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

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ABSTRACT
The *Uroglena*-like morphotype represents a prototype of a colonial naked chrysophyte, comprising 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 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 light of our findings.

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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). In particular, 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 losses, thereby optimizing resource acquisition (Lürling & Van Donk, 1996; Padisák *et al.*, 2003, 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 life as a colony (Nicholls, 1995). From this perspective, colonial flagellates are possibly among the most successful groups of chrysophytes. 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 (Kurata, 1989; Ishikawa *et al.*, 2005), as well as in numerous Canadian lakes (Watson *et al.*, 1996). In many instances, the taxonomic identity (*sensu* Boenigk *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 centre 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, the problematic taxonomy of the polyphyletic *Ochromonas* was partly resolved by rediscovery of the type species *O. triangulata* from its type locality more than 100 years after the original description (Andersen *et al.*, 2017). Consequently, the phylogenetic position of *Ochromonas sensu stricto* has been resolved, though many lineages of *Ochromonas*-like flagellates have 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, probably

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cytoplasmic, threads forming radially arranged structures. At the end of the 19th century, Lemmermann (1899) transferred all new species of *Uroglena* 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 taxonomists dealing with *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 the 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. exhibit transitional morphological states between *Synura* and *Uroglena*. Relationships of these taxa to *Uroglena*, and indeed their true status remain unknown (Kristiansen & Preisig, 2001).

In the most recent taxonomic review, Wujek & Thompson (2002) introduced emended diagnoses of *Uroglena* and *Uroglenopsis* (incl. *Eusphaerella*). Cells of *Uroglena* possess a pointed posterior that tapers to a thin, probably cytoplasmic, thread. These threads connect individual cells through a dichotomously branching system. The shorter flagellum is approximately one half the length of the longer flagellum. In contrast, cells of *Uroglenopsis* possess more variable, although predominantly truncated or rounded, cell posteriors. Colonies of *Uroglenopsis* have no visible radially arranged structures or, when visible, individual cells are connected via a dichotomously branching system of relatively thick gelatinous stalks (sometimes more visible after staining). The short flagellum is, at most, one quarter of the length of the longer flagellum.

Unfortunately, almost all the previous reviews of and shifts in *Uroglena* taxonomy have been based on the morphology only without the use of molecular data. So far, only a few *Uroglena*/*Uroglenopsis* strains have been characterized from a molecular point of view. One of the reasons may be the difficulty in isolating and subsequently cultivating these extremely fragile colonies of naked flagellates. In addition, for up to date analysis, it is usually necessary to use a large number of strains and these were not available in algal collections. Therefore, the aim of this challenging work was to conduct a modern taxonomic revision of the genera possessing the *Uroglena*-like morphotype. By using a combined methodology of studying a sufficient amount of short-term cultures and 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 of their genetics (nuclear SSU rDNA and plastid *rbc*L), morphology (light and electron microscopy) and ecology. Based on a combination of all data, we contribute significantly 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 throughout 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 selected and sampled water bodies which existed near Ehrenberg's alma mater at the time of his collection. Only Grunewaldsee in the Grunewald district, within the forest of the same name, on the outskirts of western Berlin contained *Uroglena* taxa. Sampling was predominantly, but not exclusively, carried out in the spring months. Samples were collected using a plankton net with 20 μm mesh. At 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 with 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 three 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 a sample, 10–20 washed colonies were placed individually into a well of a 96-well polypropylene plate that contained

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 + cul	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	6.5.2015	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	24.11.2015	N.A.	N.A.	N.A.
Urostipulosphaera notabilis (Mack) Pusztai *et al.*	U12-1	SC + cul	2	Velký pond in Voznice, Czech Republic	49.8185206N, 14.2169953E	20.3.2015	8.3	318	6.3
Urostipulosphaera sp.	UP-34	SC + cul	9	Lago do Viriato, Portugal	40.3135468N, 7.5661120W	4.4.2015	7.4	38	N.A.
Urostipulosphaera sp.	U5-5	SC + cul	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 + cul	4	Pool in Botanical Garden, Prague, Czech Republic	50.0710836N, 14.4206419E	6.2.2015	6.9	605	0.4
Urostipulosphaera sp.	U10-6	SC + cul	4	Unnamed pond near Kletečná, Czech Republic	49.5158761N, 15.3099022E	10.3.2015	7.7	208	8.1

In column 'Origin', SC = single-colony isolates only, SC + cul = single-colony isolates and cultures. N-isol. = number of acquired isolates with identical locality, morphology and sequences within a strain; N.A. = not available.

