307.

II. **Pusztai, M.**, & Škaloud, P. (2019). Elucidating the evolution and diversity of *Uroglena*-like colonial flagellates (Chrysophyceae): polyphyletic origin of the morphotype. *European Journal of Phycology*, 54(3), 404-416.

III. **Pusztai, M.**, & Škaloud, P. (2021). Species delimitation within the colonial flagellates *Uroglena*, *Uroglenopsis* and *Urostipulosphaera* (Chrysophyceae). *European Journal of Phycology*, 1-17.

IV. Škaloud, P., Škaloudová, M., Jadrná, I., Bestová, H., **Pusztai, M.**, Kapustin, D., & Siver, P. A. (2020). Comparing morphological and molecular estimates of species diversity in the freshwater genus *Synura* (Stramenopiles): a model for understanding diversity of eukaryotic microorganisms. *Journal of phycology*, 56(3), 574-591.

Authors' contributions:

Paper I – **PM**: drafting and editing manuscript, morphological investigations (LM), culturing; ČD: editing manuscript, acquiring molecular data; ŠM: editing manuscript, sampling and isolating cultures, morphological investigations (TEM); ŠP: original concept and study planning, editing manuscript, sampling, phylogenetic analysis

Paper II – **PM**: study planning, drafting and editing manuscript, sampling and isolating cultures, morphological investigations (LM, SEM), culturing, acquiring molecular data, phylogenetic analysis; ŠP: original concept and study planning, editing manuscript, sampling, phylogenetic analysis

Paper III – **PM**: study planning, drafting and editing manuscript, sampling and isolating cultures, morphological investigations (LM, SEM), culturing, acquiring molecular data, phylogenetic and other analysis; ŠP: original concept and study planning, editing manuscript, sampling, phylogenetic analysis

Paper IV – ŠP: original concept and study planning, drafting and editing manuscript, sampling and isolating cultures, morphological investigations (LM), phylogenetic and other analysis; ŠM: drafting manuscript, morphological investigations (SEM/TEM); JI: editing manuscript, acquiring molecular data; BH: editing manuscript, sampling, acquiring molecular data; **PM**: editing manuscript, sampling and isolating cultures, morphological investigations (LM), culturing; KD: editing manuscript, morphological investigations (SEM/TEM); SPA: drafting manuscript, morphological investigations (SEM/TEM)

On behalf of all the co-authors, I declare the keynote participation of Martin Pusztai in completing research and writing papers, as described above.

Pavel Škaloud

Abstract

Our current knowledge of chrysophyte diversity is still generally based on traditional morphospecies descriptions. Accordingly, sequence data exist for the minority of all described species. Consequently, several common morphotypes (e.g. *Ochromonas*-like flagellate) are scattered across the phylogenetic tree of Chrysophytes. It is evident that the postulated taxonomic diversity certainly does not reflect the real species richness in Chrysophytes. Moreover, recent studies on silica scaled chrysophytes (*Mallomonas*, *Paraphysomonas*, *Synura*) clearly demasked common problematics of (pseudo)cryptic species even within this group possessing relatively good species concept based on the ultrastructure of silica scales and bristles. Contrary, most of chrysophytes are naked flagellates forming stomatocysts as the only solid structure in their life cycles. While single-celled *Ochromonas* and *Spumella* were recently revisited using molecular genetic techniques, *Uroglena*-like colonials remained untreated.

Therefore, the main objective of this thesis was to provide a modern taxonomic revision of the widespread colonial chrysophytes causing conspicuous seasonal massive population booms, *Synura* s.l. and *Uroglena* s.l., by a polyphasic approach encompassing molecular phylogeny, morphology/ultrastructure and ecology. Sampling campaigns took place in Europe, North America and Japan throughout 2014–2020. More than 650 localities were explored, and more than 1,000 isolates were obtained. Moreover, type material from four type localities including type species of *Uroglena* (*U. volvox*) and *Uroglenopsis* (*U. americana*) was successfully recollected after more than 180 and 120 years, respectively.

Together with my colleagues, I revealed a polyphyletic origin of *Uroglena*-like morphotype. Following a proposed taxonomic revision, these taxa are treated as three distinct genus-level lineages exhibiting a unique combination of morphological, genetic and ecological characteristics. Accordingly, *Urostipulosphaera* gen. nov., *Urostipulosphaera granulata* sp. nov. and *Uroglena imitata* sp. nov. were described and many new taxonomic combinations were proposed. Further, an interesting case of evolutionary simplification within multi-celled colonial organisms was uncovered in *Synura synuroidea* comb. nov. formerly described as a distinct genus *Chrysodidymus*. We also revealed considerable (pseudo)cryptic diversity within *S. petersenii* s.l. (4 new species) and *S. leptorrhabda* lineages. Finally, my research illustrated the necessity of combining traditional detailed morphological investigations with gene sequence data to uncover (pseudo)cryptic species or phenotypic plasticity and to properly examine biodiversity within chrysophytes.

Abstrakt

Současný stupeň poznání diverzity zlativek bohužel stále stojí převážně na tradičních popisech morfodruhů. Trvalým problémem pak je neexistence sekvenačních dat pro většinu těchto druhů. Z toho mimo jiné plyne, že po celém fylogenetickém stromu zlativek je rozptýleno několik běžných morfotypů (*Ochromonas*-like aj.). Je zřejmé, že aktuální taxonomie třídy a obsáhnutá diverzita rozhodně neodráží skutečné druhové bohatství zlativek. Navíc nedávné studie zaměřené na zlativky s křemičitými šupinami (*Mallomonas*, *Paraphysomonas*, *Synura*) jednoznačně ukázaly, že problematika (pseudo)kryptických druhů je běžným fenoménem i pro taxony s relativně dobrým konceptem (s využitím SEM/TEM ultrastruktury). Typická zlativka nicméně vypadá jako nahý bičíkovec, jehož jedinou pevnou strukturou v rámci životního cyklu je stomatocysta. Zatímco jednobuněční bičíkovci *Ochromonas* a *Spumella* byli nedávno podrobeni taxonomické revizi s využitím technik molekulární genetiky, koloniální bičíkovci s *Uroglena*-like morfotypem nikoliv.

Hlavním cílem této práce proto bylo podrobit široce rozšířené koloniální zlativky s morfotypem *Synura* s.l. a *Uroglena* s.l., které způsobují nápadné sezónní populační boomy (vodní květy), moderní taxonomické revizi na základě znalosti jejich molekulární fylogeneze, morfologie/ultrastruktury a ekologie. V letech 2014–2020 probíhaly odběry vzorků v Evropě, Severní Americe a Japonsku. Z více než 650 prozkoumaných lokalit bylo izolováno více než 1000 kmenů. Navíc se podařilo získat cenný materiál ze čtyř typových lokalit včetně typových druhů rodu *Uroglena* (*U. volvox*) a rodu *Uroglenopsis* (*U. americana*), a to po více než 180, respektive 120, letech od jejich popisu.

Ve spolupráci s kolegy se mi podařilo odhalit polyfyletický původ koloniálních bičíkovců s *Uroglena*-like morfotypem, kteří se aktuálně dělí do třech nepříbuzných linií na úrovni rodů. Každá z těchto linií vykazuje specifickou kombinaci morfologických, genetických a ekologických charakteristik. V návaznosti na to byl nově popsán rod *Urostipulosphaera* a druhy *U. granulata* a *Uroglena imitata*, a dále bylo navrženo mnoho nových taxonomických kombinací. Dále byl odhalen zajímavý případ evolučního zjednodušení mnohobuněčných koloniálních organismů u druhu *Synura synuroidea* comb. nov., původě popsaného jako samostatný rod *Chrysodidymus*. Podařilo se nám rovněž odkrýt stále značnou (pseudo)kryptickou diverzitu v rámci *S. petersenii* agg. (4 nové druhy) a v rámci linie *S. leptorrhabda*. Výsledky mého výzkum jasně ukázaly nutnost kombinace vzájemně se doplňujících tradičních a nových metod při výzkumu reálné diverzity zlativek.

Content of the Ph.D. thesis

GENERAL INTRODUCTION

Protist diversity

One of the most fascinating aspects of life on Earth is, beside its origin, the great biodiversity. From ancient times, people try to understand to regularities of the world around. Therefore, the long-standing need to classify biodiversity arose before probably such a first published system, the Aristotle Scala Naturae (Aristotle, c. 350 BC). Until the end of 18th century biologist recognized just two kingdoms of life, Animalia and Plantae (encompassing Fungi), and considered organisms to be stable units. The 19th century brought revolutionary ideas leading to formulation of the Theory of evolution as a basis of modern biology (Darwin, 1859). At the same time, the third branch to so-called Tree of Life, protists, was added named as Protozoa (Goldfuss, 1820), Protoctista (Hogg, 1860) or later finally as Protista (Haeckel, 1866). The 20th century was not only in study of protists marked by a shift from light microscopic observations of morphology to new possibilities of electron microscopy of until then hidden ultrastructure and subsequent erection of Chromista as a second protist group (Cavalier-Smith, 1981).

Nowadays, protists are understood as paraphylum of all predominantly unicellular and microscopic eukaryotes, which are not animals, plants or fungi (Pawlowski *et al*., 2012; Burki, 2014). They naturally excel in diversity of their size, shape, nutritional and reproductive strategies and harbour a huge and still insufficiently known part of the diversity of life. The small single celled *Chlorella vulgaris* (Trebouxiophyceae) is a phototrophic protist, alga, as well as the giant kelp *Macrocystis pyrifera* (Phaeophyceae). If the massive involvement of molecular phylogenetic methods just before the turn of the millennium started revolution in study of not only biodiversity, than subsequent establishing of monophyletic supergroups (Simpson & Roger, 2004; compare to Cavalier-Smith, 2004), building on the research results of many other authors, meant probably the most significant milestone in classification of eukaryotic life on Earth.

Subsequent continuous editing and improving of the phylogenetic and later phylogenomic tree of eukaryotic life (Adl *et al*. 2005; Adl *et al*., 2012; Brown *et al*., 2018;

Adl *et al*., 2019; Burki *et al*., 2020) lead us to more accurate and robust knowledge of actual protist phylogeny as natural arrangement of their biodiversity (Fig. 1). Protists are spread over the entire eukaryote tree of life with catalogued number of tens of thousands species but with estimated number of up to over 160 million species, which illustrates discrepancy between morphological versus genetic view of protist biodiversity (Pawlowski *et al*., 2012; Larsen *et al*., 2017).

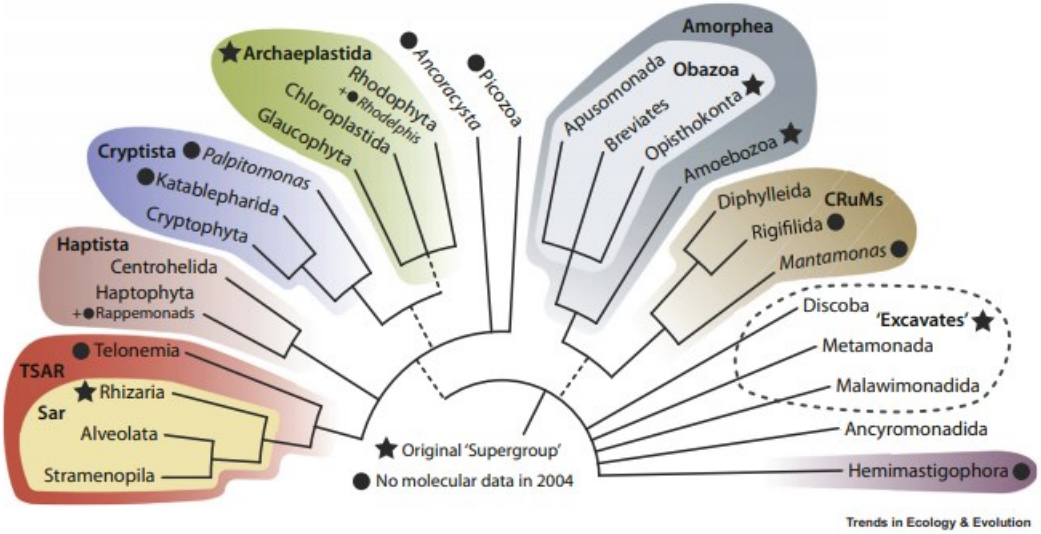

Fig. 1. The New Tree of Eukaryotes – summary based on a consensus of recent phylogenomic studies (from Burki *et al*., 2020).

Species are fundamental biodiversity units (Mayr, 1982). In my point of view, they possess dual character. Species may represent real physic entities as well as a hypothesis when using more or less transient or artificial boundaries on unstable nature (Bonde, 1977). Until recently the discussion revolved around controversial species definition and boundaries in so-called "species problem" (Hey, 2001). According to de Queiroz (2005a, 2005b, 2007) who introduced the unified species concept, "species problem" seems to be sold out by clear separation of both issues, the species conceptualisation and species delimitation. Accordingly, a separately evolving metapopulation lineage, the result of independently acting selection and drift after emergence of barrier to gene flow, is the only necessary property of a species, but the species may be delimited in a variety of ways.

Searching for a correct general species concept, however, was and will be problematic. During the process of speciation, newly acquired different properties do not necessarily arise at the same time or in a regular order, and therefore different species concepts may come into conflict (Fig. 2), especially during early stages of speciation (Leliaert *et al*., 2014). Therefore, it was suggested by Boenigk *et al*. (2012) to skip it and rather to focus on the clear species delimitation in protists, ideally by using more than one line of evidence and including a robust phylogenetic framework as a standard.

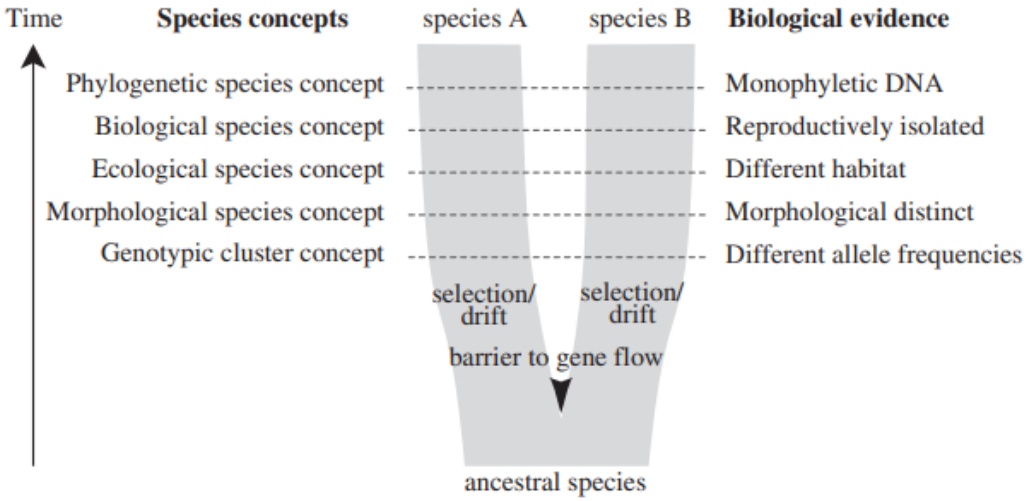

Fig. 2. Illustration of "species problem" on the example of potential conflict of different species concepts (from Leliaert *et al*., 2014).

Chrysophytes and their specifics

Chrysophytes or golden algae (Chrysophyceae) represent an important and widely studied protist (algal) group commonly observed in freshwater lentic ecosystems (Finlay & Esteban, 1998; Wolfe & Siver, 2013; Kristiansen & Škaloud, 2017). Historically chrysophytes encompass many splinter groups now considered as separate classes (Andersen *et al*., 1999; Andersen, 2007). Chrysophytes in the current definition are monophyletic and belong to SII clade of Stremenopiles (= Heterokonta) in SAR/TSAR supergroup respectively and bearing two heterokont flagella as synapomorphy (Andersen, 2004; Yang at al., 2012; Burki *et al*., 2020).

Chrysophytes are morphologically very diverse group ranging from single-celled heterokont monads or amoebas via variously composed colonies to even macroscopic forms (Starmach, 1985; Andersen *et al*., 1999). Most of chrysophytes are flagellates, often naked, but some of them produce organic or silica-scaled loricas (Kristiansen & Škaloud, 2017). The only one solid structure, which is truly present in a life cycle of every chrysophyte taxa, is a specific endogenous cyst made of silica, the stomatocyst (Fig. 3). These cysts are products of both sexual and asexual reproduction (without intraspecific difference in morphology of mature cyst) and generally exhibit great ultrastructural diversity among species (Sandgren, 1981, 1983; Firsova *et al*., 2008). However, the ultrastructure of cysts may not be always species-specific due to their simplicity in some taxa (e.g. some *Spumella* or *Synura* species), or due to the observation of the immature and not fully developed cysts in the others (Duff *et al*., 1995; Findenig *et al*., 2010).

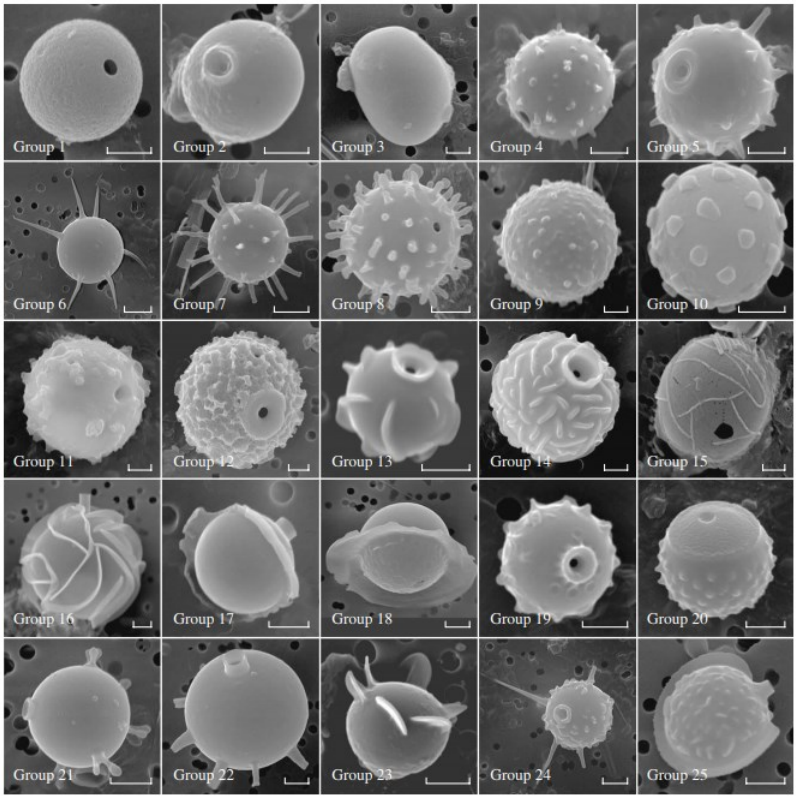

Fig. 3. Great ultrastructural diversity of chrysophycean stomatocysts from Lake Baikal (from Firsova *et al*., 2008).

Many of chrysophytes carry plastid and perform photosynthesis (Andersen *et al*., 2017; Kristiansen & Škaloud, 2017) and many are heterotrophs feeding on bacteria-sized microorganisms (Scoble & Cavalier-Smith, 2014; Grossmann *et al*., 2016). Nevertheless, the most advantageous and probably the most common strategy among chrysophytes seems to be combination of both in mixotrophy (summarized in Rottberger, 2013). There are many known mixotrophic regimes of nutrition (Jones at al., 2000) and even some heterotrophic chrysophytes, e.g. *Spumella* s.l., still possess genes involved in photosynthesis (Beisser *et al*., 2017). Moreover, according to transcriptome analysis, switch from ancestral phototrophy or mixotrophy to heterotrophy seems to have taken place many times in evolution of chrysophytes but with different intensity of pathways/genes reduction in different taxa (Graupner *et al*., 2018; Dorrell *et al*., 2019). This illustrates the real complexity of their nutritional strategies with all the ecological consequences.

Considering their known lower demands in temperature and irradiance and mixotrophy taken into account (Kamjunke *et al*., 2007; Watson & McCauley, 2010), chrysophytes often dominate in mezo-oligotrophic temperate habitats with lower nutrient concentration where they represent important grazers of bacteria-sized microorganisms as well as important primary producers (Nicholls *et al*., 1995; Reynolds, 1997; Brettum & Halvorsen, 2004). Regions with a high density of such habitat types and chrysophyte pre-dominance, e.g. Aquitaine (France), Scandinavia or Třeboňsko (Czech Republic), can be considered as biodiversity hotspots for chrysophytes harbouring a high regional taxon richness and a high number of putatively endemic taxa (Řezáčová & Neustupa, 2007; Němcová *et al*., 2012; Korkonen *et al*., 2020; Olefeld *et al*., 2020).

The chrysophyte distribution is considered to be ecologically determined mainly by temperature, pH and specific conductivity (Siver & Lott, 2012; Kristiansen & Škaloud, 2017), however the research is often focused on phototrophic chrysophytes with persistent scales, mainly from order Synurales (Siver, 1989; Siver & Hamer, 1989; Siver 1993). Furthermore, because these abiotic factors have long been preferred in studies, probably due to their ease of acquisition and overarching nature, biotic factors may in fact play an equally important role. According to comprehensive study by Bock *et al*.

(2020), the most important factors shaping the community patterns of chrysophytes on a European scale were biotic interactions, especially the co-occurrence of certain bacterioplankton groups. This finding fits perfectly into the story of the importance of mixotrophic life in the prevalence of chrysophyte taxa.

Colonial chrysophytes

Chrysophytes exhibit pronounced seasonal dynamic with vernal and autumnal maxima, but their populations may occur and dominate during any part of the year (Padisak *et al*., 1998; Bock *et al*., 2014; Kristiansen & Škaloud, 2017). In particular, the colonial chrysophytes *Dinobryon*, *Synura* and *Uroglena* are well known for their massive population booms (Fig. 1.4), which sometimes cause problems with freshwater management, well documented from Canada, Japan or Scandinavia (Kurata, 1989; Eloranta, 1994; Watson *et al*., 1996; Ishikawa *et al*., 2005). All these three genera also belong to a group of first described photosynthetic chrysophytes ever (Ehrenberg, 1834) while only few heterotrophic taxa (e.g. *Anthophysa*) were described a little earlier (summarized in Andersen *et al*., 1999).

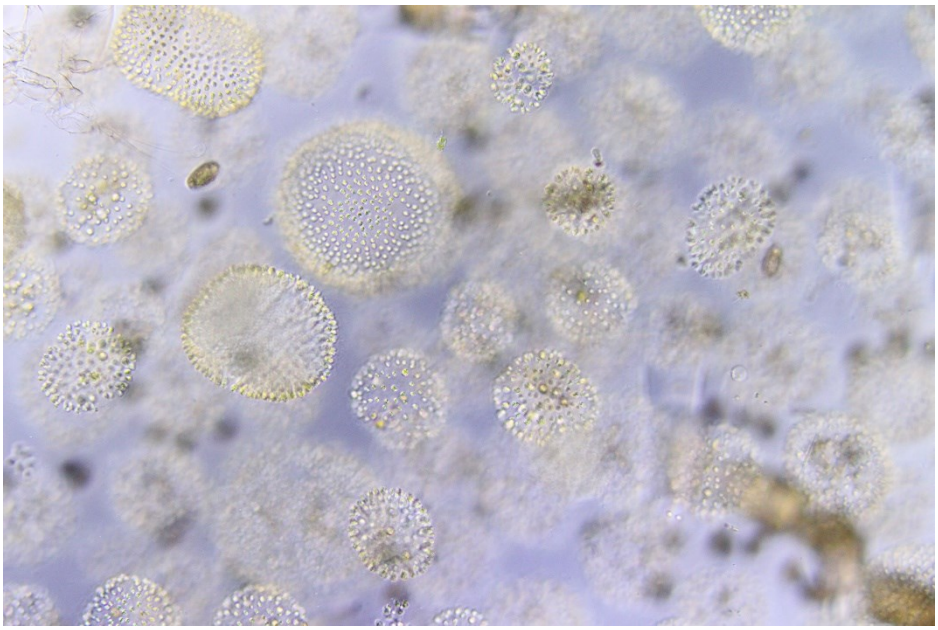

Fig. 4. *Uroglena* causing massive population boom in small spring pool, Botanical garden, Prague (photo taken by the author, 2016).

However, exact mechanisms and triggers of chrysophyte blooms are, despite years of research, still insufficiently known. It seems that they are species-specific and very complex, multi-factorial and highly interactive, requiring intersection of optimal biotic and abiotic factors (reviewed by Nicholls, 1995). Nevertheless, colonial chrysophyte blooms may be highly interconnected with subsequent density dependent sexual encystment process (Sandgren & Flanagin, 1986) since the encystment process typically takes place over a short period at the end of blooms (Agbeti & Smol, 1995).

Furthermore, living as a motile colony is one way to either reduce or avoid predation pressure and influence sinking losses, thereby optimizing resource acquisition (Lürling & Van Donk, 1996; Padisák *et al*., 2003, 2009). Colony formation further constitute important step in origin of (clonal) multicellularity, when size increase due to an accident, e.g., a mutation that prevents the daughter cells from separating (Bonner, 1998). Some chrysophyte taxa live in the form of a simple palmelloid thallus, but *Hydrurus* possess very specific, macroscopic bushy mucilaginous and multiple branched thalli, which resembles a seaweed. However, flagellates from orders Chromulinales, Ochromonadales and Synurales (e.g. *Chrysosphaerella*, *Synura* or *Uroglena*), which form spherical colonies with radially arranged cells, represent likely the most characteristic multi-celled chrysophytes (Fig. 5).

Similarly to green volvocine algae (Chlamydomonadales, Chlorophyceae) which have long been used as a model system for origin of multicellularity (review by Herron, 2016), colonial chrysophytes also possess highly variable colonial organization with body plans ranging from simple clumps of cells to spheroids with a different construction, but without or with yet undiscovered cellular differentiation. However, cellular dimorphism was recently observed in *Neotessella lapponica*, Synurales (Goldstein *et al*., 2015). Moreover, chrysophytes with spherical colonies resemble different stages of animal gastrulation process, or different stages according to the Haeckel's Gastraea hypothesis respectively (Haeckel, 1874), and encompass even blastula-like hollow colonies or gastrula-like colonies with invagination in *Uroglenopsis turfosa* [= *Eusphaerella turfosa*], Ochromonadales (Skuja, 1948). If some of the above mentioned chrysophytes really possess cellular differentiation, it could indicate that nearly all steps, except cell

junctions, leading to potential future incoming of complex multicellular organism parallel to hypothetic animal Precambrian transition (reviewed by Brunet & King, 2017, 2020) may have been achieved.

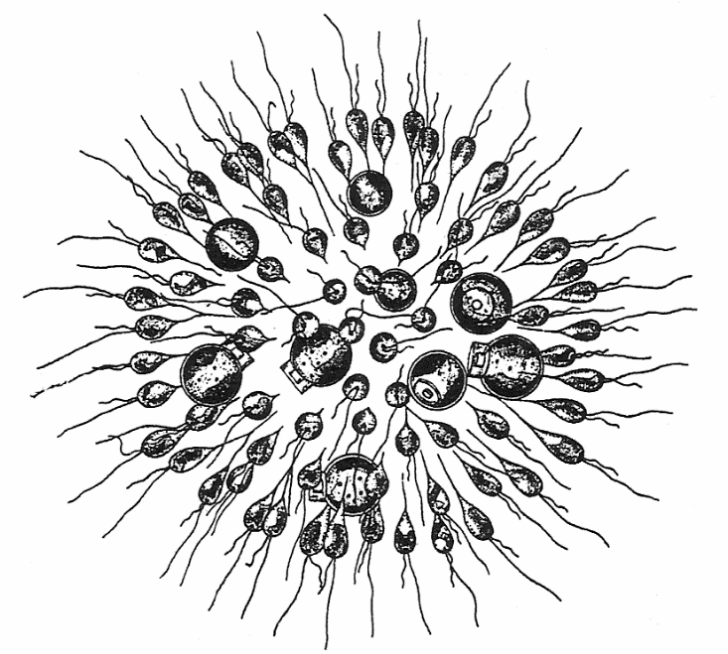

Fig. 5. Flagellates forming spherical colonies with radially arranged cells represent likely the most characteristic multi-celled chrysophytes, here *Uroglena* comprising plastid-bearing naked cells that are arranged as the surface monolayer (from Nygaard, 1945).

Diversity of chrysophytes

Our current knowledge of chrysophyte diversity is still mainly based on traditional morphospecies descriptions and the lasting problem is that sequence data exist for minority of all the described species. Taxonomy, identification and delineation of genera/species are hampered by the polyphyletic origin of several main morphotypes (e.g. *Chromulina* s.l., *Ochromonas* s.l., *Spumella* s.l. or *Uroglena* s.l.), by the low resolution of morphological features and by the potentially misleading clustering of some strains according to single gene phylogenies (Grossmann *et al*., 2016; Andersen *et al*., 2017; Bock *et al*., 2017). Consequently, the postulated taxonomic diversity certainly does not reflect the real species richness in Chrysophyceae. However, a few exceptions exist.

Silica-scaled chrysophytes, particularly Synurales and Paraphysomonadida, represent a group possessing relatively good species concept based on the ultrastructure of scales and bristles (Kristiansen & Preisig, 2007). These complex structures, which are formed of amorphous silica in silica deposition vesicles (SDVs), represent resistant imprints in time and space. Therefore, silica-scaled chrysophytes are among the most explored groups of protists in terms of their ecology and species richness. Studies on *Mallomonas matvienkoae* (Jo *et al*., 2013), *M. kalinae/rasilis* (Gusev *et al*., 2018), *Paraphysomonas vestita* (Scoble & Cavalier-Smith, 2014) and *Synura petersenii* (Kynčlová *et al*., 2010) and on loricated taxa such as *Dinobryon divergens* (Jost *et al*., 2010), however, clearly demasked common problematics of (pseudo)cryptic species within cosmopolitan and frequently found taxa.

Accordingly, these findings were clear impulses, which further accelerated their study. The diversity of Synurales represented mainly by the genera *Mallomonas* (Lavau *et al*., 1997; Jo *et al*., 2011, 2013; Siver *et al*., 2015; Čertnerová *et al*., 2019) and *Synura* (Boo *et al*., 2010; Kynčlová *et al*., 2010; Škaloud *et al*., 2012; Škaloud *et al*., 2014; Jo *et al*., 2016) has been further studied in a multidisciplinary approach providing a robust phylogenetic framework and good species-specific molecular, morphological and partly ecological congruent characters. In colonial *Synura*, however, every other study brought the discovery of new, often again (pseudo)cryptic, species. Furthermore, there still exist many taxa, even related genera, known on the basis of their morphology and ultrastructure only (e.g. *Chrysodidymus*). On the contrary, some lineages with only known sequences remain unresolved and wait for the future modern taxonomic treatment.

Among naked chrysophytes, *Kremastochrysopsis* (Remias *et al*., 2020), *Ochromonas*-like taxa bearing plastid (Andersen *et al*., 2017) and heterotrophic *Spumella*-like taxa (Findenig *et al*., 2010; Grossmann *et al*., 2016) have been evaluated using multidisciplinary approach with molecular techniques as a standard. *Ochromonas*-like and *Spumella*-like morphotypes represent 'prototypes' of a single-celled naked flagellate with a basic chrysophycean, or stramenopile, respectively, cell plan (two heterokont flagella), and as such they are scattered across the whole phylogenetic tree of

Chrysophyceae. Consequently, several new genera have been introduced (Findenig *et al*., 2010; Grossmann *et al*., 2016; Andersen *et al*., 2017) and many more will surely follow in the future.

Actual chrysophyte diversity is taxonomically treated according to Kristiansen & Škaloud (2017) as nine orders with major known diversity within mainly freshwater Ochromonadales and Synurales (Fig. 6). However, the strong evidence for hidden diversity and important role of especially picoplanktonic chrysophytes in marine ecosystems (del Campo & Massana, 2011; Lin *et al*., 2012; Kirkham *et al*., 2013) or bacteria feeding chrysophytes in soil (Boenigk *et al*., 2005; Scoble & Cavalier-Smith, 2014; Grossmann *et al*., 2016) has been shown recently. Even two new *Mallomonas* species, of the genus considered as the almost exclusively freshwater one (brackish occurrences summarized in Němcová *et al*., 2015), have been recently described from the marine environment (Jeong *et al*., 2019).

The exhaustive datamining in sequence databases by del Campo & Massana (2011) and their chrysophyte phylogeny encompassing all to date obtained environmental sequences brought very interesting results. First, about half of newly discovered marine and freshwater lineages belonged to Paraphysomonadida, a basal group of heterotrophic single-celled silica-scaled flagellates. They also discover several new clades, even order-level one sister to Hydrurales, formed exclusively by environmental sequences. Recently, it was shown that relatives of the mountain-river dwelling *Hydrurus* can unexpectedly settle other environments such as long-lasting slowly melting snow packs in both polar regions (Remias *et al*., 2013). They can cause a yellow snow similar to the other newly described but unrelated chrysophyte alga *Kremastochrysopsis austriaca* (Hibberdiales) from Alps (Remias *et al*., 2020).

Second, most environmental sequences were according to del Campo & Massana (2011) distant to sequences of cultured organisms, indicating a significant bias in the representation of taxa in culture. Such discrepancy is pretty well illustrated by a very common colonial *Uroglena* s.l. There are less than only 10 strains in the world's algal culture collections and only a few published sequences (or was in the beginning of our research). Scoble & Cavalier-Smith (2014) were facing same problem in their

comprehensive taxonomic revision of *Paraphysomonas* s.l. group and sold it out by establishing and studying of upper tens of newly acquired cultures. Accordingly, Paraphysomonadida now encompass at least three genera, *Clathromonas* (newly erected), *Paraphysomonas* and yet undescribed third genus overlapping with one of the original del Campo & Massana (2011) clades formed exclusively by environmental sequences, both marine and freshwater.

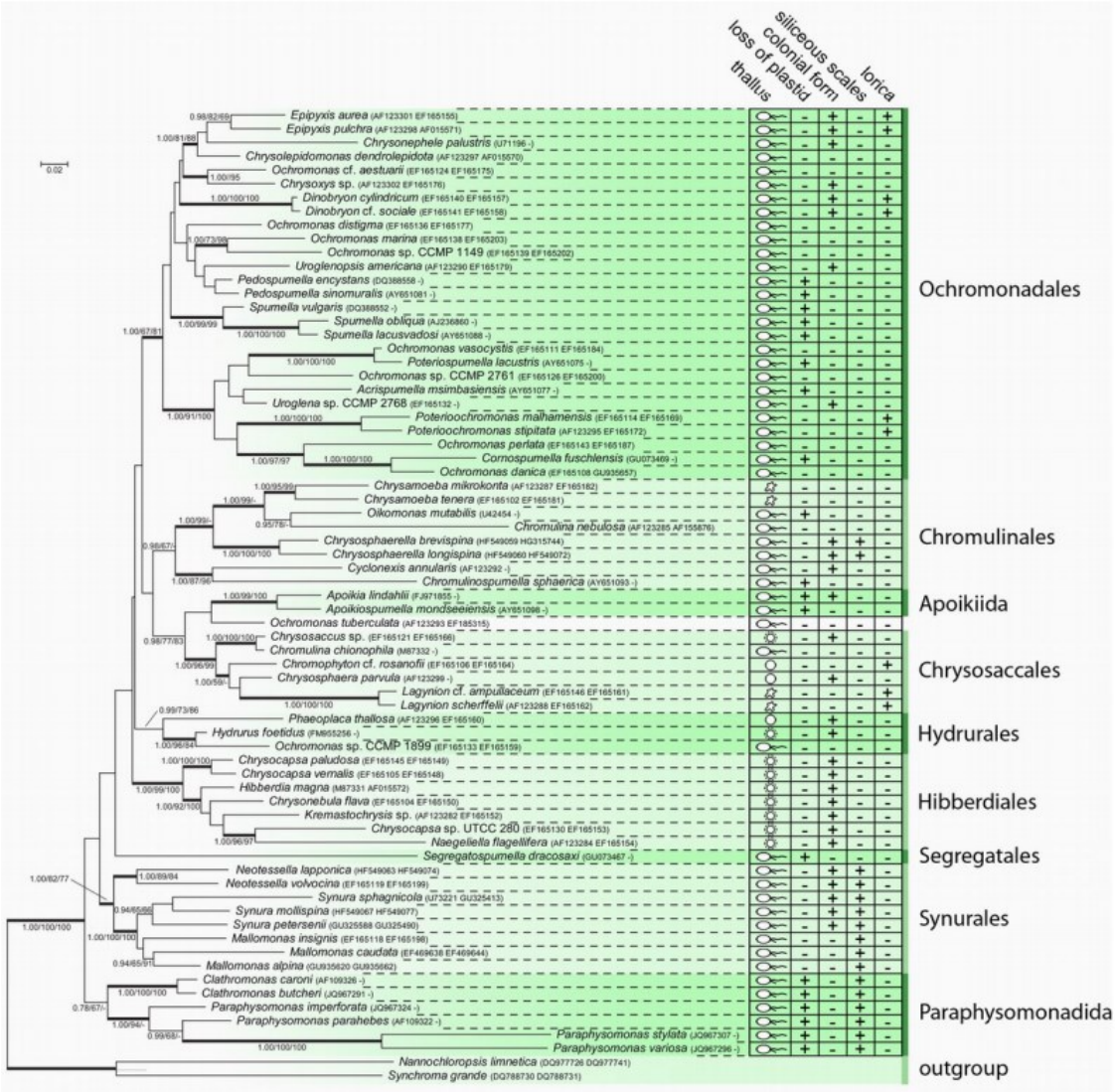

Fig. 6. Actual chrysophyte diversity is taxonomically treated as nine orders with major known diversity within mainly freshwater Ochromonadales and Synurales (from Kristiansen & Škaloud, 2017).

Similarly, even *Uroglena* sequences appeared in two non-sister clades (Andersen, 2007; del Campo & Massana, 2011) which suggests its polyphyly and gives a new impulse for its future modern taxonomic revision. So far, the main taxonomic long-standing problem was to find consensus on the presence/absence and nature of the system of dichotomously branched radial structures connecting cells in the *Uroglena* s.l. colony. Accordingly, most of taxonomists dealing with *Uroglena*-like flagellates did not recognize morphologically related genus *Uroglenopsis* Lemmermann (Skuja, 1948; Bourrelly, 1957; Starmach, 1986) while some others did (Pascher, 1913; Matvienko, 1965; Wujek & Thompson, 2002). This taxonomic topic has not been clearly resolved even in the most recent taxonomic revision (Wujek & Thompson, 2002), in which *Eusphaerella* was synonymized with *Uroglenopsis*. Some studies however indicate that *Uroglena* s.l. possess species-specific cyst (Skuja, 1948; Wujek & Thompson, 2002; Cronberg & Laugaste, 2005), which should by, under the auspices of phylogeny, starting point for its biodiversity review.

Research objectives of the thesis

I refer to the following PAPERS 1-4 as P1-4.

The main objective of this thesis was to provide a taxonomic revision of the widespread colonial chrysophytes *Synura* s.l. and *Uroglena* s.l. by a polyphasic approach. The silica-scaled chrysophytes are a long-term subject of research of our algological group, which I was happy to join (Němcová *et al*., 2013; Škaloud *et al*., 2013a; Němcová *et al*., 2016). Older research often built on premise that the genus *Synura* possess species-specific silica-scales. What is, however, the real proportion of above mentioned (pseudo)cryptic species within cosmopolitan and frequently found morphospecies? In addition, there are rare taxa whose relationship to the genus *Synura* is unknown despite the precise delimitation, mostly due to the absence of relevant genetic data. (e.g. monotypic two-celled *Chrysodidymus* with known scales).

Furthermore, some of very rare, enigmatic taxa possess a transient morphology between *Synura* and *Uroglena* (e.g. *Jaoniella* or *Syncrypta*/*Synuropsis* s.l.). However, due to the absence of any known DNA sequence, their true status remains unknown

(Kristiansen & Preisig, 2001), although some of them have been unconvincingly, only morphologically, revised (Wujek & Thompson, 2001). Contrary to *Synura*, taxa within *Uroglena*-like morphotype were never revised using molecular genetics techniques and their species concept and delimitations are vague. This is partly due to their "naked" character preventing proper morphological treatment and partly due to the likely difficult isolation and long-term cultivation of these fragile colonies (Wujek & Thompson, 2002), and subsequent lack of available cultures.

Specifically, I sought to answer the following questions:

What is the taxonomic status of the two-celled colonial monotypic genus *Chrysodidymus*? (P1) Does it really represent a separate transitional stage in the phylogeny of silica-scaled chrysophytes between the strictly unicellular genus *Mallomonas* and the strictly colonial genus *Synura*?

What is the evolutionary history of *Uroglena*-like flagellates? (P2 and 3) Do they represent sister lineages, or did this morphotype originate several times independently? What is the actual relevance of characters using for genera delimitation within these golden spherical colonials?

