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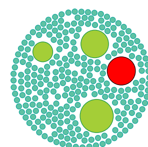

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Species delimitation within the colonial flagellates *Uroglena, Uroglenopsis* and *Urostipulosphaera* (Chrysophyceae)

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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 10 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 assign all the previously described *Uroglena*-like taxa to newly recognized genera and propose 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.

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Introduction

The term 'species' represents one of the cornerstones of both old and modern biology because of the permanent need to categorize 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 (Darwin, 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.

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The diversity of silica-scaled chrysophytes, particularly Synurales and Paraphysomonadida, has been studied by 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 fresh waters (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), focusing 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* (Supplementary 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 non-parametric tests (t-test, Mann–Whitney test).

Morphological microscopic investigations were made as described previously in Pusztai & Škaloud (2019) but using a 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 the 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 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 (Supplementary 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 1548 bp (*Uroglena*), 1566 bp (*Uroglenopsis*) and 1737 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 ~10% of which hosted a detectable population of *Uroglena*-like colonial chrysophytes at the time of sampling. We established 189 single-colony isolates (Supplementary table S1) of *Uroglena* (77), *Uroglenopsis* (67) and *Urostipulosphaera* (45). The phylogenetic position of all three genera is shown on a simplified phylogram (Supplementary fig. S1) adapted from Pusztai & Škaloud (2019). Many isolates originated from encysting populations (Supplementary 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*. Twelve 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 produced by a large colony collapsing during observation. Smaller colonies were also formed in 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 (Supplementary 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 cells (Figs 6–20) 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 with 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 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.

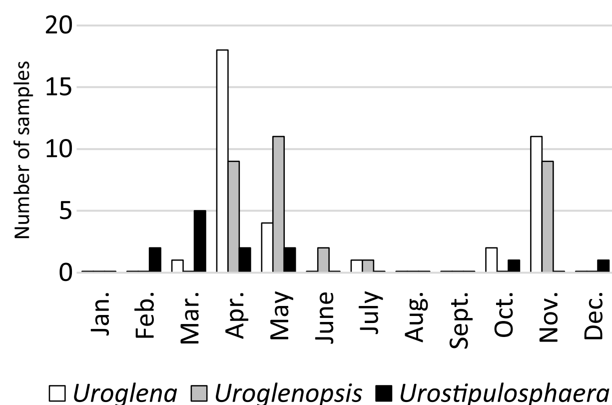


Fig. 1. Phenology of *Uroglena, Uroglenopsis* and *Urostipulosphaera* in the northern temperate zone based on all collected populations (number of samples) in the years 2014–2020. *Urostipulosphaera* seems to be an early spring taxon that peaked in March, while *Uroglenopsis* seems to be a late spring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

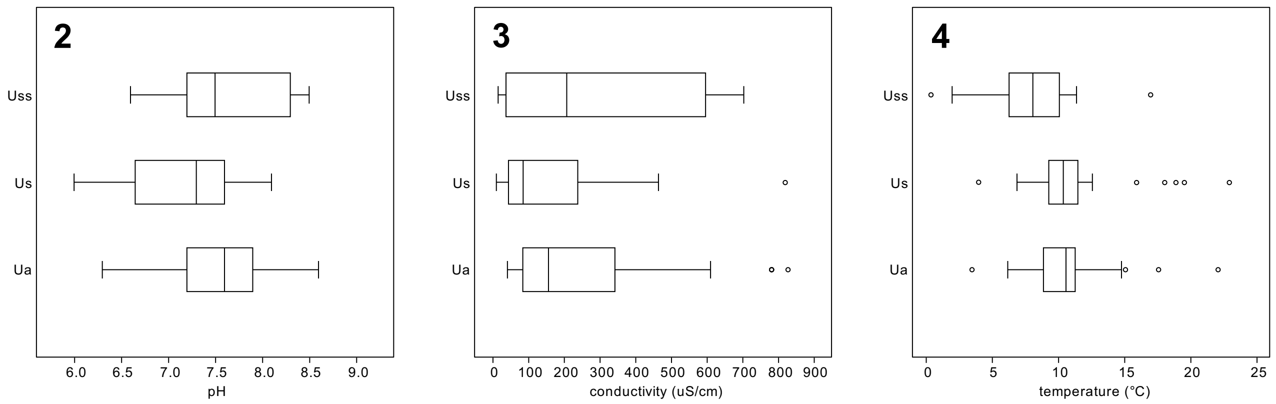
Uroglenopsis

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

Uroglenopsis colonies and cells (Figs 22–35) were of diverse shape. Colonies were usually spherical to oval, but *U. americana* and *U. botrys* also produced elongated to irregularly multi-lobed colonies. *U. turfosa* had unique morphology: fresh colonies were always closely packed together, with hexagonal cells in apical view and a conspicuous 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) anterior flagella. Cells usually contained a single parietal, gold-coloured plastid, elongated along the cell axis, with an anterior stigma. When stained with Lugol's iodine solution or methylene blue, cells were seen to be embedded into a compact jelly mantle with one to few thin short (1–3 μm) spine-like structures protruding posteriorly, most likely helping to fix cells within the jelly, as pointed out by Skuja (1948). Cells of stationary colonies exhibited characteristic jerky 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.