~400 µl Hepes-buffered DY IV liquid medium (pH ~7.5). Next, 8–16 washed colonies were put into an 8-tube strip, one colony to each tube, and frozen at −20°C for future direct use in single-colony PCR. Living isolates in plates were cultivated at 15°C, under constant illumination of 20–40 µmol photons m^{-2} s^{-1}. Owing to a low survival rate of isolated colonies, only a few isolates were successfully transferred into 50 ml Erlenmeyer flasks and maintained as short-term cultures, under the above-mentioned conditions. All cultures contained resident bacteria of natural origin, but a 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 an Olympus CX 31 light microscope at the research base just a few hours after sampling. Colonies and single cells were measured, drawn and 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 and presence of scale-like structures (e.g. silica scales) were examined with 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 with Nomarski interference contrast. The mucilaginous branching system was visualized by methylene blue staining and Lugol's iodine solution.

Sequencing and phylogenetic analysis

DNA isolation was carried out as described in Škaloudová & Škaloud (2013), slightly modified by 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 to all sequences closely related 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 xdetermined using the 'sump' command. Bootstrap analyses were performed by maximum likelihood (ML) xand 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 sensu stricto* morphotype (Figs 2, 3) were recovered in a single clade forming a monophyletic group that was sister to *Chrysonephele*, a non-motile flagellate colonial chrysophyte endemic to Tasmania. This group was also closely related to *Epipyxis* and *Chrysolepidomonas*. All strains with *Uroglenopsis* morphology formed two distant clades. The first clade, here referred to as *Uroglenopsis sensu stricto* (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 sensu stricto* clade was statistically well supported and closely related to a number of morphologically and ecologically distinct genera such as the terrestrial *Pedospumella* and the aquatic *Ochromonas triangulata* that lives in hypersaline lakes. The second clade with a *Uroglenopsis* morphology, here referred to *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 the Msimbazi River in Tanzania. This lineage