What is the real species diversity within the genera *Synura* and *Uroglena* s.l. in the light of a modern taxonomic revision? (P3 and 4) Which taxa described so far are "good species" and which represent (pseudo)cryptic species complexes or just a different expression of great phenotypic plasticity? What is the actual relevance of characters and approaches using for species delimitation within these golden spherical colonials?

To answer the above-outlined questions, I performed multipart taxonomic revision by a polyphasic approach. This included obtaining a relevant data encompassing molecular phylogeny (mainly nu ITS rDNA, nu SSU rDNA, nu LSU rDNA and pt rbcL sequences), morphology (SEM/TEM ultrastructure of silica cysts or scales, LM cell and colony features) and ecology (basic abiotic factors, habitat type, phenology). Sampling campaigns took place in Europe, North America and Japan throughout 2014–2020. The first and key step was to obtain sufficiently large number of isolates from diverse habitats, encompassing type localities, and covering broad range

of *Synura* s.l. (inc. *Chrysodidymus*) and *Uroglena* s.l. morphotypes. We proposed a combined methodology for parallel isolation (single-colony PCR plus short-term cultures) of naked and fragile *Uroglena*-like colonies to maximize future success for the molecular characterization of isolates (P2 and 3). This allowed us to overcome seemingly fundamental problem with difficult isolation and subsequent cultivation (and a general lack of available cultures and sequences).

Key results and conclusions

Together with my colleagues, I have successfully isolated colonies of broad morphological range covering *Synura* s.l. (inc. *Chrysodidymus synuroideus*), *Uroglena* s.l. (inc. *Uroglenopsis* s.s., *Eusphaerella turfosa*) and even some of their transitional forms. In total, more than 1,000 isolates of such colonial chrysophytes, gold-coloured flagellated spheres, were obtained after exploring of more than 650 various freshwater habitats including 19 type localities (Fig. 7). However, only every tenth locality hosted viable and detectable colonies of *Uroglena* s.l. while *Synura* colonies were found more often. Nevertheless, I have successfully recollected valuable material of four species from the type localities and habitats inspected – *U. americana*, *U. botrys*, *U. skujae* and *U. volvox*. I believe that these strains represent the authentic ones. *Uroglena volvox* (type species of *Uroglena* s.s.) and *Uroglenopsis americana* (type species of *Uroglenopsis* s.s.) were recollected after more than 180 and 120 years, respectively, from their original descriptions.

I acknowledge there cannot be the absolute certainty that the strains we obtained are exactly the same algae used by Ehrenberg (1834), Calkins (1892), Pascher (1913) and Matvienko (1965) for the original descriptions. However, using of microalgal or protist older type material from herbaria in the same way as it is in macroalgae (Hughey & Gabrielson, 2012) is in most cases impossible due to this type material is lost or cannot be used to extract DNA (e.g. from solely inorganic scale-cases or frustules). Therefore, the choice of material obtained from the original type localities and habitats is generally a procedure strongly recommended not only for epitypification (Hyde & Zhang 2008), as it maximizes the chances to obtain authentic material. In this way, Rindi *et al*. (2017)

have made epitypification of the green filamentous microalga *Klebsormidium flaccidum* based on new isolates from the original locality. Furthermore, in chrysophytes, Andersen *et al*. (2017) have successfully recollected the authentic material of *Ochromonas* type species, *O. triangulata*, for more than 120 years after its original description.

Fig. 7. Sampling campaigns took place in Europe, North America and Japan throughout 2014–2020. In total, more than 650 various freshwater habitats including 19 type localities were explored (visualisation of all sampling sites GPS coordinates by using Google My Maps, maps.google.com).

A colony, or there and back again

Our multigene phylogenetic analysis inferred *Chrysodidymus synuroideus* to be significantly nested deeply inside the genus *Synura* in a sister position to *S. sphagnicola* (P1), which was confirmed by subsequent studies (P4; Jadrná *et al*., 2021). This evidence led us to propose a new combination for this taxon – *Synura synuroidea* (Prowse) Pusztai *et al*., comb. nov. Bourrelly (1968) already pointed out the possible relationship between the two species. This sister position is further supported by several shared common features – relatively loose scale case, scales with simple perforated baseplate, characteristic apical scales with distinctly longer spines, presence of numerous linear or clavate scales on both flagella, frequent accumulation of red droplets in the cytoplasm

and similar ecology leading to their co-occurence (Hibberd, 1978; Nygaard, 1978; Graham *et al*., 1993; Kapustin & Gusev, 2016). We have also noticed that *S. sphagnicola* scales were present altogether with *S. synuroidea* scales within the same sample of our collections from Scotland (P1).

S. synuroidea is rarely found species with scattered distribution (Kristiansen & Preisig, 2007; Škaloud *et al*., 2013a). Most of the findings are made based only on the study of silica scales observed by EM and our study was first one linking direct observing of colonies with EM study and molecular data. Subsequent molecular studies on *S. sphagnicola* and related isolates (Škaloud *et al*., 2019) altogether with previous long-term *S. synuroidea* cultivations (Norris & Munch, 1970; Gerrath, 1974), however, support the thesis, that *S. synuroidea* is not just a two-celled curious ecomorph of otherwise multi-celled *Synura*.

Further, we clearly demonstrated that this two-celled taxon is not an intermediate evolutionary step between the single-celled *Mallomonas* and multicelled colonies of *Synura* (P1). Instead, we revealed the interesting case of evolutionary simplification in colonial organisms, which is generally considered much less common and usually associated with a significant ecological shift (Lewis & Flechtner, 2004). However, after examining a range of examples of secondary simplification and its consequences across the tree of life, O'Malley *et al*. (2016) stated in their review that the simplification has driven the diversification of many eukaryotic lineages, through the reduction of parts and even losses of hierarchical complexity. Known example is the independent loss of multicellularity in at least two yeast (Ascomycota) lineages (Cissé *et al*., 2013; Morel *et al*., 2015). Finally, I would like to notice that in phylogeny of Synurales, basal position is occupied by colonial genus *Neotessella* (Škaloud *et al*., 2013b). It is therefore easy to imagine that single-celled *Mallomonas* was such a first successful reverse transition from multi- to single-celled life in evolution of this order and *S. synuroidea* is the following much younger one, which has so far stopped at two cells.

In culture, two-celled colonies exhibited high degree of phenotypic plasticity and even three-celled (mitotic?) colonies were exceptionally observed (P1; Graham *et al*., 1993). Accordingly, colleagues from USA and Russia described two new closely related

two-celled species just few years later – *S. papillosa* and *S. prowseii* (Siver *et al*., 2017). All three species, however, favour dilute, shallow and highly acidic water bodies (e.g. sphagnum ponds and bogs). It is very likely that specific conditions of sites these species preferably inhabit may steer the evolution towards less colonial forms due to shift in nutrients availability and competitive/predation pressure.

Finally, a specific mode of movement is also associated with this two-celled colony arrangement. If colony of another *Synura* species disintegrates and only two cells remain, it will be still more or less irregularly rolling or tumbling. In contrast, *S. synuroidea* is always specifically back and forth moving (oscillate) along colony longitudinal axis (P1). Evidently, the flagellar apparatus had to be rebuilt in evolution. This is supported by EM study of Graham *et al*. (1993). They noticed the disappearance of short microtubules, which are in other Synurales taxa located in the region between proximal ends of the basal bodies. It is therefore more or less clear, that two-celled composition altogether with specific movement must have some meaning in nature. In other algae and protists, flagellar apparatus reconstructions are often associated with a change of nutritional mode or the transition from a free-living to a parasitic lifestyle (Okamoto & Keeling, 2014) or with changes in mitosis and scale deployment (McFadden & Wetherbee, 1984). However, until further specific tests are performed, we are still missing its exact significance in *S. synuroidea*.

Polyphyletic origin of a colonial morphotype

On the basis of phylogenetic analysis we showed that *Uroglena*-like colonial flagellates form at least three genetically distinct lineages within the Ochromonadales, Chrysophyceae (P2). All strains within *Uroglena* s.s. morphotype (encompassing type *U. volvox*) were recovered in a monophyletic single clade. Contrary, strains with *Uroglenopsis* morphology formed two distant clades further distinguished as *Uroglenopsis* s.s. (encompassing type *U. americana*) and a newly recognized lineage on the genus level. Finally, *Eusphaerella turfosa* was nested within *Uroglenopsis* s.s.

E. turfosa possess unique morphology with cells closely packed together, hexagonal in apical view and with a remarkable hole into colony (gastrula-like colonies

with invagination). We were therefore facing the typical 'lumpersplitter' problem (Darwin, 1857) – further split *Uroglenopsis* and conserve unique *Eusphaerella* as was done, for example, within a well-known *Hydrodyction/Pediastrum* group (Buchheim *et al*., 2005), or not? In culture, however, colonies lost their typical '*Eusphaerella*' morphology and became virtually indistinct from *Uroglenopsis* with loosely packed cells. Therefore, we decided to recognize *E. turfosa* as a member of the genus *Uroglenopsis* s.s. (P2) as it was already proposed by Wujek & Thompson, 2002.

We may only speculate on evolutionary drivers leading to production of characteristic gastrula-like compact colony with internal cavity in natural populations and its non-manifestation in culture. It would be very interesting to further explore and to test whether internal cavity could possess some virtually important functions (e.g. protection of inner reproductive cells, extracellular nutrient reservoir or culturing chamber for bacteria). And further, how many steps towards complex multicellularity have been achieved? It is very similar to the early hypothetical stage known from evolutionary models of animal Precambrian transition (Bonner, 1998; Nielsen, 2008; Niklas & Newman, 2013; Brunet & King, 2017).

All three genetically distinct genera exhibit a unique combination of morphological characteristics within chrysophytes (P2). Genera differ in cell shape (especially in cell posterior), flagellar length ratio and the character of the branched radial structures. Although cells are always arranged as the surface monolayer of the spherical colony, there are three different mechanisms by which the colony is built and the cells stick together. These different mechanisms were previously considered as a mere manifestation of the aging of colonies, i.e. young cells on thin threads and old cells on thick stalks (Conrad, 1938), which may eventually dissolve into a central mass of mucilage (Wujek & Thompson, 2002). However, none of these assumptions apply.

Strong evidence led us to describe a new genus – *Urostipulosphaera* Pusztai & Škaloud gen. nov. (P2). To avoid introduction of superfluous names, we carefully checked old descriptions of all colonial chrysophyte flagellates prior to proposing a new generic name (Starmach, 1985; Ikävalko, *et al*., 1994; Kristiansen *et al*., 2001; Kristiansen & Preisig, 2007). All of these taxa, unlike *Urostipulosphaera*, possess more or less pointed

posteriors that taper into a cytoplasmic thread, or they are embedded in a jelly mass. In other words, the invention of the colony through the joining of tapering cell posteriors or simply through cells embedded onto or in the gel has evolved more than once in the evolution of the chrysophytes, whereas the relatively thick articulated gelatinous stalks appear to be a unique feature for the newly recognized genus (P2 and 4).

Moreover, *Uroglena*, *Uroglenopsis* and *Urostipulosphaera* also differ in their ecological preferences and phenology in the northern temperate zone (P3). *Urostipulosphaera* often inhabits lowland habitats with higher pH and conductivity, such as nutrient-rich ponds, where it peaks in early spring waters with significantly lower temperature. Generally, in eutrophic waters, the seasonal distribution of chrysophytes may be restricted mainly to early spring (Kristiansen 1988). Conversely, *Uroglenopsis* prefers pristine habitats, such as drinking water reservoirs or lakes, usually situated in mountainous regions, and often among coniferous forests with lower pH and trophic states, as well as having a delayed start to the season compared with lowland ponds. *Uroglena* exhibits intermediate ecological preferences. Therefore, the usage of a single "U" codon for all *Uroglena*-like taxa in the established functional classification of the freshwater phytoplankton (Reynolds *et al*., 2002; Padisák *et al*., 2009) sounds problematic.

On the other hand, all three genera can be found in one location sharing the same planktonic habitat, but they still differ in their phenology and seasonal dynamics. This evokes the long-term core idea of plankton ecology, the paradox of the plankton (Hutchinson, 1961). Coexistence of many planktonic species was roughly conceptualised in the PEG model (Sommer *et al*., 1986; Sommer *et al*., 2012). However, changes in the chrysophyte community can take days. An example is the Vrah pond in the lowland pond system in Prague, Czech Republic (P3). For a half of April 2015, the plankton was dominated by *Urostipulosphaera notabilis*, but after just 14 days, *Urostipulosphaera* completely disappeared and the plankton began to be dominated by *Uroglena* sp. accompanied by a not so abundant *Uroglenopsis* sp. (own unpublished observations).

Finally, there remains several chrysophycean colonial enigmatic taxa with transient morphology between *Synura* (Synurales) and *Uroglena*-like taxa (Ochromonadales) prevalently known from Europe (Starmach, 1985). In the past, there

was an attempt to synonymize them into the single genus *Syncrypta* s.l. (sensu Bourrelly, 1957) or later *Synuropsis* s.l. (sensu Wujek & Thompson, 2001) despite the obvious discrepancy of important morphological features (presence of stigma, flagella length ratio). In our opinion, this synonymization is largely artificial. We can rely on our extensive dataset, which is based on examination and sequencing of thousands of samples hosting colonial chrysophytes from around the world (largely from Europe), where most of the observations of *Syncrypta* s.l. species come from (P1-5; Škaloud *et al*., 2012; Škaloud *et al*., 2013b; Škaloud *et al*., 2014; Jo *et al*., 2016; Jadrná *et al*., 2021).

In addition, we have had the opportunity to sequence many strange scale-less *Synura*-like and *Uroglena*-like taxa with unusual morphology, but never found any enigmatic unrelated sequence (except unique *Urostipulosphaera*). Therefore, *Syncrypta* s.l. or *Synuropsis* s.l. likely represent an artificial conglomerate largely consisting of atypical scale-less *Synura* spp. (almost equal flagella, no stigma) accompanied by atypical *Uroglena*-like taxa (heterokont flagella, stigma) and partly by scale-less forms of *Chrysopshaerella* and *Neotessella*, all of them living in insufficient stress conditions. Taxonomic remarks, that *Syncrypta* is no more than a temporary form of a *Synura* in mucilage and without scales, have been known for long time (Petersen & Hansen, 1956; reviewed in Wujek & Thompson, 2001). It was suggested by Kristiansen (1988b) to take type species, *Syncrypta volvox*, into synonymy with *Synura sphagnicola* according to EM works (Fott & Ludvík, 1957; Harris & Bradley, 1958). Similarly, we suggested to transfer some other species (see details in P2).

(Pseudo)cryptic species versus phenotypic plasticity

We clearly demonstrated there are evident and significant differences between natural and cultured populations of colonial chrysophytes or among populations of same species inhabiting different habitats (P1, 3 and 4). High degree of phenotypic plasticity observed in cultivated *Synura synuroidea* (P1) fully covered both species – *Chrysodidymus synuroideus* and *C. gracilis* originally described from the same locality (Prowse, 1962) and synonymized by us (P1). However, differences in cell shape and size

between algae grown in cultures and in field conditions are known from the first experiments with culturing (Andersen, 2005).

Specific "*Eusphaerella*" or poly-lobal morphology of some *Uroglenopsis* species was observed only for some populations, living in certain natural conditions, and disappeared in culture (P2 and 4). Moreover, populations of *Uroglenopsis botrys*, the most commonly observed species within the genus, were very diverse in shape of colonies and cells. It was even possible to assign different *U. botrys* populations to different previously described species (P3). We hypothesized these species represent only ecomorphs revealed by molecular phylogeny, which is further supported by observations of Wujek & Thompson (2002).

In phytoplankton, different ecomorphs production under different conditions (e.g. temperature, pH, light, predation pressure) is probably best explored for green algae (Chlorophyceae) forming cenobia (Trainor 1992; Lürling & Van Donk, 1999). In Chrysophyceae, studies are mainly focused on scale morphology of silica-scaled taxa (Němcová *et al*., 2010; Řezáčová-Škaloudová *et al*., 2010; Pichrtová & Němcová 2011; Němcová & Pichrtová, 2012). The plastic and variable shape of chrysophycean naked cells was the most striking when slides heated (during microscopic observations) and morpho-characters rapidly changed (P1, 3 and 4). Therefore, we would like to point out extremely propensity of naked chrysophytes to observational artefacts, which could play an important role in some older descriptions (summarized in P3).

However, sometimes everything is exactly the opposite. Many new (pseudo)cryptic species with a significant overlap in their morphology and ecology were recently revealed within *Synura petersenii* group mainly by molecular phylogenetics or by a combination of ultrastructural features, species-specific scale characteristics, observed in EM and statistically processed (Kynčlová *et al*., 2010; Škaloud *et al*., 2012; Škaloud *et al*., 2014; Jo *et al*., 2016). After more than 10 years of intensive research, we were still able to describe three new species just after several days of sampling at localities in a hitherto molecularly unexplored area (P4). Furthermore, it is very likely that the use of an otherwise species-specific ultrastructure of silica scales in taxonomy has its potential limits. For example, we revealed three different *S. leptorrhabda* lineages,

all of them carrying most likely identical scales (P4), which taxonomy must be further resolved. It very resemble the situation in diatom *Pinnularia borealis*, which is truly cryptic (Pinseel *et al*., 2019), in contrast to the majority of other diatom species complexes, which turned out to be pseudo-cryptic following detailed morphological analysis (Poulíčková *et al*., 2010).

Based on our molecular analyses and morphological observations, we assign all the previously described *Uroglena*-like taxa to newly recognized genera and propose a key to identification. Within these naked colonials, *Uroglena* and *Urostipulosphaera* species are well defined by the ultrastructure of their species-specific stomatocysts (P2 and 4). Despite the fact, that cysts are used for species identification and for the inferring of past climate conditions, only minority of stomatocysts described so far are reliably linked with vegetative stages using modern identification standards employing SEM and/or molecular phylogeny (Findenig *et al*., 2010; Piątek *et al*., 2020). It is, however, very likely that even usage of otherwise species-specific ultrastructure of cysts in taxonomy has again its limits. For example, we revealed several different *U. zachariasii* s.l. lineages, all of them carrying almost identical cysts (P3), which taxonomy and (pseudo)cryptic character must be further resolved. Contrary, in *Uroglenopsis* species are defined by the cell and colony characteristics as it cysts are probably invariant (P3).

In summary, it is obvious that significant part of chrysophyte, or protist respectively, diversity is still uncovered (Pawlowski *et al*., 2012) and new taxa are continuously described even among long-term studied genera with a robust taxonomy and species concept, e.g. silica-scaled chrysophytes (Gusev *et al*., 2019; Kapustin *et al*., 2019; Němcová & Kapustin, 2019). As was stated in the introduction, one of the main problems and challenges in biology is the persistent incompleteness of reference DNA databases due to the lack of molecular data in numerous morphologically described species (Leray and Knowlton, 2015). However, molecular and morphological data are mutually reinforcing, both are needed for evaluating diversity of *Mallomonas* (Jo *et al*., 2013; Gusev *et al*., 2018), *Paraphysomonas* (Scoble & Cavalier-Smith, 2014), *Synura* (P1 and 5) or *Uroglena*-like flagellates (P2 and 4).

In our last case study (P4), only one third of identified silica-scaled species was recorded by both molecular and morphological techniques, showing both approaches are complementary in estimating species diversity within this genus. Our current overall work connects the comprehensive pool of older knowledge on diversity of *Synura*-like and *Uroglena*-like colonial chrysophytes (traditionally defined morphospecies) with modern approaches, and brings graspable species-specific morphological characters in a robust phylogenetic framework, useful and essential for future challenging studies in the field. Therefore, according to Boenigk *et al*. (2012), we strongly recommend focusing on more than one line of evidence for correct species delimitation, whether it is naked flagellate of silica-scaled taxa. This must always include combination of a robust multigene phylogeny, which is an essential standard, and clear morphological and/or ecological evidence.

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PAPER I

Martin Pusztai, Dora Čertnerová, Magda Škaloudová & Pavel Škaloud

Elucidating the phylogeny and taxonomic position of the genus *Chrysodidymus* Prowse (Chrysophyceae, Synurales)

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Elucidating the phylogeny and taxonomic position of the genus *Chrysodidymus* Prowse (Chrysophyceae, Synurales)

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Abstract

Chrysodidymus represents a monotypic genus of silica-scaled chrysophytes, with well characterised morphology and ultrastructure, as well as pretty known ecology. However, the taxonomic status of this genus remains ambiguous due to the absence of relevant sequence data. In this study, we have aimed to genetically characterize a newly established *C. synuroideus* culture to elucidate the taxonomy of *Chrysodidymus*. Our multigene SSU rDNA + LSU rDNA + rbcL phylogenetic analysis inferred *C. synuroideus* to be significantly nested deeply inside the genus *Synura*. This convincing evidence led us to propose a new combination for this taxon - *Synura synuroidea* (Prowse) Pusztai *et al*., comb. nov.

Keywords: *Chrysodidymus synuroideus*, Chrysophyceae, multigene phylogeny, Silica-scaled chrysophytes, *Synura synuroidea* comb. nov., Synurales, taxonomy

Introduction

Chrysophytes (Chrysophyceae, Stramenopiles) represent a monophyletic group (Yang *et al*., 2012) of predominantly freshwater microalgae. They often dominate the phytoplankton of the oligotrophic temperate lentic ecosystems (Nicholls, 1995). However, the evidence for undiscovered diversity and important role of especially picoplanktonic chrysophytes in marine ecosystems has been shown recently (del Campo & Massana, 2011; Kirkham *et al*., 2013).

Chrysophyte taxa bearing silica scales constitute a group possessing relatively good species concept based on the ultrastructure of scales and bristles (Kristiansen & Preisig, 2007). These complex structures, which are formed of amorphous silica in silica deposition vesicles (SDVs), represent resistant imprints in time and space. Therefore, silica-scaled chrysophytes are among the most explored groups of chrysophytes in terms of their ecology and species richness. Interestingly, the production of silica scales evolved at least three times during the evolution of Chrysophyceae, with the vast of scale-bearing organisms occurring in two unrelated orders: Paraphysomonadida and Synurales (Škaloud *et al*., 2013a; Scoble & Cavalier-Smith, 2014).

In a recent taxonomic treatment (Kristiansen & Preisig, 2007), the Synurales comprise five well-recognized genera as well as the highly dubious *Jaoniella* Skvortzov and *Pseudosyncrypta* Kisselev. Besides *Mallomonas* Perty, *Synura* Ehrenberg and *Neotessella* (Playfair) B.Y. Jo, J.I. Kim, W. Shin, P. Škaloud & P. Siver belonging to molecularly well-defined genera, taxonomic position of *Chrysodidymus* Prowse and *Conradiella* Pascher remains unresolved (Škaloud *et al*., 2013a). In the case of the enigmatic genus *Conradiella* there is suspicion that it represents a species of *Mallomonas* (Kristiansen, 1988a; Kristiansen & Preisig, 2007). Conversely, *Chrysodidymus* has a well characterised morphology and ultrastructure (Wujek & Wee, 1983; Graham *et al*., 1993), a distinctive ecology, and a wide distribution (Kristiansen & Preisig, 2007; Škaloud *et al*., 2013b). This flagellate is free-living and autotrophic. Typically it forms two-celled "stretched out sausages-like" colonies where the cells are united at their broad posterior bases. The colony swims back and worth along its longitudinal axis, yielding a very characteristic swimming behavior. Cells are covered with a number of small imbricate

plate-shaped scales each with a short apical spine. Each cell contains two golden brown plastids without pyrenoids and bears two unequal flagella covered by linear or clavate scales. *Chrysodidymus* is an acidophilic alga with a cosmopolitan, but scattered distribution.

The genus was erected by Prowse from Malayan acid swamps as "*Chrysodidyma*" (Prowse, 1960) and validly described two years later (Prowse, 1962). However, the description was made without any illustrations of the siliceous scales. Originally, *Chrysodidymus* encompassed two distinct species – *C. synuroideus* Prowse and *C. gracilis* Prowse differing in cell shape and size. Later, these two taxa were synonymized based on the high degree of phenotypic plasticity observed within a single *C. synuroideus* colony covering morphological characteristics of both species (Wujek & Wee, 1983; Kristiansen & Preisig, 2007). The first electron micrographs of *C. synuroideus* scales and scale-case were published just ten years after its original description, based on the collections from Canada (Puytorac *et al*., 1972). In 2000, *Chrysodidymus synuroideus* became the first photosynthetic stramenopile where a complete mitochondrial genome sequence was recovered (Chesnick *et al*., 2000). Unfortunately, the original culture deposited in the UTEX Algal Culture Collection, Austin TX, USA (LB 2713) has not survived. Moreover, no sequences of the generally used nuclear- or plastid-encoded molecular markers have been obtained to date, and consequently the taxonomy of this "golden-twins" microalga has not been elucidated correctly.

The general goal of this study was to establish a culture of *Chrysodidymus synuroideus*, characterize its genetic makeup, and resolve its taxonomic status within the Chrysophyceae.

Material and Methods

Collection, isolation and cultivation of a Chrysodidymus *strain*

On September 19th 2012 the samples of phytoplankton containing *Chrysodidymus* were collected from a small, unnamed lake near the Loch Garten in the Grampian Mountains, Scotland (57° 13′ 32.55″ N, 3° 43′ 20.71″ W). A plankton net with 20μm mesh was used. Standard measurement of abiotic factors on the sampling site encompassing

water temperature: 11.0 °C, pH: 5.9 and specific conductivity: 27 μS cm-1 was carried out using a combined pH/conductometer (WTW 340i; WTW GmbH, Weilheim, Germany). Collected samples were kept in a polystyrene box containing cooling gel pad during the sampling day. Samples were examined with an Olympus CX 31 light microscope and *Chrysodidymus* colonies were isolated immediately after returning to a research base. In the effort to establish uni-algal cultures, the individual colonies were isolated by micropipetting. Each colony was washed 3-5 times with distilled water to minimize the risk of contamination. Finally, the colony was placed into a separate well of a 96-well polypropylene plate. Each well was filled with approximately 400 μl of MES-buffered DY IV liquid medium (pH ≈ 6; Andersen *et al*., 1997). The well plates were transported to the lab safely stored in a fridge bag (TK 51, Ardes SpA, Ponte Nossa, Italy). Climatic conditions in a fridge bag were maintained as approximately 15°C and constant illumination of 50–200 μmol m−2 s−1 provided by 6 W LED diodes (LB115A-6W-X, Yuyao Lianliang Electric Appliance Co Ltd, Ningbo, China). In the laboratory, the uni-algal "pre-cultures" were transferred from wells into 50 ml Erlenmeyer flasks filled with the same MES-buffered DY IV liquid medium (pH ≈ 6). Thereafter they were cultivated in cooling box (C5G, Helkama Oy, Helsinki, Finland) at 15°C, under the permanent illumination of 40 μmol m−2 s−1 (TLD 18W/33 fluorescent lamps, Philips, Amsterdam, the Netherlands). The cultures were periodically checked and reinoculated into fresh medium as necessary.

Morphological investigations

Chrysodidymus strains were determined based on the species specific scales using transmission electron microscopy (TEM). Samples from each culture were dropped onto Formvar-coated copper grids. Grids were dried, rinsed in 5 drops of distilled water, dried, and examined with a JEOL 1011 transmission electron microscope equipped by CCD camera Veleta with acquisition software (Olympus Soft Imaging Solution GmbH, Müenster, Germany). Morphological diversity of two-celled colonies from cultures with different age and condition was investigated in detail using Olympus BX 51 light microscope equipped by Nomarski interference contrast.

Sequencing and phylogenetic analysis

DNA isolations were carried out as described in Škaloudová & Škaloud (2013). Three molecular markers were amplified by PCR: nuclear SSU rDNA, nuclear LSU rDNA and plastidial rbcL. The amplification of SSU rDNA was performed as described by Škaloud *et al*. (2013a), using the primers 18S-F and 18S-R (Katana *et al*. 2001) and 528F (Montresor *et al*., 2004). The amplification of LSU rDNA was performed as described by Jo *et al*. (2011), using the primers 28S_25F, 28S_861R and 28S_2160R (Jo *et al*., 2011). Additionally, new primers 28S_732F2 (5′-CCC GAA AGA TGG TGA ACT-3′) and 28S_1435R (5′-GTT CAC ATG GAA CCT TTC TCT AC-3′) were designed for this study using the Primer3 software (Untergasser *et al*. 2007). The amplification of the rbcL marker was performed using newly designed primers S_IF (5′-GTT TAT GAA GGA TTA AAA GGT GG-3′) and S_IR (5′-GAC ATT CTC ATC CAT TTA CAA AT-3′). The PCR products were purified and sequenced at Macrogen Inc. in Seoul, Korea.

The newly determined sequences were aligned to other sequences from the GenBank database. The GenBank accession numbers of all strains used in this study are provided in Table 1. A concatenated SSU rDNA, LSU rDNA, and rbcL alignment was produced, including a total of 43 sequences of Synurales taxa. The sequences were aligned using MAFFT v. 6 software (Katoh *et al*. 2002) under the Q-INS-I strategy, and checked for obvious sequencing errors. For each of the alignment partitions, the most appropriate substitution model was estimated using the Bayesian information criterion (BIC) as implemented in jModelTest 2.1.4 (Darriba *et al*. 2012). This BIC-based model selection procedure selected the following models: (1) TIM2 + I + Γ for SSU rDNA, (2) GTR + I + Γ for LSU rDNA and the first codon position of the rbcL gene, (3) TPM3 + I for the second codon position of the rbcL gene, and (4) TIM3 + I + Γ for the third codon position of the rbcL gene. The phylogenetic tree was inferred by Bayesian inference (BI) using MrBayes version 3.2.1 (Ronquist *et al*. 2012). The analysis was carried out on partitioned datasets using the substitution models best matching those selected by jModelTest 2.1.4. All parameters were unlinked among partitions. Two parallel MCMC runs were carried out for eight million generations, each with one cold and three heated

chains. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was assessed during the run by calculating the average standard deviation of split frequencies (SDSF). The SDSF value was 0.0013. Finally, the burn-in value was determined using the 'sump' command. Bootstrap analyses were performed by maximum likelihood (ML) and weighted maximum parsimony (wMP) criteria using GARLI, version 2.01 (Zwickl 2006) and PAUP*, version 4.0b10 (Swofford 2002), respectively. ML analyses consisted of rapid heuristic searches (100 pseudo-replicates) using automatic termination (genthreshfortopoterm command set to 100,000). The analysis was performed on partitioned datasets using the different substitution models selected by jModelTest 2.1.4. The wMP bootstrapping (1,000 pseudo-replicates) was performed using heuristic searches with 100 random sequence addition replicates, tree bisection reconnection swapping, random addition of sequences, and gap characters treated as missing data. Character weights were assigned using the rescaled consistency index on a scale of 0 to 1,000. New weights were based on the mean fit values for each character over all trees in the memory.

Results

Morphology, ultrastructure and molecular systematics

A novel strain of *Chrysodidymus* S95.E4 was successfully isolated from a sampled material, and a uni-algal culture for long-term cultivation was established. The strain was determined as *C. synuroideus* based on ultrastructure of scales and the scale-case (Figs. 1-4). Small elliptical scales bearing an apical spine (0.4-1.5 μm) were 1.5-2.0 μm long and 0.6-0.9 μm broad. Two-celled colonies exhibited high degree of phenotypic plasticity (Figs. 5-16). There were colonies consisted of bigger elongated cells through trapezoid, pyriform, ellipsoidal or ovoid cells to smaller almost spherical cells presented in the cultures of different age and condition. Cells were 10.0-27.0 μm long and 6.5-16.0 μm broad. Three-celled colonies were exceptionally presented (Figs. 17, 18). Multigene phylogenetic analysis based on three molecular markers (nuclear SSU rDNA, nuclear LSU rDNA, plastidial rbcL) clearly demonstrated the phylogenetic position of *C. synuroideus* lying deeply inside the genus *Synura* (Fig. 19). *Chrysodidymus synuroideus*

was inferred in a sister position to the clade composed of two *Synura sphagnicola* (Korshikov) Korshikov strains, within the statistically well supported monophyletic clade additionally including *S. curtispina* (J.B. Petersen & J.B. Hansen) Asmund, *S. longitubularis* B.Y. Jo, W. Shin, J.I. Kim & P. Siver, *S. mollispina* (J.B. Petersen & J.B. Hansen) Péterfi & Momeu and *S. spinosa* Korshikov. We therefore propose a new combination for this taxon - *Synura synuroidea* (Prowse) Pusztai, Čertnerová, Škaloudová & Škaloud, comb. nov.

Taxonomic conclusion

***Synura synuroidea* (Prowse) Pusztai, Čertnerová, Škaloudová & Škaloud, comb. nov. (Figs. 1-18)**

Basionym: *Chrysodidymus synuroideus* Prowse 1962, in Garden Bull., Singapore, 19: 128-129, Plate IV, fig. n. Type locality: Malacca - in acid swamps, Malaya

Synonyms: *Chrysodidymus gracilis* Prowse (1962: 128); *Synura microcrepis* Nygaard (1978: 200)

Reference strain: The live culture of strain S95.E4 has been deposited as CAUP B712 in the Culture Collection of Algae of Charles University in Prague, Czech Republic (http://botany.natur.cuni.cz/algo/caup.html).

Discussion

On the basis of colony character, cell morphology and scale ultrastructure provided by Puytorac *et al*. (1972) our isolated strain distinctly belongs to the description of the *Chrysodidymus synuroideus* Prowse. High degree of phenotypic plasticity exhibited by our strain (e.g. from bigger elongated cells to smaller almost spherical cells) is in agreement with previous observations (Wujek & Wee, 1983; Graham *et al*., 1993; Khondker *et al*., 2007). This plasticity can be detected in a natural sample as well within a cultured strain. Stress conditions and maturity of cells seem to be the common denominator that makes the plasticity noticeable. In the same sample from Bangladesh, Khondker *et al*. (2007) observed *C. synuroideus* colonies composed of typically elongated cells as well as of smaller ellipsoidal ones corresponding to description of already

synonymized *C. gracilis*. It resembles the situation of the original simultaneous erection of both species by Prowse from the same locality, "Malacca – in acid swamps". Wujek & Wee (1983) reported the same morphological divergence as a product of stress caused progressive changes in *Chrysodidymus* cells shape during microscopic observation. They suggested merging the two former species into a single valid species *C. synuroideus* on the basis of priority. Moreover, Graham *et al*. (1993) revealed that colonies of typically elongated cells are more mature, while colonies of smaller oval cells represent more recently divided cells. They further reported a smooth transition between these two frequently mentioned morphotypes which is in concordance with our findings. We therefore agree with the presumption, that Prowse observed only phenotypic plasticity within the single species. Notwithstanding the above mentioned, on the basis of subtle variations in scales ultrastructure between temperate and (sub)tropic populations of *C. synuroideus*, Kapustin a Gusev (2016) suggested that there could be more than one species within the genus *Chrysodidymus*. However, a comparative molecular and morphological investigation of several isolated strains is needed to decipher the real species diversity within this genus.

The ultrastructural analogy of *Synura* and *Chrysodidymus* silica scales led several authors to consider their close taxonomic relationship (Bourrelly, 1968; Nicholls & Gerrath, 1985; Graham *et al*., 1993). Nygaard (1978) even described *C. synuroideus* as a distinct species of *Synura*, *S. microcrepis*, although in "appendix" he mentioned a question regarding the synonymy of these taxa. According to Nicholls & Gerrath (1985), the principal differences between *Chrysodidymus* and *Synura* comprise colony formation and movement characteristics. In *Synura*, the colonies are generally multi-celled, although two-celled young stages or colony fragments with irregular tumbling can be seen, as well. On the contrary, *Chrysodidymus* consistently forms two-celled colonies in both natural conditions and laboratory cultures, rarely forming three-celled, probably mitotic stages (Norris & Munch, 1970; Gerrath, 1974; Graham *et al*., 1993; own observation). Therefore, the main distinguishing feature remains a little bit peculiar back and worth moving along colony longitudinal axis in *C. synuroideus*.

Nevertheless, our multigene phylogenetic analysis clearly demonstrated that the *C. synuroideus* is a member of the genus *Synura*, forming a distinct clade together with *S. sphagnicola* (Fig. 19). This position is furthermore supported by the fact that these two taxa share several common features (Hibberd, 1978; Graham *et al*., 1993). First, both species bear relatively loose scale case consisting of scales with very similar simple perforated baseplate, although the scales are distinct in the number and size. Second, both species form characteristic apical scales with distinctly longer spines. Furthermore, *C. synuroideus* and *S. sphagnicola* share the presence of numerous linear or clavate scales on both flagella (Hibberd, 1978; Graham *et al*., 1993). Another common feature is the frequent accumulation of red droplets in the cytoplasm. Finally, both species exhibit a similar ecology are often found together, and are reported from freshwater sites with low pH, including sphagnum ponds and bogs (Nygaard, 1979; Škaloud *et al*., 2013b). We have also noticed that *S. sphagnicola* scales were present altogether with *C. synuroideus* scales within the same sample of our collections from Scotland.

The above-mentioned, well supported observations warrant to place the genus *Chrysodidymus* into synonymy with the genus *Synura*. Therefore, we propose new combination *Synura synuroidea* (Prowse) Pusztai, Čertnerová, Škaloudová & Škaloud, comb. nov. We clearly demonstrated that this two-celled taxon is not an intermediate evolutionary step between the single-celled *Mallomonas* and multi-celled colonies of *Synura*. Instead, we revealed the interesting case of evolutionary simplification in colonial organisms. Coloniality in microalgae is usually perceived as an evolutionary innovation that increases protection from predation or improve acquisition of resources (Lürling & Van Donk, 1996; Siver & Trainor, 1981). Independent origins of coloniality have been revealed in many algal lineages, including green algae (Herron *et al*., 2009), chrysophytes (Němcová & Pichrtová, 2009), diatoms (Yamaoka *et al*., 2016) or dinoflagellates (Matsuoka & Fukuyo, 1986). However, colony simplification is much less common, usually associated with a significant ecological shift, e.g., a transition from the aquatic to the terrestrial biotope (Lewis & Flechtner, 2004). We may only speculate on evolutionary drivers of colony simplification in *S. synuroidea*. For example, acidic

conditions of sites this species strictly inhabits may steer the evolution towards less colonial forms due to shift in nutrients availability and predation pressure.

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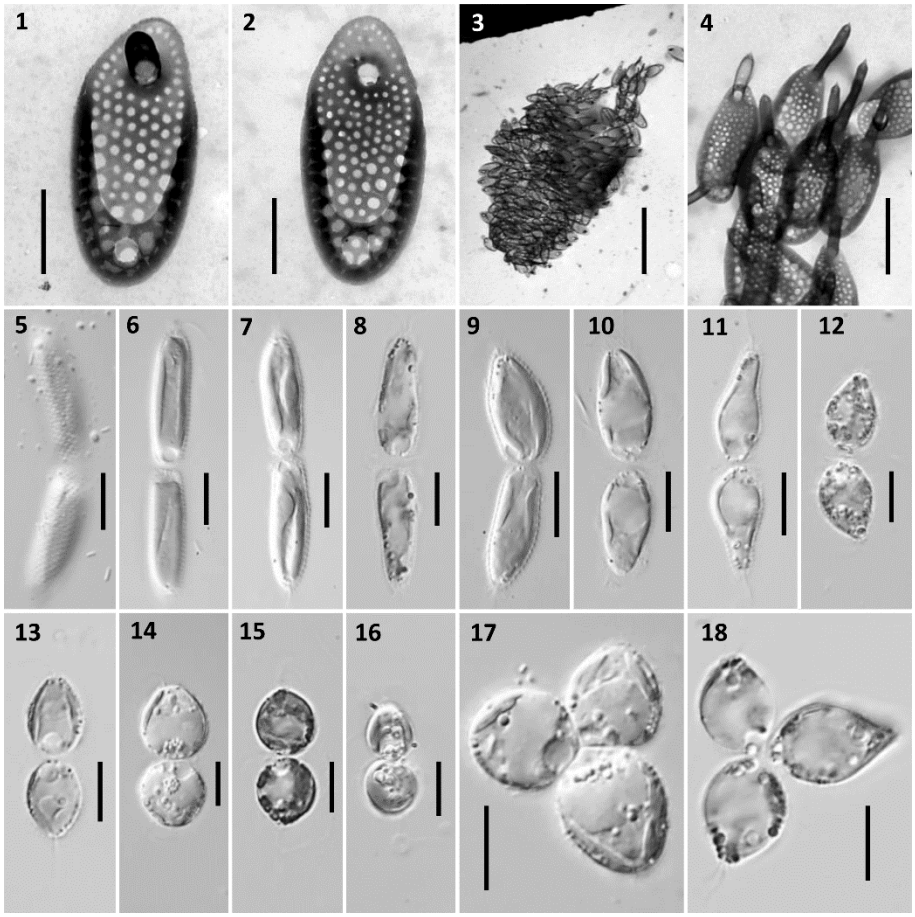
Table 1. Specific names, strain numbers and GenBank accession numbers of the Synurales taxa used in this study.