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).

Urostipulosphaera (Figs 37–57) cells were usually obovate, with two distinctly unequal (ratio ≥ 1:4) anterior flagella. Cells usually contained a single girdle-shaped or slightly spiral, or ribbon-shaped, bi-lobed, gold-coloured plastid with an anterior stigma. Predominantly truncate or rounded cell posteriors

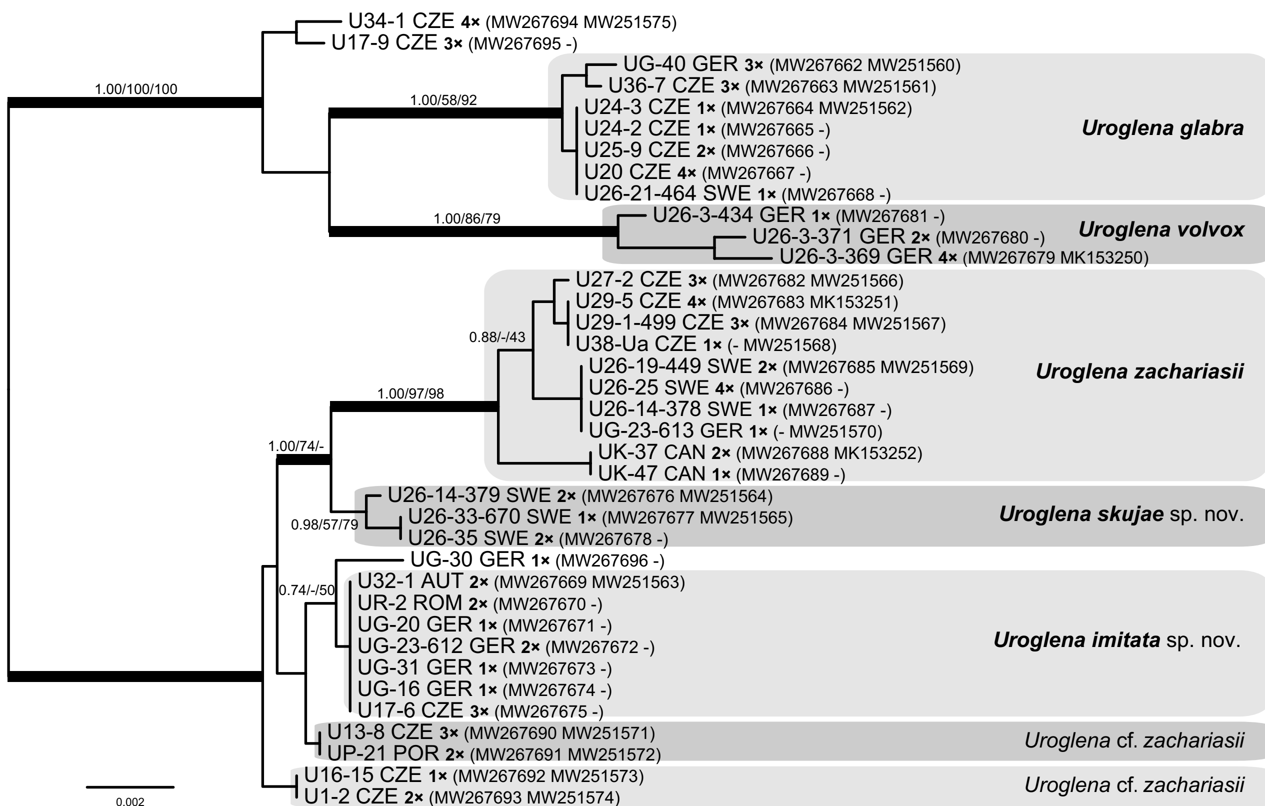
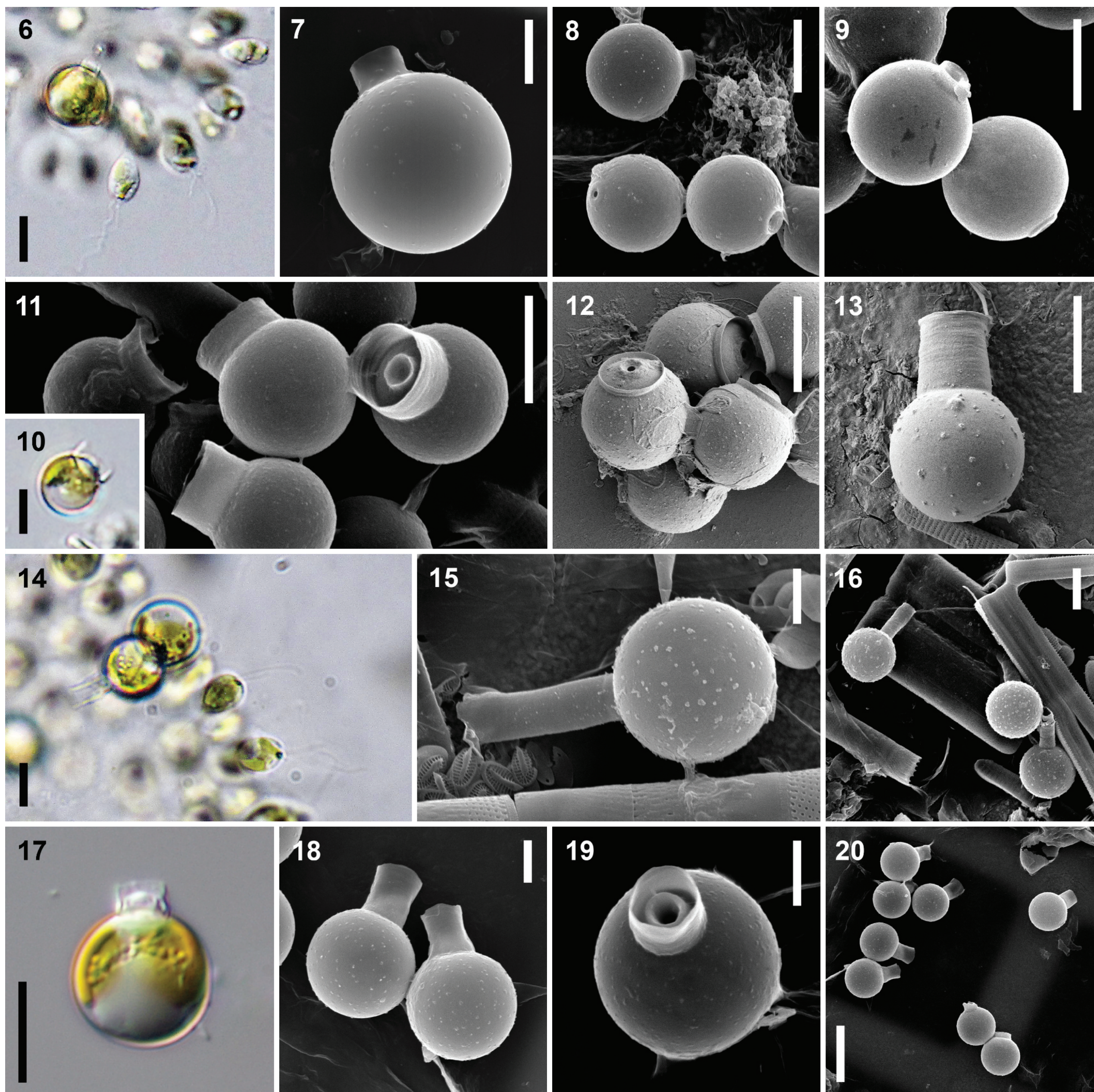