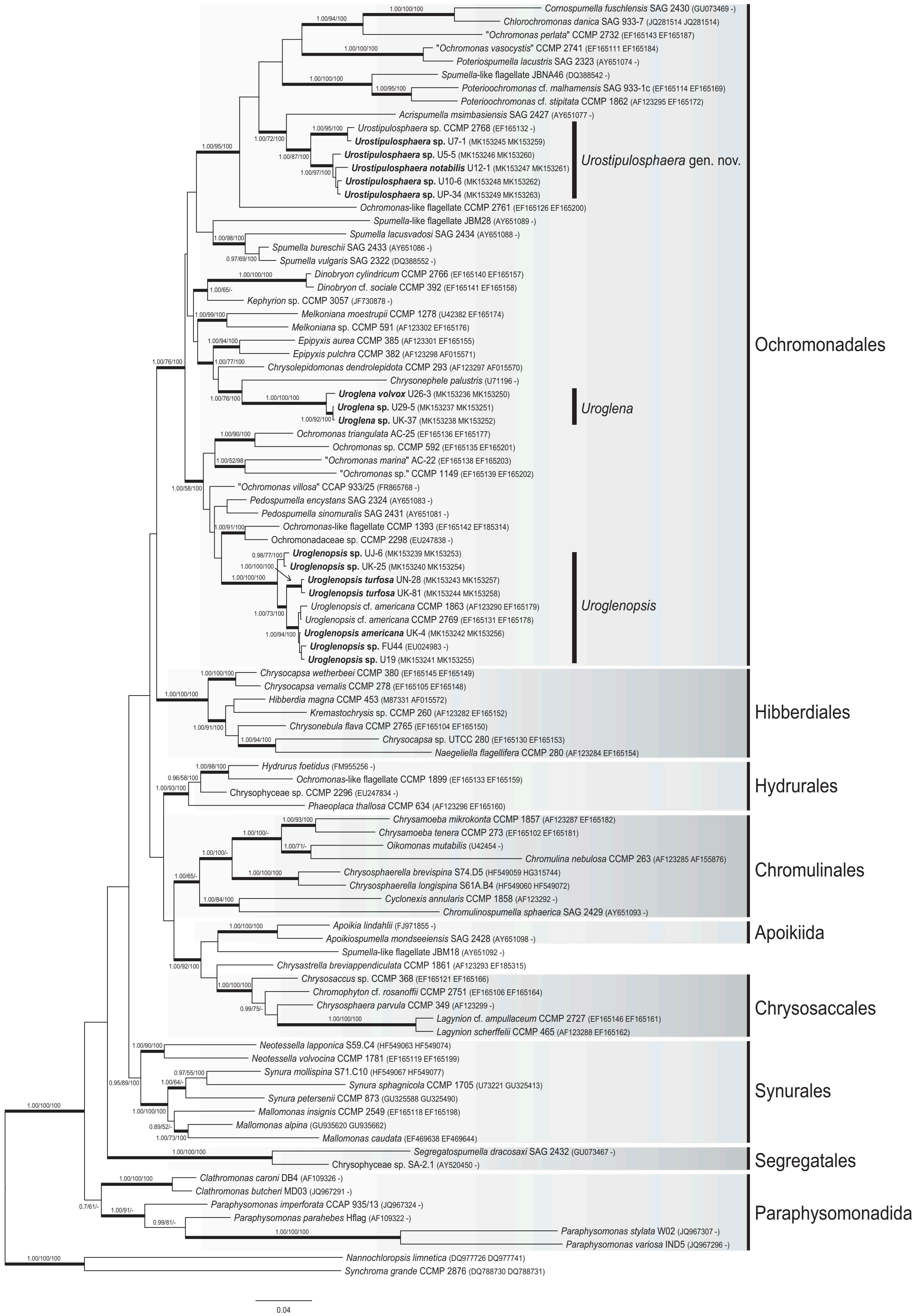
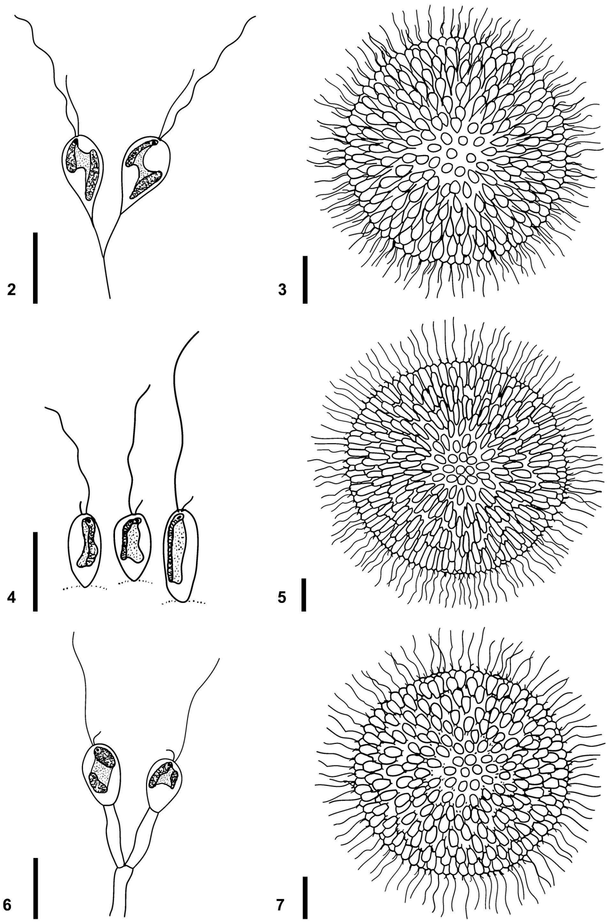


Fig. 1. Phylogeny of the Chrysophyceae obtained by Bayesian inference of the concatenated SSU rDNA and *rbc*L dataset. The analysis was performed under a partitioned model, using different substitution models for each partition. Values at the nodes indicate statistical support estimated by three methods: 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 on the emended diagnosis – cell shape, cell posterior, short: long flagella length ratio, presence/absence and characters of branched radial structures. **Fig. 2**. *Uroglena* cells of inverse tear-drop shape with sharply pointed cell posterior passing into a thin, probably cytoplasmic thread, shorter flagellum approx. half length of longer flagellum, usually one girdle-shaped, bilobed, slightly spiral plastid. **Fig. 3**. *Uroglena* colony. **Fig. 4**. *Uroglenopsis* cells of diverse shape embedded in a compact jelly, shorter flagellum < 0.25 of longer flagellum, usually one girdle-shaped, plate plastid. **Fig. 5**. *Uroglenopsis* colony. **Fig. 6**. *Urostipulosphaera* obovate cells attached by their truncate or rounded cell posterior to relatively thick articulated gelatinous stalks, shorter flagellum < 0.25 length longer flagellum, usually one girdle-shaped, broadly ribboned, bi-lobed, slightly spiral plastid. **Fig. 7**. *Urostipulosphaera* colony. Scale = 10 µm (Figs 2, 4, 6) and 20 µm (Figs 3, 5, 7).

was also related to *Cornospumella, Chlorochromonas, Poteriospumella* and *Poteriochromonas*.