Taxon	Strain	GenBank accession numbers		
		SSU rDNA	LSU rDNA	rbcL
Chrysodidymus synuroideus Prowse	S 95.E5	KX815882	KX815883	KX815884
Mallomonas acaroides Perty	SYJMAc	JX946333	JX946341	JX946349
Mallomonas akrokomos Ruttner	Posan012608J	GU935625	GU935647	GU935667
Mallomonas caudata Ivanov	Dangje060207A	GU935629	GU935651	GU935671
Mallomonas heterospina Lund	Posan012608A	GU935617	GU935639	GU935659
Mallomonas insignis Penard	Beopsu033107D	GU935634	GU935656	GU935676
Mallomonas matvienkoae Asmund & Kristiansen	Muryeong112807B	GU935628	GU935650	GU935670
Mallomonas punctifera Korshikov	Angumal032010C	JQ955667	JQ955672	JQ955662
Neotessella lapponica (Skuja) Jo *et al*.	S 59.C4	HF549063	-	HF549074
Neotessella volvocina (Playfair) Jo *et al*.	CCMP 1782	EF165119	-	EF165199
Synura americana Kynčlová & Škaloud	CCMP 862	GU325582	-	GU325485
Synura americana Kynčlová & Škaloud	Johae010508F	JX455151	JX455155	JX455147
Synura asmundiae (Cronberg & Kristiansen) Škaloud *et al*.	S 90.D10	HF549069	-	HF549079
Synura bjoerkii (Cronberg & Kristiansen) Škaloud *et al*.	SC 57.A6	HF549070	-	HF549080
Synura conopea Kynčlová & Škaloud	NIES 1007	GU325578	-	GU325479
Synura conopea Kynčlová & Škaloud	CCMP 859	GU325580	-	GU325482
Synura curtispina (Petersen & Hansen) Asmund	SAG 29.92	GU325515	-	GU325415
Synura echinulata Korshikov	SAG 15.92	GU325513	-	GU325414
Synura glabra Korshikov	NIES 233	GU325577	-	GU325480
Synura glabra Korshikov	Dohak111107C	JX455149	JX455153	JX455145
Synura heteropora Škaloud *et al*.	WA18K_U	GU325597	-	GU325499
Synura heteropora Škaloud *et al*.	CCMP 2898	GU325596	-	GU325498
Synura longitubularis Jo *et al*.	Jeongsan070607A	KM590580	KM590646	KM590867
Synura macracantha (Petersen & Hansen) Asmund	S 90.B5	HF549064	-	HF549075
Synura mammillosa Takahashi	S89C3	HF549066	KM590652	-
Synura mammillosa Takahashi	SIE105A	HF549065	KM590654	HF549076
Synura mollispina (Petersen & Hansen) Péterfi & Momeu	S 71.C10	HF549067	-	HF549077
Synura multidentata (Balonov & Kuzmin) Péterfi & Momeu	S 90.C11	HF549068	-	HF549078
Synura petersenii Korshikov	KNU 09	GU325525	-	GU325426

Synura petersenii Korshikov	Youngji101407A	JX455150	JX455154	JX455146
Synura soroconopea Jo *et al*.	CNU 01	GU325530	-	GU325431
Synura sp.	CCMP 847	EF165128	-	EF165196
Synura sp.	CCAC 0052	GU325606	-	GU325508
Synura sp.	CCMP 869	GU325587	-	GU325489
Synura sp.	UTEX LB 239	GU325591	-	GU325493
Synura sphagnicola (Korshikov) Korshikov	CCMP 1705	U73221	-	EF165197
Synura sphagnicola (Korshikov) Korshikov	JYS001	DQ980485	DQ980475	-
Synura spinosa Korshikov	S 74.D2	-	-	HF549081
Synura splendida Korshikov	S 90.E4	HF549071	-	HF549082
Synura truttae (Siver) Škaloud & Kynčlová	Nemcova 2	GU325598	-	GU325500
Synura truttae (Siver) Škaloud & Kynčlová	Nemcova D5	GU325600	-	GU325502
Synura uvella Ehrenberg	CNU 53	GU325514	-	GU325416
Synura uvella Ehrenberg	CCMP 871	U73222	-	EF165192



Figs 1-18. Morphology and ultrastructure of *Synura synuroidea* ('Chrysodidymus synuroideus'); Figs 1-4: TEM; Figs 5-18: LM. **1-2.** Body scales with different pattern of base-plate pores. **3.** Whole scale case. **4**. Apical scales with distinctly longer spines. **5.** Arrangement of scales on the cell surface. **6-16.** Phenotypic plasticity of two-celled colonies in the cultures of different age and condition. **17-18.** Rare three-celled colonies. Scale bars represent: 0.5 μm (Figs 1-2), 5 μm (Fig 3), 1 μm (Fig 4), 10 μm (Figs 5-18).

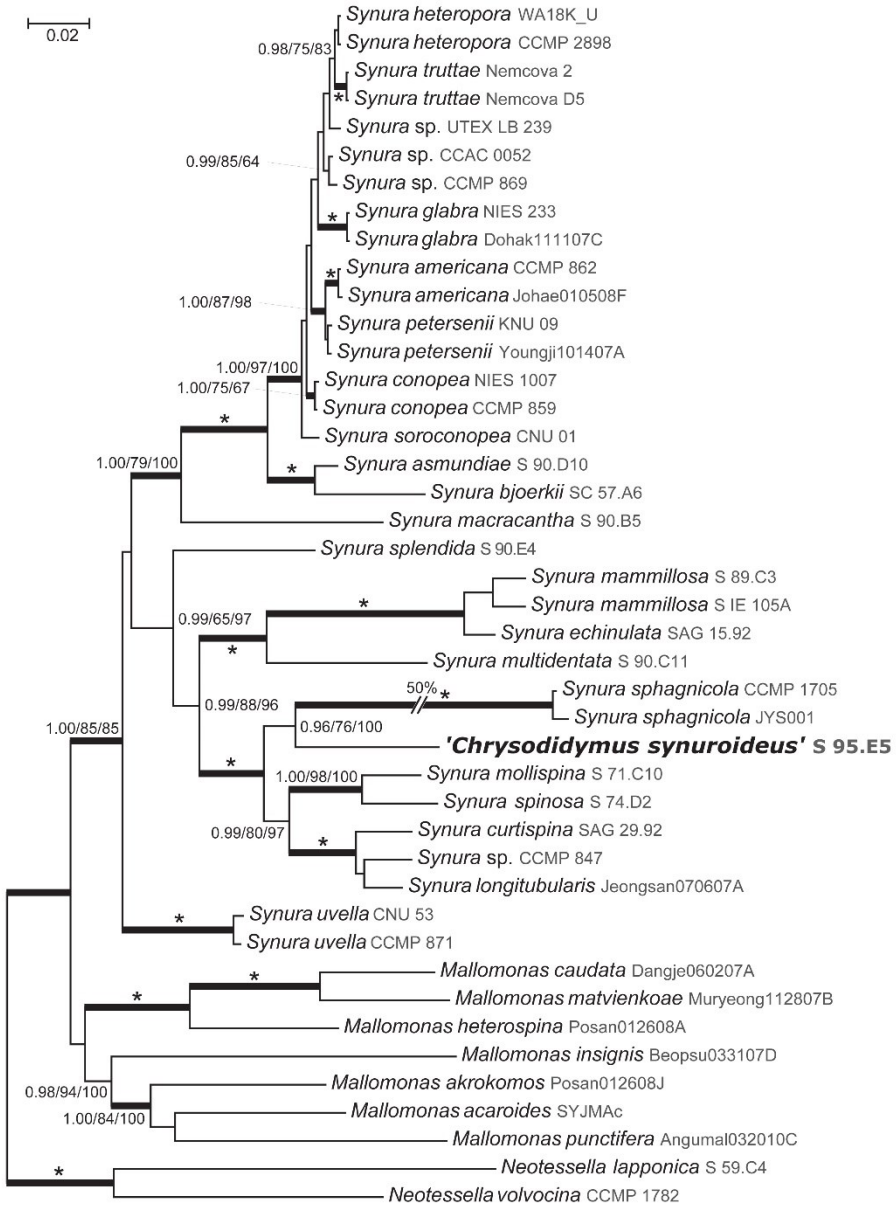


Fig 19. Phylogeny of the Synurales obtained by Bayesian inference of the concatenated SSU rDNA, LSU rDNA and rbcL dataset. The analysis was performed under a partitioned model, using different substitution models for each partition. Values at the nodes indicate statistical support estimated by three methods; MrBayes posterior node probability (left), maximum likelihood bootstrap (middle), and maximum parsimony bootstrap (right). Only statistical supports higher than 0.95/60/60 are shown. Thick branches highlight nodes receiving the highest posterior probability (PP) support (1.00). Nodes receiving the absolute statistical support 1.00/100/100 are marked by asterisks. *C. synuroideus* strain is marked in bold. Scale bar represents the expected number of substitutions per site.

PAPER II

Martin Pusztai & Pavel Škaloud

Elucidating the evolution and diversity of *Uroglena*-like colonial flagellates (Chrysophyceae), polyphyletic origin of the morphotype

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Elucidating the evolution and diversity of *Uroglena*-like colonial flagellates (Chrysophyceae), polyphyletic origin of the morphotype

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Running title: *Uroglena*-like colonial flagellates (Chrysophyceae)

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Abstract

The *Uroglena*-like morphotype represents a prototype of a colonial naked chrysophyte, comprised of plastid-bearing cells that are arranged as the surface monolayer of the spherical colony. So far, insufficient molecular characterization appears to be the most significant brake on the modern taxonomic revision of this ecologically and morphologically coherent group of organisms. The general aim of this work was to conduct a modern taxonomic revision of *Uroglena*-like flagellates by using the combined molecular, morphological and ultrastructural methodology, complemented by exploring type localities of *Uroglena volvox* and *Uroglenopsis americana* in Europe and North America, respectively. On the basis of phylogenetic analysis of concatenated nuclear SSU rDNA and plastid *rbc*L sequences we show that *Uroglena*-like colonial flagellates form three genetically and morphologically distinct lineages within the Ochromonadales (Chrysophyceae), distinguished here as *Uroglena*, *Uroglenopsis* and *Urostipulosphaera* gen. nov. The taxonomic status of the other chrysophyte genera with spherical colonies is discussed in the light of our findings.

Keywords: Chrysophyceae, colonial flagellates, Ochromonadales, phylogeny, protist taxonomy, *Uroglena volvox*, *Uroglenopsis americana*, *Urostipulosphaera notabilis*.

Introduction

Chrysophytes or golden algae (Chrysophyceae, Stramenopiles) represent a monophyletic and diverse protist group commonly observed in planktonic freshwater communities (Finlay & Esteban, 1998; Wolfe & Siver, 2013; Kristiansen & Škaloud, 2017). Especially photosynthetic colonial flagellates, such as the genera *Dinobryon*, *Synura* and *Uroglena,* often dominate in the spring and autumn phytoplankton (Anneville *et al.*, 2005; Bock *et al.*, 2014). Life as a motile colony is one way to either reduce or avoid predation pressure and influence sinking loses, and thereby how to optimize resource acquisition (Lürling & Van Donk, 1996; Padisák *et al.*, 2003; Padisák *et al.*, 2009). The well-known spring and autumnal blooms of *Dinobryon*, *Synura* and *Uroglena* are facilitated by their lower growth optima in water temperature, light conditions and amounts of nutrients, along with the phenomenon of the life in the colony (Nicholls, 1995). From this perspective, colonial flagellates are possibly among the most successful groups of chrysophytes. An unpleasant water taste and odour, and potential fish deaths, are drawbacks of chrysophyte blooms from a water management perspective worldwide (Nicholls, 1995; Watson *et al.*, 2001). Agencies struggle annually with *Uroglena* blooms in Lake Biwa, Japan (Kutata, 1989; Ishikawa, 2005), as well as in numerous Canadian lakes (Watson *et al.*, 1996). In many instances, the taxonomic identity (sensu Boenig *et al.*, 2012; Pawlowski *et al.*, 2012) of the problematic species remains unresolved.

Taxa possessing the *Uroglena*-like morphotype resemble a simple spherical colony of *Ochromonas*-type cells arranged in a monolayer on the surface periphery. Individual cells may or may not be connected by a system of dichotomously branched structures (cytoplasmic threads or gelatinous stalks) radiating from the center of the colony. Whereas the *Ochromonas*-like morphotype represents a "prototype" of a single-celled naked flagellate with a basic chrysophycean cell plan (two heterokont flagella, parietal plastid), the *Uroglena*-like morphotype serves as a colonial "prototype". This is one of the possible reasons why the taxonomy of both above-mentioned morphotypes is so complicated. Nevertheless, problematic taxonomy of the polyphyletic *Ochromonas* was partly resolved by rediscovery of the type species *O. triangulata* from its type locality after more than 100 years from the original description (Andersen *et al.*, 2017).

Consequently, the phylogenetic position of *Ochromonas* s. str. has been resolved, though many lineages of *Ochromonas*-like flagellates remained taxonomically untreated (reviewed in Andersen *et al.*, 2017).

The type species of *Uroglena*, *U. volvox* Ehrenberg, was described in 1834 by Ehrenberg from a sampling campaign nearby his alma mater in Berlin, Germany. Ehrenberg precisely described cells with pointed cell posteriors that continued as thin, likely cytoplasmic, threads forming radially arranged structures. At the end of the 19th century, Lemmermann (1899) transferred all *Uroglena* sp. nov. taxa previously described from Massachusetts, USA by Calkins (1892) to the newly established genus *Uroglenopsis*, with the type species *U. americana* (Calkins) Lemmermann. Lemmermann (1899) introduced the presence of many oil droplets within the cell and the absence of radially arranged structures connecting cells in the colony as the main distinguishing characters for his new genus. Subsequently, some ongoing taxonomists dealing with the *Uroglena*-like flagellates did not recognize *Uroglenopsis* while others did (reviewed in Wujek & Thompson, 2002). The main problem was to find consensus on the presence/absence and nature of the system of dichotomously branched radial structures connecting cells in the colony.

Based on old original chrysophycean descriptions, there are additional enigmatic and often monotypic taxa adding to confusion when identifying colonial chrysophytes. For example, *Eusphaerella turfosa* Skuja has a typical hexagonal formation of cells, and the poorly described *Jaoniella planctonica* Skvortzov or *Syncrypta*/*Synuropsis* spp. possess transient morphologic states between *Synura* and *Uroglena.* Relationships of these taxa to *Uroglena*, and indeed their true existence, remain unknown (Kristiansen & Preisig, 2001).

In the most recent taxonomic review, Wujek & Thompson (2002) introduced emended diagnosis of *Uroglena* and *Uroglenopsis* (incl. *Eusphaerella*). Cells of *Uroglena* possess a pointed posterior that tapers to a thin, likely cytoplasmic, thread. These threads connect individual cells through a dichotomously branching system. The shorter flagellum is approximately one half the length of longer flagellum. In contrast, cells of *Uroglenopsis* possess more variable, although predominantly truncated or rounded, cell

posteriors. Colonies of *Uroglenopsis* possess no visible radially arranged structures or when visible, individual cells are connected via dichotomously branching system of relatively thick gelatinous stalks (sometimes better visible after staining). The short flagellum is at most one quarter the length of the longer flagellum.

Unfortunately, almost all the previous reviews and shifts in *Uroglena* taxonomy have been based only on the morphology without the use of any molecular data. So far, only a few *Uroglena*/*Uroglenopsis* strains have been characterized from a molecular point of view. One of the reasons may lie in a difficult isolation and subsequent cultivation of these extremely fragile colonies made of naked flagellates. In addition, for today's analysis it is usually necessary to use a large number of strains and these were not available in world's algae collections. Therefore, the aim of this challenging work was to conduct a modern taxonomic revision of the genera possessing a *Uroglena*-like morphotype. By using a combined methodology of studying sufficient amount of short-term cultures along with single colony isolates coupled with exploration of isolates from the type localities of *Uroglena volvox* in Europe and *Uroglenopsis americana* in North America we obtained data characterizing these taxa on the basis their genetics (nuclear SSU rDNA and plastid rbcL), morphology (light and electron microscopy) and ecology. Based on a combination of all data, we significantly add to the evolutionary history and taxonomic delineation of *Uroglena*-like colonial chrysophytes.

Materials and methods

Sampling

Sampling campaigns (Table 1) took place in Europe and North America through the years 2014-2017. Isolates of *Uroglena*-like flagellates were obtained from various freshwater bodies as well as from the type localities of *Uroglena volvox* (Grunewaldsee, Grunewald district, Berlin, Germany) and *Uroglenopsis americana* (Buckmaster pond, Norwood, Massachusetts, USA) after more than 180 and 120 years, respectively. In Berlin we have selected and sampled those water bodies which had already existed nearby to Ehrenberg's alma mater at the time of his collection. Only Grunewaldsee in Grunewald district within the same name forest in western Berlin periphery was hosting *Uroglena*

taxa. Sampling was predominantly, but not exclusively, carried out in the spring months. Samples were collected using plankton net with 20μm mesh. In each site, abiotic factors including water pH, temperature and specific conductivity were measured using a combined pH/conductometer (WTW 340i; WTW GmbH, Weilheim, Germany). Collected samples were kept in a polystyrene box equipped by a cooling gel pad for a few hours until they were processed at the research base. Phytoplankton communities were examined with an Olympus CX 31 (Olympus Corporation, Shinjuku, Tokyo, Japan) light microscope. Colonies of *Uroglena*-like chrysophytes were morphologically characterized and then isolated by micropipetting. Each colony was washed only 3 times with Hepes-buffered DY IV liquid medium (pH ≈ 7.5; Andersen *et al.*, 1997) to minimize the risk of colony disintegration and loss. Colonies often disintegrated during isolation, significantly reducing success of establishing cultures compared with similar efforts for isolation of other colonial chrysophytes such as *Synura petersenii* (Škaloud *et al.*, 2014).

A combined methodology was used to maximize future success for the molecular characterization of isolates. For each morphotype found in sample, 10-20 washed colonies were individually placed into a well of a 96-well polypropylene plate that contained approx. 400 μl Hepes-buffered DY IV liquid medium (pH ≈ 7.5). Next 8-16 washed colonies were put into 8-tube strip, one colony to each tube, and were frozen at -20°C for future direct use in single-colony PCR. Living isolates in plates were cultivated in 15°C, under constant illumination of 20-40 μmol m^{-2} s^{-1}. Due to low survival rate of isolated colonies, only a few of isolates were successfully transferred into 50ml Erlenmeyer flasks and maintained as short-term cultures under the above-mentioned conditions. All the cultures contained resident bacteria of natural origin, but sterile technique was used throughout to avoid further contamination. One of the cultures (U7-1) is still successfully maintained as a long-term culture.

Morphological investigations

Colonies of *Uroglena*-like chrysophytes were thoroughly checked under Olympus CX 31 light microscope at the research base just a few hours after the sampling. Colonies and single-cells were measured, drawn and sometimes photographed (if

possible). The cell posterior, flagella length ratio, and presence/absence and nature of the system of dichotomously branched radial structures were used to distinguish between *Uroglena* and *Uroglenopsis* (sensu Wujek & Thompson, 2002). One *Uroglena*-like culture was also encysting. The ultrastructure of cysts as well as presence of scale-like structures (e.g. silica scales) were examined with a JEOL 6380 LV (JEOL, Ltd., Akishima, Tokyo, Japan) and FEI Helios NanoLab G3 UC (FEI Company, Hillsboro, Oregon, USA) scanning electron microscopes (SEM) and with a JEOL 1011 (JEOL, Ltd., Akishima, Tokyo, Japan) transmission electron microscope (TEM). All types of samples (field samples, single colony isolates and cultures) were examined by electron microscopy. The Morphology of *Uroglena*-like chrysophytes, which were successfully maintained in short-term cultures, was examined with an Olympus BX 51 (Olympus Corporation, Shinjuku, Tokyo, Japan) light microscope equipped by Nomarski interference contrast. The mucilaginous branching system was visualized by methylene blue staining and Lugol's iodine solution.

Sequencing and phylogenetic analysis

DNA isolations were carried out as described in Škaloudová & Škaloud (2013) with the only difference in using 10 mL of InstaGene matrix (Bio-Rad Laboratories) for single-colony isolates. Two molecular markers were amplified by PCR: nuclear SSU rDNA and plastid *rbc*L. These molecular markers provide sufficient genus-level taxonomic resolution within the Chrysophyceae (Andersen *et al*., 2017; Kristiansen & Škaloud, 2017). The amplification of SSU rDNA was partly performed as described by Škaloud *et al.* (2013), using the primers 18SF and 18SR (Katana *et al.* 2001). Additionally, new primers Chryso_SSU_F2 (5′-TGT CTC AAA GAT TAA GCC AT-3′) and Chryso_SSU_R2 (5′-CTA CGG AAA CCT TGT TAC GA-3′) were designed for this study. The amplification of the *rbc*L marker was performed according to Jo *et al.* (2011), using the newly designed primers Chryso_*rbc*L_F4 (5′-TGG ACD GAY TTA TTA ACD GC-3′) and Chryso_*rbc*L_R7 (5′-CCW CCA CCR AAY TGT ARW A-3′). The PCR products were purified and sequenced at Macrogen Inc. in Seoul, Korea or in Amsterdam, The Netherlands.

The newly determined sequences were aligned to other sequences of Chrysophyceae from the GenBank database. The sequences were selected according to Andersen *et al.* (2017) and Kristiansen & Škaloud (2017) to encompass all chrysophycean lineages. This selection was extended by all closely related sequences to the newly determined sequences using BLAST (Altschul *et al.*, 1990). The GenBank accession numbers of all strains used in this study are provided in Supplementary table S1. A concatenated 2592 bp long SSU rDNA and *rbc*L alignment was produced, including sequences from a total of 94 chrysophyceaen taxa plus two outgroup taxa – *Synchroma* and *Nannochloropsis*. The outgroup taxa were selected based on the results of the multigene phylogenetic analysis of Stramenopiles published by Yang *et al.* (2012). The SSU rDNA sequences were aligned using MAFFT v. 6 software (Katoh *et al.*, 2002) under the Q-INS-I strategy and checked for obvious sequencing errors. Poorly aligned positions were eliminated using the program Gblocks, ver. 0.91b (Talavera & Castresana 2007). The *rbc*L sequences were manually aligned using MEGA 6 (Tamura *et al.*, 2013). The site-stripping method was used to remove over-saturated nucleotide positions from the *rbc*L dataset according to Škaloud *et al.* (2013).

For each of the alignment partitions, the most appropriate substitution model was estimated using the Bayesian information criterion (BIC) as implemented in jModelTest 2.1.4 (Darriba *et al.*, 2012). This procedure selected the following models: (1) GTR + I + G for SSU rDNA, (2) GTR + G for the first codon position of the *rbc*L gene, (3) TVM + I + G for the second codon position of the *rbc*L gene, and (4) GTR + G for the third codon position of the *rbc*L gene. The phylogenetic tree was inferred by Bayesian inference (BI) using MrBayes version 3.2.1 (Ronquist *et al.*, 2012). The analysis was carried out on partitioned datasets using the substitution models best matching those selected by jModelTest 2.1.4. All parameters were unlinked among partitions. Two parallel MCMC runs were carried out for 10 million generations, each with one cold and three heated chains. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was assessed during the run by calculating the average standard deviation of split frequencies (SDSF). The SDSF value was 0.00637. Finally, the burn-in value was determined using the "sump" command. Bootstrap

analyses were performed by maximum likelihood (ML) and weighted maximum parsimony (wMP) criteria using GARLI, version 2.01 (Zwickl, 2006) and PAUP*, version 4.0b10 (Swofford, 2002), respectively, as described in Pusztai *et al.* (2016).

Results

We successfully established 53 single-colony isolates and the cultures of these corresponded morphologically to *Uroglena* and *Uroglenopsis* (Table 1). In addition, isolates from the type localities for *Uroglena volvox* in Berlin, Europe (7 isolates) and *Uroglenopsis americana* in Norwood, North America (5 isolates) were successfully established. Moreover, we also isolated colonies into culture that exhibited the distinct morphology of the rare *Eusphaerella turfosa* (Table 1).

Molecular evidence

Phylogenetic analysis of the concatenated nuclear SSU rDNA and plastid *rbc*L sequences revealed a polyphyletic origin for the *Uroglena*-like morphotypes (Fig. 1). These organisms were inferred in three distinct, statistically well supported clades within the Ochromonadales, Chrysophyceae. All strains with *Uroglena* s. str. morphotype (Figs 2, 3) were recovered in a single clade forming a monophyletic group that was sister to *Chrysonephele*, a non-motile flagellated colonial chrysophyte endemic of Tasmania. This group was also closely related to *Epipyxis* and *Chrysolepidomonas*. All strains with a *Uroglenopsis* morphology formed two distant clades. The first clade, here referred to as *Uroglenopsis* s. str. (Figs 4, 5), included *Uroglenopsis americana* and other *Uroglenopsis* spp. that lacked any visible radial structures connecting the individual cells. Interestingly, *Eusphaerella turfosa* was nested within this clade as well. The *Uroglenopsis* s. str. clade was statistically well supported and closely related to a number of morphologically and ecologically distinct genera such as the terrestrial *Pedospumella* and aquatic *Ochromonas triangulata* living in hypersaline lakes. The second clade with a *Uroglenopsis* morphology, here referred as *Urostipulosphaera* gen. nov. (Figs 6, 7), was genetically distinct. Based on the phylogenetic analysis, this second clade formed a monophyletic lineage sister to *Acrispumella msimbasiensis*, a heterotrophic chrysophyte

found in Msimbazi River in Tanzania. This lineage was also related to *Cornospumella*, *Chlorochromonas*, *Poteriospumella* and *Poteriochromonas*.

Morphology

Uroglena Ehrenberg

Uroglena volvox was recollected from its type locality and organisms related to *Uroglena volvox* were collected from two other locations in Canada and the Czech Republic (Table 1). Cells of *Uroglena* were radially arranged as a monolayer coat at the colony periphery and individual cells possessed pointed cell posteriors that continued as thin, likely cytoplasmic, threads (Figs 2,3). These threads connected individual cells through a dichotomously branching system into a spherical colony. Colony dimensions ranged from 50 μm to 250 μm in diameter, most commonly 70-150 μm in diameter. The smaller colonies with fewer cells were usually a product of large colony collapsing during observation. Colonies consisted of tens to hundreds of cells. Cells were inverse tear-drop in shape with a sharply pointed cell posterior. Cell size varied from 9-12.5 μm long to 6-10 μm wide. Each cell had two unequal anterior flagella. The longer flagellum ranged from 15 μm to 25 μm. The shorter flagellum ranged from 7.5 μm to 12.5 μm in length, or and was approximately half the length of the longer flagellum. Cells usually had a single girdle-shaped, bilobed, slightly spiral gold-colored plastid that possessed an anterior stigma. Cell shape and plastid number changed when microscope slides heated and dried during observation. Electron microscopy did not confirm the presence of any scale-like structures, which is in accordance with the finding of Wujek (1976).

Uroglenopsis Lemmermann

Uroglenopsis americana was recollected from its type locality and organisms closely related to *Uroglenopsis* were collected from five other localities in Canada, the Czech Republic and Norway (Table 1). Cells of *Uroglenopsis* possessed a predominantly truncate or rounded cell posterior (Figs 4, 5). No branching system of any radially arranged thin cytoplasmic threads or thick gelatinous stalks was present even when stained with Lugol's iodine solution and or methylene blue. Instead, cells were

embedded onto a compact jelly mantle as a monolayer coat at the colony periphery. This compact jelly mantle was in normal condition invisible and appeared after staining by methylene blue (Fig. 8). Colonies possessed high degree of phenotypic plasticity in their shape – from spherical to oval, elongated or characteristically irregularly poly-lobal (Fig. 9) observed in *U. americana* (UK-4) and *U.* sp. (U19) populations. Dimensions of explored colonies ranged from 50 μm to 350 μm in diameter, most commonly 100-200 μm in diameter. The smaller colonies with fewer cells were usually a product of large colony collapsing during observation. Colonies consisted of tens to hundreds of cells. Cells were of diverse shape (obovate, oval, elongated to cylindrical) with a predominantly truncate or rounded cell posterior. Cell size varied from 10-12.5 μm long to 5-7.5 μm wide. Each cell had two distinctly unequal anterior flagella. The longer flagellum ranged from 15 μm to 25 μm. The shorter flagellum ranged from 2 μm to 3 μm in length, or and was approximately at most one quarter of the longer flagellum. Cells usually had a single girdle-shaped, plate gold-colored plastid that possessed an anterior stigma. Cell shape and plastid number changed when microscope slides heated and dried during observation. Electron microscopy did not confirm the presence of any scale-like structures, which is in accordance with the finding of Wujek (1976).

We found colonies that were morphologically indistingishable from *Eusphaerella turfosa*, and these organisms were nested within the *Uroglenopsis* clade. Cells and colonies agreed in all ways with *Uroglenopsis* except for they were closely packed together and hexagonal from apical view with remarkable hole into spherical colony (Fig. 10). Cultured colonies lost their typical "*Eusphaerella*" morphology and became virtually indistinct from *Uroglenopsis* when their cells became more loosely packed (Fig. 11).

Urostipulosphaera gen. nov.

Finally, we discovered a third clade of colonial flagellates that was morphologically (Figs 6, 7), as well as genetically (Fig. 1), distinct from *Uroglenopsis*. Cells in the colony exhibited truncate or rounded cell posterior and they were connected via dichotomously branching system of relatively thick articulated gelatinous stalks,

sometimes covered with bacteria and thus made more visible (Figs 12-16). Colonies were usually spherical, sometimes oval, in their shape. Dimensions of explored colonies ranged from 40 μm to 200 μm in diameter, most commonly 90-200 μm in diameter. The smaller colonies with fewer cells were usually a product of large colony collapsing during observation. Colonies consisted of tens to hundreds of cells. Cells were usually obovate in shape with a predominantly truncate or rounded cell posterior. Cell size varied from 7.5-10 μm long to 5-7.5 μm wide. Each cell had two distinctly unequal anterior flagella. The longer flagellum ranged from 12.5 μm to 20 μm. The shorter flagellum ranged from 2.5 μm to 3 μm in length, or and was approximately at most one quarter of the longer flagellum. Cells usually had a single girdle-shaped, broadly ribbon, bilobed, slightly spiral gold-colored plastid that possessed an anterior stigma. The strain U7-1 collected from small pool filled with decomposing plant material exhibited reduced plastids (distinctly smaller and pale) which became normal right after few days of culturing. This may indicate mixotrophic nutrition. Cell shape and plastid number changed when microscope slides heated and dried during observation (e.g. frequently observed two or three biconcave disk plastids). Electron microscopy did not confirm the presence of any scale-like structures, which is in accordance with the finding of Wujek (1976).

Some of these organisms were morphologically identical to the previously desecribed *Uroglena notabilis* Mack. In particular, the stomatocyst (12.5-14 μm in diameter) had a characteristic curved, collapsed, tubular neck formed by rolled up sheet, and ranged from almost smooth-walled to embellished cyst wall with wart-like processes ("verrucae") of irregular number and shape (Figs 17-19). Based on the study of previously published records of colonies with characteristic morphology corresponding to the newly recognized *Urostipulosphaera*, we can further state that the potential size of *Urostipulosphaera* is in the range of 100-300 μm in diameter with cells of 10-15 μm long, 5-8 μm wide.

Taxonomic conclusions

***Urostipulosphaera* Pusztai & Škaloud, gen. nov.** (Figs 6, 7, 12-19)

Description

Photosynthetic, non-scaled chrysophycean bi-flagellates forming colonies. Colonies free-swimming, spherical to oval, (40-)90-200(-300) μm in diameter, consisting of tens to hundreds of cells. Cells obovate, 7.5-10(-15) μm long, 5-7.5(-8) μm wide, united by their truncate or rounded cell posterior to relatively thick articulated gelatinous stalks. Stalks forming dichotomously branched system gradually merging to the center of the colony. Cells radially arranged as a monolayer coat at the colony periphery. Two heterokont distinctly unequal flagella located anteriorly. Shorter flagellum (2.5-3 μm) approx. at most one quarter of longer flagellum (12.5-20 μm). Longer flagellum approx. once to twice of cell length. Usually one girdle-shaped, broadly ribbon, bilobed, slightly spiral gold-colored plastid with anterior stigma.

TYPE SPECIES: *Urostipulosphaera notabilis* (Mack) Pusztai & Škaloud, comb. nov.

ETYMOLOGY: "uro" refers to morphologically related and previously described taxa *Uroglena* and *Uroglenopsis*, and it means to glow or to live, "stipulo" refers to presence of gelatinous stalks, "sphaera" refers to usually perfectly spherical colonies in comparison with sometimes oval or poly-lobal colonies in *Uroglenopsis*.

Urostipulosphaera notabilis (Mack) Pusztai & Škaloud, comb. nov. (Figs 12, 17-19)

BASIONYM: *Uroglena notabilis* Mack, Österr. Bot. Z. 98: 266, 274, Fig. 3h-k (1951).

SYNONYMS: *Uroglenopsis notabilis* (Mack) Thompson & Wujek 2002: 301.

TYPE LOCALITY: Prater and Perchtoldsdorf, Wien, Austria

REFERENCE STRAIN LOCALITY: Strain U12-1 was isolated from a Velký pond in Voznice, Czech Republic (49.8185206N, 14.2169953E).

REPRESENTATIVE DNA SEQUENCES: GenBank accessions no. MK153247, MK153261.

Discussion

The independent development of similar or identical phenotypes can be determined, in part, by experiencing similar selective pressures (Neiva *et al.*, 2012). There

are several examples of planktonic protists with a similar phenotype of individuals grouped in more or less spherical colonies: *Dictyosphaerium* (Trebouxiophyceae), *Ophrydium* (Ciliophora), *Pseudodendromonas* (Bicosoecida), *Sphaeroeca* (Choanoflagellatea), *Spongomonas* (Cercozoa), *Synura* (Chrysophyceae), and *Volvox* (Chlorophyceae). Growth as a colony may reduce or avoid predation pressure and influence sinking loses and, thereby, may optimize free resources acquisition (Lürling & Van Donk, 1996; Padisák *et al.*, 2003; Padisák *et al.*, 2009). Living in colony is also one of the first steps on the path to complex multicellularity. It was demonstrated by Herron & Michod (2008) that the *Volvox*-like morphotype evolved independently several times within Volvocaceae (Chlorophyceae). On the other hand, Pusztai *et al*. (2016) revealed the interesting case of retrospective simplification in the colonial chrysophyte *Synura synuroidea* (Prowse) Pusztai, Čertnerová, Škaloudová & Škaloud.

It is evident that not only different species, but also distinct genera can share the same morphotype. Recently, revised problematic taxonomy of the polyphyletic genus *Ochromonas* was partly resolved by precisely fixing the phylogenetic position of the type species (Andersen *et al.*, 2017). Nevertheless, many understudied lineages of *Ochromonas*-like flagellates remain to be characterized. On the other hand, comprehensive taxonomic revisions of the heterotrophic taxa *Spumella* (Findenig *et al.*, 2010; Grossmann *et al.*, 2016) and *Paraphysomonas* (Scoble & Cavalier-Smith, 2014) were published recently. Polyphyletic origin of *Uroglena*-like colonial flagellates was previously shown by Andersen (2007), recognizing that a single *Uroglena* isolate was unrelated to a larger cluster of strains. Even after adding several environmental sequences of chrysophytes to a larger dataset (del Campo & Massana, 2011), the story of uncovering *Uroglena*/*Uroglenopsis* evolutionary history remained unresolved, including within other chrysophyte phylogenetic investigations (Klaveness, 2011; Andersen *et al.*, 2017; Bock *et al.*, 2017). In this paper, we show that *Uroglena*-like colonial flagellates form three genetically and morphologically distinct lineages, distinguished here as genera *Uroglena*, *Uroglenopsis* and *Urostipulosphaera* gen. nov.

Ehrenberg described the genus *Uroglena*, with the type species *U. volvox*, in 1834. The description was based on the sampling campaign nearby his alma mater, Humboldt

University of Berlin, Germany. Along with *Uroglena*, colonial *Synura* and *Syncrypta* were described as well (Ehrenberg, 1834, 1838). In contrast to *Synura* and *Syncrypta*, *Uroglena* was characterized to exhibit pronounced red stigma in cell anterior. Nevertheless, in his drawings, Ehrenberg (1838) sketched a stigma even in some cells of *Synura*. This schism was probably introduced by the fact he did not pay attention to the presence of stigma in his earlier observations. Accordingly, he referred some colonies possessing stigmata wrongly as *Synura*. Ehrenberg (1834, 1838) characterized *U. volvox* by the cells forming a coat of a spherical motile colony where the cells posteriorly pass into connected threads, which radiate out from the center of the colony. He further stated that it is hard to recognize whether the cells possessed one or two plastids. Later, Skuja (1948) identified that the cells contain single, girdle-shaped, ribbon, bilobed and slightly spiral plastid. Ehrenberg (1834, 1838) further observed that flagella serve not only for locomotion, but also for procuring food. This is in accordance with the mixotrophic character of these taxa (Kristiansen & Preisig, 2001).

Although Ehrenberg did not specify the exact water body nearby Berlin where he collected *Uroglena volvox* (he only wrote "*in Torfwasser bei Berlin*"), we have selected and sampled those water bodies which had already existed nearby to his alma mater in the time of his collection. Only Grunewaldsee in Grunewald district within the same name forest in western Berlin periphery was hosting *Uroglena* taxa. The phenology and morphology of the *U. volvox* population we collected in Grunewaldsee fully corresponds to the Ehrenberg's protologue of this species. Ehrenberg found *U. volvox* from April to June. Our collections were carried out on 28th April. Moreover, colonies of Ehrenberg's *U. volvox* were approximately 282 μm in diameter (1/8'''), which is congruent with our findings (colonies of approximately 250 μm in diameter).

At the end of the 19th century, Lemmermann (1899) transferred "*Uroglena*" taxa described previously from USA by Calkins (1892) into a newly established genus *Uroglenopsis*, with the type species *Uroglenopsis americana*. Lemmermann's decision to erect *Uroglenopsis* was based on the works of other taxonomists (Calkins, 1892; Zacharias, 1895; Moore, 1897) and, as he wrote, without any direct observation of *Uroglena* s.l. taxa under the microscope, since colonies in provided fixed samples were no longer present

(Lemmermann, 1899). The main morphological features characterizing the new genus were the presence of numerous oil droplets in the cells and the absence of any radially arranged structures connecting cells. The first discriminating feature is questionable as the presence and the number of droplets in cells is not a stable and valuable character (own observations). The second feature is, however, fully congruent with the observations provided by Calkins (1892). Calkins stated that upon crushing colonies of *U. americana* found in Norwood and Plymouth with a coverslip, the monads possessed no tails or stalks, separated and formed an amorphous mass with the jelly. The species of *Uroglenopsis* found by us at the type locality possessed cells embedded in a compact jelly coat at the colony periphery and without radial structures. This is in accordance with original description of "*Uroglena*" *americana* as well as with the key characters of later newly erected genus *Uroglenopsis*. "*Uroglena*" *americana* found by Calkins (1892) exhibited cells of 5-7 μm wide, longer flagellum of 13 μm and shorter flagellum of 2 μm in length, which is congruent with our findings (cells of 5-7.5 μm wide, length of longer and shorter flagellum 12.5 and 2.5 μm, respectively).

Based on electron micrographs of *Uroglena* and *Uroglenopsis* cysts, it seems that the cyst ultrastructure is species specific (Cronberg & Laugaste, 2005). Unfortunately, both Ehrenberg and Calkins did not illustrate any cyst in their descriptions of *U. volvox* and *U. americana*, respectively. The cyst morphology has been provided by later taxonomists, however, based on observations of encysting populations collected far from the type locality (reviewed in Wujek & Thompson, 2002). The result of this effort was an assignment of several different cyst-morphotypes to the original description of *U. volvox* with the most cited smooth-walled cyst with a simple pore sensu Kent (1881) and smooth-walled cyst with a tubular neck and wider collar sensu Zacharias (1895). Therefore, we reject the concept of choosing the firstly described cyst from all previous records, as proposed by Wujek & Thompson (2002). In the effort to fix and complete the modern and useful *U. volvox* and *U. americana* descriptions precisely, we propose to add information about the ultrastructure of its cyst altogether with its molecular characterization on the basis exploring the encysting populations from the type localities. As both the populations of *U. volvox* from Grunewaldsee and *U. americana*

from Buckmaster pond had not produced cysts, further effort to find encysting populations genetically identical to our re-discovered species will be of great value and lead to more complete descriptions.

Our findings are, in some respect, an expected consequence of the taxonomic bias in distinguishing between genera *Uroglena* and *Uroglenopsis*, as the consensus on the presence/absence and the nature of the radial structures was previously never reached. Though Skuja (1948) did not distinguish between these genera, recognizing only *Uroglena* s. l., he likely observed organisms belonging to all three newly recognized lineages. Based on his detailed drawings, it is now possible to assign his *U. europea* (Pascher) Skuja and *U. volvox* to *Uroglena* (species with sharply pointed cell posteriors passing into a thin thread), *U. americana* and *U. irregularis* Rodhe & Skuja to *Uroglenopsis* (species without any radial structures and sometimes poly-lobal colonies), and *U. eustylis* Skuja presumably to *Urostipulosphaera* gen. nov. (species with cells united by their truncate cell posterior to relatively thick articulated gelatinous stalks).