Fig. 5. Phylogeny of the genus *Uroglena* obtained by Bayesian inference of the concatenated ITS rDNA and *rbc*L dataset. The analysis was performed under a partitioned model, using different substitution models for each partition. Values at the nodes indicate statistical support estimated by three methods: 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 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 clearly 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).

were always connected via a 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 but sometimes with poorly visible stalks. Cultured colonies were sometimes oval but always with clearly 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* had a significantly higher survival rate than the two above-mentioned genera. Therefore, we were able to morphologically

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.

characterize in detail all species-level clades. Accordingly, we evaluated the usability of cell morphological features in species delineation. Our analyses show that *U. notabilis* (U12-1), *U. articulata* (U5-5) and *U. lindiae* (UP-34) had similar ranges of cell length and width (Figs 58–60).

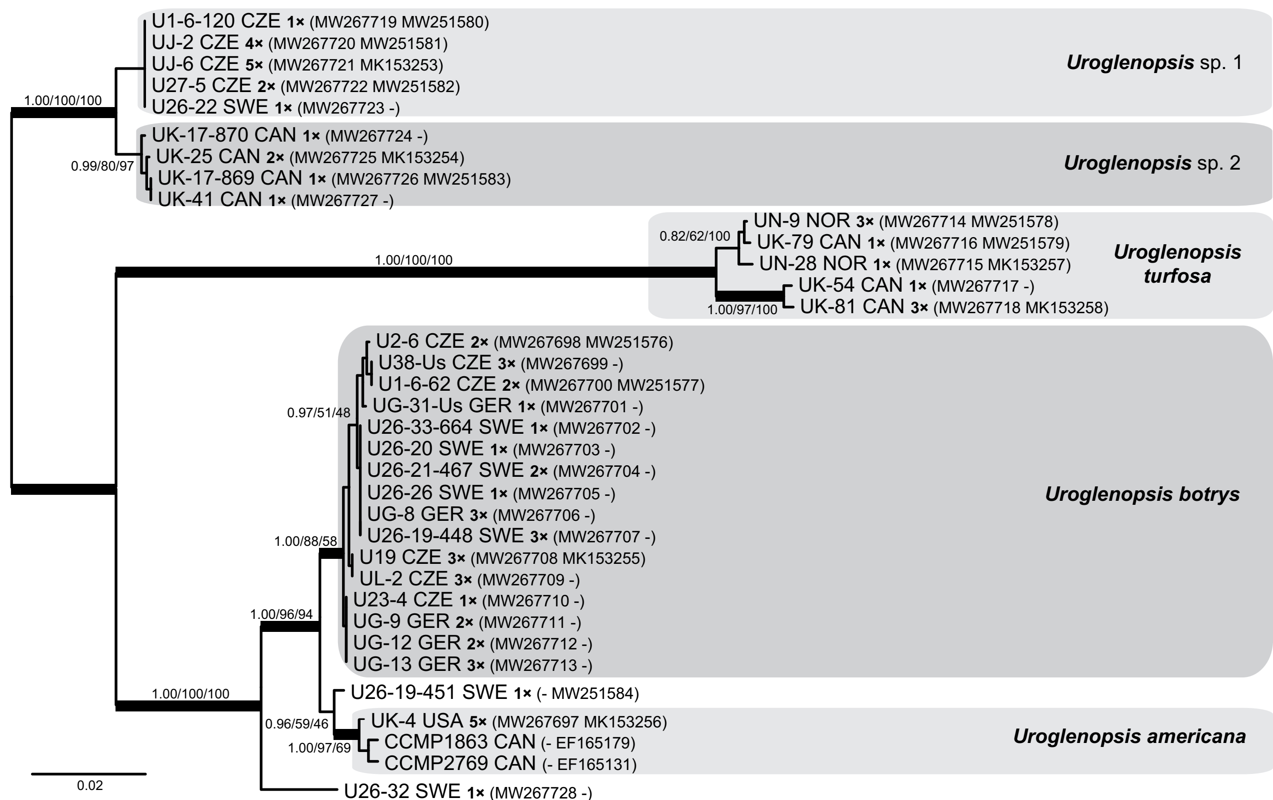
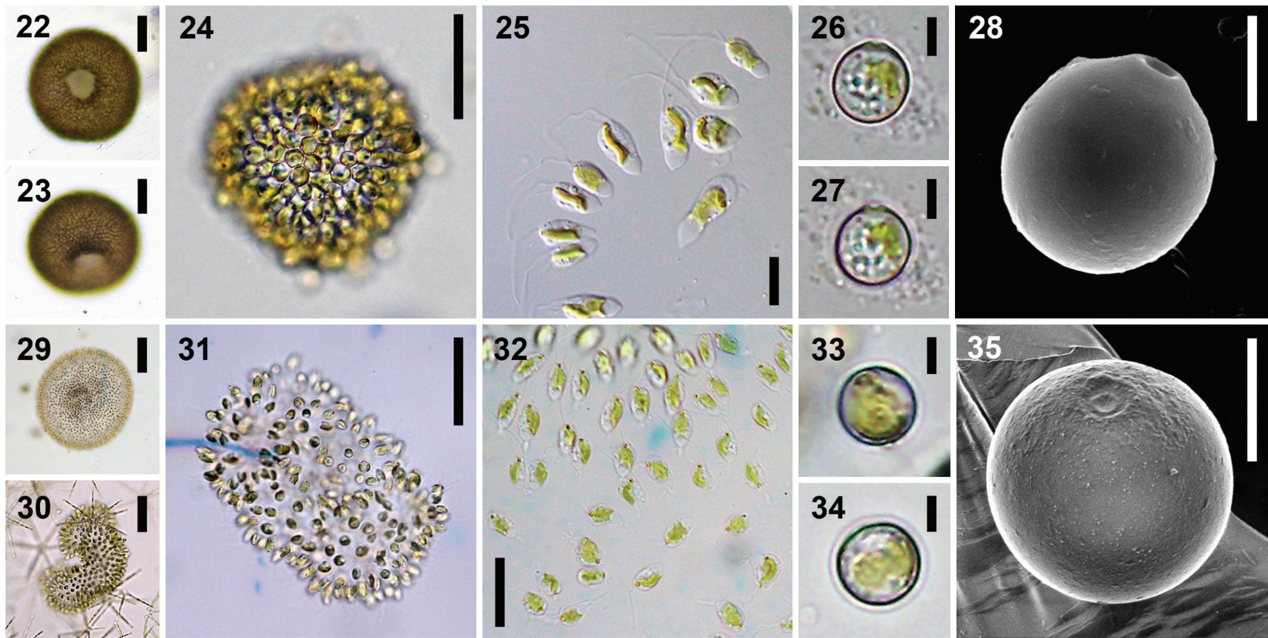


Fig. 21. Phylogeny of the genus *Uroglenopsis* obtained by Bayesian inference of the concatenated ITS rDNA and *rbc*L dataset. The analysis was performed under a partitioned model, using different substitution models for each partition. Values at the nodes indicate statistical support estimated by three 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 cyst characteristics within the genus *Uroglenopsis*. **Figs 22–28**. *U. turfosa* – colonies in fresh natural samples with a remarkable hole in the spherical closely packed 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) which are very diverse in shape, mature cysts exhibiting very simple ultrastructure (Figs 33–35).