Morphology

Uroglena *Ehrenberg*

Uroglena volvox was re-collected from its type locality and organisms related to it 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, probably cytoplasmic, threads (Figs 2, 3). These threads connected individual cells through a dichotomously branching system into a spherical colony. Colonies ranged from 50 µm to 250 µm in diameter, most commonly 70–150 µm. The smaller colonies with fewer cells were usually a product of a 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 was 9–12.5 µm long and 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, and/or was approximately half the length of the longer flagellum. Cells usually had a single girdle-shaped, bi-lobed, slightly spiral, gold-coloured 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 re-collected 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 observed even when stained with Lugol's iodine solution and/or methylene blue. Instead, cells were embedded into a compact jelly mantle as a monolayer coat at the colony periphery. This compact jelly mantle was, in normal conditions, invisible and appeared after staining with methylene blue (Fig. 8). Colonies possessed a 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 *Uroglenopsis* 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 a 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, and/or was approximately, at most, one quarter of the longer flagellum. Cells usually had a single girdle-shaped, gold-coloured 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 indistinguishable from *Eusphaerella turfosa*, and these organisms were nested within the *Uroglenopsis* clade. Cells and colonies agreed in all ways with *Uroglenopsis* except that they were closely packed together and hexagonal in apical view with a remarkable hole in the 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 a truncate or rounded cell posterior and they were connected via a 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 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 a 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 and 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, and/or was approximately, at most, one quarter of the longer flagellum. Cells usually had a single, girdle-shaped, broadly ribboned, bi-lobed, slightly spiral, gold-coloured plastid with an anterior stigma. The strain U7-1 collected from a small pool filled with decomposing plant material exhibited reduced plastids (distinctly smaller and pale) which became normal 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. two or three biconcave disk plastids were frequently observed). 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 described *Uroglena notabilis* Mack. In particular, the stomatocyst (12.5–14 µm in diameter) had a characteristic curved, collapsed, tubular neck formed by a rolled up sheet, and the cyst wall ranged from almost smooth-walled to embellished 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 10–15 µm long and 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 centre 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) < 0.25 length of longer flagellum (12.5–20 µm). Longer flagellum approx. once to twice cell length. Usually one girdle-shaped, broadly ribboned, bi-lobed, slightly spiral, gold-coloured plastid with anterior stigma.

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

ETYMOLOGY: 'uro' refers to the 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.

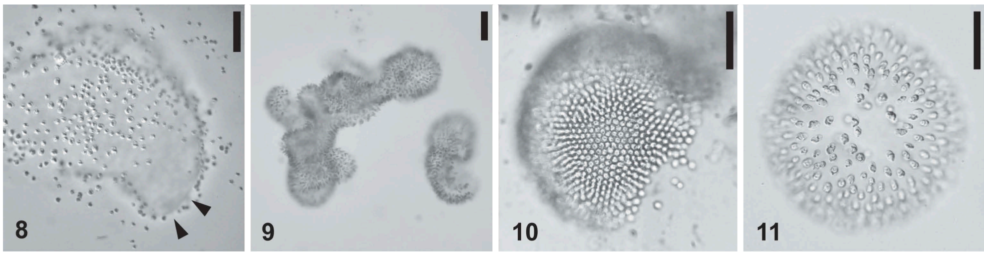
TYPE LOCALITY: Prater and Perchtoldsdorf, Wien, Austria.