It is ironic that in this work Skuja (1948) also erected a new monotypic genus *Eusphaerella*, which is, based on our phylogenetic analysis, significantly nested within *Uroglenopsis*. However, *Eusphaerella turfosa* possesses a highly distinctive morphology characterized by a remarkable hole in the hemispherical colony and the closely packed cells of hexagonal shape as observed in apical view. We are therefore facing the typical "lumper-splitter" problem (Darwin, 1857) resulting in establishing number of new monophyletic genera to accommodate morphologically distinct paraphyletic taxa, as it was done for example within a well-known *Hydrodyction/Pediastrum* group (Buchheim *et al.*, 2005). However, we decided to recognize *E. turfosa* as a member of the genus *Uroglenopsis*, as already proposed by Wujek & Thompson (2002) who established a new combination *U. turfosa* (Skuja) Wujek & Thompson. First, *Eusphaerella* and *Uroglenopsis* share a common absence of any visible radial structures between the colony center and periphery. Second, based on our observations of cultured *E. turfosa* from samples taken in Scandinavia and Canada, we recognized that old colonies lose their typical "*Eusphaerella*" morphology and become virtually indistinct from *Uroglenopsis* when their cells become more loosely packed.

To avoid introduction of superfluous names, we carefully checked old descriptions of all colonial chrysophyte flagellates prior to proposing a new generic name for the *Urostipulosphaera* lineage. The monotypic genus *Jaoniella* Skvortzov, despite its inadequate description, resembles newly emended *Uroglena* with the only one exception of the presence of equally length flagella. However, this difference might be caused by an observation error. Another monotypic genus, *Lepidochrysis* Ikävalko, Kristiansen & Thomsen, lives in brackish water and its cells bear organic scales. Scales were not found in *Uroglena* or *Uroglenopsis* (Wujek, 1976). The genus P*seudosyncrypta* Kisselev exhibits eight or more plastids per cell, a dubious character when compared with other chrysophytes that usually have only one or two plastids per cell. The higher number of plastids may represent a unique character as well as it could be an artefact caused by extreme conditions in situ or during processing the samples (e.g. common change in plastid number in *Uroglena*-like flagellates by heating and drying microscope slides). If the latter is true and by considering almost equal flagellar length, the lack of stigma, and the presence of mucilage with small bodies (possibly scales?) surrounding the colonies, P*seudosyncrypta* resembles the genus *Neotessella* (Playfair) Jo, Kim, Shin, Škaloud & Siver (Synurales). Colonies of *Chrysomoron* Skuja and *Chrysobotriella* Strand were described as consisting of just a few cells. It is a question whether they are just transient clusters of single-celled *Ochromonas* s. l., or if they represent colony fragments of *Synuropsis* s. l. as proposed by Wujek & Thompson (2001).

The rest of chrysophycean colonial genera – *Pseudosynura* Kisselev, *Syncrypta*, *Synochromonas* Korshikov, *Synuropsis* Schiller and *Volvochrysis* Schiller – represent enigmatic taxa with transient or chimeric morphology between *Uroglena* (Ochromonadales) and *Synura* (Synurales) in general. Therefore, they were altogether synonymized into one genus – *Syncrypta* s. l. (sensu Bourrelly, 1957) or later *Synuropsis* s. l. (sensu Wujek & Thompson, 2001). Even though synonymy is controversial, all of these synonymized taxa, contrary to *Urostipulosphaera*, possess more or less pointed posteriors that taper into cytoplasmic thread or they are embedded into jelly mass. In other words, the invention of the colony through the joining of tapering cell posteriors or simply through cells embedded onto or in the gel has evolved more than once in the

evolution of the chrysophytes, whereas the relatively thick articulated gelatinous stalks appear to be a unique feature for the newly recognized *Urostipulosphaera*. In our opinion, based on thousands examined samples hosting colonial chrysophytes from around the world (e.g. Škaloud *et al*., 2013; Škaloud *et al*., 2014; Němcová *et al*., 2016; Pusztai *et al*., 2016; this study) with subsequent sequencing of many "strange scale-less *Synura*-like" taxa, *Syncrypta* s. l. (or *Synuropsis* s. l.) represents an artificial conglomerate largely consisting of atypical scale-less *Synura* spp. living in insufficient conditions (the taxa lacking stigma with almost equal flagella), atypical *Uroglena* spp. (likely *Synochromonas elaeochrus* Jane, *Synochromonas gracilis* Korshikov and *Synochromonas perlata* Skuja), *Uroglenopsis* spp. (likely *Syncrypta dubia* Bourrelly), scale-less *Chrysosphaerella* Lauterborn (likely *Volvochrysis globosa* Schiller) and true, but certainly very rare, *Syncrypta* s. l. (or *Synuropsis* s. l.) possessing morphology as emended *Synuropsis danubiensis* Schiller (Wujek & Thompson, 2001).

The newly proposed *Urostipulosphaera* therefore represent a distinct genus, exhibiting a unique combination of morphological and genetic characteristics within chrysophytes. We have successfully obtained several cultures belonging to the *Urostipulosphaera*, including one culture of an encysting population. Cysts possessed very specific ultrastructure: they were spherical, bearing wart-like processes ("verrucae") of irregular number and shape and pronounced curved tubular neck formed by rolled up sheet, distinct from other known *Uroglena* cysts bearing rather monolithic necks (Cronberg & Laugaste, 2005). According to this specific cyst ultrastructure, we have unambiguously identified this strain as "*Uroglena*" *notabilis*, proposing it as a type species of the newly erected *Urostipulosphaera*, *Urostipulosphaera notabilis* (Figs 17-19). Subsequently, future re-evaluation of the other previously described *Uroglena*/*Uroglenopsis* species should happen in accordance to detailed genetic, morphological, and ultrastructural characterization of cultures established from encysting populations.

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Table 1. Origin and sampling details of newly acquired strains.

Taxon	Strain	Origin	N-isol.	Locality	GPS	Sampling date	pH	Conductivity (µS cm^{-1})	Temperature (°C)
Uroglena volvox Ehrenberg	U26-3	SC	7	Grunewaldsee, Berlin, Germany	52.4668203N, 13.2589594E	28.4. 2016	7.9	782	11.3
Uroglena sp.	UK-37	SC	2	Paddys Pond, Newfoundland, Canada	47.4734824N, 52.8791929W	25.5. 2017	7.6	117	8.0
Uroglena sp.	U29-5	SC	4	Cep I pool, Czech Republic	48.9180044N, 14.8837858E	8.10. 2016	7.2	46	10.9
Uroglenopsis americana (Calkins) Lemmermann	UK-4	SC	5	Buckmaster pond, Norwood, Massachusetts, USA	42.2075884N, 71.2286782W	21.5. 2017	7.4	823	23.0
Uroglenopsis turfosa (Skuja) Wujek & Thompson	UK-81	SC + Cu	3	Expoits River oxbow lake, Newfoundland, Canada	48.9423473N, 55.7692623W	29.5. 2017	7.8	37	11.0
Uroglenopsis turfosa (Skuja) Wujek & Thompson	UN-28	SC	1	unnamed pool in wetland, Norway	60.50695N, 8.09486E	6.5.2 015	6.0	29	12.6
Uroglenopsis sp.	UJ-6	SC	2	Souš dam, Czech Republic	50.7944681N, 15.3194992E	12.6. 2015	6.7	39	18.7
Uroglenopsis sp.	UK-25	SC	2	unnamed lake, Newfoundland, Canada	47.3336095N, 53.0417669W	24.5. 2017	7.4	49	8.0
Uroglenopsis sp.	U19	SC	3	Mšeno dam, Jablonec nad Nisou, Czech Republic	50.7337736N, 15.1780583E	24.1 1.20 15	N. A.	N.A.	N.A.
Urostipulosphaera notabilis(Mack) Pusztai *et al*.	U12-1	SC + Cu	2	Velký pond in Voznice, Czech Republic	49.8185206N, 14.2169953E	20.3. 2015	8.3	318	6.3
Urostipulosphaera sp.	UP-34	SC + Cu	9	Lago do Viriato, Portugal	40.3135468N, 7.5661120W	4.4.2 015	7.4	38	N.A.

Urostipulosphaera sp.	U5-5	SC + Cu	5	Kříž pond in PP Na Plachtě, Czech Republic	50.1827819N, 15.8702700E	3.12. 2014	8.4	704	2.0
Urostipulosphaera sp.	U7-1	SC + Cu	4	pool in Botanical Garden, Prague, Czech Republic	50.0710836N, 14.4206419E	6.2.2 015	6.9	605	0.4
Urostipulosphaera sp.	U10-6	SC + Cu	4	unnamed pond near Kletečná, Czech Republic	49.5158761N, 15.3099022E	10.3. 2015	7.7	208	8.1

In column Origin, SC means single-colony isolates only, SC + Cu means single-colony isolates and cultures. N-isol. means number of acquired isolates with identical locality, morphology and sequencies within a strain.

Supplementary table S1. Taxa selected according to Andersen *et al*. (2017) and Kristiansen & Škaloud (2017) used in current Chrysophyceae phylogeny. Outgroup taxa selected according to Yang *et al*. (2012). Newly acquired strains are highlighted in bold.

Order	Taxon	Strain	GenBank accession number	
			SSU	*rbc*L
Apoikiida	*Apoikia lindahlii*	-	FJ971855	-
Apoikiida	*Apoikiospumella mondseeiensis*	SAG 2428	AY651098	-
Apoikiida	*Spumella*-like flagellate	JBM18	AY651092	-
Hibberdiales	*Hibberdia magna*	CCMP 453	M87331	AF015572
Hibberdiales	*Chrysocapsa* sp.	UTCC 280	EF165130	EF165153
Hibberdiales	*Chrysocapsa vernalis*	CCMP 278	EF165105	EF165148
Hibberdiales	*Chrysocapsa wetherbeei*	CCMP 380	EF165145	EF165149
Hibberdiales	*Chrysonebula flava*	CCMP 2765	EF165104	EF165150
Hibberdiales	*Kremastochrysis* sp.	CCMP 260	AF123282	EF165152
Hibberdiales	*Naegeliella flagellifera*	CCMP 280	AF123284	EF165154
Hydrurales	*Hydrurus foetidus*	-	FM955256	-
Hydrurales	*Chrysophyceae* sp.	CCMP 2296	EU247834	-
Hydrurales	*Ochromonas*-like flagellate	CCMP 1899	EF165133	EF165159
Hydrurales	*Phaeoplaca thallosa*	CCMP 634	AF123296	EF165160

Chromulinales	*Cyclonexis annularis*	CCMP 1858	AF123292	-
Chromulinales	*Chromulina nebulosa*	CCMP 263	AF123285	AF155876
Chromulinales	*Chromulinospumella sphaerica*	SAG 2429	AY651093	-
Chromulinales	*Chrysamoeba mikrokonta*	CCMP 1857	AF123287	EF165182
Chromulinales	*Chrysamoeba tenera*	CCMP 273	EF165102	EF165181
Chromulinales	*Chrysosphaerella brevispina*	S74.D5	HF549059	HG315744
Chromulinales	*Chrysosphaerella longispina*	S61A.B4	HF549060	HF549072
Chromulinales	*Oikomonas mutabilis*	-	U42454	-
Chrysosaccales	*Chromophyton* cf. *rosanoffii*	CCMP 2751	EF165106	EF165164
Chrysosaccales	*Chrysastrella breviappendiculata*	CCMP 1861	AF123293	EF185315
Chrysosaccales	*Chrysosaccus* sp.	CCMP 368	EF165121	EF165166
Chrysosaccales	*Chrysosphaera parvula*	CCMP 349	AF123299	-
Chrysosaccales	*Lagynion* cf. *ampullaceum*	CCMP 2727	EF165146	EF165161
Chrysosaccales	*Lagynion scherffelii*	CCMP 465	AF123288	EF165162
Ochromonadales	*Acrispumella msimbasiensis*	SAG 2427	AY651077	-
Ochromonadales	*Cornospumella fuschlensis*	SAG 2430	GU073469	-
Ochromonadales	*Dinobryon* cf. *sociale*	UTCC 392	EF165141	EF165158
Ochromonadales	*Dinobryon cylindricum*	CCMP 2766	EF165140	EF165157
Ochromonadales	*Epipyxis aurea*	CCMP 385	AF123301	EF165155

Ochromonadales	*Epipyxis pulchra*	CCMP 382	AF123298	AF015571
Ochromonadales	*Chlorochromonas danica*	SAG 933-7	JQ281514	GU935657
Ochromonadales	*Chrysolepidomonas dendrolepidota*	CCMP 293	AF123297	AF015570
Ochromonadales	*Chrysonephele palustris*	-	U71196	-
Ochromonadales	*Kephyrion* sp.	CCMP 3057	JF730878	-
Ochromonadales	*Melkoniana moestrupii*	CCMP 1278	U42382	EF165174
Ochromonadales	*Melkoniana* sp.	CCMP 591	AF123302	EF165176
Ochromonadales	*Ochromonadaceae* sp.	CCMP 2298	EU247838	-
Ochromonadales	*Ochromonas* sp.	CCMP 592	EF165135	EF165201
Ochromonadales	*Ochromonas triangulata*	AC-25	EF165136	EF165177
Ochromonadales	"*Ochromonas marina*"	AC-22	EF165138	EF165203
Ochromonadales	"*Ochromonas perlata*"	CCMP 2732	EF165143	EF165187
Ochromonadales	"*Ochromonas* sp."	CCMP 1149	EF165139	EF165202
Ochromonadales	"*Ochromonas vasocystis*"	CCMP 2741	EF165111	EF165184
Ochromonadales	"*Ochromonas villosa*"	CCAP 933/25	FR865768	-
Ochromonadales	*Ochromonas*-like flagellate	CCMP 1393	EF165142	EF185314
Ochromonadales	*Ochromonas*-like flagellate	CCMP 2761	EF165126	EF165200
Ochromonadales	*Pedospumella encystans*	SAG 2324	AY651083	-
Ochromonadales	*Pedospumella sinomuralis*	SAG 2431	AY651081	-

Ochromonadales	*Poterioochromonas* cf. *malhamensis*	SAG 933-1c	EF165114	EF165169
Ochromonadales	*Poterioochromonas* cf. *stipitata*	CCMP 1862	AF123295	EF165172
Ochromonadales	*Poteriospumella lacustris*	SAG 2323	AY651074	-
Ochromonadales	*Spumella bureschii*	SAG 2433	AY651086	-
Ochromonadales	*Spumella lacusvadosi*	SAG 2434	AY651088	-
Ochromonadales	*Spumella vulgaris*	SAG 2322	DQ388552	-
Ochromonadales	*Spumella*-like flagellate	JBNA46	DQ388542	-
Ochromonadales	*Spumella*-like flagellate	JBM28	AY651089	-
Ochromonadales	***Uroglena volvox***	**U26-3**	**MK153236**	**MK153250**
Ochromonadales	***Uroglena* sp.**	**U29-5**	**MK153237**	**MK153251**
Ochromonadales	***Uroglena* sp.**	**UK-37**	**MK153238**	**MK153252**
Ochromonadales	*Uroglenopsis* cf. *americana*	CCMP 1863	AF123290	EF165179
Ochromonadales	*Uroglenopsis* cf. *americana*	CCMP 2769	EF165131	EF165178
Ochromonadales	***Uroglenopsis americana***	**UK-4**	**MK153242**	**MK153256**
Ochromonadales	*Uroglenopsis* sp.	FU44	EU024983	-
Ochromonadales	***Uroglenopsis* sp.**	**UJ-6**	**MK153239**	**MK153253**
Ochromonadales	***Uroglenopsis* sp.**	**UK-25**	**MK153240**	**MK153254**
Ochromonadales	***Uroglenopsis* sp.**	**U19**	**MK153241**	**MK153255**
Ochromonadales	***Uroglenopsis turfosa***	**UN-28**	**MK153243**	**MK153257**
Ochromonadales	***Uroglenopsis turfosa***	**UK-81**	**MK153244**	**MK153258**
Ochromonadales	***Urostipulosphaera notabilis***	**U12-1**	**MK153247**	**MK153261**
Ochromonadales	*Urostipulosphaera* sp.	CCMP 2768	EF165132	-
Ochromonadales	***Urostipulosphaera* sp.**	**U7-1**	**MK153245**	**MK153259**
Ochromonadales	***Urostipulosphaera* sp.**	**U5-5**	**MK153246**	**MK153260**
Ochromonadales	***Urostipulosphaera* sp.**	**U10-6**	**MK153248**	**MK153262**
Ochromonadales	***Urostipulosphaera* sp.**	**UP-34**	**MK153249**	**MK153263**
Paraphysomonadida	*Clathromonas butcheri*	MD03	JQ967291	-
Paraphysomonadida	*Clathromonas caroni*	DB4	AF109326	-

Paraphysomonadida	*Paraphysomonas imperforata*	CCAP 935/13	JQ967324	-
Paraphysomonadida	*Paraphysomonas parahebes*	Hflag	AF109322	-
Paraphysomonadida	*Paraphysomonas stylata*	W02	JQ967307	-
Paraphysomonadida	*Paraphysomonas variosa*	IND5	JQ967296	-
Segregatales	*Chrysophyceae* sp.	SA-2.1	AY520450	-
Segregatales	*Segregatospumella dracosaxi*	SAG 2432	GU073467	-
Synurales	*Mallomonas alpina*	-	GU935620	GU935662
Synurales	*Mallomonas caudata*	-	EF469638	EF469644
Synurales	*Mallomonas insignis*	CCMP 2549	EF165118	EF165198
Synurales	*Neotessella lapponica*	S59.C4	HF549063	HF549074
Synurales	*Neotessella volvocina*	CCMP 1781	EF165119	EF165199
Synurales	*Synura mollispina*	S71.C10	HF549067	HF549077
Synurales	*Synura petersenii*	CCMP 873	GU325588	GU325490
Synurales	*Synura sphagnicola*	CCMP 1705	U73221	GU325413
OUTGROUP	*Nannochloropsis limnetica*	-	DQ977726	DQ977741
OUTGROUP	*Synchroma grande*	CCMP 2876	DQ788730	DQ788731

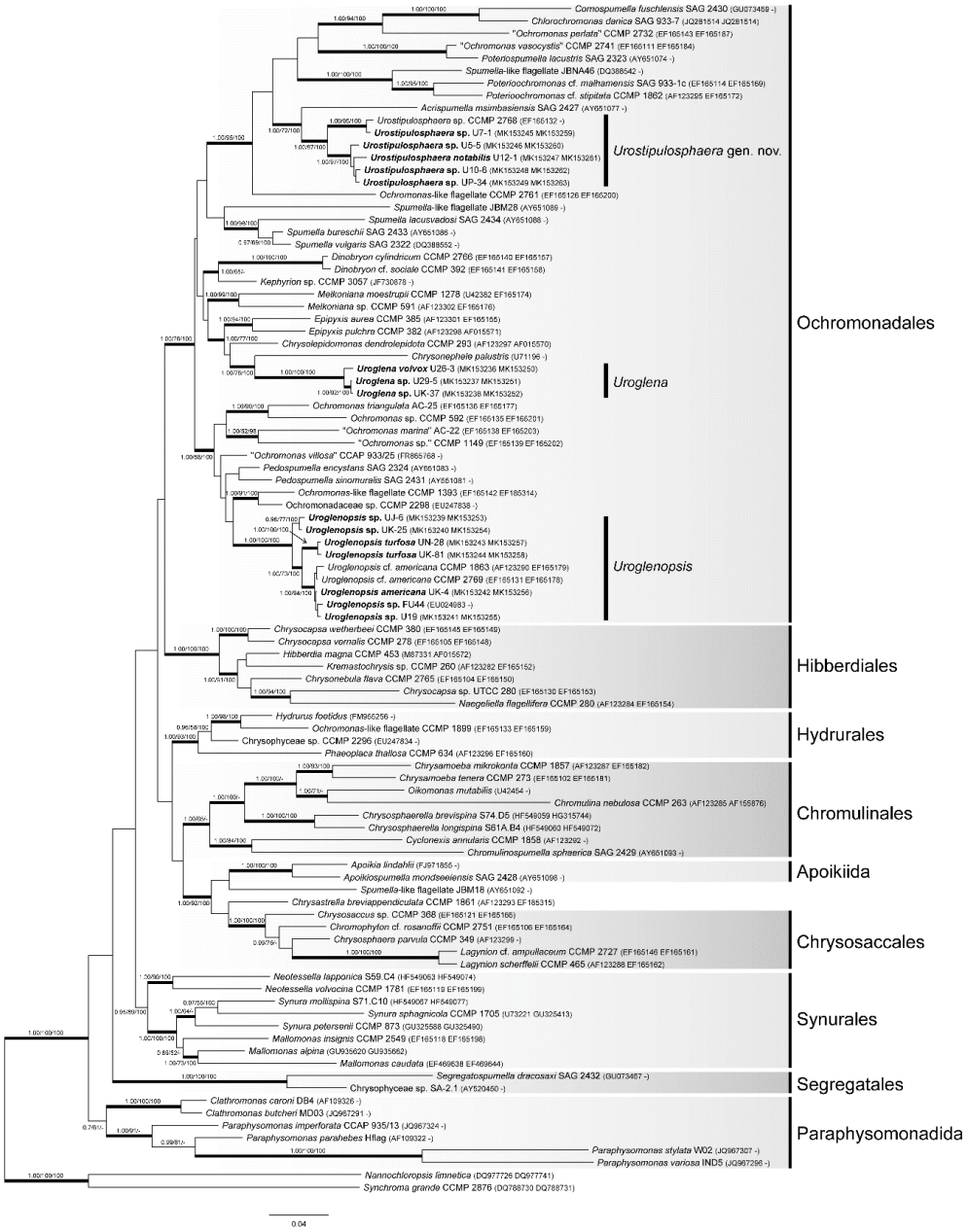
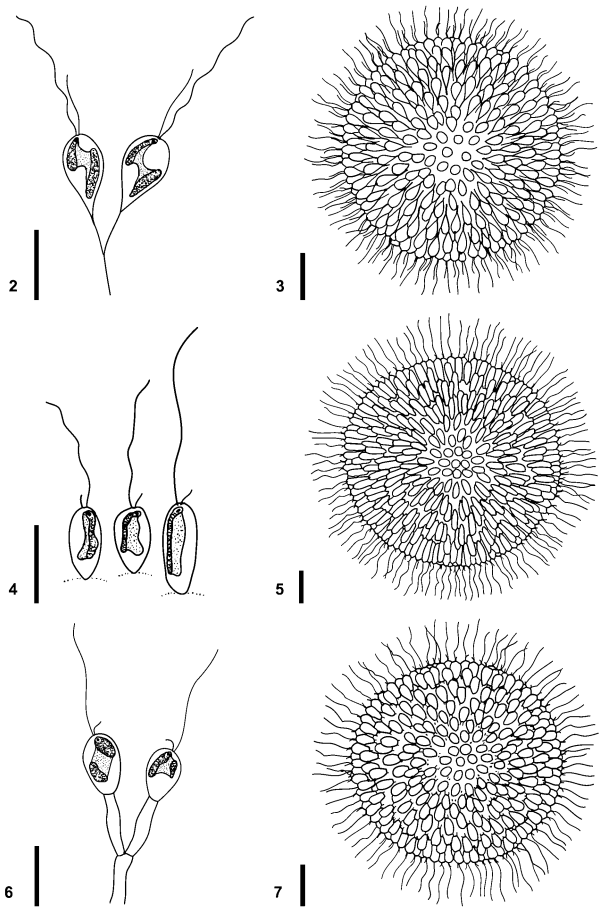
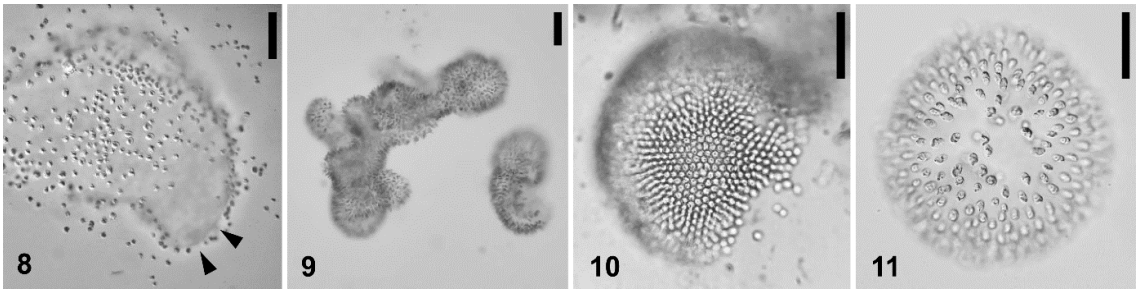


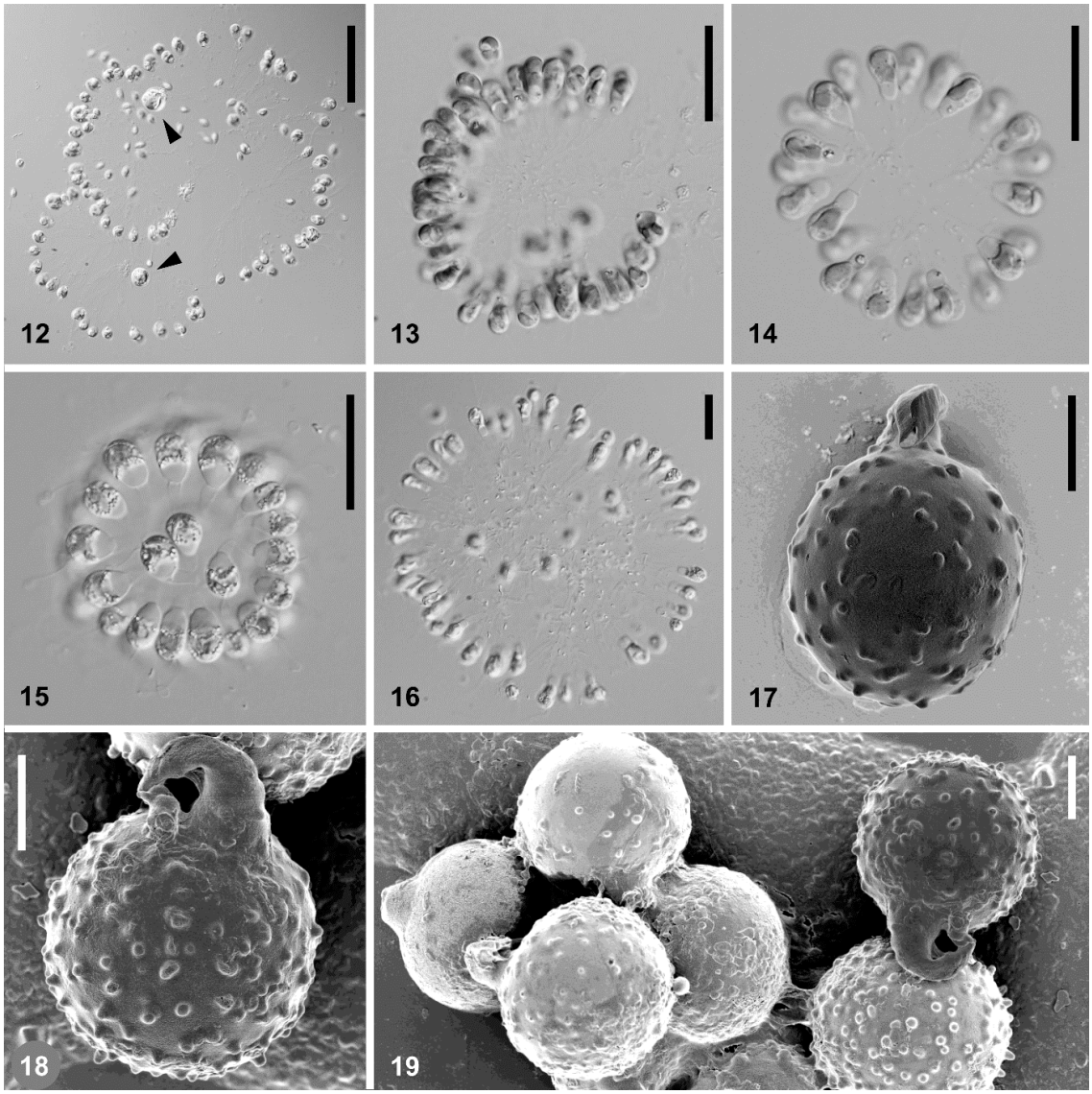
Fig. 1. Phylogeny of the Chrysophyceae obtained by Bayesian inference of the concatenated SSU rDNA and *rbc*L dataset. The analysis was performed under a partitioned model, using different substitution models for each partition. Values at the nodes indicate statistical support estimated by three methods; MrBayes posterior node probability (left), maximum likelihood bootstrap (middle), and weighted maximum parsimony bootstrap (right). Only statistical supports higher than 0.7/50/95 are shown. Thick branches highlight nodes receiving the highest posterior probability (PP) support (1.00). Newly obtained *Uroglena*, *Uroglenopsis* and *Urostipulosphaera* gen. nov. strains are marked in bold. Scale bar represents the expected number of substitutions per site.



Figs 2-7. Morphology of *Uroglena* (Figs 2, 3), *Uroglenopsis* (Figs 4, 5) and *Urostipulosphaera* gen. nov. (Figs 6, 7) focusing emended diagnosis – cell shape, cell posterior, flagella length ratio, presence/absence and character of branched radial structures. **Fig. 2.** *Uroglena* cells of inverse tear-drop in shape with sharply pointed cell posterior passing into a thin, likely cytoplasmic thread, shorter flagellum approx. one half of longer flagellum, usually one girdle-shaped, bilobed, slightly spiral plastid. **Fig. 3.** *Uroglena* colony. **Fig. 4.** *Uroglenopsis* cells of diverse shape embedded onto a compact jelly, shorter flagellum approx. at most one quarter of longer flagellum, usually one girdle-shaped, plate plastid. **Fig. 5.** *Uroglenopsis* colony. **Fig. 6.** *Urostipulosphaera* obovate cells united by their truncate or rounded cell posterior to relatively thick articulated gelatinous stalks, shorter flagellum approx. at most one quarter of longer flagellum, usually one girdle-shaped, broadly ribbon, bilobed, slightly spiral plastid. **Fig. 7.** *Urostipulosphaera* colony. Scale = 10 μm (Figs 2, 4, 6) and 20 μm (Figs 3, 5, 7).



Figs 8-11. Morphology of *Uroglenopsis* shown on natural population U19 (Figs 8, 9), natural population UK-81 (Fig. 10) and cultured strain UK-81 (Fig. 11). **Fig. 8.** Colony after staining by methylene blue – cells are embedded onto a compact jelly mantle (black arrows). **Fig. 9.** Irregularly poly-lobal colonies. **Fig. 10.** Cells of *U. turfosa* (formerly *Eusphaerella*) are closely packed together and hexagonal from apical view. **Fig. 11.** Cultured colonies of *U. turfosa* lost their typical morphology when their cells became more loosely packed. Scale = 50 μm.



Figs 12-19. Cultured strains of *Urostipulosphaera* gen. nov. – cells with truncate or rounded posteriors connected to relatively thick articulated gelatinous stalks. (Figs 12-16) and SEM micrographs of *U. notabilis* encysting strain U12-1 (Figs 17-19). **Fig. 12.** *U. notabilis* strain U12-1 colony bearing cysts (black arrows). **Fig. 13.** *Urostipulosphaera* sp. strain UP-34. **Fig. 14.** *Urostipulosphaera* sp. strain U5-5. **Fig. 15.** *Urostipulosphaera* sp. strain U7-1. **Fig. 16.** *Urostipulosphaera* sp. strain U10-6. **Fig. 17.** *U. notabilis* fully developed cyst with well visible collapsed neck formed by rolled up sheet. **Fig. 18.** *U. notabilis* fully developed cyst with well visible pronounced curved tubular neck. **Fig. 19.** *U. notabilis* cysts ranged from almost smooth-walled to embellished cyst wall with wart-like processes ("verrucae") of irregular number and shape. Scale = 50 μm (Fig. 12), 20 μm (Figs 13-16) and 5 μm (Figs 17-19).

Supplementary table S1. Taxa selected according to Andersen *et al*. (2017) and Kristiansen & Škaloud (2017) used in current Chrysophyceae phylogeny. Outgroup taxa selected according to Yang *et al*. (2012). Newly acquired strains are highlighted in bold.

Order	Taxon	Strain	GenBank accession number	
			SSU	*rbc*L
Apoikiida	*Apoikia lindahlii*	–	FJ971855	–
Apoikiida	*Apoikiospumella mondseeiensis*	SAG 2428	AY651098	–
Apoikiida	*Spumella*-like flagellate	JBM18	AY651092	–
Hibberdiales	*Hibberdia magna*	CCMP 453	M87331	AF015572
Hibberdiales	*Chrysocapsa* sp.	UTCC 280	EF165130	EF165153
Hibberdiales	*Chrysocapsa vernalis*	CCMP 278	EF165105	EF165148
Hibberdiales	*Chrysocapsa wetherbeei*	CCMP 380	EF165145	EF165149
Hibberdiales	*Chrysonebula flava*	CCMP 2765	EF165104	EF165150
Hibberdiales	*Kremastochrysis* sp.	CCMP 260	AF123282	EF165152
Hibberdiales	*Naegeliella flagellifera*	CCMP 280	AF123284	EF165154
Hydrurales	*Hydrurus foetidus*	–	FM955256	–
Hydrurales	*Chrysophyceae* sp.	CCMP 2296	EU247834	–
Hydrurales	*Ochromonas*-like flagellate	CCMP 1899	EF165133	EF165159
Hydrurales	*Phaeoplaca thallosa*	CCMP 634	AF123296	EF165160

Chromulinales	*Cyclonexis annularis*	CCMP 1858	AF123292	–
Chromulinales	*Chromulina nebulosa*	CCMP 263	AF123285	AF155876
Chromulinales	*Chromulinospumella sphaerica*	SAG 2429	AY651093	–
Chromulinales	*Chrysamoeba mikrokonta*	CCMP 1857	AF123287	EF165182
Chromulinales	*Chrysamoeba tenera*	CCMP 273	EF165102	EF165181
Chromulinales	*Chrysosphaerella brevispina*	S74.D5	HF549059	HG315744
Chromulinales	*Chrysosphaerella longispina*	S61A.B4	HF549060	HF549072
Chromulinales	*Oikomonas mutabilis*	–	U42454	–
Chrysosaccales	*Chromophyton* cf. *rosanoffii*	CCMP 2751	EF165106	EF165164
Chrysosaccales	*Chrysastrella breviappendiculata*	CCMP 1861	AF123293	EF185315
Chrysosaccales	*Chrysosaccus* sp.	CCMP 368	EF165121	EF165166
Chrysosaccales	*Chrysosphaera parvula*	CCMP 349	AF123299	–
Chrysosaccales	*Lagynion* cf. *ampullaceum*	CCMP 2727	EF165146	EF165161
Chrysosaccales	*Lagynion scherffelii*	CCMP 465	AF123288	EF165162
Ochromonadales	*Acrispumella msimbasiensis*	SAG 2427	AY651077	–
Ochromonadales	*Cornospumella fuschlensis*	SAG 2430	GU073469	–
Ochromonadales	*Dinobryon* cf. *sociale*	UTCC 392	EF165141	EF165158
Ochromonadales	*Dinobryon cylindricum*	CCMP 2766	EF165140	EF165157
Ochromonadales	*Epipyxis aurea*	CCMP 385	AF123301	EF165155

Ochromonadales	*Epipyxis pulchra*	CCMP 382	AF123298	AF015571
Ochromonadales	*Chlorochromonas danica*	SAG 933-7	JQ281514	GU935657
Ochromonadales	*Chrysolepidomonas dendrolepidota*	CCMP 293	AF123297	AF015570
Ochromonadales	*Chrysonephele palustris*	–	U71196	–
Ochromonadales	*Kephyrion* sp.	CCMP 3057	JF730878	–
Ochromonadales	*Melkoniana moestrupii*	CCMP 1278	U42382	EF165174
Ochromonadales	*Melkoniana* sp.	CCMP 591	AF123302	EF165176
Ochromonadales	*Ochromonadaceae* sp.	CCMP 2298	EU247838	–
Ochromonadales	*Ochromonas* sp.	CCMP 592	EF165135	EF165201
Ochromonadales	*Ochromonas triangulata*	AC-25	EF165136	EF165177
Ochromonadales	"*Ochromonas marina*"	AC-22	EF165138	EF165203
Ochromonadales	"*Ochromonas perlata*"	CCMP 2732	EF165143	EF165187
Ochromonadales	"*Ochromonas* sp."	CCMP 1149	EF165139	EF165202
Ochromonadales	"*Ochromonas vasocystis*"	CCMP 2741	EF165111	EF165184
Ochromonadales	"*Ochromonas villosa*"	CCAP 933/25	FR865768	–
Ochromonadales	*Ochromonas*-like flagellate	CCMP 1393	EF165142	EF185314
Ochromonadales	*Ochromonas*-like flagellate	CCMP 2761	EF165126	EF165200
Ochromonadales	*Pedospumella encystans*	SAG 2324	AY651083	–
Ochromonadales	*Pedospumella sinomuralis*	SAG 2431	AY651081	–

Ochromonadales	*Poterioochromonas* cf. *malhamensis*	SAG 933-1c	EF165114	EF165169
Ochromonadales	*Poterioochromonas* cf. *stipitata*	CCMP 1862	AF123295	EF165172
Ochromonadales	*Poteriospumella lacustris*	SAG 2323	AY651074	–
Ochromonadales	*Spumella bureschii*	SAG 2433	AY651086	–
Ochromonadales	*Spumella lacusvadosi*	SAG 2434	AY651088	–
Ochromonadales	*Spumella vulgaris*	SAG 2322	DQ388552	–
Ochromonadales	*Spumella*-like flagellate	JBNA46	DQ388542	–
Ochromonadales	*Spumella*-like flagellate	JBM28	AY651089	–
Ochromonadales	***Uroglena volvox***	**U26-3**	**MK153236**	**MK153250**
Ochromonadales	***Uroglena* sp.**	**U29-5**	**MK153237**	**MK153251**
Ochromonadales	***Uroglena* sp.**	**UK-37**	**MK153238**	**MK153252**
Ochromonadales	*Uroglenopsis* cf. *americana*	CCMP 1863	AF123290	EF165179
Ochromonadales	*Uroglenopsis* cf. *americana*	CCMP 2769	EF165131	EF165178
Ochromonadales	***Uroglenopsis americana***	**UK-4**	**MK153242**	**MK153256**
Ochromonadales	*Uroglenopsis* sp.	FU44	EU024983	–
Ochromonadales	***Uroglenopsis* sp.**	**UJ-6**	**MK153239**	**MK153253**
Ochromonadales	***Uroglenopsis* sp.**	**UK-25**	**MK153240**	**MK153254**
Ochromonadales	***Uroglenopsis* sp.**	**U19**	**MK153241**	**MK153255**
Ochromonadales	***Uroglenopsis turfosa***	**UN-28**	**MK153243**	**MK153257**
Ochromonadales	***Uroglenopsis turfosa***	**UK-81**	**MK153244**	**MK153258**
Ochromonadales	***Urostipulosphaera notabilis***	**U12-1**	**MK153247**	**MK153261**
Ochromonadales	*Urostipulosphaera* sp.	CCMP 2768	EF165132	–
Ochromonadales	***Urostipulosphaera* sp.**	**U7-1**	**MK153245**	**MK153259**
Ochromonadales	***Urostipulosphaera* sp.**	**U5-5**	**MK153246**	**MK153260**
Ochromonadales	***Urostipulosphaera* sp.**	**U10-6**	**MK153248**	**MK153262**
Ochromonadales	***Urostipulosphaera* sp.**	**UP-34**	**MK153249**	**MK153263**
Paraphysomonadida	*Clathromonas butcheri*	MD03	JQ967291	–
Paraphysomonadida	*Clathromonas caroni*	DB4	AF109326	–

Paraphysomonadida	*Paraphysomonas imperforata*	CCAP 935/13	JQ967324	–
Paraphysomonadida	*Paraphysomonas parahebes*	Hflag	AF109322	–
Paraphysomonadida	*Paraphysomonas stylata*	W02	JQ967307	–
Paraphysomonadida	*Paraphysomonas variosa*	IND5	JQ967296	–
Segregatales	*Chrysophyceae* sp.	SA-2.1	AY520450	–
Segregatales	*Segregatospumella dracosaxi*	SAG 2432	GU073467	–
Synurales	*Mallomonas alpina*	–	GU935620	GU935662
Synurales	*Mallomonas caudata*	–	EF469638	EF469644
Synurales	*Mallomonas insignis*	CCMP 2549	EF165118	EF165198
Synurales	*Neotessella lapponica*	S59.C4	HF549063	HF549074
Synurales	*Neotessella volvocina*	CCMP 1781	EF165119	EF165199
Synurales	*Synura mollispina*	S71.C10	HF549067	HF549077
Synurales	*Synura petersenii*	CCMP 873	GU325588	GU325490
Synurales	*Synura sphagnicola*	CCMP 1705	U73221	GU325413
OUTGROUP	*Nannochloropsis limnetica*	–	DQ977726	DQ977741
OUTGROUP	*Synchroma grande*	CCMP 2876	DQ788730	DQ788731

PAPER III

Martin Pusztai & Pavel Škaloud

Species delimitation within the colonial flagellates *Uroglena, Uroglenopsis* and *Urostipulosphaera* (Chrysophyceae)

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Species delimitation within the colonial flagellates *Uroglena*, *Uroglenopsis* and *Urostipulosphaera* (Chrysophyceae)

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Abstract

Until recently, there was no agreement on species delimitation within the morphologically similar chrysophycean genera *Uroglena*, *Uroglenopsis* and *Urostipulosphaera*. In this study, we aimed at a modern taxonomic revision based on the combination of morphological characters (ultrastructure of cysts, cell and colony features) and a multigene phylogeny (SSU, ITS rDNA and *rbc*L sequences), with ecology taken into account. Of more than 650 explored localities, only approximately one in ten hosted a viable and detectable population of these colonial chrysophytes at the time of sampling. We established and examined 189 short-term cultures along with single colony isolates, derived mostly from blooming or encysting populations. We obtained the cyst morphology for four species and two lineages of *Uroglena*, two species of *Uroglenopsis*, and four species of *Urostipulosphaera*. A total of 12 resolved lineages could be attributed to previously described species or new species (*Uroglena imitata* sp. nov., *Urostipulosphaera granulata* sp. nov.). Based on our molecular analyses and morphological observations, we assigned all the previously described *Uroglena*-like taxa to newly recognized genera and proposed a key to identification. Consequently, *Uroglena* now includes 16 species and two varieties, *Uroglenopsis* contains four species, and *Urostipulosphaera* encompasses nine species. Within *Uroglena* and *Urostipulosphaera*, species are defined by the ultrastructure of their cysts. On the contrary, as *Uroglenopsis* has simple cysts, species are defined by cell and colony characteristics.