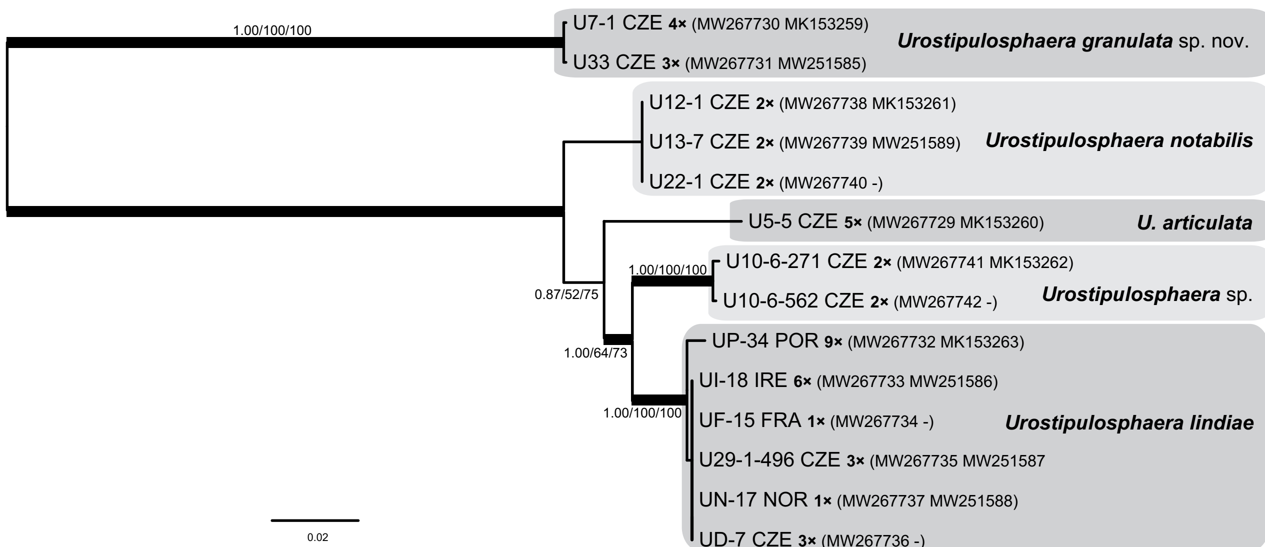
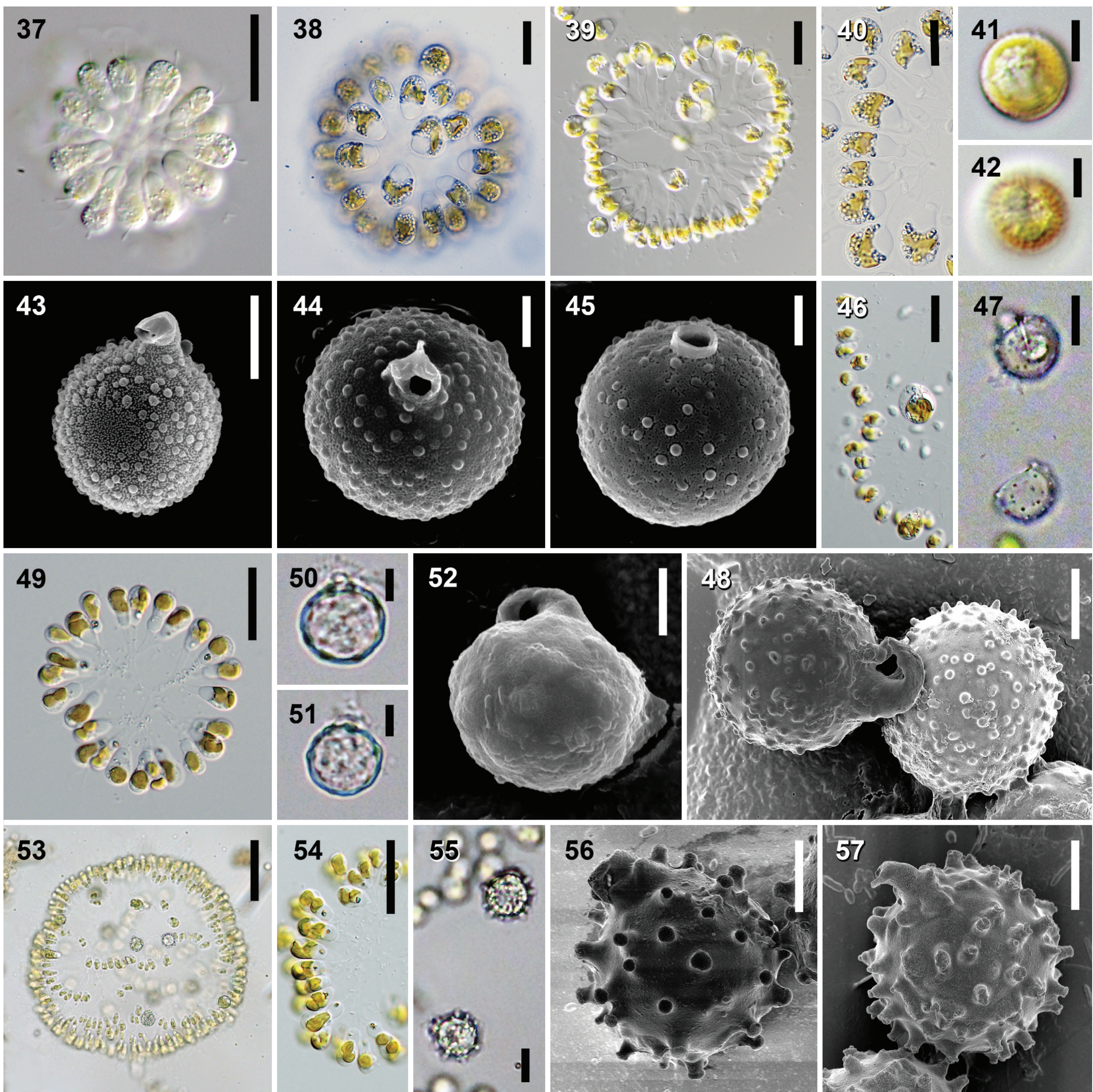