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

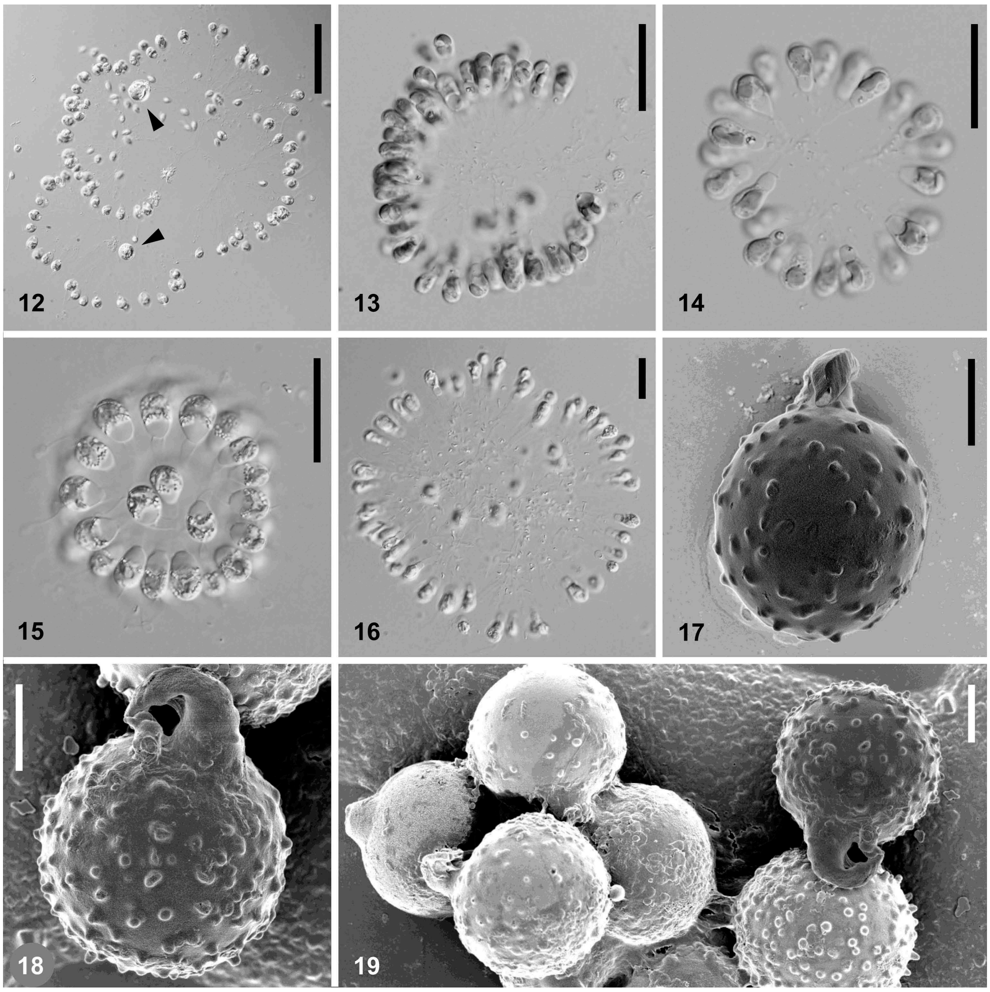
REPRESENTATIVE DNA SEQUENCES: GenBank accession nos. MK153247, MK153261.

Discussion

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



Figs 8–11. Morphology of *Uroglenopsis* shown in natural population U19 (Figs 8, 9), natural population UK-81 (Fig. 10) and cultured strain UK-81 (Fig. 11). **Fig. 8**. Colony after staining with methylene blue – cells are embedded into 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 in apical view. **Fig. 11**. Cultured colonies of *U. turfosa* lose 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 arrowheads). **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 visible collapsed neck formed by rolled up sheet. **Fig. 18**. *U. notabilis* fully developed cyst with visible pronounced curved tubular neck. **Fig. 19**. *U. notabilis* cysts ranged from almost smooth-walled to embellished cyst walls 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).

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 losses and, thereby, may optimize free resources acquisition (Lürling & Van Donk, 1996; Padisák *et al.*, 2003, 2009). Living in a 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, revision of the 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 under-studied lineages of *Ochromonas*-like flagellates have yet 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. The 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), uncovering the story of the *Uroglena*/*Uroglenopsis* evolutionary history remained unresolved (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 the 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 near Humboldt University of Berlin, Germany. Along with *Uroglena*, the colonial *Synura* and *Syncrypta* were also described (Ehrenberg, 1834, 1838). In contrast to *Synura* and *Syncrypta, Uroglena* was characterized as exhibiting a pronounced red stigma in the cell anterior. Nevertheless, in his drawings, Ehrenberg (1838) sketched a stigma in some cells of *Synura*. Accordingly, he wrongly referred to some colonies possessing stigmata 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 centre 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 a single, girdle-shaped, ribboned, 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 existed near there at the time of his collection. *Uroglena* taxa were only found in the Grunewaldsee in the Grunewald district within the forest of the same name, on the outskirts of western Berlin. The phenology and morphology of the *U. volvox* population we collected in Grunewaldsee fully correspond to Ehrenberg's protologue of this species. Ehrenberg found *U. volvox* from April to June. Our collections were made on 28 April. Moreover, colonies of Ehrenberg's *U. volvox* were ~282 µm in diameter (1/8‴), which is congruent with our findings (colonies of ~250 µm in diameter).