Keywords: Chrysophyceae; colonial flagellates; cysts, phylogeny; taxonomy; species delimitation; *Uroglena*; *Uroglenopsis*; *Urostipulosphaera*.

Introduction

The term 'species' represents one of the cornerstones of both the old and the modern biology because of permanent need to categorise and identify organisms. Nevertheless, alternative taxonomy-independent methods of biodiversity research have grown (Sun *et al*., 2012; Apotheloz-Perret-Gentil *et al*., 2017). Since the introduction of binomial nomenclature by Linnaeus (1753), the nature of species changed with evolutionary concepts. Darwin's theory of evolution (1859) accelerated the so-called 'species problem' and the discussion continues. Although we consider 'species' as a hypothesis (Bonde, 1977) when using more or less transient or artificial boundaries in nature, 'species' acts as the fundamental framework in many fields of biological research. Different species concepts (from the morphological species concept to multidisciplinary approaches) have been introduced, and are usually applied differently to particular taxonomic groups.

Hey (2001) stated that "the species problem is the long-standing failure of biologists to agree on how we should identify species and how we should define the word species". de Queiroz (2005, 2007) introduced the unified species concept which clearly separates the issues of species conceptualization and species delimitation. In this view, a separately evolving metapopulation lineage is the only necessary property of a species, but the species may be delimited in a variety of ways. In protists, it has been suggested that we should skip problematic searching for a correct general species concept and rather focus on clear species delimitation, ideally using more than one line of evidence and including a robust phylogenetic framework as a standard (Boenigk *et al*., 2012).

Protist taxonomy is still dealing with a high proportion of cryptic taxa within morphospecies (Howe *et al*., 2009; Škaloud & Rindi, 2013) and one of the main problems and challenges is incomplete reference DNA databases due to the lack of molecular data for numerous morphologically described species (Leray & Knowlton, 2015). The use of both molecular and morphological techniques is essential in the correct estimation of species diversity as both approaches are complementary (Škaloud *et al*., 2020). In Chrysophyceae (Stramenopiles, SAR), a diverse protist group commonly observed in

planktonic freshwater communities (Finlay & Esteban, 1998; Wolfe & Siver, 2013), current knowledge of diversity is mainly based on traditional morphology, with a few exceptions.

The diversity of silica-scaled chrysophytes, particularly Synurales and Paraphysomonadida, has been studied in a multidisciplinary approach providing a robust phylogenetic framework and good species-specific morphological characters (Jo *et al*., 2013; Scoble & Cavalier-Smith, 2014; Škaloud *et al*., 2014). In naked chrysophytes, however, only *Kremastochrysopsis* (Remias *et al*., 2020), *Ochromonas*-like (Andersen *et al*., 2017) and *Spumella*-like (Findenig *et al*., 2010; Grossmann *et al*., 2016) morphotypes have been evaluated using molecular techniques. These morphotypes represent 'prototypes' of a single-celled naked flagellate with a basic chrysophycean, or stramenopile, respectively, cell plan (two heterokont flagella), and as such they are scattered across the whole phylogenetic tree of Chrysophyceae.

Due to the absence of solid surface structures (e.g. silica scales) and a variable cell shape, the taxonomy of naked flagellates is very problematic. Consequently, the postulated taxonomic diversity certainly does not reflect the real species richness. Fortunately, all the chrysophytes possess one solid structure in their life cycles suitable for precise morphological delineation, the stomatocyst. These silica cysts are products of both asexual and sexual reproduction and usually exhibit great ultrastructural diversity – cyst wall decoration and shape of collar(s) surrounding the pore (Sandgren, 1991). However, encysting populations are rarely observed since the encystment process typically takes place over a short period at the end of blooms (Agbeti & Smol, 1995).

Photosynthetic colonial *Dinobryon*, *Synura* and *Uroglena*-like flagellates often cause the well-known spring and autumn plankton blooms in meso-oligotrophic freshwaters (Anneville *et al*., 2005; Bock *et al*., 2014). Recently, Pusztai & Škaloud (2019) taxonomically revised the polyphyletic *Uroglena*-like morphotype, which has resulted in at least three genetically and morphologically distinct lineages within the Ochromonadales (Chrysophyceae), distinguished as *Uroglena* Ehrenberg, *Uroglenopsis* Lemmermann and *Urostipulosphaera* Pusztai & Škaloud. So far, 35 taxa of *Uroglena* (the majority), *Uroglenopsis* and *Urostipulosphaera* have been validly described (Cronberg &

Laugaste, 2005; Pusztai & Škaloud 2019; Guiry & Guiry, 2020; Index Nominum Algarum, 2020). Cells of these three genera are always radially arranged as a monolayer coat at the periphery of the predominantly spherical colony. Nevertheless, the genera differ in cell shape (especially in cell posterior), flagellar length ratio, and the character of the branched radial structures.

Unfortunately, these morphological characters that clearly delimit three *Uroglena*-like genera seem to be useless in species delimitation. The colonies and cells of species within each of the genera are generally uniform and/or exhibit the same trends in phenotypic plasticity (Wujek & Thompson, 2002; Pusztai & Škaloud, 2019). Moreover, *Uroglena*, *Uroglenopsis* and *Urostipulosphaera* seem to have a similar ecology. Finally, previous work has not confirmed the presence of any scale-like structures (Wujek, 1976; Pusztai & Škaloud, 2019). Therefore, in these naked chrysophytes the ultrastructure of cysts seems to be the only applicable and relatively stable morphological character for species delineation. In general, *Uroglena*-like taxa have smooth or decorated spherical cysts with or without a straight/curved collar or two concentric collars.

The present study represents a follow-up to our previous paper showing that the *Uroglena*-like morphotype includes three separate genera (Pusztai & Škaloud, 2019), focussing on species diversity. It is based on the examination of short-term cultures along with single colony isolates, derived mostly, but not exclusively, from blooming and encysting populations of *Uroglena*, *Uroglenopsis* and *Urostipulosphaera*. The goal of this work was to conduct a modern taxonomic revision at species level, based on the combination of both morphological (ultrastructure of cysts, cell and colony features) and genetic evidence (SSU, ITS rDNA and *rbc*L sequences), taking ecology into account.

Material and Methods

Sample processing and morphological investigations

Sampling was carried out predominantly in the northern temperate zone (throughout Europe and part of North America) in 2014-2020. Isolates of *Uroglena*, *Uroglenopsis* and *Urostipulosphaera* (Table S1) were obtained from various freshwater bodies mostly during the spring and autumn chrysophyte blooms. Samples were

collected and processed as described previously in Pusztai & Škaloud (2019) but using TES-buffered WC liquid medium (pH ~ 7.5; Andersen *et al*., 1997) additionally. Measured values of abiotic factors (water pH, temperature, specific conductivity) were further visualized by boxplots and ecological differences between taxa were tested by parametric and nonparametric tests (t-test, Mann-Whitney test).

Morphological microscopic investigations were made as described previously in Pusztai & Škaloud (2019) but using FE-SEM ZEISS Ultra Plus (ZEISS Oberkochen, Germany) scanning electron microscope (SEM) additionally. Moreover, 50 cells from each of the six successfully maintained cultures of five different species of *Urostipulosphaera* strains were photographed and their shape and size analysed with ImageJ 1.45s (Schneider *et al*. 2012) for potential use in species delineation. Species determination of encysting populations was carried out according to information on ultrastructure in original descriptions.

Sequencing and phylogenetic analysis

DNA isolation was carried out as described in Škaloudová & Škaloud (2013) but using 10 ml of InstaGene matrix (Bio-Rad Laboratories) for single-colony isolates. Three loci were amplified by PCR: nuclear SSU rDNA, entire nuclear ITS rDNA region (ITS1-5.8S-ITS2) and plastid *rbc*L. These molecular markers should provide sufficient genus-level taxonomic resolution as well as species-level taxonomic resolution within the Chrysophyceae (Scoble & Cavalier-Smith, 2014; Grossmann *et al*., 2016; Andersen *et al*., 2017; Bock *et al*., 2017; Kristiansen & Škaloud, 2017). In addition, ITS rDNA is one of the most frequently used chrysophyte-specific barcodes (Pawlowski *et al*., 2012). It is preferred over COI (*cox*1) in order to avoid the potentially misleading clustering of some strains (Jost *et al*., 2010; Bock *et al*., 2017).

The amplification of SSU rDNA and *rbc*L markers followed Pusztai & Škaloud (2019), using the primers 18SF and 18SR (Katana *et al*. 2001) and our previously designed primers Chryso_SSU_F2 (5′-TGT CTC AAA GAT TAA GCC AT-3′), Chryso_SSU_R2 (5′-CTA CGG AAA CCT TGT TAC GA-3′), Chryso_*rbc*L_F4 (5′-TGG ACD GAY TTA TTA ACD GC-3′) and Chryso_*rbc*L_R7 (5′-CCW CCA CCR AAY TGT ARW A-3′). The

amplification of the ITS marker was performed as described by Kynčlová *et al*. (2010), using the newly designed primers Chryso_ITS_F (5′-ATC ATT TAG AGG AAG GTG A-3′) and Chryso_ITS_R (5′-GCT TCA CTC GCC GTT ACT-3′). The PCR products were purified and sequenced at Macrogen Inc. Sequencing of additional molecular loci was not possible due to the limited amount of DNA obtained using our single-colony isolation method. Newly determined sequences were aligned with sequences of *Uroglena*, *Uroglenopsis* and *Urostipulosphaera* from GenBank (Table S1) to produce three multigene alignments, one for each genus. The SSU rDNA sequences were not used in species-level analyses because they were invariant within each genus. ITS rDNA (586/604/775 bp) and *rbc*L (962 bp) were concatenated as alignments of 1,548 bp (*Uroglena*), 1,566 bp (*Uroglenopsis*) and 1,737 bp (*Urostipulosphaera*). Single-locus alignments were used to evaluate congruence, including 18/77 unique/total sequences of *Uroglena*, 20/67 unique/total sequences of *Uroglenopsis* and 8/45 unique/total sequences of *Urostipulosphaera* taxa. *rbc*L sequences were manually aligned using MEGA 6 (Tamura *et al*., 2013), and ITS alignments were constructed using MAFFT v6, applying the Q-INS-i strategy (Katoh *et al*., 2002). Positions with deletions in a majority of sequences were removed from the alignment.

The best-fit nucleotide substitution model for each of the alignment partitions was estimated using the Bayesian Information Criterion (BIC) and Akaike Information Criterion (AIC) in jModelTest 2.1.4 (Darriba *et al*., 2012). For the ITS region, boundaries of the ITS1, 5.8S and ITS2 regions were determined by comparing them with the published 5.8S sequence of *Dinobryon pediforme* strain LO2_36_1 (KJ579347). These procedures selected for *Uroglena* the following models: HKY for ITS1 and 5.8S, HKY + I for ITS2, GTR + I for the first and second codon position of the *rbc*L gene, GTR + G for the third codon position of the *rbc*L gene; for *Uroglenopsis* HKY + G for ITS1 and ITS2, GTR + I for 5.8S and the *rbc*L second codon position, GTR for the *rbc*L first codon position, GTR + G for the *rbc*L third codon position; for *Urostipulosphaera* HKY + I for ITS1, GTR + I for 5.8S and the *rbc*L first and second codon positions, GTR + G for ITS2 and the *rbc*L third codon position.

Phylogenetic trees were inferred by Bayesian inference (BI) using MrBayes version 3.2.1 (Ronquist *et al*., 2012). BI analyses were run on the CIPRES Science Gateway v.3.3 web portal (Miller *et al*., 2010) with partitioned datasets using the substitution models specified above. All parameters were unlinked among partitions. Two parallel MCMC runs were carried out for 10 million generations, each with one cold and three heated chains. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was assessed during the run by calculating the average standard deviation of split frequencies (SDSF). The SDSF value was 0.0012 for *Uroglena*, 0.0010 for *Uroglenopsis* and 0.0001 for *Urostipulosphaera*. Finally, the burn-in value was determined using the "sump" command. Bootstrap analyses were performed by maximum likelihood (ML) and weighted maximum parsimony (wMP) criteria using GARLI, version 2.01 (Zwickl, 2006) and PAUP*, version 4.0b10 (Swofford, 2002), respectively, as described in Pusztai *et al*. (2016). Topologies of all clades in single-locus ITS and *rbc*L trees were congruent with the exception of UK-37 and UK-41 isolates with unsupported positions in the ITS tree.

Results

Distribution and ecology

More than 650 localities were explored, only approximately 10% of which hosted a detectable population of *Uroglena*-like colonial chrysophytes at the time of sampling. We established 189 single-colony isolates (Table S1) of *Uroglena* (77), *Uroglenopsis* (67) and *Urostipulosphaera* (45). The phylogenetic position of all three genera is shown on a simplified phylogram (Fig. S1) adapted from Pusztai & Škaloud (2019). Many isolates originated from encysting populations (Table S1) and we successfully collected material from type localities of four taxa – *Uroglena skujae* Matvienko ex Pusztai & Škaloud, sp. nov. [= *Uroglena europaea* (Pascher) Skuja], *Uroglena volvox* Ehrenberg, *Uroglenopsis americana* (Calkins) Lemmermann and *Uroglenopsis botrys* (Pascher) Pascher. Further, we obtained the cyst morphology for four species and two lineages of *Uroglena*, two species of *Uroglenopsis*, and four species of *Urostipulosphaera*. 12 resolved lineages could be clearly attributed to previously or newly described species. Colonies usually consisted

of tens to hundreds of cells, but smaller colonies with fewer cells might be often produced by a large colony collapsing during observation. Smaller colonies were also produced in the culture.

Although all three genera exhibited a similar ecology in the northern temperate zone, some differences were discovered (Figs 1-4). *Uroglena* and *Uroglenopsis* both exhibit spring and autumn population maxima, but not *Urostipulosphaera*. *Urostipulosphaera* occurred in colder waters than *Uroglena* (Mann-Whitney, $p = 0.021$) and *Uroglenopsis* (Mann-Whitney, $p = 0.017$). *Uroglenopsis* occupied waters with significantly lower pH than *Uroglena* (t-test, $p = 0.014$) and *Urostipulosphaera* (t-test, $p = 0.026$) and with significantly lower conductivity than *Uroglena* (Mann-Whitney, $p = 0.034$). Values of measured environmental factors (Table S1) are shown as habitat differences at the generic level (Figs 2-4). Unfortunately, statistical evaluation of species-level differences is not possible due to insufficient numbers of observations.

Uroglena

Phylogenetic analysis revealed two strongly supported major lineages within *Uroglena* (Fig. 5). The first lineage consisted of a well-resolved clade of *Uroglena glabra* Matvienko and *U. volvox*, and two isolates (U34-1 and U17-9) forming an unsupported basal clade. The second lineage included two well-resolved sister clades of *Uroglena zachariasii* Thompson & Wujek and *Uroglena skujae* Matvienko ex Pusztai & Škaloud, sp. nov., plus a group of genetically identical isolates here referred to as *Uroglena imitata* sp. nov., two clades both termed *Uroglena* cf. *zachariasii*, and a single isolate UG-30.

Uroglena (Figs 6-20) cells were always inverse-teardrop shaped, with a sharply pointed cell posterior and two unequal (ratio 1:2) anterior flagella. Cells usually contained a single girdle-shaped, bi-lobed, slightly spiral, gold-coloured plastid that possessed an anterior stigma. Cell posterior continuing as thin, probably cytoplasmic, threads connecting individual cells by a dichotomously branching system into a more or less spherical colony; threads at colony centre sometimes thicker. Cysts were always spherical and smooth or imperfectly smooth (i.e., regularly coated with almost

imperceptible very small particles) with simple or complex concentric straight collar(s). Morphological characteristics of individual species are summarized in Table 1.

Uroglenopsis

Phylogenetic analysis revealed three strongly supported major lineages within *Uroglenopsis* (Fig. 21). The first lineage consisted of two sister clades, referred here to as *Uroglenopsis* sp. 1 and sp. 2. We were not able to assign these two clades to any of previously described species. The second lineage encompassed only isolates determined as *Uroglenopsis turfosa* (Skuja) Thompson & Wujek. The third lineage was composed by two well-recognized clades of *Uroglenopsis americana* and *Uroglenopsis botrys*, and two single-sequence isolates U26-32 and U26-19-451. *U. americana* isolates were related to the strains CCMP1863 and CCMP2769 from Canada. *U. botrys* was the most common species recovered in this study.

Uroglenopsis (Figs 22-35) colonies and cells were of diverse shape. Colonies were usually spherical to oval, but *U. americana* and *U. botrys* further produced elongated to irregularly poly-lobal colonies. *U. turfosa* possessed unique morphology, fresh colonies were always closely packed together, with hexagonal cells in apical view and a remarkable hole in the spherical colony. Cells were mostly spindle-shaped, oval to slightly obovate or elongated and cylindrical, with a predominantly bluntly tapering cell posterior and two distinctly unequal (ratio 1:4 or more significant) anterior flagella. Cells usually contained a single parietal, gold-coloured plastid that possessed an anterior stigma. Plastid was elongated and oriented in cell axis direction. No branching system of any radially arranged structures was observed, even when stained with Lugol's iodine solution or methylene blue. Instead, cells were embedded into a compact jelly mantle and possessed one to few thin and short (1-3 μm) spine-like structures protruding from posterior part, most likely helping to fix cells within the jelly mantel, as previously pointed out by Skuja (1948). Accordingly, cells of stationary colonies exhibited characteristic jerking and yanking movements. Cysts were almost spherical to slightly oval or oblate, smooth and without a collar. Morphological characteristics of individual species are summarized in Table 1.

Urostipulosphaera

Of two strongly supported major lineages (Fig. 36), the first encompassed a single clade, here referred to as *Urostipulosphaera granulata* sp. nov. Based on the concatenated SSU rDNA and *rbc*L phylogeny (Pusztai & Škaloud, 2019), *Urostipulosphaera* sp. CCMP 2768 is the sister clade to *U. granulata* within the first lineage. The second lineage was composed of four clades, here referred to as *U. notabilis* (Mack) Pusztai & Škaloud, *U. articulata* (Korshikov) Pusztai & Škaloud comb. nov., *U. lindiae* (Bourrelly) Pusztai & Škaloud comb. nov., and *Urostipulosphaera* sp.

Urostipulosphaera (Figs. 37-57) cells were usually obovate in shape, carried two distinctly unequal (ratio 1:4 or more significant) anterior flagella. Cells usually contained a single girdle-shaped or slightly spiral, broadly ribbon, bilobed, gold-coloured plastid that possessed an anterior stigma. Predominantly truncate or rounded cell posteriors were always connected via dichotomously branching system of relatively thick articulated gelatinous stalks, sometimes covered with bacteria and thus made more visible. In fresh samples, colonies were usually perfectly spherical in their shape but sometimes with poorly visible stalks. Cultured colonies were sometimes oval in their shape but always with well visible stalks. Cysts were almost spherical to slightly oval or oblate, rough or embellished and with a curved collar. Morphological characteristics of individual species are summarized in Table 1.

Isolates of *Urostipulosphaera* possessed significantly higher survival rate as compared to two above-mentioned genera. Therefore, we were able to morphologically characterise in detail all species-level clades. Accordingly, we evaluated the usability of cell morphological features in the species delineation. Our analyses show that species of *U. notabilis* (U12-1), *U. articulata* (U5-5) and *U. lindiae* (UP-34) had similar range of cell length and width (Figs 58-60). *U.* sp. (U10-6) possessed possessed very elongated cells. Further, *U. granulata* possessed larger cells and smaller cysts than other *Urostipulosphaera* species. Within the single species, however, isolates of *U. granulata* (U7-1 and U33) differed in their cell length/width range though they are virtually genetically identical. Moreover, the U7-1 isolate possessed generally longer cells in natural sample (12-16 × 6-8,5 μm) than in the culture (7-14 × 6-10.5 μm).

Taxonomic revisions and diagnoses

***Uroglena imitata* Pusztai & Škaloud sp. nov.** (Figs 17-20)

DESCRIPTION: Colonies are 120-180 μm in diameter with cells 10-12.5 μm long and 5.5-7.5(-9) μm wide. Cysts spherical, 13.3-14.8 μm in diameter with 1 μm wide pore and complex collars. Cysts usually smooth (LM) or imperfectly smooth (SEM), regularly coated with very small particles. Primary collar is 1.5-2 μm high, 2.4-2.8 μm wide. Secondary collar is (1.9-)4.8-7.9 μm high, 4.6-6.3(-8.1) μm wide. Cyst diameter/secondary collar width ratio is 2.1-2.6.

HOLOTYPE (here designated): Portion of a single gathering of cysts (strain UR-2) on SEM stub deposited at the Culture Collection of Algae of Charles University, Prague (CAUP). Figure 19 presents an illustration of the holotype.

TYPE LOCALITY: Lacul Noua, Romania (45.61429N, 25.63962E).

ETYMOLOGY: The specific epithet 'imitata' reflects that cysts of *U. imitata* highly resemble those of *U. zachariasii*, the most common cyst morphotype among *Uroglena*, but possessed significantly narrower and higher secondary collar.

REPRESENTATIVE DNA SEQUENCES: GenBank accessions no. MW267669, MW251563.

DISTRIBUTION: Currently known from Austria, Czech Republic, Portugal and Romania.

***Uroglena rotundata* (Skvortzov) Pusztai & Škaloud comb. nov.**

BASIONYM: *Uroglenopsis rotundata* Skvortzov Philip. J. Sc. 86: 183, pl. 6: fig. 53 (1958).

TYPE LOCALITY: swamp near Harbin, China.

NOTE: We did not have the opportunity to observe living material of this species. However, the original drawings of the colony with characteristic flagella length ratio unequivocally assign the species to the genus *Uroglena*.

***Uroglena skujae* Matvienko ex Pusztai & Škaloud sp. nov.** (Figs 14-16)

DESCRIPTION: Colonies range 100-150 μm in diameter with cells 8.5-11.5 μm long and 7-8.5 μm wide. Cysts spherical, (11-)12.5-14.5 μm in diameter with a pronounced very long collar of 8.5-14.5 μm high and 3.5-4.5 μm wide. Cysts usually smooth (LM) or imperfectly smooth (SEM), regularly coated with very small particles.

HOLOTYPE (here designated): original drawings of *U. europaea* by Skuja, Symb. Bot. Upsal. 9(3): p. 272, pl. 30: fig. 10-12 (1948).

SYNONYM: *Uroglena europaea* (Pascher) Skuja 1948: 267

TYPE LOCALITY: Ubby-Langsjön, Sweden.

ETYMOLOGY: The specific epithet 'skujae' was originally proposed by Matvienko (1965) for the species to be named in honour of Latvian phycologist Heinrich Leonhards Skuja (1892–1972), who first described cysts with such a morphology in Sweden.

REPRESENTATIVE DNA SEQUENCES: GenBank accessions no. MW267676, MW251564.

DISTRIBUTION: Currently known from Sweden and Ukraine.

***Uroglenopsis troitzkajae* (Korshikov) Pusztai & Škaloud comb. nov.**

BASIONYM: *Uroglena troitzkajae* Korshikov in Korshikov & Matwienko, Uchen. Zap. Kharkovsk. Derzh, Univ., Trudy Inst. Bot. 4: 13 (1941).

SYNONYM: *Uroglenopsis americana* (Calkins) Lemmermann sensu Troitzkaja 1924: 266

TYPE LOCALITY: Environs of Saint Petersburg, Russia.

NOTE: We did not have the opportunity to observe living material of this species. However, the original description and drawings of the colonies and cells unequivocally assign the species into the genus *Uroglenopsis*.

***Urostipulosphaera articulata* (Korshikov) Pusztai & Škaloud comb. nov.** (Figs 49-52)

BASIONYM: *Uroglena articulata* Korshikov in Korshikov & Matwienko, Uchen. Zap. Kharkovsk. Derzh, Univ., Trudy Inst. Bot. 4: 5-9, figs 1-4 (1941).

SYNONYM: *Uroglenopsis articulata* (Korshikov) Thompson & Wujek 2002: 302.

TYPE LOCALITY: Bogged lake near the village Kovda, Karelia, Russia.

REFERENCE STRAIN LOCALITY: Strain U5-5 was isolated from Kříž pond in PP Na Plachtě, Czech Republic (50.1827819N, 15.8702700E).

REPRESENTATIVE DNA SEQUENCES: GenBank accessions no. MW267729, MK153260.

***Urostipulosphaera conimamma* (Nygaard) Pusztai & Škaloud comb. nov.**

BASIONYM: *Uroglena conimamma* Nygaard, K. Danske Vid. Selsk. Biol. Skr. 21(1): 10, fig. 7 (1977).

SYNONYM: *Uroglenopsis conimamma* (Nygaard) Wujek & Thompson 2002: 303; *U. americana* (Calkins) Lemmermann sensu Nygaard 1945: 26.

TYPE LOCALITY: Lille Gribsø, Denmark.

NOTE: We did not have the opportunity to observe living material of this species. However, the original description and drawings of the colonies and cysts unequivocally assign the species into the genus *Urostipulosphaera*.

***Urostipulosphaera europaea* (Pascher) Pusztai & Škaloud comb. nov.**

BASIONYM: *Uroglenopsis europaea* Pascher, Osterr. Bot. Z. 60: 4, pl. I: figs. 15-17 (1910).

SYNONYM: non *Uroglena europaea* (Pascher) Skuja 1948: 267

TYPE LOCALITY: 'Olsch' bei Mugrau (pond or stream near villages Olšina or Olšov), Šumava mountains, Czech Republic.

NOTE: We did not have the opportunity to observe living material of this species. However, the original description and drawings of the colonies and cells unequivocally assign the species into the genus *Urostipulosphaera*.

***Urostipulosphaera eustylis* (Skuja) Pusztai & Škaloud comb. nov.**

BASIONYM: *Uroglena eustylis* Skuja, Symb. Bot. Upsal. 9(3): p. 272, pl. 30: fig. 16-18 (1948).

SYNONYM: *Uroglenopsis eustylis* (Skuja) Thompson & Wujek 2002: 302.

TYPE LOCALITY: Ämsjön, Uppland, Sweden.

NOTE: We did not have the opportunity to observe living material of this species. However, the original description and drawings of the colonies unequivocally assign the species into the genus *Urostipulosphaera*.

***Urostipulosphaera granulata* Pusztai & Škaloud sp. nov.** (Figs 37-45)

DESCRIPTION: Colonies range 40-80(-100) μm in diameter with cells (7-)10-16 μm long and 6-9.5(-10.5) μm wide. Cysts almost spherical to slightly oblate or slightly oval, 9.5-

12 μm wide and 7-11(-12.5) μm in length. Cysts usually equally embellished with numerous regularly shaped granules. Granules are (0.3-)0.4-0.6(-0.7) μm in diameter and well visible in both LM and SEM. Pore (0.4-0.9 μm in diameter) is surrounded by 1.7-2.3 μm wide, curved, collapsed, tubular collar.

HOLOTYPE (here designated): Portion of a single gathering of cysts (strain U7-1) on SEM stub deposited at the Culture Collection of Algae of Charles University, Prague (CAUP). Figure 43 presents an illustration of the holotype.

REFERENCE STRAIN: The culture of the strain U7-1 has been deposited as CAUP B 801 in the Culture Collection of Algae of Charles University in Prague, Czech Republic.

TYPE LOCALITY: Small pool in the Botanical Garden of Charles University, Prague, Czech Republic (50.0710836N, 14.4206419E), ca 50 m far from our office.

ETYMOLOGY: The specific epithet 'granulata' reflects that cysts of *U. granulata* are decorated by numerous small granules.

REPRESENTATIVE DNA SEQUENCES: GenBank accessions no. MW267730, MK153259.

DISTRIBUTION: Currently only known from two localities in Prague, Czech Republic.

***Urostipulosphaera lindiae* (Bourrelly) Pusztai & Škaloud comb. nov.** (Figs 53-57)

BASIONYM: *Uroglena lindiae* Bourrelly, Rev. Alg., Mém. Hors-sér. 1: 155, pl. 1: figs. 35-38 (1957).

SYNONYM: *Uroglenopsis lindiae* Bourrelly in Thompson & Wujek 2002: 302.

TYPE LOCALITY: Forêt de Sénart, Paris, France.

REFERENCE STRAIN LOCALITY: Strain U29-1-496 was isolated from Vydýmač pond, Czech Republic (48.9617636N, 14.9525025E).

REPRESENTATIVE DNA SEQUENCES: GenBank accessions no. MW267732, MK153263.

***Urostipulosphaera proxima* (Korshikov & Matvienko) Pusztai & Škaloud comb. nov.**

BASIONYM: *Uroglena proxima* Korshikov & Matvienko, Uchen. Zap. Kharkovsk. Derzh, Univ., Trudy Inst. Bot. 4: 9-14, figs 5-9 (1941).

TYPE LOCALITY: near Kharkiv, Ukraine.

NOTE: We did not have the opportunity to observe living material of this species. However, the original description and drawings of the colonies unequivocally assign the species into the genus *Urostipulosphaera*.

***Urostipulosphaera soniaca* (Conrad) Pusztai & Škaloud comb. nov.**

BASIONYM: *Uroglena soniaca* Conrad, Bull. Mus. R. Hist. Nat. Belg. 14(42): 1, figs. A-E, H, pl. I, II (1938).

SYNONYM: *Uroglenopsis soniaca* (Conrad) Thompson & Wujek 2002: 301

TYPE LOCALITY: Forêt de Soignes, Belgium.

NOTE: We did not have the opportunity to observe living material of this species. However, the original description and drawings of the colonies unequivocally assign the species into the genus *Urostipulosphaera*.

Discussion

Morphological features and species delimitation

Originally, *Uroglena*-like taxa were predominantly defined by the morphology of colonies, cells, and plastids. However, this often proved to be insufficient to distinguish amongst species. Comparing five *Urostipulosphaera* species cultivated under the same conditions, their dimensions overlap quite a lot (Figs 58-60). In addition, these features are plastic and variable during ontogenesis, due to environmental conditions or due to stress from heating and drying of the sample during LM observations (Wujek & Thompson, 2002; Pusztai & Škaloud, 2019). Differences in cell shape and size between algae grown in cultures, and in the field conditions, are also known from the first experiments with culturing (Andersen, 2005). Cultured cells (e.g. *U. granulata* sp. nov.) are generally smaller in cell length and more globular when compared to fresh natural samples or dimensions given by other authors (summarised in Starmach, 1985). Even *Uroglenopsis* and *Urostipulosphaera* can produce thin, but short and unbranched threads when stressed (Fig. 40) and the use of fixations or dyes can cause the formation of

artefacts (Conrad, 1938). Therefore, the precise examination of cyst using SEM is not only a great advantage, but a necessity.

The use of cysts in taxonomy is not without complications. Cysts are not known for all the described species. For such species, it is still possible, even usually challenging, to recollect encysting material from the type locality. The intriguing question is whether the cyst ultrastructure of *Uroglena*-like colonial flagellates really represents the species-specific character (Skuja, 1948; Wujek & Thompson, 2002; Cronberg & Laugaste, 2005). We are aware of the fact that in some chrysophyte genera (e.g. some *Synura* species), the cysts are not species-specific due to their simplicity, resembling immature and not fully developed cysts (Duff *et al*., 1995). In addition, Holen (2014) showed that in monoclonal chrysophyte cultures, the cyst diameter may be relatively stable in some populations but possess a huge range in others. Moreover, it is known that sexual and asexual cysts possess, in general, the same morphology, differing only in their diameter (Sandgren, 1983). On the other hand, the length of both the spines and the collars is markedly variable (Bourrelly, 1957; Nygaard, 1977) and influenced by temperature during the encystment process (Sandgren, 1983).

According to our observations and taxonomic revision, *Urostipulosphaera* and *Uroglena* cysts seem to be truly species-specific when observed by SEM. *Uroglenopsis* species generally possess morphologically highly similar cysts, differing only in their diameter. Despite that, it seems that many *Uroglenopsis* species may be determined by the cell and colony characteristics.

Ecological differences among lineages

An overall task in this study was to obtain a sufficient number of single colony isolates (or short-term cultures) derived from encysting populations. The proportion of encysting populations within all the sampled populations was, however, different between the genera. The highest proportion was observed in *Urostipulosphaera* and *Uroglena* where cysts were successfully acquired for nearly all the revealed lineages. On the contrary, in *Uroglenopsis* we only obtained cysts for *U. turfosa* (strain UK-81) encysting in culture, and for *U. botrys* (UL-2). *U. botrys* was collected directly from the

encysting population in summer after many regular inspections at the site since its spring population bloomed in April.

One possible explanation of such an encystment difference lies in our newly discovered ecological preference of *Uroglenopsis* in the north temperate zone. *Uroglenopsis* seems to be a predominantly late spring to summer taxon, peaking in May with an occurrence from April to July. Since our sampling effort was typically focussed on spring and autumn chrysophytes maxima, the summer under-sampling could have easily happened and affected our dataset. The long-term examination of encysting *Uroglenopsis* populations by Skuja (1948, 1956) in Sweden support this explanation. He found *U. americana* sensu Skuja and *U. turfosa* from spring, but they peaked and encysted later, from summer to autumn. He further found that *U. irregularis*, which had already peaked during the spring, produced cysts again from summer to autumn.

According to our observations of ecological (Figs 1-4) and habitat (Table S1) preferences in the north temperate zone, it is further evident that *Urostipulosphaera* rather inhabits man-made and strongly influenced habitats, such as ponds, where it peaks in early spring waters with significantly lower temperature when compared to *Uroglena* and *Uroglenopsis*. The vast majority of *Urostipulosphaera* isolates came from the ponds in the Czech Republic, distinctive by their high productivity, trophic state, and phytoplankton biomass (IUCN, 1997). Conversely, *Uroglenopsis* rather inhabits pure habitats, such as drinking water reservoirs or lakes, usually situated in mountain regions or northward, and in both cases, often in the area of coniferous forests. Therefore, these habitats usually possess a low pH and a trophic state, as well as a later start of the season when compared to lowland ponds. This is in accordance with our presumption of a late summer encystment process in *Uroglenopsis* mentioned above. Finally, *Uroglena* exhibits intermediate ecological preferences. On the other hand, all three genera can be found in one location sharing the same planktonic habitat, but they still differ in their phenology and seasonal dynamics.

Taxonomy

From the past taxonomical systems of *Uroglena*-like flagellates, the most elaborated and closest to the present one was the system of Korshikov & Matvienko (Korshikov & Matvienko, 1941; Matvienko, 1965), where *Urostipulosphaera* species were placed into *Uroglena* s.l. The common element was the presence of the system of dichotomously branched radial structures connecting cells in the colony. Two or three sections (formally unestablished), differing in thread/stalk thickness, were distinguished within *Uroglena* s.l. In contrast, *Urostipulosphaera* species were placed into *Uroglenopsis* s.l. by Wujek & Thompson (2002), pointing thin threads as *Uroglena* synapomorphy, and reflecting sometimes poorly visible stalks of (yet unrecognised) *Urostipulosphaera*.

The long-standing discrepancy of the concepts "all are *Uroglena*" vs. "*Uroglenopsis* exist", has been resolved by the latest taxonomical revision and by the introduction of *Urostipulosphaera* (Pusztai & Škaloud 2019). According to our molecular analyses and morphological observations, we were able to assign all the previously described taxa to recognised genera (Table S2). Consequently, *Uroglena* includes 16 species and two varieties, *Uroglenopsis* contains four species, and *Urostipulosphaera* encompasses nine species. Some of previously described species were placed in synonymy. Below, we provide a taxonomic overview for all three genera. In addition, the key to the determination of genera and species, mainly based on differences in cyst morphology, is listed as Table S3.

***Uroglena* Ehrenberg, 1834**

The type species *U. volvox* was recollected from its type locality in Berlin (Germany), and was determined according to its original description given by Ehrenberg (for more details see Pusztai & Škaloud, 2019).

Although Skuja (1948, 1956) recognised only the genus *Uroglena*, here, based on his detailed drawings, it is possible to affiliate his recorded taxa to newly circumscribed genera. Accordingly, *U. europaea,* with a newly associated species-specific smooth cyst with a very long collar is, with no doubt, a new *Uroglena* species for science. Unfortunately, *Uroglena* cyst and colony types were incorrectly assigned by Skuja (1948)

to a previously described species *Uroglenopsis europaea*, and characteristics of both taxa were mixed in the new 'hybrid' combination *Uroglena europaea* as a further often recognised species. The study pointing out this problem was put forward by Matvienko (1965) and the new species was, unfortunately incorrectly (missing latin diagnosis), described according to Skuja's previous observations as *Uroglena skujae*. Therefore, species *Uroglena europaea* should be taken into synonymy with newly proposed *Uroglena skujae* Matvienko ex Pusztai & Škaloud sp. nov. *U. skujae* was recollected from its type locality (Ubby-Langsjön, Sweden) for more than 60 years after cysts with such morphology were first described by Skuja (1948).

In the second species with a newly assigned cyst sensu Skuja, *U. botrys* (Pascher) Conrad, the newly associated species-specific cyst is identical with the previously described species *U. glabra*. It is evident that the same cyst type was, however, later incorrectly assigned by previous authors to a species from the genus *Uroglenopsis*, *U. botrys*, and characteristics of both genera were mixed in the 'hybrid' new combination *Uroglena botrys* as a further often recognised species with a characteristic smooth cyst with a low collar. Conrad (1938) knew that Schiller (1926) added a different cyst type to *U. botrys,* but he ignored this cyst as immature. Therefore, species *Uroglena botrys* and findings of *Uroglena* with such cysts, sensu Skuja, should be taken into synonymy with *Uroglena glabra*. This cyst type given by Skuja was originally described from Sweden, and we found such cysts in Swedish locations as well. Our SEM findings of the cyst ultrastructure further indicate that in *U. glabra*, the collar production starts as a very low thick-walled rounded marginal rim around the pore (immature cysts) and is followed by the production of a low collar with an acute rim, or with a false complex collar. This may explain deviations in the collar characteristics (mainly length) given by different authors.

Based on comparison of our material with the dimensions and figures of material originally examined by Zacharias (1895) and later on by Wujek & Thompson (2002), we were able to undoubtedly assign one well-supported clade to *U. zachariasii* [= *U. volvox* sensu Zacharias]. *U. zachariasii* represents a genetically diverse clade encompassing three lineages which may belong to different populations (as considered here) or different

species. Interestingly, all three lineages are geographically distinct with the first lineage (UK-37, UK-41) coming from North America, not Europe. In the second lineage, mainly from Sweden, cysts of the *U. zachariasii* var. *uplandica* were recovered in one natural sample (U26-14). However, since we don't have enough data, further evaluation is needed.

Furthermore, based on genetic data and a specific cyst diameter/secondary collar width ratio, we proposed a new species with a cyst morphology similar to *U. zachariasii*, *U. imitata* sp. nov. All populations of *U. zachariasii* showed a ratio between 1.3-1.8, corresponding to earlier findings in the literature. Conversely, populations of *U. imitata* showed a ratio between 2.1-2.6. According to older examinations given by other authors, only the examination given by Geissbühler (1933) fits to this newly recognised species. The remaining two clades with cysts similar to *U. zachariasii* or *U. imitata*, whose isolates originated from poorly encysting populations and therefore we were not able to precisely evaluate their characteristics in SEM, were left in uncertainty as *U.* cf. *zachariasii* and will need further examination.