Fig. 36. Phylogeny of the genus *Urostipulosphaera* obtained by Bayesian inference of the concatenated ITS rDNA and *rbc*L dataset. The analysis was performed under a partitioned model, using different substitution models for each partition. Values at the nodes indicate statistical support estimated 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.

Urostipulosphaera sp. (U10-6) possessed very elongated cells. *U. granulata* exhibited larger cells and smaller cysts than other *Urostipulosphaera* species. 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 cells in the natural sample were generally longer (12–16 × 6–8.5 µm) than in the culture (7–14 × 6–10.5 µm).



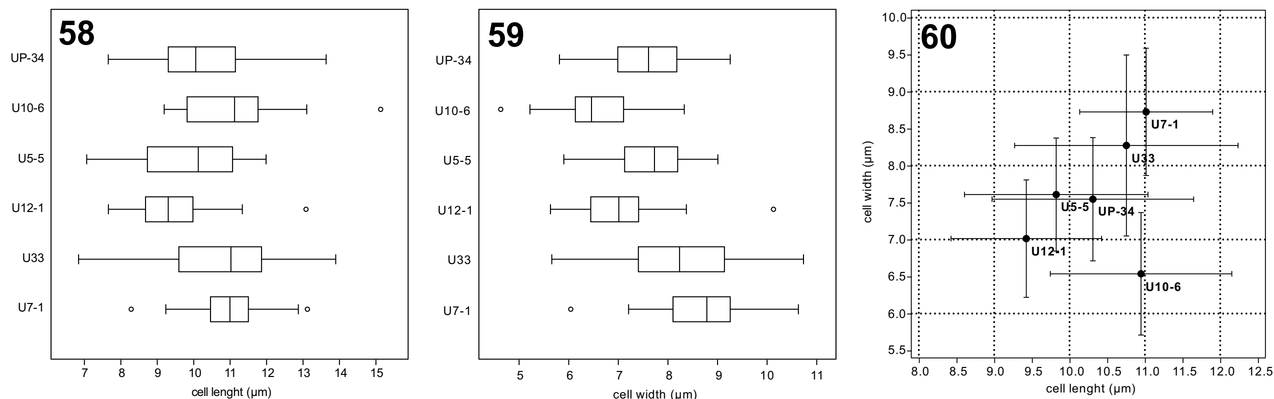
Figs 37–57. Species-specific cysts and colony characteristics within the genus *Urostipulosphaera*. **Figs 37–45**. *U. granulata* – strain U7-1 in a fresh natural sample with reduced plastids (Fig. 37), the same strain after one week of culturing (Fig. 38), strain U33 with clearly visible articulated 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).

Taxonomic revisions and diagnoses

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

DESCRIPTION: Colonies 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 illustrates the holotype.



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 favour 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).

TYPE LOCALITY: Lacul Noua, Romania (45.61429'N, 25.63962'E).
ETYMOLOGY: The specific epithet 'imitata' reflects that cysts of *U. imitata* closely resemble those of *U. zachariasii*, the most common cyst morphotype among *Uroglena*, but had a 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.
Notes: 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 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 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: figs 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, named in honour of Latvian phycologist Heinrich Leonhards Skuja (1892–1972), who first described this 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.
Notes: 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 to 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) (Wujek & thompson, 2002: 302).
TYPE LOCALITY: Boggy lake near the village Kovda, Karelia, Russia.
REFERENCE STRAIN LOCALITY: Strain U5-5 was isolated from Kříž pond in PP Na Plachtě, Czech Republic (50.1827819'N, 15.8702700'E).