At the end of the 19th century, Lemmermann (1899) transferred '*Uroglena*' taxa described previously from the USA by Calkins (1892) into a newly established genus *Uroglenopsis*, with the type species *U. 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 sensu lato* taxa under the microscope, since colonies were no longer present in the fixed samples (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 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 had 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 the later newly erected genus *Uroglenopsis*. '*Uroglena*' *americana* found by Calkins (1892) had cells 5–7 µm wide, a longer flagellum of 13 µm and shorter flagellum of 2 µm in length, which is congruent with our findings (cells 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, neither Ehrenberg nor Calkins illustrated any cysts in their descriptions of *U. volvox* and *U. americana*, respectively. The cyst morphology has been provided by later taxonomists, 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 being a smooth-walled cyst with a simple pore *sensu* Kent (1881) and a smooth-walled cyst with a tubular neck and wider collar *sensu* Zacharias (1895). Therefore, we reject the concept of choosing the originally described cyst from all previous records, as proposed by Wujek & Thompson (2002). In an effort to correct and complete useful modern *U. volvox* and *U. americana* descriptions precisely, we propose to add information about the ultrastructure of the cyst together with its molecular characterization on the basis of exploring the encysting populations from type localities. As the populations of *U. volvox* from Grunewaldsee and *U. americana* from Buckmaster pond had not produced cysts, further efforts to find encysting populations that are 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 no consensus on the presence/absence and the nature of the radial structures was reached. Though Skuja (1948) did not distinguish between these genera, recognizing only *Uroglena sensu lato*, he probably 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 establishment of a number of new monophyletic genera in order to accommodate morphologically distinct paraphyletic taxa, as 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, for two reasons. Firstly, *Eusphaerella* and *Uroglenopsis* share the common absence of any visible radial structures between the colony centre and periphery. Secondly, 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 exception being the presence of equal 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 *Pseudosyncrypta* 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, or it could be an artefact caused by extreme conditions *in situ* or during sample processing (e.g. common change in plastid number in *Uroglena*-like flagellates by heating and drying microscope slides). If the latter is true, and considering the almost equal flagellar length, the lack of stigma and the presence of mucilage, with small bodies (possibly scales?) surrounding the colonies, *Pseudosyncrypta* 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. The question is whether they were just transient clusters of single-celled *Ochromonas sensu lato*, or if they represent colony fragments of *Synuropsis sensu lato* as proposed by Wujek & Thompson (2001).

The rest of the chrysophycean colonial genera – *Pseudosynura* Kisselev, *Syncrypta, Synochromonas* Korshikov, *Synuropsis* Schiller and *Volvochrysis* Schiller – represent enigmatic taxa with transient or chimaeric morphology between *Uroglena* (Ochromonadales) and *Synura* (Synurales) in general. Therefore, they were all synonymized into one genus – *Syncrypta sensu lato* (*sensu* Bourrelly, 1957) or later *Synuropsis sensu lato* (*sensu* Wujek & Thompson, 2001). Even though the synonymy is controversial, all of these synonymized 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 *Urostipulosphaera*. In our opinion, based on examination of thousands of samples hosting colonial chrysophytes from around the world (e.g. Škaloud *et al*., 2013, 2014; Němcová *et al*., 2016; Pusztai *et al*., 2016; this study) and with subsequent sequencing of many 'strange scale-less *Synura*-like' taxa, *Syncrypta sensu lato* (or *Synuropsis sensu lato*) 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. (probably *Synochromonas elaeochrus* Jane, *Synochromonas gracilis* Korshikov and *Synochromonas perlata* Skuja), *Uroglenopsis* spp. (probably *Syncrypta dubia* Bourrelly), scale-less *Chrysosphaerella* Lauterborn (probably *Volvochrysis globosa* Schiller) and true, but certainly very rare, *Syncrypta sensu lato* (or *Synuropsis* s.l.) possessing morphology as emended *Synuropsis danubiensis* Schiller (Wujek & Thompson, 2001).

The newly proposed *Urostipulosphaera* therefore represents a distinct genus, exhibiting a unique combination of morphological and genetic characteristics within chrysophytes. We have successfully obtained several cultures belonging to *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 had a pronounced curved tubular neck formed by a 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 occur in accordance with detailed genetic, morphological and ultrastructural characterization of cultures established from encysting populations.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Author contributions

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

Supplementary Information

The following supplementary material is accessible via the Supplementary Content tab on the article's online page at http://10.1080/09670262.2019.1574030

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).

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