Based on the original descriptions made on material carrying species-specific cysts, species *U. collaris* Thompson & Wujek, *U. dendracantha* Cronberg, *U. estonica* Cronberg & Laugaste, *U. kukkii* Cronberg & Laugaste, *U. marina* Büttner, *U. nygaardii* Bourrelly, *U. pikamae* Cronberg & Laugaste, and *U. spinosa* Cronberg & Laugaste represent well delimited species with precise descriptions distinguishing them from any other *Uroglena* species. The taxonomic status of *U. conradii* Schiller and *U. conradii* var. *gallica* Bourrelly will need further evaluation. Their cysts were described (the first one only verbally) as globular and smooth in LM without any collars, only slightly thickened around the pore in the second species. Thus, they resemble any *Uroglena* immature cyst. Similarly, *U. volvox* var. *verrucosa* (Mack) Thompson & Wujek [= *U. botrys* var. *verrucosa* Mack] with variable cysts (only LM knowledge) resembling *U. pikamae* and *U. glabra*, will need further evaluation of its taxonomical status. In *U. radiata* Calkins, which was originally described from the USA on material lacking cysts, further efforts to find encysting populations will be of great value to provide a more detailed description. However, the original description and drawings of the colonies unequivocally assign the

species into the genus *Uroglena,* and its later displacement into *Uroglenopsis* by Lemmermann (1899) is in conflict with the current taxonomic revision. According to the original description, *U. radiata* Calkins possessed thin threads unlike *Uroglenopsis americana* (Calkins) Lemmermann in which no such structures were observed.

For *U. rotundata* comb. nov., which was originally described from China on material lacking cysts, further efforts to find encysting populations will be of great value to provide a more detailed description. Despite the original description and drawings being vague, this species can be unequivocally assigned into the genus *Uroglena* according to its characteristic flagella length ratio.

***Uroglenopsis* Lemmermann, 1899**

The type species *U. americana* was recollected from the type locality and determined according to its original description given by Calkins (1892); for more details see Pusztai & Škaloud (2019). *U. americana* is closely related to *U. botrys* according to the molecular genetic data as well as the specific morphology of the poly-lobal colonies, which they share. Considering our isolates of *U. americana* obtained from the type locality (USA), as well as older sequenced isolates (only from Canada), it seems that *U. americana* is not common in Europe. This is in accordance with observations on *Uroglenopsis* given by Schiller (1926) who stated that unlike other species, *U. americana* very rarely occurred in Europe. Therefore, many of the previously recorded European observations of *U. americana* very likely belonged to the widespread *U. botrys* and related species (see below). It was recognised by Matvienko (1965) that *U. americana* sensu Skuja possessed cells significantly differing in shape and dimensions from true *U. americana,* with generally smaller cells. Therefore, she erected a new species, *Uroglenopsis skujae* Matvienko.

Pascher (1910, 1913) clearly distinguished *Uroglenopsis* in the way it was originally described, thus without any system of dichotomously branched radial structures that are well visible in *Uroglena*. Therefore, it is no doubt that Pascher was observing *Uroglenopsis botrys*. Our isolated *U. botrys* recollected from the type locality Máchovo jezero, Czech Republic, and from other localities, was in accordance with the

original description. Unfortunately, Pascher did not observe cysts and our material from the type locality also lacked any cysts. Fortunately, we collected *U. botrys* from many other localities and one population (UL-2) was producing cysts. These cysts correspond to cysts additionally assigned to *U. botrys* by Schiller (1926), or to cysts found later by Skuja (1948, 1956) in Scandinavian *U. skujae* and *U. irregularis*.

Interestingly, colonies and cells of different *U. botrys* populations were very diverse in shape and therefore it was even possible to assign different *U. botrys* populations to different previously described species – *U. apiculata*, *U. irregularis* and *U. skujae* (Table S4). In the light of such natural variability of colony, cell shape and dimensions within a single *U. botrys* species, the probability that *U. apiculata*, *U. irregularis* and *U. skujae* are different species seems to be lower than the probability that they are only ecomorphs. This hypothesis is further supported by previous findings given by Thompson & Wujek (2002). Moreover, *U. botrys* was the most commonly observed species within *Uroglenopsis* representing nearly every second *Uroglenopsis* sequence obtained within this survey. Therefore, we propose *U. apiculata*, *U. irregularis* and *U. skujae* should be taken into synonymy with *U. botrys*.

U. turfosa [= *Eusphaerella turfosa* Skuja] colonies were unequivocally determined according to their species-specific morphology. The cyst of *U. turfosa* was originally only verbally described as almost spherical to slightly roundly obovate with the dimensions 13-15 μm in diameter, and with a very low, 3.5-3.8 μm wide, collar (marginal rim). Cysts of *U. turfosa* found by us (produced in culture) were almost spherical to slightly oval and smooth, 9.5-10 μm wide and 10-10.5 μm in length. The concave pore was surrounded by a 2 μm wide, rounded and slightly conical marginal rim, lower than 1 μm. However, it is known from other chrysophytes that the cyst diameter may be generally invariant among the populations (Sandgren, 1983). Two main lineages were resolved within *U. turfosa*, considered here as different populations of the single species.

According to cell and plastid characteristics, *U. troitzkajae* comb. nov. certainly belongs to the *Uroglenopsis*. This species was originally described from Russia on material lacking cysts, and further efforts to find encysting populations will be of great value and will lead to a more detailed description. However, *U. troitzkajae* possess

unique invaginations of the gel matrix among cells. According to Conrad (1938), the 'fibrous' structures observed by some authors were merely an artifact of the method due to the use of unsuitable dyes. Very likely, this could lead to observations of wrinkles caused in the shrunken gelatinous mass of the colony due to the loss of water by the dye. Whether this is true or not for *U. troitzkajae*, there is other evidence for colony invaginations (gel matrix with the cells) in *U. turfosa*.

Finally, we cannot assign *Uroglenopsis* sp. 1 and *Uroglenopsis* sp. 2 to any previously described species. However, partly due to lacking knowledge of their cyst morphology and partly due to the extreme fragility causing rapid disintegration of the colonies and preventing their proper examination, we do no treat these lineages taxonomically.

***Urostipulosphaera* Pusztai & Škaloud, 2019**

Based on either morphological and molecular data, or the original descriptions and drawings, we may unequivocally assign five previously described species (*U. articulata*, *U. lindiae*, *U. notabilis*, *U. conimamma* and *U. eustylis*) into the genus *Urostipulosphaera* (see Taxonomic revisions and diagnoses).

U. proxima comb. nov. was originally described from Ukraine on a material carrying species-specific cysts, different from any other *Urostipulosphaera* species. *U. proxima* possesses all the characteristics of *Urostipulosphaera* except for the articulated nature of the stalks. Korshikov & Matvienko (1941) have done a proper investigation and stained colonies with several dyes, but they did not find any septa within the stalks. When examining cultured material, we have found articulated stalks in all *Urostipulosphaera* lineages genetically characterised in our study. However, in fresh material from natural samples, whole stalks were sometimes nearly invisible and therefore, septa were not detected. From this perspective, *U. proxima* certainly belongs to *Urostipulosphaera*, but it may form a separate lineage possessing unarticulated stalks.

U. soniaca comb. nov. was originally described from Belgium on a material carrying species-specific cysts with a hook-like projection, different from any other *Urostipulosphaera* species. Interestingly, *U. soniaca* was erected on the material containing

both *Uroglena* and *Urostipulosphaera* taxa mixed in the sample and was, unfortunately, confusingly interpreted by Conrad (1938) as young and old colonies.

In *U. europaea*, comb. nov., which was originally described from Czech Republic on a material lacking cysts, further efforts to find encysting populations will be of great value and lead to a more detailed description. However, the original description and drawings unequivocally assign the species into the genus *Urostipulosphaera* according to plastid and cell characteristics, together with the flagella length ratio (but with not visible stalks as it sometimes can happen with fresh material). Pascher (1910) further listed one plastid in smaller cells, while two plastids in larger cells. These larger cells were probably already deformed due to microscopy (heating stress, etc.), and their plastid was typically split into two smaller ones.

U. granulata sp. nov. was newly erected from the Czech Republic on a material carrying species-specific cysts clearly different, morphologically, from all previously described *Urostipulosphaera* species, and it is therefore described as a new species.

Finally, we cannot assign *Urostipulosphaera* sp. to any previously described species, but due to lacking knowledge of its cyst morphology, we cannot be sure if it is a new species or an already described species. In order to finally decide this issue, further examination of an encysting *Urostipulosphaera* sp. population is needed.

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Supplementary Information

The following supplementary material is accessible via the Supplementary Content tab on the article's online page at http://dx.doi.org/10.1080/09670262.2021.1892196

Supplementary Table S1. Strains of the genera *Uroglena*, *Uroglenopsis* and *Urostipulosphaera* used in this study. Additional information is provided for each strain: number of identical isolates (N-isol.), sampling site (Locality) along with geographic coordinates (GPS), date, physico-chemical water parameters (pH, conductivity, temperature) and the GenBank accession numbers for their ITS, rbcL and SSU gene sequences. Those strains sharing identical DNA sequences are marked with lower case letters.

Supplementary Table S2. Taxonomic overview for all three genera (*Uroglena*, *Uroglenopsis* and *Urostipulosphaera*). According to our molecular analyses and morphological observations, we were able to assign all the previously described taxa to recognised genera.

Supplementary Table S3. Key to the determination of genera and species within *Uroglena*-like chrysophytes.

Supplementary Table S4. Cell characteristics of different *U. botrys* populations.

Supplementary Fig. S1. Phylogeny of the Chrysophyceae showing position of *Uroglena*, *Uroglenopsis* and *Urostipulosphaera* within the Ochromonadales clade (adopted from Pusztai & Škaloud, 2019).

Author Contributions

M. Pusztai: drafting and editing manuscript, sampling, morphological investigations (LM, SEM), culturing, acquiring molecular data, phylogenetic analysis; P. Škaloud: original concept, editing manuscript, sampling, phylogenetic analysis.

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Table 1. Morphological characteristics of individual *Uroglena, Uroglenopsis* and *Urostipulosphaera* species.

Taxon	Colony diameter (μm)	Cell dimensions (μm)	Cyst diameter (μm)	Primary collar dimensions (μm)	Secondary collar dimensions (μm)	Pore diameter (μm)
Uroglena glabra	50-220	7.5-13 × 6.5-10	11-17	0.5-3 × 3-5	absent	1.0
Uroglena imitata sp. nov.	120-180	10-12.5 × 5.5-9	13.3-14.8	1.5-2 × 2.4-2.8	1.9-7.9 × 4.6-8.1	1.0
Uroglena skujae sp. nov.	100-150	8.5-11.5 × 7-8.5	11-14.5	8.5-14.5 × 3.5-4.5	absent	n.a.
Uroglena volvox	120-250	12.5 × 10	n.a.	n.a.	n.a.	n.a.
Uroglena zachariasii	60-130	7.5-12.5 × 5-10	10.9-14	0.5-2 × 2-3.5	0.5-10.5 × 5.5-9	0.7-1.0
Uroglena cf. zachariasii	60-190	9.5-12.5 × 6-9.5	12.5-16	n.a.[1]	n.a.[1]	n.a.
Uroglenopsis americana	40-500	5-11.5 × 5-7.5	n.a.	n.a.	n.a.	n.a.
Uroglenopsis botrys	80-280	8.5-16.1 × 4.5-7.5	9.7-11.3	absent[2]	absent	1.0
Uroglenopsis turfosa	50-350	7.5-16 × 5-9	9.5-10.5	absent[3]	absent	n.a.
Uroglenopsis sp. 1	150-200	6.6-9.2 × 4.8-6.8	n.a.	n.a.	n.a.	n.a.
Uroglenopsis sp. 2	500-1000	7.5 × 5	n.a.	n.a.	n.a.	n.a.
Urostipulosphaera articulata comb. nov.	50-90	7-12 × 6-9	13-14.5	4.5-7 × 1.9-2.8	absent	n.a.
Urostipulosphaera granulata sp. nov.	40-100	7-16 × 6-10.5	7-12.5	3.6-6.2 × 1.7-2.3	absent	0.4-0.9
Urostipulosphaera lindiae comb. nov.	60-200	7.5-13.5 × 7-8.5	12-14.5	4.9-7.2 × 1.5-2.8	absent	n.a.

Urostipulosphaera notabilis	90-200	7.5-11.5 × 5.5-8.5	12.5-14	6.3-8.3 × 1.6-3.2	absent	n.a.
Urostipulosphaera sp.	100-200	9-13 × 5-8.5	n.a.	n.a.	n.a.	n.a.

[1] Poorly encysting populations, cysts were observed only in LM, at least two different types (more details in the text).

[2] Concave pore was surrounded by a 2 μm wide and very low, irregular and almost imperceptible marginal rim.

[3] Concave pore was surrounded by a 2 μm wide and less than 1 μm high rounded, slightly conical marginal rim.

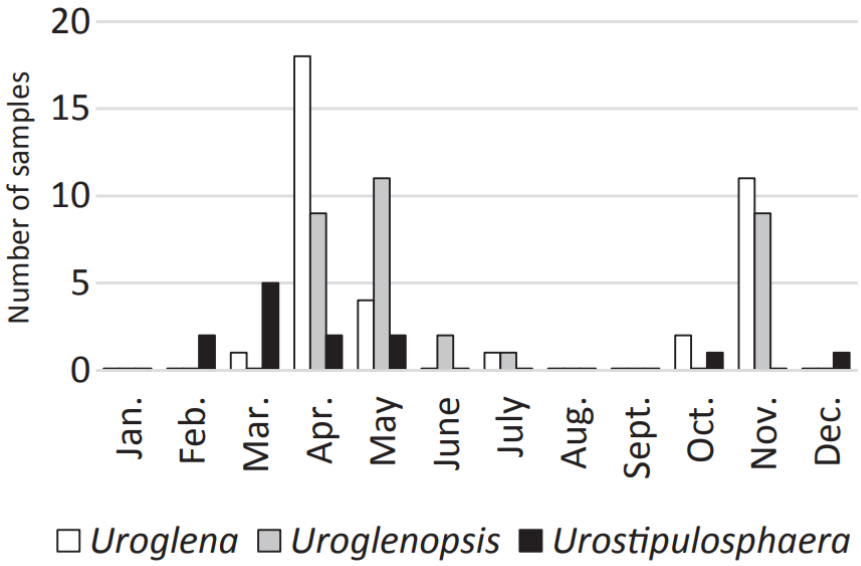
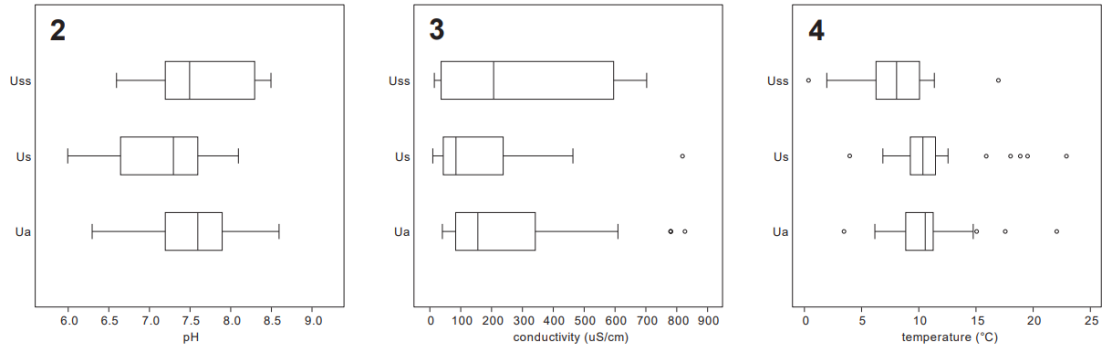


Fig. 1. Phenology of *Uroglena*, *Uroglenopsis* and *Urostipulosphaera* in the north temperate zone based on all collected populations (Number of samples) through the years 2014-2020. *Urostipulosphaera* seems to be early spring taxon peaked in March, while *Uroglenopsis* seems to be late spring taxon peaked in May. *Uroglena* peaked in April. *Uroglena* and *Uroglenopsis* exhibit significant spring and autumnal population maxima, while *Urostipulosphaera* not.



Figs 2-4. Habitat differences between *Uroglena* (Ua), *Uroglenopsis* (Us) and *Urostipulosphaera* (Uss) in terms of measured environmental factors for all collected populations. **Fig. 2.** pH. **Fig. 3**. Conductivity. **Fig. 4.** Temperature. *Urostipulosphaera* occurred in waters with significantly lower temperature than *Uroglena* (Mann-Whitney, p = 0.021) and *Uroglenopsis* (Mann-Whitney, p = 0.017). *Uroglenopsis* occurred in waters with significantly lower pH than *Uroglena* (t-test, p = 0.014) and *Urostipulosphaera* (t- test, p = 0.026) and with significantly lower conductivity than Uroglena (Mann-Whitney, p = 0.034).

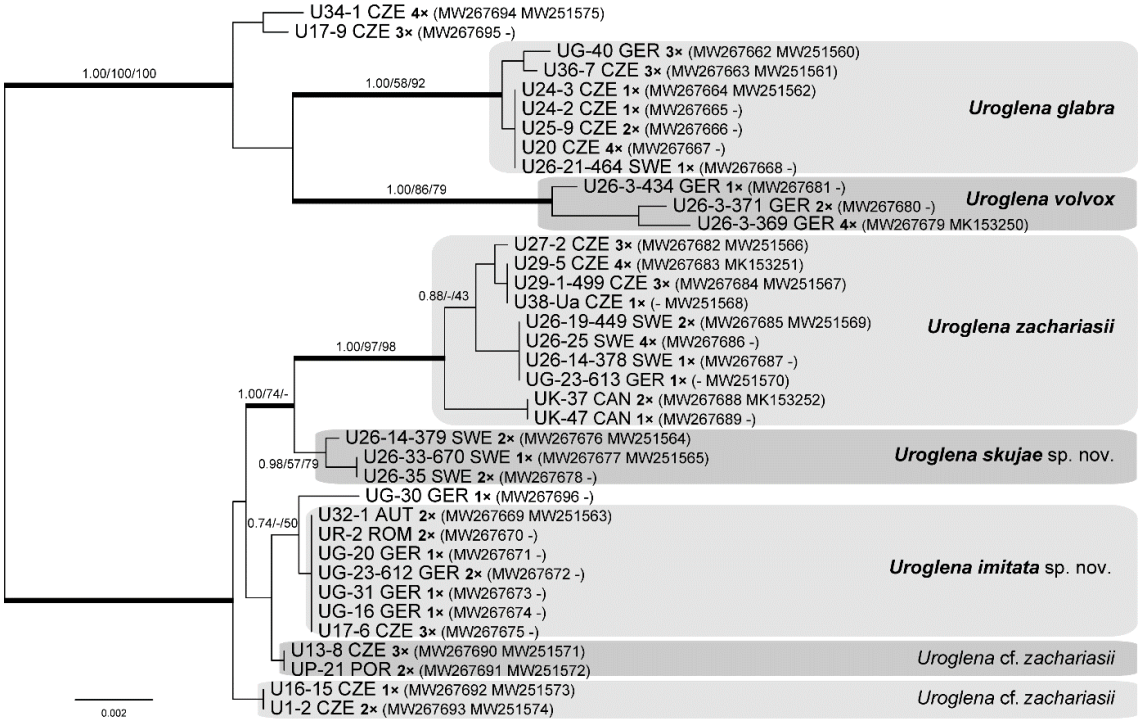
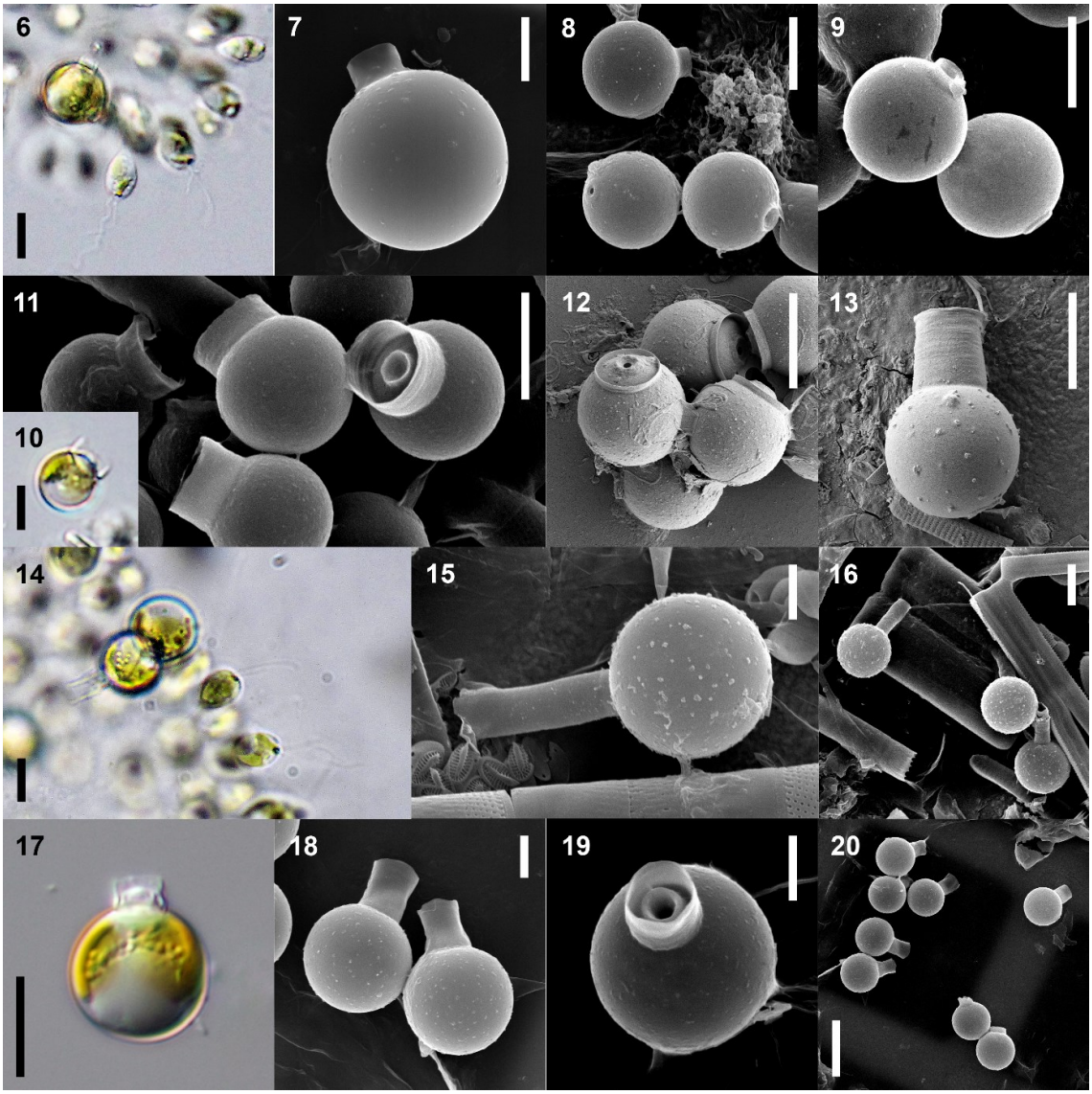


Fig. 5. Phylogeny of the genus *Uroglena* obtained by Bayesian inference of the concatenated ITS rDNA and *rbc*L dataset. The analysis was performed under a partitioned model, using different substitution models for each partition. Values at the nodes indicate statistical support estimated by three methods; MrBayes posterior node probability (left), maximum likelihood bootstrap (middle), and weighted maximum parsimony bootstrap (right). Only statistical supports with posterior probability higher than 0.7 are shown. Thick branches highlight nodes receiving the highest posterior probability support (1.00). Number of isolates sharing identical DNA sequences within a strain is indicated as "**1-4×**". Scale bar represents the expected number of substitutions per site.



Figs 6-20. Species-specific cysts as the main morphological character for the species delimitation within the genus *Uroglena*. **Figs 6-9.** *U. glabra* – mature cysts (Figs 6, 7) and group of immature cysts with not fully developed collar (Figs 8, 9). **Figs 10-13.** *U. zachariasii* – mature cysts (Figs 10, 11), group of immature cysts with not fully developed collars (Fig. 12) and cysts with very high secondary collar morphologically fitting *U. zachariasii* var. *uplandica* (Fig. 13). **Figs 14-16.** *U. skujae* – mature cysts (Figs 14, 15) and group of cysts with different collar lengths (Fig. 16). **Figs 17-20.** *U. imitata* – mature cysts with well visible primary collar (Figs 17, 19) and fully developed secondary collar (Fig. 18), group of cysts with different secondary collar lengths (Fig. 20). Scale = 20 μm (Fig. 20), 10 μm (Figs 6, 8-14, 16, 17) and 5 μm (Figs 7, 15, 18, 19). LM investigations (Figs 6, 10, 14, 17), SEM investigations (Figs 7-9, 11-13, 15, 16, 18-20).

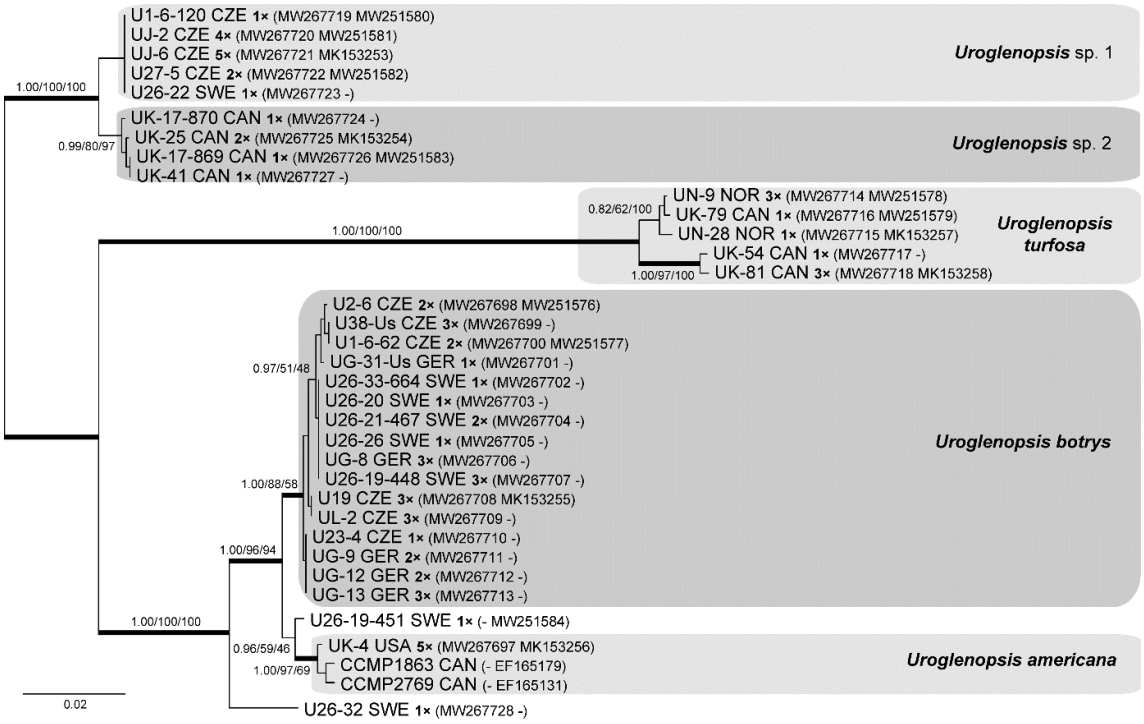
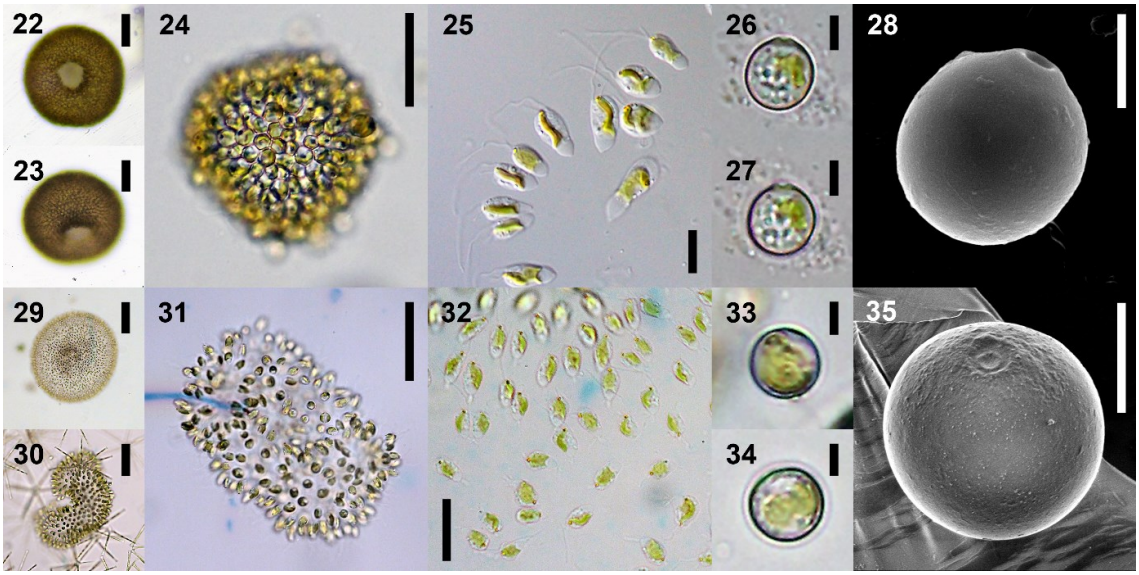


Fig. 21. Phylogeny of the genus *Uroglenopsis* obtained by Bayesian inference of the concatenated ITS rDNA and *rbc*L dataset. The analysis was performed under a partitioned model, using different substitution models for each partition. Values at the nodes indicate statistical support estimated by three methods; MrBayes posterior node probability (left), maximum likelihood bootstrap (middle), and weighted maximum parsimony bootstrap (right). Only statistical supports with posterior probability higher than 0.8 are shown. Thick branches highlight nodes receiving the highest posterior probability support (1.00). Number of isolates sharing identical DNA sequences within a strain is indicated as "**1-5×**". Scale bar represents the expected number of substitutions per site.



Figs 22-35. Colony, cell and cysts characteristics within the genus *Uroglenopsis*. **Figs 22-28.** *U. turfosa* – colonies in fresh natural samples with a remarkable hole in the spherical closely packed together colony (Figs 22, 23), hexagonal cells in apical view still closely packed together in young cultures (Fig. 24), colonies with cells loosely packed in old cultures (Fig. 25), mature cysts with a characteristic marginal rim surrounding the pore (Figs 26-28). **Figs 29-35.** *U. botrys* – colonies (Figs 29-31) and cells (Fig. 32) very diverse in shape, mature cysts exhibiting very simple ultrastructure (Figs. 33-35).

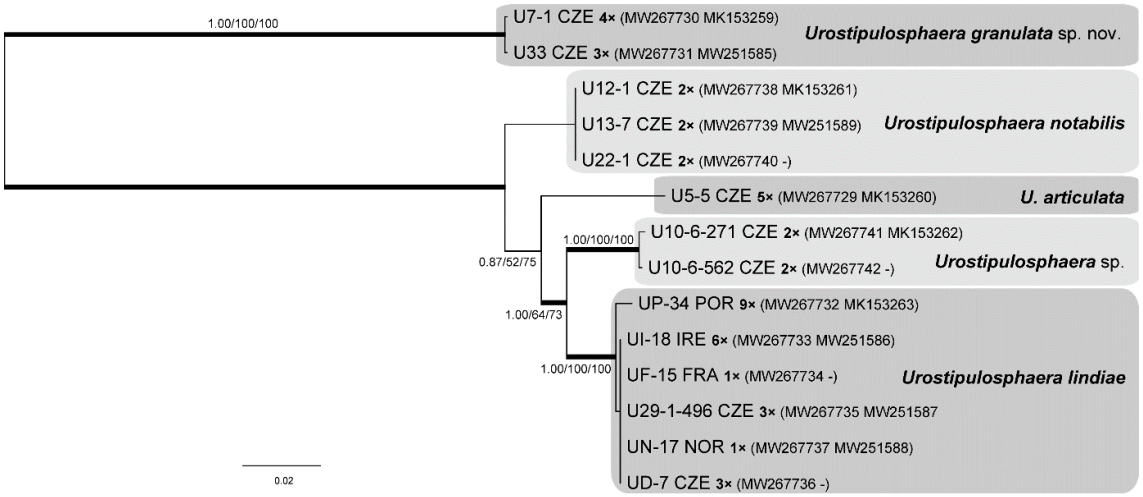
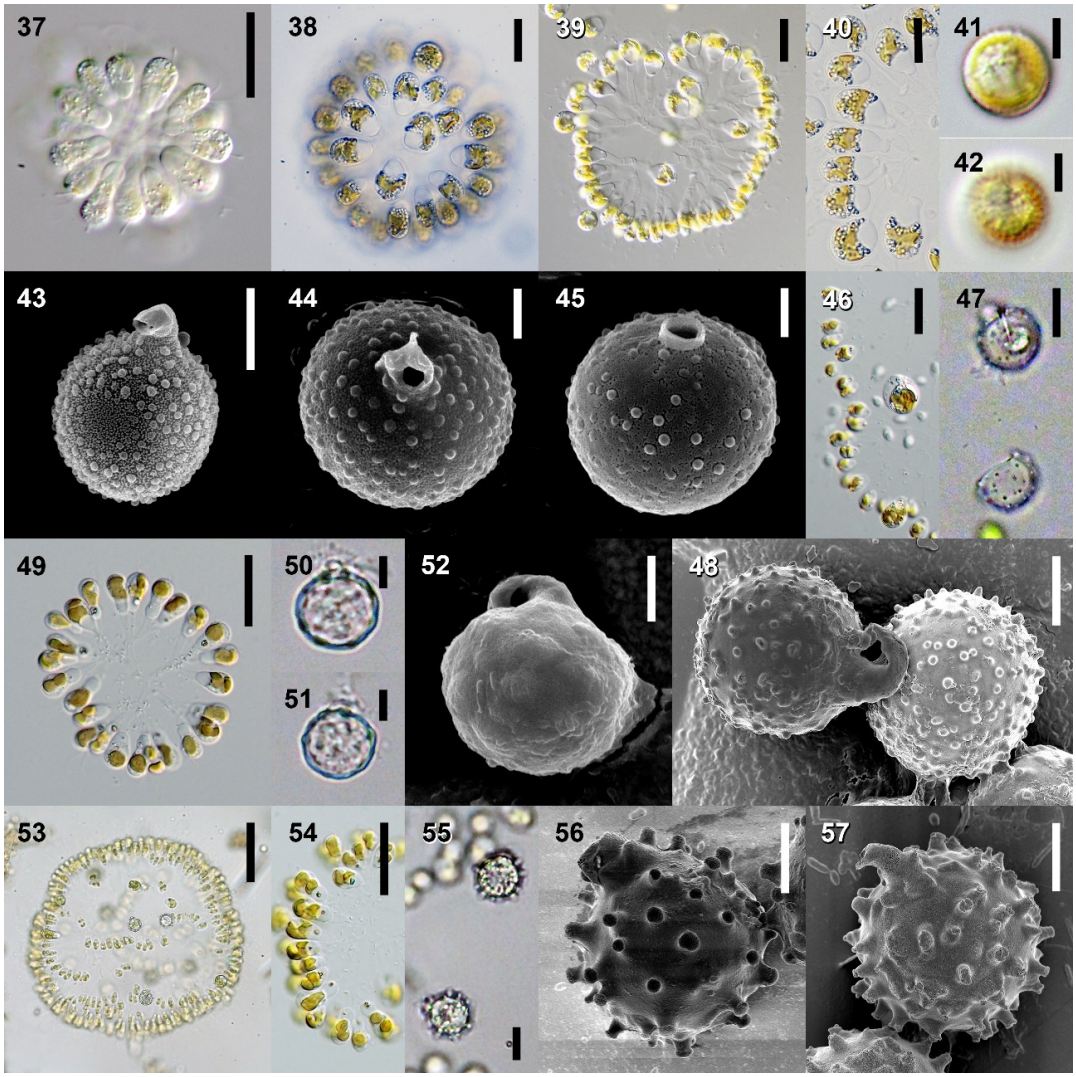
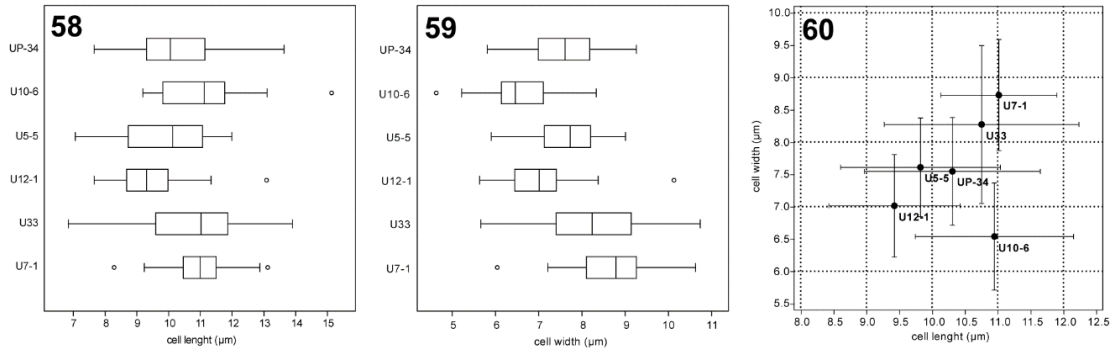


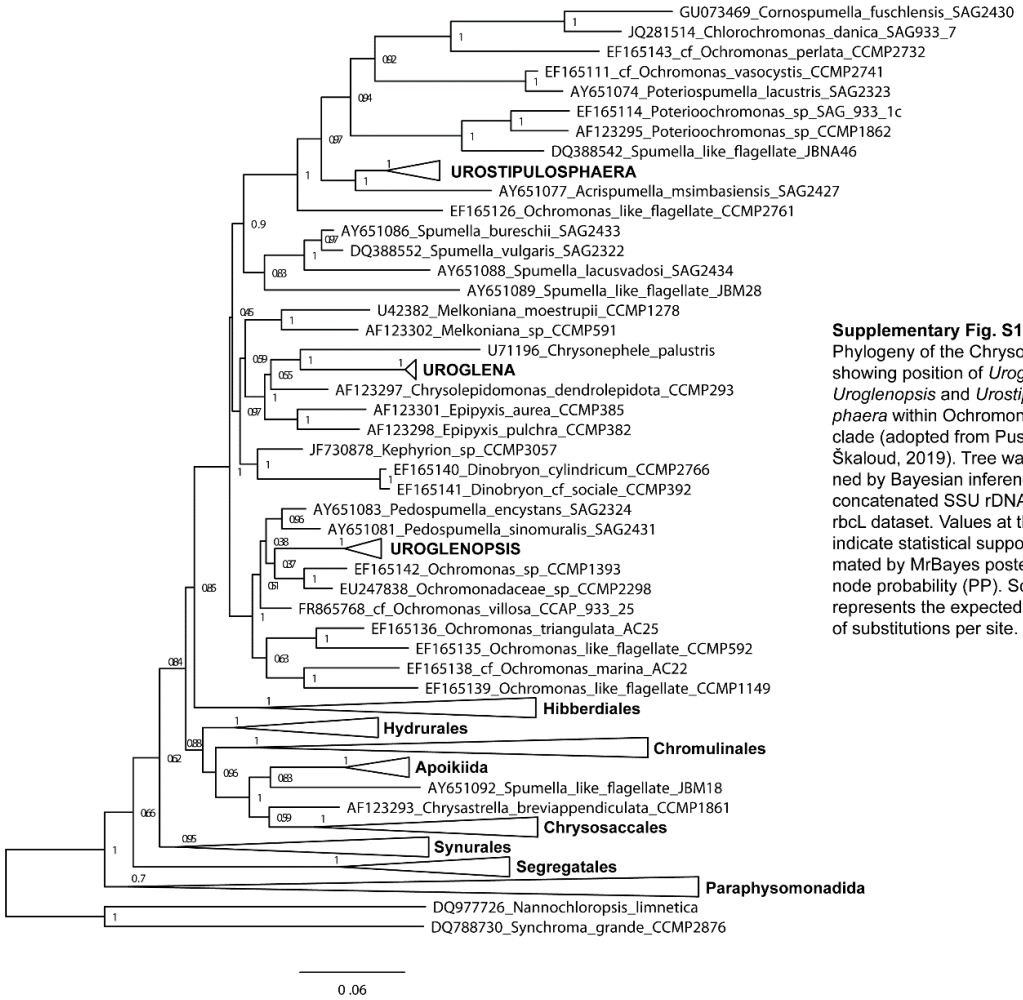
Fig. 36. Phylogeny of the genus *Urostipulosphaera* obtained by Bayesian inference of the concatenated ITS rDNA and *rbc*L dataset. The analysis was performed under a partitioned model, using different substitution models for each partition. Values at the nodes indicate statistical support estimated by three methods; MrBayes posterior node probability (left), maximum likelihood bootstrap (middle), and weighted maximum parsimony bootstrap (right). Thick branches highlight nodes receiving the highest posterior probability support (1.00). Number of isolates sharing identical DNA sequences within a strain is indicated as "**1-9×**". Scale bar represents the expected number of substitutions per site.