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.
Notes: 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 to 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.
Notes: 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 to the genus *Urostipulosphaera*.

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

BASIONYM: *Uroglena eustylis* Skuja, Symb. Bot. Upsal. 9(3): p. 272, pl. 30: figs 16–18 (1948).
SYNONYM: *Uroglenopsis eustylis* (Skuja) (Wujek & Thompson, 2002: 302).
TYPE LOCALITY: Ämsjön, Uppland, Sweden.
Notes: 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 to the genus *Urostipulosphaera*.

Urostipulosphaera granulata Pusztai & Škaloud, sp. nov. (Figs 37–45)

Description: Colonies 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 regular granules (0.3–)0.4–0.6(–0.7) µm in diameter and clearly 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). Fig. 43 illustrates the holotype.
REFERENCE STRAIN: The culture of 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 (50.0710836'N, 14.4206419'E), ~50 m from our office.
ETYMOLOGY: The specific epithet 'granulata' refers to cysts of *U. granulata* being 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 (Wujek & Thompson, 2002: 302).
TYPE LOCALITY: Forêt de Sénart, Paris, France.
REFERENCE STRAIN LOCALITY: Strain U29-1-496 was isolated from Vydýmač pond, Czech Republic (48.9617636'N, 14.9525025'E).
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.
Notes: 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 to 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) (Wujek & Thompson, 2002: 301.
TYPE LOCALITY: Forêt de Soignes, Belgium.
Notes: 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 to 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 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 field conditions, are also known from the first experiments with culturing (Andersen, 2005). Cultured cells (e.g. *U. granulata* sp. nov.) are generally smaller and more globular when compared with fresh natural samples or dimensions given by other authors (summarized in Starmach, 1985). Even *Uroglenopsis* and *Urostipulosphaera* can produce thin, short unbranched threads when stressed (Fig. 40) and the use of fixation or dyes can cause artefacts (Conrad, 1938). Therefore, the precise examination of cysts 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 possible, though challenging, to re-collect encysting material from the type locality. The intriguing question is whether the cyst ultrastructure of *Uroglena*-like colonial flagellates really has species-specific characters (Skuja, 1948; Wujek & Thompson, 2002; Cronberg & Laugaste, 2005). We are aware that in some chrysophyte genera (e.g. some *Synura* species), the cysts are not species-specific due to their simplicity, resembling immature not fully developed cysts (Duff *et al*., 1995). Holen (2014) showed that in monoclonal chrysophyte cultures, the cyst diameter may be relatively stable in some populations but show a huge range in others. Moreover, sexual and asexual cysts share, 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 have morphologically highly similar cysts, differing only in their diameter. Neverthless, it seems that cysts of many *Uroglenopsis* species may be determined by cell and colony characteristics.

Ecological differences among lineages

An overall goal of this study was to obtain a sufficient number of single colony isolates (or short-term cultures) from encysting populations. The proportion of encysting populations in sampled populations differed 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 this difference in encystment lies in our newly discovered ecological preference of *Uroglenopsis* in the northern temperate zone. *Uroglenopsis* seems to be a predominantly late spring to summer taxon, peaking in May and occurring from April to July. Since our sampling effort was typically focused on spring and autumn chrysophyte maxima, potential summer under-sampling could have affected our dataset. The long-term examination of encysting *Uroglenopsis* populations by Skuja (1948, 1956) in Sweden supports this explanation. He found *U. americana sensu* Skuja and *U. turfosa* from spring onwards, 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 (Supplementary table S1) preferences in the northern temperate zone, it is evident that *Urostipulosphaera* inhabits man-made and strongly influenced habitats, such as ponds, where it peaks in early spring waters with significantly lower temperature when compared with *Uroglena* and *Uroglenopsis*. The vast majority of *Urostipulosphaera* isolates came from ponds in the Czech Republic, distinctive by their high productivity, trophic state, and phytoplankton biomass (IUCN, 1997). Conversely, *Uroglenopsis* inhabits pristine habitats, such as drinking water reservoirs or lakes, usually situated in mountainous regions, and often among coniferous forests with low pH and trophic states, as well as having a delayed start to the season compared with lowland ponds. This is in accordance with our presumption of a late summer encystment process in *Uroglenopsis*. 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 differ in their phenology and seasonal dynamics.

Taxonomy

Of previous taxonomies for *Uroglena*-like flagellates, the closest to the present one was the system of Korshikov & Matvienko (1941) and 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 (not formally established), 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 to thin threads as a synapomorphy for *Uroglena*, and reflecting the sometimes poorly visible stalks of (still undescribed) *Urostipulosphaera*.