Figs 37-57. Species-specific cysts and colony characteristics within the genus *Urostipulosphaera*. **Figs 37-45.** *U. granulata* – strain U7-1 in a fresh natural sample with reduced plastids (Fig. 37), the same strain after one week of culturing (Fig. 38), strain U33 with well visible articulated stalks (Fig. 39), changes in cell shape and posterior under stress conditions during microscopy (Fig. 40), mature cysts with fully developed collar and granules (Figs 41-44) and immature cyst (Fig. 45). **Figs 46-48.** *U. notabilis* – formation of cysts within a colony in culture (Fig. 46), mature cysts (Figs 47, 48). **Figs 49-52.** *U. articulata* – cultured colony (Fig. 49), cysts possessing a typical acute rim surrounding the pore (probably immature) or only slightly incrusted collar (Figs 50, 51), a mature cyst (Fig. 52). **Figs 53-57.** *U. lindiae* – formation of cysts within a colony in natural sample (Fig. 53), colony in culture (Fig. 54), mature cysts with various paw-like hooked processes (Figs 55-57). Scale = 50 μm (Fig. 53), 20 μm (Figs 37, 39, 46, 49, 54), 10 μm (Figs 38, 40, 47, 55), 5 μm (Figs 41-43, 48, 50-52, 56, 57) and 2.5 μm (44, 45). LM investigations (Figs 37-42, 46, 47, 49-51, 53-55), SEM investigations (Figs 43-45, 48, 52, 56, 57).



Figs 58-60. Comparison of cell length and cell width between cultured *Urostipulosphaera* species. *U. notabilis* (U12-1), *U. articulata* (U5-5) and *U. lindiae* (UP-34) shared very similar range of cell length and width. *Urostipulosphaera* sp. (U10-6) possessed cells with clearly skewed length/width ratio in favor of length. *U. granulata* (U7-1 and U33) possessed generally larger cells than all the other species belonging to the second *Urostipulosphaera* lineage. On the other hand, two isolates of *U. granulata* (U7-1 and U33) differed in their cell length/width range though they are virtually genetically identical. Average values and standard deviations are given (Fig. 60).



Supplementary Fig. S1. Phylogeny of the Chrysophyceae showing position of *Uroglena*, *Uroglenopsis* and *Urostipulosphaera* within Ochromonadales clade (adopted from Pusztai & Škaloud, 2019). Tree was obtained by Bayesian inference of the concatenated SSU rDNA and rbcL dataset. Values at the nodes indicate statistical support estimated by MrBayes posterior node probability (PP). Scale bar represents the expected number of substitutions per site.

PAPER IV

Pavel Škaloud, Magda Škaloudová, Iva Jadrná, Helena Bestová, **Martin Pusztai**, Dimitry Kapustin & Peter A. Siver

Comparing morphological and molecular estimates of species diversity in the freshwater genus *Synura* (Stramenopiles): a model for understanding diversity of eukaryotic microorganisms

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COMPARING MORPHOLOGICAL AND MOLECULAR ESTIMATES OF SPECIES DIVERSITY IN THE FRESHWATER GENUS *SYNURA* (STRAMENOPILES): A POTENTIAL MODEL FOR UNDERSTANDING DIVERSITY OF EUKARYOTIC MICROORGANISMS[1]

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Running Title: Diversity of *Synura* in Newfoundland

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Abstract

We performed a comparison of molecular and morphological diversity in a freshwater colonial genus *Synura* (Chrysophyceae, Stramenopiles), using the island of Newfoundland (Canada) as a case study. We examined the morphological species diversity in collections from 79 localities, and compared these findings to diversity based on molecular characters for 150 strains isolated from the same sites. Of 27 species or species-level lineages identified, only one third was recorded by both molecular and morphological techniques, showing both approaches are complementary in estimating species diversity within this genus. Eight taxa, each representing young evolutionary lineages, were recovered only by sequencing of isolated colonies, whereas ten species were recovered only using standard microscopical techniques. Our complex investigation, involving both morphological and molecular examinations, indicates that our knowledge of *Synura* diversity is still poor, limited only to a few well-studied areas. We revealed considerable cryptic diversity within the core *S. petersenii* and *S. leptorrhabda* lineages. We further resolve the phylogenetic position of two previously described taxa, *S. kristiansenii* and *S. petersenii* f. *praefracta*, propose species-level status for *S. petersenii* f. *praefracta*, and describe three new species, *S. vinlandica*, *S. fluviatilis* and *S. cornuta*, based on a combination of molecular and morphological evidence. Including the species uncovered in this study, the number of taxa in *Synura* recorded from Newfoundland waterbodies has increased from 14 to 31. Our findings add to the growing body of literature detailing distribution patterns observed in the genus, ranging from cosmopolitan species, to highly restricted taxa, to species such as *S. hibernica* found along coastal regions on multiple continents. Finally, our study illustrates the usefulness of combining detailed morphological information with gene sequence data to examine species diversity within chrysophyte algae.

Keywords: algae, biogeography, chrysophytes, diversity, molecular phylogeny, morphology, *Synura*, protists, taxonomy.

Introduction

Protists represent a wide diversity of organisms that are distributed across the eukaryote tree of life and play critical roles in ecological and biogeochemical processes, including carbon fixation, decomposition, elemental transformations, energy transfer, and animal and plant diseases (Adl *et al*. 2019). Despite their significance to the functioning of aquatic and terrestrial ecosystems, species diversity, biogeography and ecological importance are poorly known for many groups of protists. In addition, contrasting views regarding protist diversity have emerged over the last several decades. On the one hand, global protist diversity is believed to be extraordinarily high and represented by a wide range of distribution patterns (Foissner 1999). In contrast, other studies suggest that protist diversity is much lower and fundamentally different than that of macroorganisms (Fenchel and Finlay 2003). The majority of recent surveys based on environmental DNA (eDNA) support the former opinion, often reporting an extremely high proportion of SSU rDNA sequences that could not be assigned to described species (e.g., Šlapeta *et al*. 2005, Howe *et al*. 2009, Behnke *et al*. 2011). Indeed, projections of the number of protist species globally have ranged from several tens of millions (Adl *et al*. 2012) to over 160 million, especially when parasitic and symbiotic taxa are considered (Larsen *et al*. 2017).

Tools used to estimate protist diversity, including advances in microscopical and molecular techniques, have evolved rapidly since the 1950's (Pawlowski *et al*. 2012, Caron and Hu 2019). Morphology has always been and remains the central criterion for delineating protist species. However, several investigations indicate that for some groups morphospecies can fail to differentiate all species due to both the lack of discriminating characters, and convergent morphological evolution (Von Der Heyden *et al*. 2004, Krienitz *et al*. 2010, Škaloud and Rindi 2013, Pinseel *et al*. 2019). Recent development of molecular tools has advanced our ability to discriminate among cryptic taxa, improving overall diversity estimates. For some cryptic complexes a re-evaluation of the morphological characters supported the molecular findings (Škaloud *et al*. 2014).

Without question, eDNA metabarcoding surveys have yielded deep insights into the composition of protist communities in soil (Mahé *et al*. 2017), marine (De Vargas *et al*.

2015) and freshwater (Boenigk *et al*. 2018) habitats. However, despite its increasing application in estimating protist biodiversity, there are drawbacks to using metabarcoding to estimate species richness. First, the lack of morphological data associated with metabarcoding sequences prevents investigation of other facets of diversity, such as structural and functional aspects. Second, generation of short single loci sequences makes it difficult or even impossible to determine appropriate species boundaries. Therefore, the taxonomic interpretation of generated sequences greatly relies on the completeness and quality of existing reference databases. Third, environmental sequencing may often lead to creating molecular chimaeras and amplification of a number of alien organisms, transported into study sites from other systems. Studies that can improve our understanding of species boundaries based on molecular data, and effectively link the molecular data to morphospecies, would advance the use of metabarcoding in determining protist diversity, especially within closely related groups of organisms, and improve comparison with previous and historical studies based solely on morphological data.

Synura is a species-rich freshwater genus (Chrysophyceae, Stramenopiles) that has one of the best morphological species concepts among protists. A total of 90 *Synura* taxa have been described so far, from which 54 are recognized as currently accepted (see Škaloud *et al*. 2012 for the list of synonyms and taxa *nomen nudum*). Cells of *Synura* are covered with an organized layer of morphologically complex siliceous scales, each of which is produced under highly controlled conditions within a silica deposition vesicle (Leadbeater 1990, Kristiansen 2005). Scale shape, size and design are primary characters used to distinguish between species. Consistent differences in scale morphology have largely aligned nicely with differences between taxa identified using multiple gene sequences (Škaloud *et al*. 2014, Jo *et al*. 2016). The alignment of morphological and molecular traits makes *Synura* an excellent model organism for comparing the two methodologies as tools used to distinguish between species.

Newfoundland (Canada) is a large island (area 108 860 km^2) situated off the east coast of the North American mainland. The morphological diversity of *Synura* in Newfoundland was documented by four previous studies, investigating 37 different

water bodies by means of transmission or scanning electron microscopy (Wawrzyniak and Andersen 1985, Siver and Lott 2016, 2017, Siver *et al*. 2018). These studies recorded 14 *Synura* taxa (*S. bjoerkii*, *S. curtispina, S. echinulata*, *S. kristiansenii, S. leptorrhabda, S. mammillosa, S. mollispina, S. papillosa, S. petersenii* sensu lato, *S. sphagnicola, S. spinosa*, *S. spinosa* f. *nygaardii, S. synuroidea*, *S. uvella*). A high morphological diversity of scales within several *Synura* species complexes was reported by Siver and Lott (2017), who mentioned the perforce of future molecular analyses of these cryptic species complexes to enhance our understanding of scaled chrysophyte diversity.

The aim of the current study is to estimate species diversity of *Synura* in Newfoundland using a combination of morphological and molecular techniques. We characterized morphological and molecular data for 150 *Synura* strains isolated from 79 localities on the island in order to estimate species diversity, and examine how the morphological diversity reported in the previous works compares with an investigation based on a combined data set. A total number of 16 *Synura* species has been molecularly detected (*S. americana, S. borealis, S. conopea, S. hibernica, S. kristiansenii, S. lanceolata, S. leptorrhabda/ S. mammillosa* sensu lato, *S. petersenii, S. sphagnicola, S. splendida*, *S. truttae* and four new taxa within *Synura petersenii* species complex (section Petersenianae). In addition, the *Synura* species diversity was further investigated using electron microscopy, and the list of *Synura* species in Newfoundland waterbodies has increased to 31.

Materials and methods

Collection, isolation and cultivation of Synura *strains*

On May 24-29th 2017 samples of phytoplankton were collected from 79 lakes and ponds in Newfoundland (Fig. 1 and Table S1 in the Supporting Information) using a plankton net with 20μm mesh. Standard measurements of water temperature, pH and specific conductivity was carried out using a combined pH/conductometer (WTW 340i; WTW GmbH, Weilheim, Germany). Samples were examined with an Olympus CX 31 light microscope and the individual *Synura* colonies were isolated by micropipetting. Each colony was placed into a separate well of a 96-well polypropylene plate filled with

approximately 300 µl of MES buffered DY IV liquid medium (pH ≈ 6; Andersen *et al*. 2005). In the laboratory, the well growing cultures were transferred from wells into 50 ml Erlenmeyer flasks filled with the same medium. They were cultivated in a cooling box (C5G, Helkama Oy, Helsinki, Finland) at 15°C, under constant illumination of 40 µmol m−2 s−1 (TLD 18W/33fluorescent lamps, Philips, Amsterdam, the Netherlands).

Sequencing and phylogenetic analysis

For DNA isolation, 100-200 ml of living cultures were centrifuged in PCR tubes (6,000 rpm for 3 minutes), and 30 mL of InstaGene matrix (Bio-Rad Laboratories) was added to the pellet. The solution was vortexed for 10 s, incubated at 56 °C for 30 min, and heated at 99 °C for 8 min. After vortexing a second time, the tubes were centrifuged at 12,000 rpm for 2 min, and the supernatant was directly used as a PCR template. A total of seven molecular loci were sequenced. First, all strains were genetically characterized by sequencing their ITS rDNA. This molecular locus has been shown to represent an ideal DNA barcode to distinguish among Chrysophycean species, including those belonging to the genus *Synura* (Jost *et al*. 2010, Škaloud *et al*. 2012, Bock *et al*. 2017). For the selection of strains having a unique ITS rDNA barcode, additional loci were amplified to obtain robust, well-resolved phylogenies. For the strains belonging to Petersenianae, we additionally sequenced *rbc*L and *cox*I loci. For other strains, we further sequenced nuclear 18S and 26S rDNA, and plastid 23S rDNA, *psa*A and *rbc*L loci. The amplifications were performed as described in Škaloud *et al*. (2014) and Jo *et al*. (2016), using the primers and amplification conditions listed in Table S2 in the Supporting Information. The PCR products were purified by NucleoMag® NGS Clean-up and Size Select kit (Macherey-Nagel) and sequenced with an ABI3730XL DNA Analyzer at Macrogen Inc. in Seoul, Korea.

Multiple alignments of nuclear ITS, 18S, 28S rDNA and organellar *cox*I, *rbc*L, *psa*A and 23S rDNA loci sequences were either manually built in MEGA6 (Tamura *et al*. 2013) or constructed using MAFFT v6, applying the Q-INS-i strategy (Katoh *et al*. 2002). The newly determined sequences were aligned to other sequences from the GenBank database, selected to encompass all known lineages (Tables S3 and S4 in the Supporting

Information). The positions with deletions prevailing in a majority of sequences were removed from the alignment. Two alignments were constructed for the phylogenetic analyses: (i) a concatenated ITS rDNA + *rbc*L + *cox*I alignment of 60/187 unique/total sequences of Petersenianae, and (ii) a concatenated ITS rDNA + 18S rDNA + 28S rDNA + 23S rDNA + *rbc*L + *psa*A alignment of 39/54 unique/total sequences of the genus *Synura*. In Synurales, the ITS rDNA alignment consisted of the 5.8S rDNA and ITS2 rDNA regions only, due to high genetic divergence among the strains. ITS2 rDNA sequences were aligned with the help of their secondary structure information, using the ITS2 database V (Ankenbrand *et al*. 2015). The ITS2 secondary structures of *Synura americana* (HG514166.1), *S. petersenii* (AF308832.1), *S. conopea* (FM178506.1), *S. truttae* (FM178508.1), *S. borealis* (HG514174.1), *S. glabra* (FM178511), *S. macropora* (FM178494.1), *Pedospumella encystans* (EF577176), *P. sinomuralis* (EF577170) and *Ellipsoidion* sp. (HE586522) were used as a template for homology modelling. Homology modelling was performed by the custom modelling option, using the ITS 2 PAM 50 matrix and 20% threshold for the transfer of helices. Secondary structures were successfully obtained for all analysed species, with the exception of *S. longitubularis*, *S. curtispina*, *S. sphagnicola*, *S. synuroidea*, and *S. spinosa*. These were manually folded with the help of modelled secondary structures of closely related taxa. The alignments were generated using the ITS2 database V, by both sequences and structures. DNA alignments are freely available on Mendeley Data: http://dx.doi.org/10.17632/jjfmp6nv4b.1.

The Bayesian evolutionary analyses were performed to infer a phylogeny and simultaneously estimate branch divergence times for the investigated strains, using the program BEAST v1.10.4 (Suchard *et al*. 2018). The analyses were performed on the two concatenated and partitioned alignments as specified above. For each of the 15 specified alignment partitions, the most appropriate substitution model was estimated using the Bayesian information criterion (BIC) as implemented in jModelTest 2.1.4 (Darriba *et al*. 2012). This BIC-based model selection procedure selected the following models: (i) GTR + Γ for the ITS1 rDNA, the first codon positions of the *psa*A gene, and the third codon positions of the *rbc*L and *psa*A genes, (ii) GTR + I for the first and second codon positions of the *cox*I and *psa*A genes, respectively, (iii) GTR + I + Γ for the ITS2 rDNA, 18S rDNA,

28S rDNA, 23S rDNA, and the first and third codon positions of the *rbc*L and *cox*I genes, respectively, (iv) JC for 5.8S rDNA and the second codon positions of the *rbc*L gene, and (v) F81 for the second codon positions of the *cox*I gene. Lognormal relaxed clock models were selected for the partitions, and a birth-death diversification process was used as a prior on the distribution of node heights. Three temporal constraints were used to calibrate the Synurales phylogeny, based on the fossil scales found in lacustrine mudstones from the Giraffe (Siver *et al*. 2015) and Wombat (Siver *et al*. 2013) cores, respectively. These constrains include (i) the lineage comprising *S. uvella* and *S. splendida* (Giraffe core), (ii) the stem of *S. curtispina* + *S. longitubularis* lineage (Giraffe core), and (iii) the lineage of all Petersenianae taxa including *S. macracantha* (Wombat core). The splits were based on an offset of either 48 (Giraffe core) or 83 Ma (Wombat core), a mean of 8.0 and a standard deviation of 6.0. Six Markov Chain Monte Carlo (MCMC) analyses were run for 50 million generations, sampling every 10,000 generation. After the diagnosis for convergence using Tracer 1.6, the log files were merged using the burn-in set to 10 million generations. Accordingly, the substitution rates were obtained for the ITS2 rDNA (8.857E-4) and three codon partitions of the *rbc*L gene (1.875E-4, 4.318 E-5, 0.001663), respectively. The estimated rates were then applied to infer the Petersenianae phylogeny, since the fossil calibrations are unrealistic due to high morphological similarity of cryptic species. The Bayesian evolutionary analyses were performed as described above, with the exception of fixing substitution rates of ITS2 and *rbc*L partitions instead of defining temporal constrains. The analyses were run on the CIPRES ScienceGateway v.3.3 web portal (Miller *et al*. 2010).

Morphological investigations and statistical analyses

To assess the morphological diversity of *Synura*, selected samples (those with numerous living *Synura* colonies detected by light microscopic examinations) were investigated with transmission electron microscopy (TEM). After a gentle mix, a drop of the sample was placed onto formvar-coated copper grids and dried. After washing in a series of distilled water droplets, the grids were examined in a TEM JEOL 1011 electron microscope. In four new taxa, morphology of colonies and ultrastructure of silica scales

were observed by light microscopy (LM), as well as by TEM and scanning electron microscopy (SEM). For TEM investigations, a drop from the living cultures was placed onto formvar-coated copper grids, dried, and investigated as described above. For SEM investigations, aliquots of each *Synura* culture were air dried onto heavy duty aluminium foil. The aluminium foil samples were trimmed, attached to aluminium stubs with Apiezon® wax, coated with a mixture of gold and palladium for 2 min with a Polaron Model 5100E sputter coater, and examined with a FEI Nova NanoSEM 450 field emission SEM. For each strain, seven morphological characters of 30 randomly selected silica scales were measured using the program ImageJ 1.45s (Schneider *et al*. 2012). The seven morphological characters, as described in Škaloud *et al*. (2014), include: (1) scale length; (2) scale width; (3) area of a base hole; (4) average area of a keel pore; (5) average area of a base-plate pore; (6) keel width; and (7) number of struts. Measured data were compared with those we obtained in our previous investigations (Škaloud *et al*. 2014, Jo *et al*. 2016). Data visualisation and statistical analyses (principal component analysis, linear discrimination analysis and phylomorphospace plots) were performed in R 3.5.2 (R Development Core Team), using the packages phytools (Revell 2012), and MASS (Venables and Ripley 2002). Principal component analysis (PCA) and linear discrimination analysis (LDA) were performed using the functions prcomp and lda, respectively.

Results

Analyses of molecular data

Our six-loci phylogeny of Synurales resolved three major clades identified here as sections Synura, Curtispinae, and Petersenianae (Fig. 2). All three clades were strongly supported (Bayesian posterior probabilities 1.00), but their relationship remain unresolved probably due to their concurrent origin. On the basis of our time calibration, the genus *Synura* originated near the onset of the Cretaceous (approximately 145 Mya), and split into the three major clades during the Early Cretaceous at about 117 Mya. During the late Neogene, the major radiation occurred within the core Petersenianae, leading to the origin of about twenty species-level lineages (Fig. 3).

We have successfully sequenced a total of 150 *Synura* strains, forming 17 well-resolved lineages. Ten of these lineages were well attributed to previously described and genetically characterized species *S. americana*, *S. borealis*, *S. conopea*, *S. hibernica*, *S. lanceolata*, *S. leptorrhabda*, *S. petersenii*, *S. sphagnicola*, *S. splendida* and *S. truttae*. We resolved the phylogenetic position of *S. kristiansenii*, a putative Newfoundland endemic species recently described by Siver and Lott (2016). It represents a distinct lineage within the section Petersenianae that originated ca 51 Mya (Fig. 3). Significant cryptic diversity has been detected within the *S. leptorrhabda* clade of section Curtispinae and within the core Petersenianae. We identified three and four genetically novel lineages from these two sections, respectively. The ones from Petersenianae are proposed here as *S. praefracta* comb. nov., *S. vinlandica* sp. nov., *S. fluviatilis* sp. nov. and *S. cornuta* sp. nov.

Morphological analyses of natural populations

Based on TEM investigations of natural samples, we identified a total of 19 *Synura* morphotypes (Fig. 4, Table S5 in the Supporting Information). Seven morphotypes were identified within the section Petersenianae, of which only four might be assigned to any of described species (*S. conopea*, *S. hibernica*, *S. kristiansenii*, *S. petersenii*). One morphotype was distinct by a very narrow keel (Fig. 4e) and corresponds well to the scales found by Wujek and Igoe (1989) in Michigan, USA (Little Tom Lake, Fig. 12) determined as *Synura petersenii* f. *praefracta*. However, this morphotype does not correspond to the iconotype of *S. petersenii* f. *praefracta* (Asmund 1968) in both keel morphology and strut number and therefore very probably represents a novel yet undescribed species. In addition, two Petersenianae morphotypes did not fit into any of previously described, morphologically similar taxa. *Synura* sp. 1 (Fig. 4f) differs by having medium-sized scales possessing a large number of struts (29-32). *Synura* sp. 2 (Fig. 4g) is distinct by rather broad scales with a wide keel and a low number of struts (26-28).

A remarkable diversity has been recognized within the section Curtispinae, as well. Along with well-characterized *S. curtispina*, *S. echinulata*, *S. leptorrhabda*, *S. mammillosa*, *S. papillosa*, *S. sphagnicola*, *S. spinosa* f. *longispina* and *S. synuroidea* we observed some yet undescribed species or taxa with uncertain taxonomic status. *S. curtispina* f. *reticulata*

(Fig. 4k) was found in several investigated localities. Although this taxon has been synonymized with *S. curtispina* (Kristiansen and Lind 1995), we are convinced it represents a distinct taxon since the honeycomb reticulation extends to the proximal end of the scale. Probably the most conspicuous morphotype was found in Great Rattling Brook (locality K70), distinct by a curved spine and a large area of distinctive labyrinthic pattern, spreading almost to the proximal part of the scale (Fig. 4m). The scale corresponds well to those presented by Nicholls and Gerrath (1985) from Ontario, Canada, determined there as *S. echinulata*. However, the labyrinthic pattern differs a lot from the sculpture present in the iconotype of *S. echinulata* (Korshikov 1929). We therefore presume the morphotype shown in Fig. 4m, which has been observed in other North American localities (Siver, *unpublished data*), represents a distinct species, not identical to *S. echinulata*.

Morphological analyses of cultured strains

From a morphological perspective, the majority of strains fit the circumscription of described taxa, forming single, genetically distinct lineages. However, our investigations revealed a striking morphological similarity of strains belonging to the novel lineages within the *S. leptorrhabda* clade of section Curtispinae and within the core Petersenianae. Three lineages morphologically corresponding to *S. leptorrhabda* were highly similar in their sculpture and dimensions of silica scales (Fig. 5). In addition, we revised the morphology of closely related strains we originally determined as *S. mammillosa* in Škaloud *et al*. (2013a), concluding they either morphologically better fit with *S. leptorrhabda* (strains S89.C3 and S96.B5) or represent a transient morphotype between these two taxa (the strain SIE.105A). Accordingly, the *S. leptorrhabda* clade probably consists of numerous cryptic lineages. Since we do not currently possess enough material to investigate in detail the morphological properties of *S. leptorrhabda* lineages, we do not treat them taxonomically in this paper, and will focus on this cryptic complex in a separate study.

On the other hand, we analysed in detail the morphological properties of four novel lineages inferred within the core Petersenianae, along with all previously described,

closely related taxa (Fig. 6). Morphological comparisons of silica scales revealed the general similarity of all novel clades to the previously described taxa (Fig. 6, a and b). Indeed, only 73, 60, 70 and 63 percent of *S. praefracta*, *S. vinlandica*, *S. fluviatilis* and *S. cornuta* scales were correctly recognized by the discrimination function, respectively (Table S6 in the Supporting Information). However, all four novel lineages could be clearly differentiated by the combination of scale dimensions, basal/keel pore sizes and specific morphological features. The scales of *S. vinlandica* are characterized by the shortened, eccentrically positioned keel, observed in the majority of apical and even some of the body scales. The three remaining lineages can be well recognized by the unique shapes of their keel tips. Whereas the scales of *S. praefracta* possess a rounded tip terminated by several short teeth, the keels of *S. fluviatilis* and *S. cornuta* protrude into acute tips, which are either very long and tapering (*S. fluviatilis*) or shorter and much narrow (*S. cornuta*), respectively (see the taxonomic revision below for more details). The toothed keel tips observed in the scales of *S. praefracta* are characteristic of *Synura petersenii* f. *praefracta*, described by Asmund (1968) from Alaska. Indeed, the scale morphology is in correspondence with the taxon iconotype, showing the morphology of three apical silica scales (Fig. 6c). Therefore, we can unquestionably assign this species to *Synura petersenii* f. *praefracta*. Since this lineage represents a distinct species within the core Petersenianae, we are proposing a new combination, *S. praefracta*, comb. nov. (see below). The remaining three novel lineages could not be assigned to any *Synura* taxon with known morphology of silica scales but lacking molecular characterization (*S. obesa*, *S. australiensis*, *'S. petersenii' f. columnata*, and *'S. petersenii' f. taymyrensis*). To avoid introduction of superfluous names, we also carefully considered all previously described species with unknown ultrastructure of silica scales. According to Škaloud *et al*. (2012), six of these taxa can be affiliated to the section Petersenianae according to either the presence of keel or the absence of distal spines on the scales: *S. adamsii*, *S. adamsii* f. *malabrica*, *S. caroliniana*, *S. elipidosa*, *S. intermedia* and *S. virescens*. The former three taxa are well differentiated by their very long cells, *S. elipidosa* is distinct by very small cell dimensions (up to 12 ◎m in length), and *S. virescens* has very large colonies (up to 137 ◎m in diameter). *S. intermedia* can be distinguished by a strongly prolonged keel

resembling the spine. Even though a similarly prominent keel tip has been observed in *S. cornuta*, the spiny keel in *S. intermedia* is much longer, exceeding in its length the entire length of the scale. Consequently, given the fact three novel lineages are not identical to any previously described species, we are proposing that they represent three new species (*S. vinlandica*, *S. fluviatilis* and *S. cornuta*) described below.

Consequently, the core Petersenianae now includes 17 ultrastructurally and molecularly well-defined species distributed in four clades (Fig. 3). The phylomorphospace plots (projections of the species trees into the morphospaces based on silica scale morphology) show the morphological similarity of species belonging to particular clades, though in *S. macropora* and *S. borealis* a significant morphological shift occurred during the evolution of the genus (Fig. 6d). In general, whereas clade 1 is composed by the species possessing rather broad scales, clade 2 comprises those species having the smallest scale dimensions (Fig. 6, e and f).

Taxonomic revisions and diagnoses

***Synura praefracta* (Asmund) Škaloud & Škaloudová comb. nov.** (Fig. 7, a-i)

Basionym: *Synura petersenii* Korshikov f. *praefracta* Asmund (1968), Hydrobiologia, 31: 501.

Observation: Colonies are spherical, up to 57 μm in diameter, consisting of approximately 6–28 cells associated by their posterior ends (Fig. 7a). Cells are elongated, anteriorly cylindrical, posteriorly tapering into the long tail, 21–29 μm long and 6.5–8.5 μm wide (Fig. 7b). Each cell is surrounded by a layer of imbricate siliceous scales (Fig. 7, c and d). Body scales are 2.8–3.9 μm long and 1.5–2.0 μm wide, consisting of a basal plate with a centrally raised keel protruding into a very short acute tip (Fig. 7e). The keel is cylindrical and ornamented by larger pores (diameter, 51–93 nm). The basal plate is ornamented by numerous small pores (diameter 19–37 nm), and anteriorly perforated by an elongated or rounded base hole (diameter 0.19–0.74 μm). Numerous struts (27–36, rarely 40), interconnected by transverse ribs, extend regularly from the keel to the scale perimeter (Fig. 7f). Apical scales are 2.6-3.3 μm long and 1.6-1.8 μm wide (Fig. 7g). The

keel of the apical scales with rounded spine terminated by several short teeth (Fig. 7, g and h). Rear scales are 2.5-2.6 μm long and x 1 x 0.9-1.1 μm wide (Fig. 7i).

Holotape: material deposited in Statens Naturhistoriske Museum, Copenhagen, Denmark (currently lost).

Epitype (here designated): Strain I7 permanently cryopreserved in a metabolic inactive state in the Culture Collection of Algae of Charles University in Prague (CAUP) as the item TYPE-B714.

Reference strain: The live culture of the epitype (strain I7) has been deposited as CAUP B714 in the Culture Collection of Algae of Charles University in Prague, Czech Republic.

Distribution: The scales with rounded spine terminated by minute teeth at the apex designated as *Synura petersenii* f. *praefracta* have previously been reported from different areas, e.g. Canada (Nicholls and Gerrath 1985), USA (Siver 1987), Chile (Dürrschmidt 1982), Hungary (Barreto 2005), Ireland (Řezáčová and Škaloud 2005), Netherlands (Wujek and Van Der Veer 1976, Roijackers and Kessels 1981), Russia (Balonov 1976).

***Synura vinlandica* Škaloud, Škaloudová & Siver sp. nov.** (Fig. 7, j-r)

Description: Colonies are spherical, up to 65 μm in diameter, consisting of approximately 20–42 cells associated by their posterior ends (Fig. 7j). Cells are drop-shaped, anteriorly cylindrical, posteriorly tapering into the tail, 21–32 μm long and 7–12 μm wide (Fig. 7k). Each cell is surrounded by a layer of imbricate siliceous scales (Fig. 7, l and m). Body scales are 2.9-4.1 μm long and 1.4-2.2 μm wide, consisting of a basal plate with a centrally raised keel, which is rounded or mostly protruding into an acute tip (Fig. 7n). The keel is cylindrical, usually narrow, rarely slightly widened anteriorly, and ornamented by larger pores (diameter 56–115 nm). The basal plate is ornamented by numerous medium-sized pores (diameter 25–37 nm), and anteriorly perforated by an elongated base hole (diameter 0.17–0.46 μm). Struts (23–34), sometimes interconnected by transverse ribs, extend regularly from the keel to the scale perimeter (Fig. 7, n-p). Apical scales are 2.9-3.6 μm long and 1.6-2.2 μm wide (Fig. 7q). The keel of the apical scales usually ends in a prominent, acute tip. The keel of apical scales is shortened and positioned eccentrically to one side of the scale (Fig. 7q). Such eccentric keel positioning

was observed in some of the body scales, as well (Fig. 7p) Rear scales are 1.9-4.6 μm long and 0.8-1.5 μm wide (Fig. 7r). Differs from other *Synura* species by the ultrastructure of silica scales and by ITS rDNA (GenBank Accession MN782206), *rbc*L (MN783119) and *cox*I (MN783144) sequences.

Holotype (here designated): Strain I82 permanently cryopreserved in a metabolic inactive state (cryopreservation in liquid nitrogen) at the Culture Collection of Algae of Charles University in Prague (CAUP) as the item TYPE-B715. Figure 7n presents an illustration of the holotype.

Reference strain: The live culture of the epitype (strain I82) has been deposited as CAUP B715 in the Culture Collection of Algae of Charles University in Prague, Czech Republic.

Etymology: The specific epithet "*vinlandica*" refers to the Viking name for the Canadian Island of Newfoundland (Vinland), where the species has been discovered.

Type locality: Shoe Cove Pond, Newfoundland, Canada (47.74186, -52.74175).

Distribution: Currently only known from Newfoundland, Canada.

***Synura fluviatilis* Škaloud, Škaloudová & Siver sp. nov.** (Fig. 8, a-i)

Description: Colonies are spherical, up to 56 μm in diameter, consisting of approximately 16–24 cells associated by their posterior ends (Fig. 8a). Cells are lanceolate, widest in their middle part, posteriorly tapering into the tail, 20–28 μm long and 7–10 μm wide (Fig. 8b). Each cell is surrounded by a layer of imbricate siliceous scales (Fig. 8c). Body scales are 2.7-4.0 μm long and 1.3-1.8 μm wide, consisting of a basal plate with a centrally raised rounded keel. Body scales in the anterior part of the cell have a keel which protruding into an acute tip (Fig. 8d). The keel is cylindrical, occasionally slightly widened anteriorly, and ornamented by larger pores (diameter, 64–105 nm) (Fig. 8, e and f). The basal plate is ornamented by numerous medium-sized pores (diameter 22–39 nm), and anteriorly perforated by a rounded or elongated base hole (diameter 0.15–0.38 μm). Numerous struts (26–37), sometimes interconnected by transverse folds, extend regularly from the keel to the scale perimeter (Fig. 8e). Apical scales are 2.0-2.9 μm long and 1.4-1.8 μm wide. The keel of the apical scales ends in a

long, prominent, usually acute tip (Fig. 8, g and h). Rear scales are 1.9-2.5 μm long and 0.9-1.1 μm wide (Fig. 8i). Differs from other *Synura* species by the ultrastructure of silica scales and by ITS rDNA (GenBank Accession MN782209), *rbc*L (MN783121) and *cox*I (MN783146) sequences.

Holotype (here designated): Strain J87 permanently cryopreserved in a metabolic inactive state (cryopreservation in liquid nitrogen) at the Culture Collection of Algae of Charles University in Prague (CAUP) as the item TYPE-B716. Figure 8f presents an illustration of the holotype.

Reference strain: The live culture of the epitype (strain J87) has been deposited as CAUP B716 in the Culture Collection of Algae of Charles University in Prague, Czech Republic.

Etymology: The specific epithet "*fluviatilis*" refers to the common habitat of the species, i.e., various running water bodies such as rivers and brooks.

Type locality: Oxbow lake of Exploits River, Newfoundland, Canada (48.94234, -55.76928).

Distribution: Currently only known from Newfoundland, Canada.

***Synura cornuta* Škaloud, Škaloudová & Siver sp. nov.** (Fig. 8, j-r)

Description: Colonies are spherical, up to 55 μm in diameter, consisting of approximately 8–16 cells associated by their posterior ends (Fig. 8j). Cells are spherical, anteriorly rounded, posteriorly tapering into the tail, 13– 27 μm long and 9–15 μm wide (Fig. 8k). Each cell is surrounded by a layer of imbricate siliceous scales (Fig. 8l). Body scales are 3.2-4.9 μm long and 1.4-1.9 μm wide, consisting of a basal plate with a centrally raised rounded keel, which protruding into either a short, tapering, acute tip (Fig. 8, m and n), or rarely a very specific, narrow and prominent tip resembling a horn (Fig. 8o). The keel is cylindrical, occasionally slightly widened anteriorly, and ornamented by larger pores (diameter, 47–92 nm). The basal plate is ornamented by numerous small pores (diameter 17–30 nm), and anteriorly perforated by a rounded or elongated base hole (diameter 0.16–0.39 μm). Numerous struts (22–35), not interconnected by transverse rims, extend regularly from the keel to the scale perimeter. Apical scales are 2.2-3.2 μm

long and 1.3-1.6 μm wide (Fig. 8, p and q). Similar to some of the body scales, the keel of the apical scales ends in a very prominent, narrow tip resembling a horn (Fig. 8, p and q). Rear scales are 2.3-3.1 μm long and 0.8-1.2 μm wide (Fig. 8r). Differs from other *Synura* species by the ultrastructure of silica scales and by ITS rDNA (GenBank Accession MN782210), *rbc*L (MN783122) and *cox*I (MN783147) sequences.

Holotype (here designated): Strain K6 permanently cryopreserved in a metabolic inactive state (cryopreservation in liquid nitrogen) at the Culture Collection of Algae of Charles University in Prague (CAUP) as the item TYPE-B717. Figure 8o presents an illustration of the holotype.

Reference strain: The live culture of the epitype (strain K6) has been deposited as CAUP B717 in the Culture Collection of Algae of Charles University in Prague, Czech Republic.

Etymology: The specific epithet "*cornuta*" refers to the specific shape of the keel tip on the silica scales.

Type locality: Unnamed lake, Newfoundland, Canada (48.94364, -55.82329).

Distribution: Currently only known from Newfoundland, Canada.

Discussion

Comparing morphological and molecular diversity estimates

Despite increasing popularity of cultivation-independent molecular methods to determine the overall diversity and distribution of protists, accurate comparative studies between morphological and molecular approaches remain very rare. DNA metabarcoding, primarily focusing on overall diversity of aquatic protist communities, usually reveals five to ten times higher diversity than the microscopic examinations (Abad *et al*. 2016, Groendahl *et al*. 2017, Rippin *et al*. 2018), though some studies have reported comparable taxon richness (Bazin *et al*. 2014). However, only a limited number of taxa are being identified by both molecular and microscopical approaches. For example, of 180 protist taxa morphologically determined by Groendahl *et al*. (2017) in the estuary of Bilbao River, only 44 of them were detected in the DNA-based datasets consisting of hundreds to thousands of OTUs. Obviously, metabarcoding of protist communities is severely limited by i) overestimation of species diversity by accounting

erroneous sequences and PCR chimaeras as new, distinct species (Behnke *et al*. 2011, Lücking *et al*. 2014), ii) incompleteness of reference DNA databases linked to morphology of described species (Leray and Knowlton 2015), and iii) a still high portion of cryptic taxa in protist morphospecies (Howe *et al*. 2009, Škaloud and Rindi 2013).

More accurate molecular estimations of protist diversity are obtained when distinct, morphologically well recognized protist lineages are analysed. For example, Bachy *et al*. (2013) studied the diversity of the marine ciliate order Tintinnida, characterized by production of a species-specific secreted shell, the lorica. The morphological observations were supplemented by classical DNA cloning and metabarcoding, using the modern, more complex algorithms of OTU generation. The molecular approaches congruently detected the vast majority of morphologically observed taxa, and additionally revealed numerous novel lineages hidden to the traditional approaches.

Our study utilized a similar approach to Bachy *et al*. (2013) in combining molecular information with detailed morphological data for a taxon that forms highly distinctive siliceous scales. Although we did not employ the DNA metabarcoding approach, we investigated the molecular diversity by ITS rDNA sequencing of 150 isolated *Synura* colonies grown over a short time period. Contrary to Bachy *et al*. (2013), we focused on a much more narrowly defined protist lineage - the single genus *Synura*, comparing the molecular diversity with the traditional ultrastructural investigations of silica scales retrieved from water samples. Of the 27 identified species or species-level lineages, only one third were recorded by both molecular and morphological investigations (Fig. 9a). Eight taxa were recovered just by sequencing of isolated colonies. All of them represented rather young evolutionary lineages within the *S. petersenii* and *S. leptorrhabda* clades (Fig. 9b). Their presence in the samples might be masked by their morphological similarity to closely related species discovered by morphological investigations. A total of ten species were recovered only microscopically, four of them lack previous molecular characterization. These four taxa may represent rare, locally distributed, ecologically specialized, seasonally restricted, or hard-to-culture species. However, the remaining six taxa generally represented common species, widely distributed in temperate or boreal regions of North Hemisphere. It is possible that we

were unsuccessful isolating colonies representing these ten taxa because they were rare, difficult to establish in culture, or absent altogether in the collections at the time of sampling. It is worth noting that silica scales, typically used as a sole sign of species occurrences in many algal diversity studies, can remain in the water column after the demise of their carrier cells. Consequently, isolated silica scales can be effectively used to provide detailed insight into the species composition at a given locality, however their applicability to study the short-term temporal dynamics of species composition can be limited.

In conclusion, morphological and molecular approaches are clearly complementary in estimating the species diversity of protists, even for a narrowly defined, morphologically distinct lineage. When used separately, both approaches have their limitations. Molecular approaches using metabarcoding can be highly sensitive to species abundances at the time of sampling. Indeed, protist species richness, composition and abundance may fluctuate greatly due to changes in environmental factors, grazing pressure, and parasitism. Morphological approaches, on the other hand, can fail to recognize cryptic taxa and lead to underestimation of overall species diversity. In the case of *Synura*, morphological differences, especially of siliceous scales, have been successfully assigned to the majority of the cryptic species detected by molecular techniques. For cryptic *Synura* species, the morphological differences were always present, but simply needed to be recognized as important characters. Building datasets that combine morphological and molecular species data, as we strive to do for *Synura*, will provide a more complete reference baseline that will ultimately aid future metabarcoding investigations.

***Towards a more complete understanding of the global diversity within the genus* Synura**

With respect to global diversity estimates, there remains a significant biogeographical bias in chrysophyte studies, where many more studies have taken place in Europe and North America than in most other regions of the world (Kristiansen 2005). Even in regions such as Newfoundland previously thought to be relatively well-studied, we documented much higher diversity using a combination of morphological and

molecular techniques. The combination of techniques gave a better understanding of the boundaries used to delineate between either morphospecies or molecular species, thus improving the estimate of species diversity. Based on previous works using morphology alone (Wawrzyniak and Andersen 1985, Siver and Lott 2016, 2017, Siver *et al*. 2018), species diversity for *Synura* was considered high for Newfoundland with 14 species recorded. By simultaneously examining molecular data with a finer analysis of differences in morphological structure, the number of *Synura* species has more than doubled to 31 (Table S7 in the Supporting Information), illustrating the advantage of combining both techniques. Given the considerable cryptic diversity within the *S. leptorrhabda* lineage of the section Curtispinae that remains to be described, and finding additional distinct morphotypes that remain molecularly uncharacterized, Newfoundland waterbodies undoubtedly harbour even greater *Synura* species diversity.

In addition to differences used to distinguish between species (i.e. morphology versus molecular data), we recognize potential limitations in comparing species diversity estimates made between studies that employ different sampling methods. We further recognize that since only a small fraction of the aquatic habitats has been investigated to date, that it is highly likely additional *Synura* taxa reside in Newfoundland waterbodies. In the current study, and in the earlier investigation by Wawrzyniak and Andersen (1985), sampling was based on organisms actively growing at the time of collection. Since many chrysophytes present seasonal growth strategies and do not actively grow over the entire year, the time of collection can bias species diversity estimates (Siver 2015). This issue was less of a problem in the studies of Siver and colleagues (Siver and Lott 2016, 2017, Siver *et al*. 2018) since they incorporated remains of organisms found in both plankton and surface sediment samples. However, even though the top cm of surface sediments usually contains remains of organisms that grew over the last few years, only isolated scales are uncovered making it potentially difficult to capture the full complement of species. Given differences in methodologies between studies, differences in the seasonal occurrences between species, and since many waterbodies on the island have not been sampled, the full complement of species residing in Newfoundland is most likely still not realized.

Care also needs to be taken when comparing between morphological studies from different time periods, because the degree of variation in scale morphology used to differentiate between some taxa may have changed. We now recognize that the range of morphological scale variation associated with some species concepts was overly broad and representative of multiple taxa, and that other characters, such as size and distribution of base plate pores, can further aid in distinguishing between species. This was especially true for taxa in section Petersenianae where small, but consistent, differences in scale morphology are now recognized at the species level (e.g. Škaloud *et al*. 2012, 2014, Jo *et al*. 2016). Since the majority of published surveys illustrate only a few scales (and often only one) for each taxon, even for species found in numerous sites, it is highly unlikely that newly recognized differences could be fully evaluated using only the published illustrations in these previous works.

Despite thousands of localities investigated and thousands of sequences generated so far, our knowledge of global *Synura* diversity remains incomplete (Siver *et al*. 2010, Škaloud *et al*. 2013b). Geographic coverage needs to be broaden, and whenever possible investigations should strive to provide both morphological and molecular data. Most floristic and diversity studies for scaled chrysophytes are based solely on morphological investigations of silica scales, with no sequence data provided (Kristiansen and Preisig 2007). Accordingly, the molecular data are available only for about 16% of all currently accepted species and infraspecific taxa of silica-scaled chrysophytes. Considering the low genetic characterization of described taxa, and the here documented level of unknown genetic diversity, we propose that generating sequence data should become a gold standard in diversity studies of silica-scaled chrysophytes.

Biogeographic implications

The biogeography of protists has become a highly controversial topic over the last two decades (Martiny *et al*. 2006, Caron 2009, Ryšánek *et al*. 2015). Finally, supported by a number of studies based on detailed molecular investigations, the ubiquitous dispersal hypothesis has been rejected in favour of a moderate endemicity model (van der Gast 2015), proposing that although some protists may have cosmopolitan distributions,

others are restricted in their distribution to particular regions and/or specific habitats (Foissner 2006). Indeed, very different distributional patterns have been highlighted previously for *Synura* (Boo *et al*. 2010). For chrysophytes, including the genus *Synura*, distribution patterns depend on dispersal capacity of the species, resistance level of resting cysts, available vectors, and suitable available habitats (Boo *et al*. 2010, Siver and Lott 2012, Kristiansen and Škaloud 2017).

Distribution patterns of particular *Synura* species are especially diverse, ranging from cosmopolitan distribution (e.g., *S. petersenii*, *S. glabra*) to much restricted patterns. In this respect, we point out the restricted distributional patterns of two taxa found in Newfoundland, *S. kristiansenii* and *S. hibernica*. The former species, which has a highly distinctive keel and base plate pore, was described quite recently by Siver and Lott (2016) from a small oligotrophic and highly acidic (pH 3.9) bog in Newfoundland. During our investigations, we found this species at three additional oligotrophic localities, with pH ranging 7.3-7.6. This species thus seems to occur quite frequently in Newfoundland, spanning various habitats. Interestingly, it was never found outside of Newfoundland in numerous studies performed on the North American continent in the past, despite its very distinct scale morphology. Though *S. kristiansenii* represents a deep and evolutionary old lineage that originated ca 51 Mya, it seems to be highly restricted in its distribution to a small area in North America, possibly due to limited dispersal capacities.

Even most striking was our frequent observation of *S. hibernica* in Newfoundland localities. Indeed, this species represented one of the most observed and sequenced species, being detected in 11 localities. Since its description in 2014, *S. hibernica* was considered to have a very restricted distribution pattern, occurring only in western Ireland (Škaloud *et al*. 2014). Despite our extensive sampling in Europe, including a total of 71 ecologically highly similar and geographically close localities in north-western Scotland, we did not discover a single colony of *S. hibernica* outside of Ireland. However, our present results show that *S. hibernica* has a much broader distribution, extending to North America. Moreover, it is possible that *S. hibernica* was observed in North America much earlier, almost a hundred years ago. The distinctive shape of colonies resembles

that of *S. adamsii*, a species originally described by Smith (1924) from two ponds in the Palisades Interstate Park, New York, USA. *Synura adamsii* was subsequently reported in several ponds along coastal North Carolina (Whitford and Schumacher 1973). Since the morphology of silica scales was neither illustrated nor described in the protologue given by Smith (1924), the identity of *S. adamsii* is unclear relative to *S. hibernica* and it is possible that these taxa are conspecific. Notwithstanding the taxonomic status of *S. hibernica*, the distribution pattern of this species now spans coastal sites along both sides of the North Atlantic. Perhaps the distribution may be related to ionic chemistry as influenced by proximity to the ocean.

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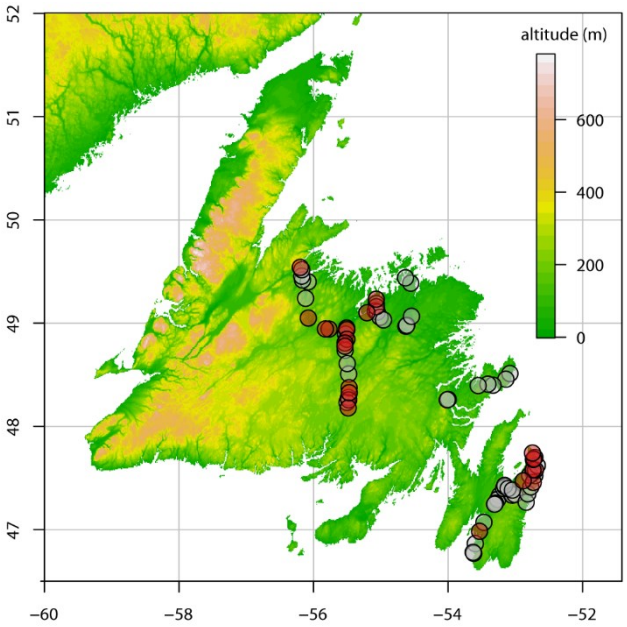

Fig. 1. Map of Newfoundland (Canada) showing the location of sampling sites. Those sites where *Synura* colonies were detected by light microscopy, and subsequently isolated into the cultures, are given by red circles.

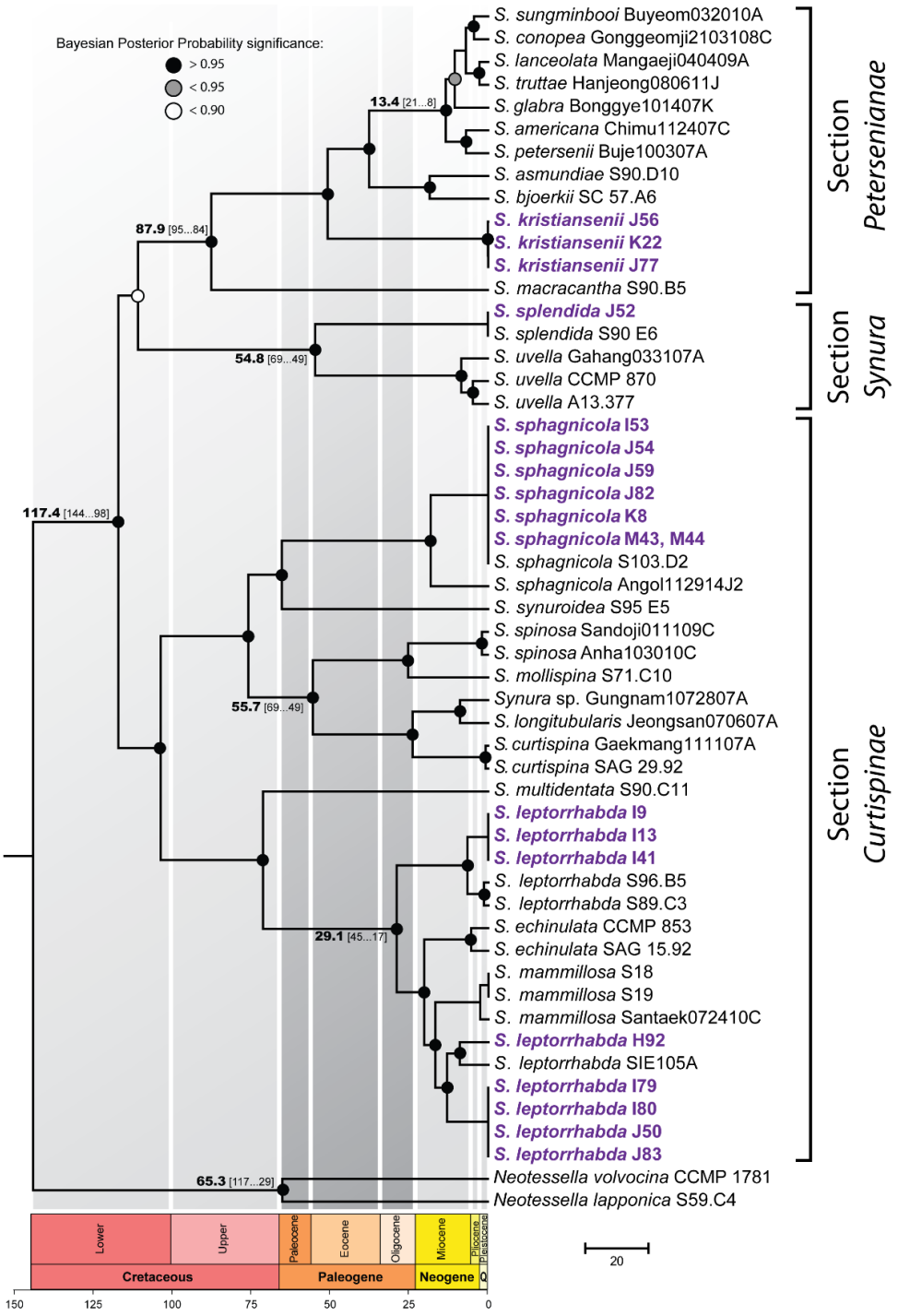

Fig. 2. Time-calibrated phylogeny of the genus *Synura* based on concatenated ITS rDNA, 18S rDNA, 28S rDNA, *rbc*L, *psa*A and 23S rDNA sequences. Newly generated sequences are given in bold. Mean divergence times are given for selected nodes, along with 95% highest posterior density (HPD) values in square brackets. Time axis is Mya, along with chronological dating of geologic intervals.

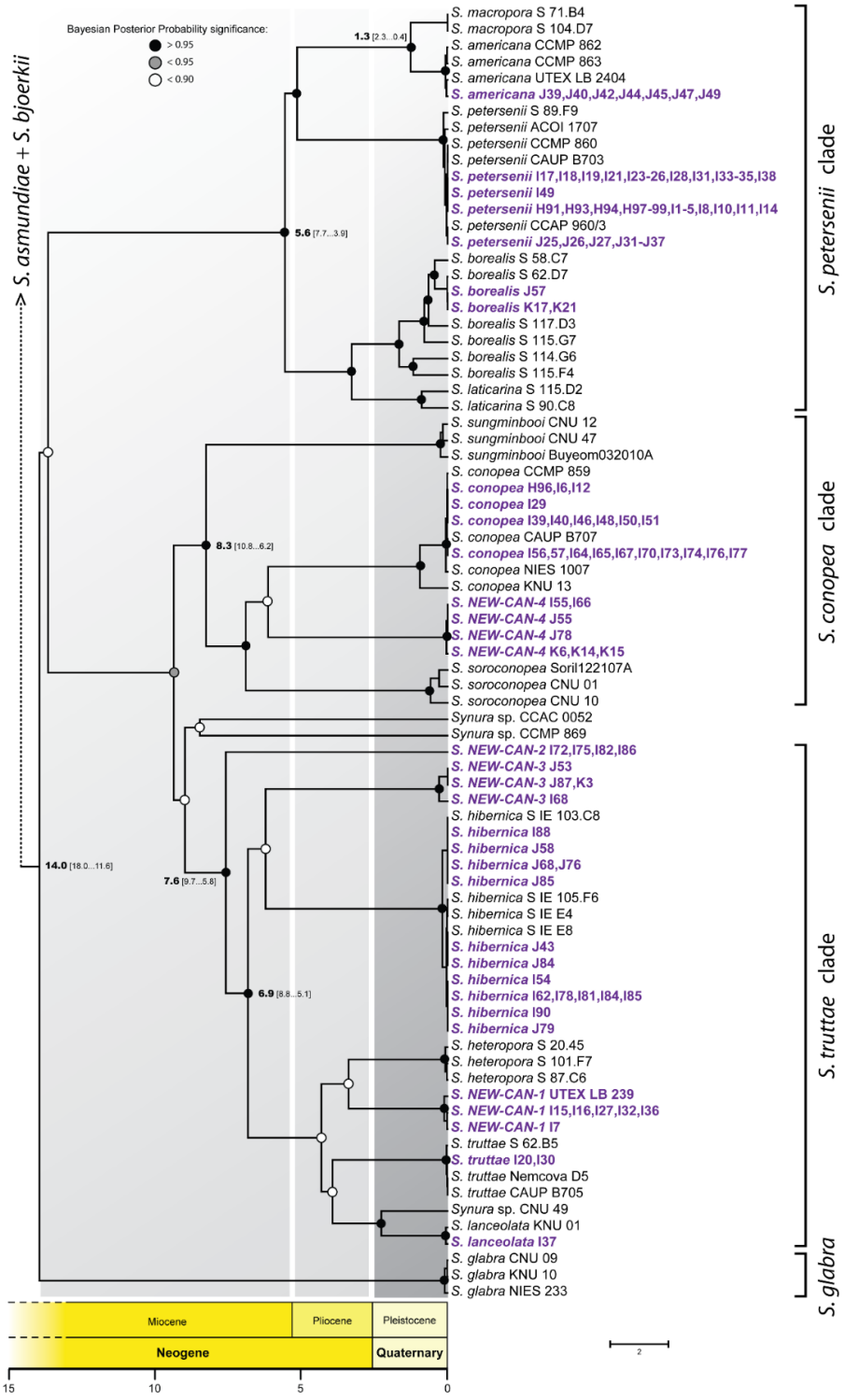

Fig. 3. Time-calibrated phylogeny of the genus *Synura*, section Petersenianae, based on concatenated ITS rDNA, *rbc*L and *cox*I sequences. Newly generated sequences are given in bold. Mean divergence times are given for selected nodes, along with 95% highest posterior density (HPD) values in square brackets. Time axis is Mya, along with chronological dating of geologic intervals.

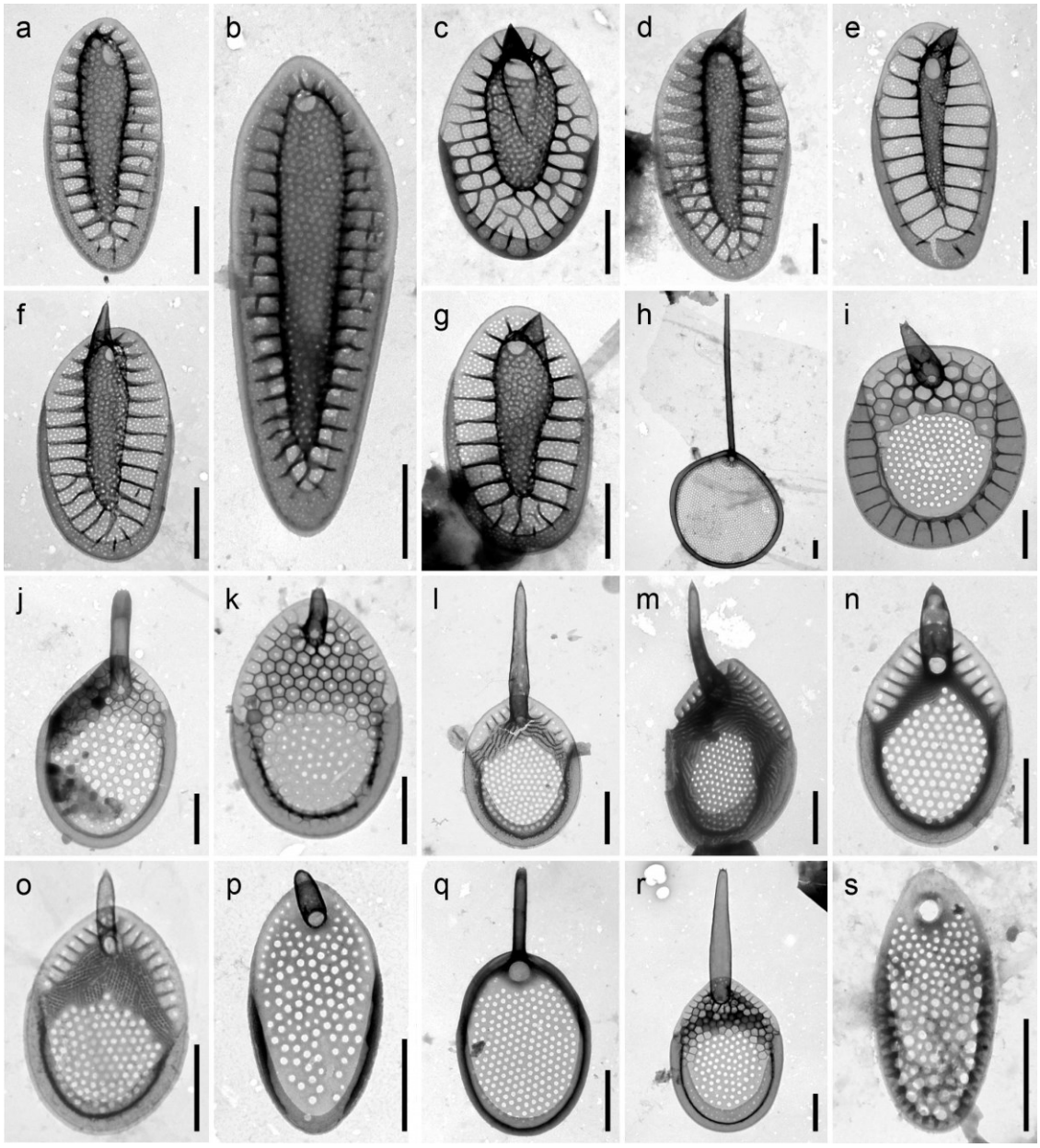

Fig. 4. *Synura* morphotypes identified in natural populations. **a**. *S. conopea*. **b**. *S. hibernica*. **c**. *S. kristiansenii*. **d**. *S. petersenii*. **e**. *S. petersenii* f. *"praefracta"* sensu Wujek & Igoe 1989, **f**. *Synura* sp. 1. **g**. *Synura* sp. 2. **h**. *S. splendida*. **i**. *S. uvella*. **j**. *S. curtispina*. **k**. *S. curtispina* f. *reticulata*. **l**. *S. echinulata*. **m**. *S. "echinulata"* sensu Nicholls & Gerrath 1985. **n**. *S. leptorrhabda*. **o**. *S. mammillosa.* **p**. *S. papillosa*. **q**. *S. sphagnicola*, **r**. *S. spinosa* f. *longispina*. **s**. *S. synuroidea*. Scale bars represent 1 μm.

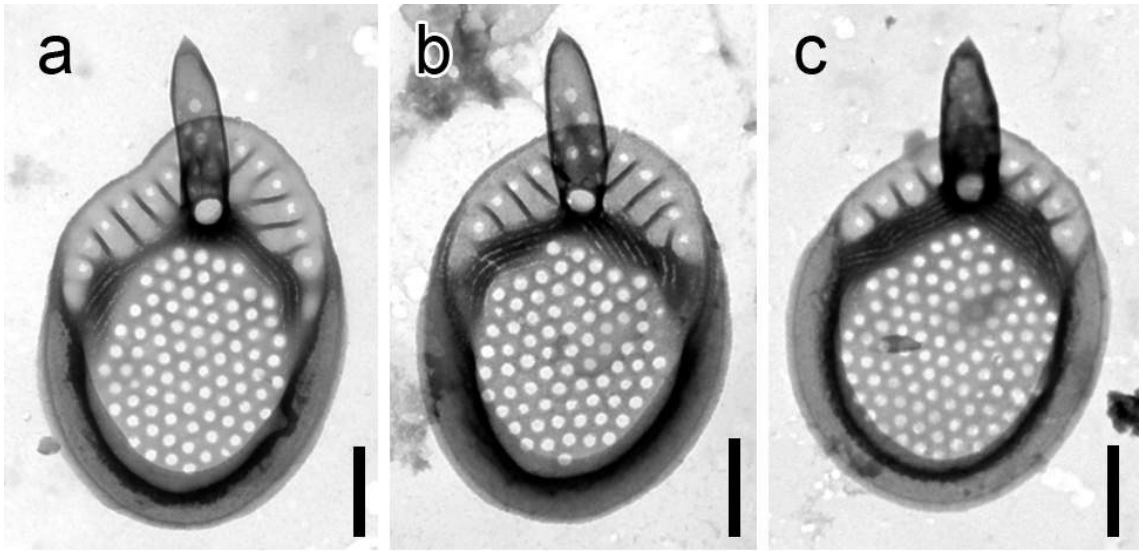

Fig. 5. Silica scales of three *S. leptorrhabda* lineages. **a**. strain I13. **b**. strain H92. **c**. strain J83. Scale bars represent 0.5 μm.

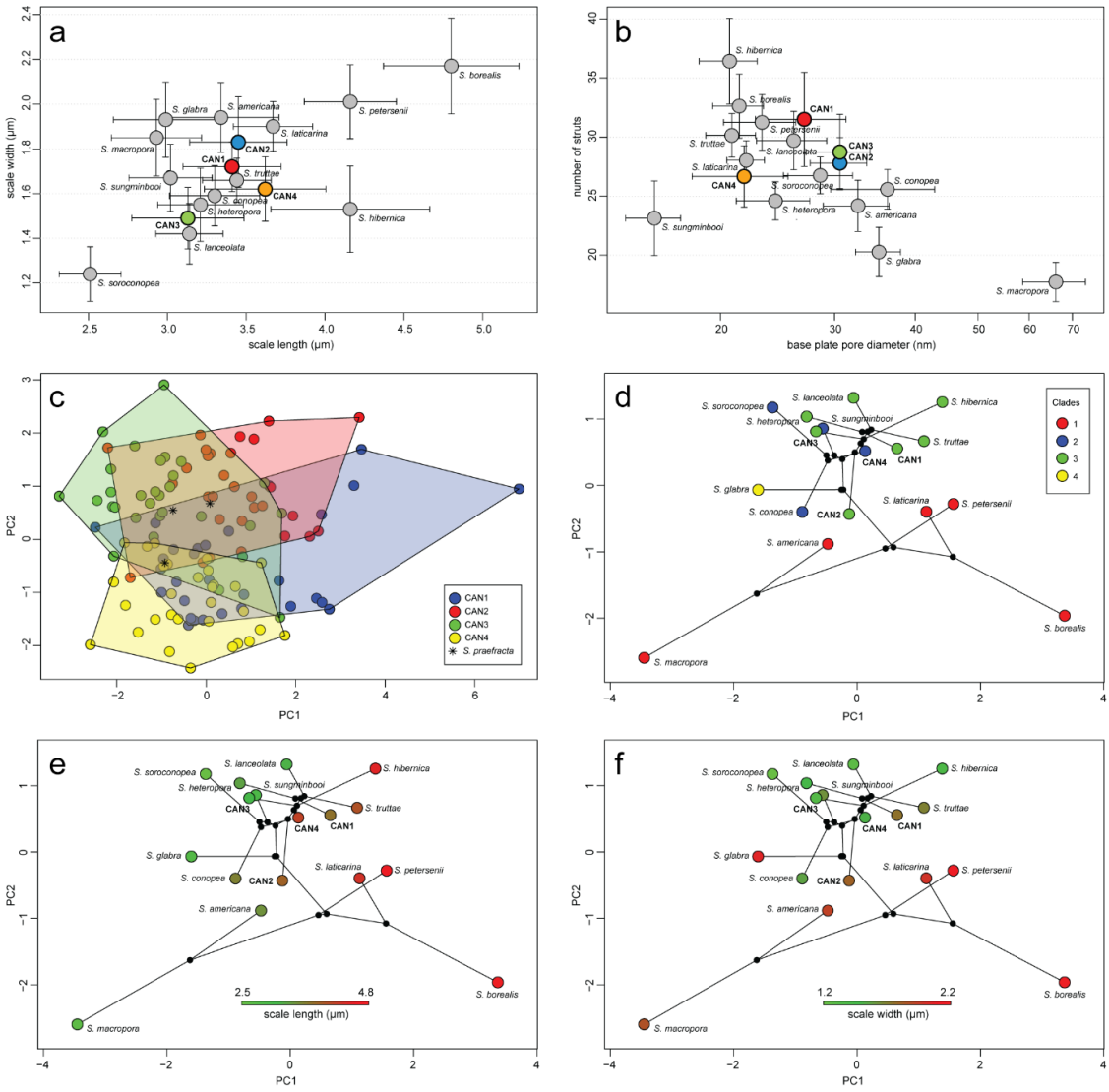

Fig. 6. Morphological analyses of 17 core Petersenianae species. **a-b**. Comparison of four morphological traits (scale length, scale width, base plate pore diameter, number of struts); average values and standard deviations are given, with the four newly characterized species highlighted. **c**. PCA ordination diagram showing the morphological diversity of 120 silica scales belonging to four novel lineages, along with the three iconotype scales of *S. petersenii* f. *praefracta*. **d-f**. Phylomorphospace plots of PCA axes obtained by the analysis of seven measured morphological traits. The circles represent individual species coloured by their clade affiliation (**d**), scale length (**e**) and scale width (**f**). Lines connect related species through hypothetical ancestors (black dots).

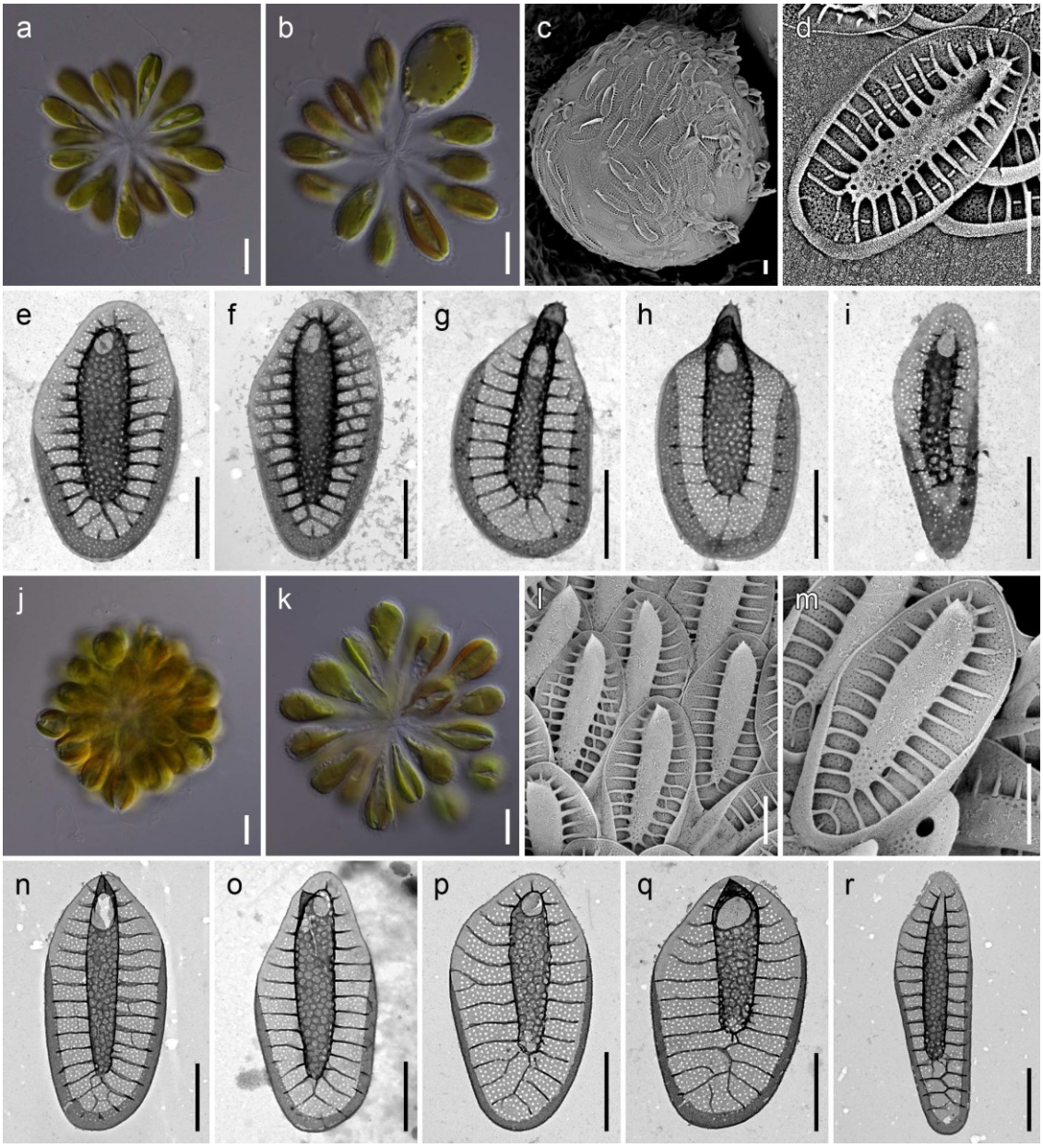

Fig. 7. Scale morphology of *Synura* species. **a-i.** *S. praefracta.* **a.** Colony consisting of elongated, drop-shaped cells. **b.** Colony with one encysting cell. **c.** Single cell surrounded by a layer of siliceous scales. **d-f.** Body scales. **g-h.** Apical scales with rounded spine terminated by several short teeth. **i.** Rear scale. **j-r.** *S. vinlandica.* **j-k.** Colonies consisting of spherical, drop-shaped cells. **l.** Layer of silica scales covering one cell. **m-o.** Body scales. **p.** Body scale with eccentrical keel. **q.** Apical scale with shortened and eccentrical keel. **r.** Rear scale. Scale bars represent 10 μm (**a-b**, **j-k**) and 1μm (**c-i**, **l-r**). **a-b**, **j-k**: LM; **c-d**, **l-m**: SEM; **e-i**, **n-r**: TEM.

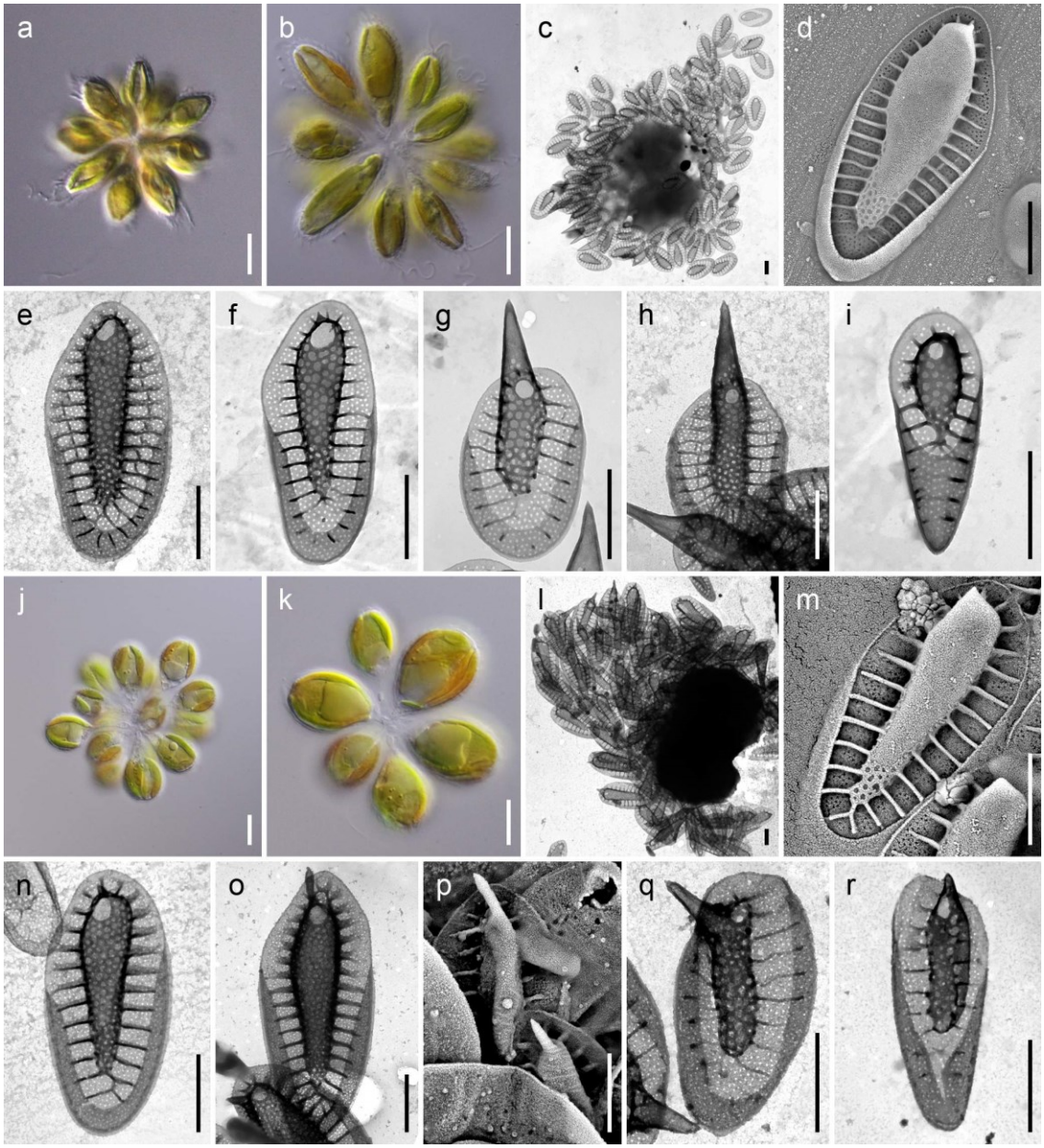

Fig. 8. Scale morphology of *Synura* species. **a-i.** *S. fluviatilis.* **a-b.** Colonies consisting of lanceolate cells. **c.** Single cell surrounded by a layer of siliceous scales. **d-f.** Body scales. **g-h.** Apical scales with prominent spines. **i.** Rear scale. **j-r.** *S. cornuta.* **j-k.** Colonies consisting of spherical cells. **l.** Single cell surrounded by a layer of siliceous scales. **m-n.** Body scales. **o.** Body scale with a prominent, narrow tip resembling a horn. **p-q.** Apical scales with the keels ending by prominent horn-like tips. **r.** Rear scale. Scale bars represent 10 μm (**a-b**, **j-k**) and 1μm (**c-i**, **l-r**). **a-b**, **j-k**: LM; **d**, **m, p**: SEM; **c**, **e-i**, **l**, **n, o, q-r**: TEM.

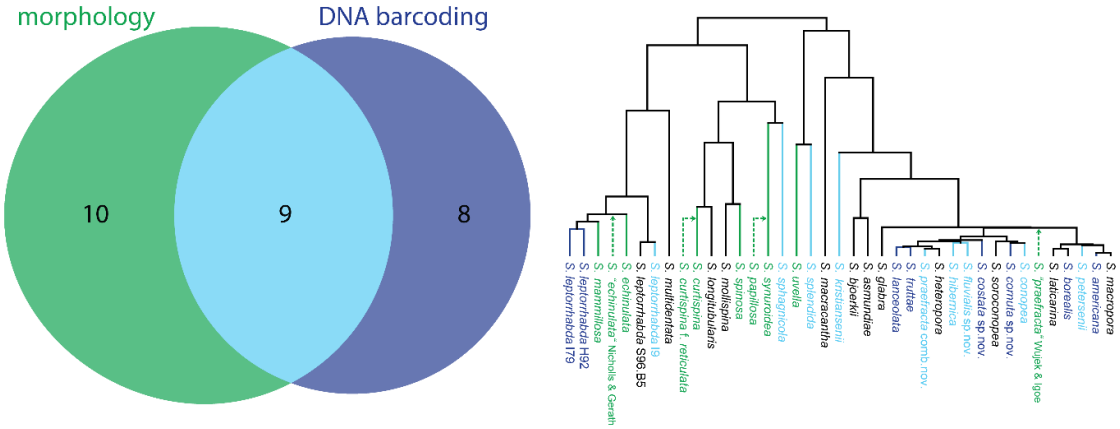

Fig. 9. Summary of taxa found by morphology and ITS rDNA sequencing (**a**) and their phylogenetic position along the *Synura* species tree (**b**). Estimated positions of taxa lacking molecular data (according to the morphology of silica scales) are visualized by dashed arrows. Taxa are color-coded in accordance to the Venn's diagram: Morphologically detected taxa are given in green, those recovered by DNA barcoding are given in violet, and those detected by both approaches are given in light blue. Unrecovered taxa are given in black.

Supporting Information:

Additional Supporting Information may be found in the online version of this article at the publisher's web site: https://doi.org/10.1111/jpy.12978

Table S1. List of sampling sites along with geographic coordinates, physico-chemical water parameters as well as the indication of those sites where *Synura* colonies were detected.

Table S2. Primers for amplifying and sequencing of the nuclear 18S rDNA, 26S rDNA and ITS rDNA, the mitochondrion-encoded *cox*I gene, and the plastid-encoded *rbc*L, *psa*A and 23S rDNA genes.

Table S3. Strains of the genus *Synura* used in this study and the GenBank accession numbers for their nu ITS rDNA, nu SSU rDNA, nu LSU rDNA, pt LSU rDNA, pt *psa*A and pt *rbc*L gene sequences. Those strains sharing identical DNA sequences are marked with lower case letters.

Table S4. Strains of the genus *Synura*, section Petersenianae, used in this study and the GenBank accession numbers for their nu ITS rDNA, pt *rbc*L and mt *cox*I gene sequences. Those strains sharing identical DNA sequences are marked with lower case letters.

Table S5. List of species recovered by TEM investigations of selected localities.

Table S6. The linear discriminant analysis confusion matrix summarizing the reclassification of the silica scales based on their morphological features. The proportions of correctly classified scales are given in the diagonal.

Table S7. A summary of *Synura* taxa recorded in Newfoundland island, Canada.

DNA alignments are freely available on Mendeley Data: http://dx.doi.org/10.17632/jjfmp6nv4b.1.