The long-standing discrepancy of the concepts 'all are *Uroglena*' vs. '*Uroglenopsis* exist', has been resolved by the latest taxonomic 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 recognized genera (Supplementary table S2). *Uroglena* now includes 16 species and two varieties, *Uroglenopsis* contains four species, and *Urostipulosphaera* encompasses nine species. Some previously described species were placed in synonymy. Below, we provide a taxonomic overview for all three genera. In addition, a key to the determination of genera and species, mainly based on differences in cyst morphology, is provided in Supplementary table S3.

Uroglena Ehrenberg, 1834

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

Although Skuja (1948, 1956) recognized only the genus *Uroglena*, based on his detailed drawings, it is possible to assign his 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. Unfortunately, these *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*. This problem was pointed out by Matvienko (1965) and the new species was, invalidly (missing Latin diagnosis), described according to Skuja's previous observations as *Uroglena skujae*. Therefore, *Uroglena europaea* should be placed in synonymy with the newly proposed *Uroglena skujae* Matvienko ex Pusztai & Škaloud, sp. nov. *U. skujae* was re-collected from its type locality (Ubby-Langsjön, Sweden) 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 species-specific cyst is identical to that of the previously described species *U. glabra*. The same cyst type was, however, later incorrectly assigned by previous authors to a species of *Uroglenopsis, U. botrys*, and characteristics of both genera were mixed in the 'hybrid' new combination *Uroglena botrys* as a 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, *Uroglena botrys* and *Uroglena* with such cysts, *sensu* Skuja, should be placed in synonymy with *Uroglena glabra*. Skuja's cyst type was originally described from Sweden, and we found such cysts in Swedish locations. Our SEM findings of the cyst ultrastructure further indicate that in *U. glabra*, 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 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. All three lineages are geographically distinct with the first lineage (UK-37, UK-41) from North America, not Europe. In the second lineage, mainly from Sweden, cysts of *U. zachariasii* var. *uplandica* were recovered in one natural sample (U26-14); further evaluation is needed.

Based on genetic data and a specific cyst diameter: secondary collar width ratio, we propose a new species with a cyst morphology similar to *U. zachariasii, U. imitata* sp. nov. All populations of *U. zachariasii* showed a ratio of 1.3–1.8, corresponding to the literature. Conversely, populations of *U. imitata* showed a ratio of 2.1–2.6. According to older publications, only the description by Geissbühler (1933) fits this newly recognized species. The remaining two clades with cysts similar to *U. zachariasii* or *U. imitata* had isolates originating from poorly encysting populations so we were unable to precisely evaluate their characteristics in SEM, and will need further examination.

Based on the original descriptions of material with species-specific cysts, *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 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 (LM only) resembling *U. pikamae* and *U. glabra*, will need further evaluation of its taxonomic status. In *U. radiata* Calkins, which was originally described from the USA from material lacking cysts, finding 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 to 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 exhibited thin threads unlike *Uroglenopsis americana* (Calkins) Lemmermann in which no such structures were observed.

For *U. rotundata* comb. nov., originally described from China from material lacking cysts, a more detailed description is required. Despite the original description and drawings being vague, this species can be unequivocally assigned to the genus *Uroglena* according to its characteristic flagella length ratio.

Uroglenopsis Lemmermann, 1899

The type species *U. americana* was re-collected 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 molecular genetic data as well as the specific morphology of the multi-lobed colonies, which they share. Considering our isolates of *U. americana* from the type locality, as well as older sequenced isolates (from Canada), it seems that *U. americana* is not common in Europe. This is in accordance with observations on *Uroglenopsis* by Schiller (1926) who stated that *U. americana* very rarely occurred in Europe. Therefore, many previous European observations of *U. americana* very likely belonged to the widespread *U. botrys* and related species (see below). Matvienko (1965) recognized that cells of *U. americana sensu* Skuja differed significantly in shape and dimensions from true *U. americana*, so she erected a new species, *Uroglenopsis skujae* Matvienko.

Pascher (1910, 1913) distinguished *Uroglenopsis*, lacking the system of dichotomously branched radial structures that is clearly visible in *Uroglena*, and he was clearly observing *Uroglenopsis botrys*. Our isolated *U. botrys* re-collected 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* (Supplementary table S4). In the light of such natural variability of colony, cell shape and dimensions within a single species, it is more likely that *U. apiculata, U. irregularis* and *U. skujae* are ecomorphs. This hypothesis is further supported by Wujek & Thompson (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 that *U. apiculata, U. irregularis* and *U. skujae* should be placed in 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, 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 (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 < 1 µm. In other chrysophytes 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 *Uroglenopsis*. This species was originally described from Russia from material lacking cysts. However, *U. troitzkajae* has unique invaginations of the gel matrix among cells. According to Conrad (1938), the 'fibrous' structures observed by some authors were an artefact of the use of unsuitable dyes, which could lead to wrinkles in the shrunken gelatinous mass of the colony. 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, so we do not treat these lineages taxonomically.

Urostipulosphaera Pusztai & Škaloud, 2019

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

U. proxima comb. nov. was originally described from Ukraine from material with species-specific cysts differing from any other *Urostipulosphaera* species. *U. proxima* has all the characteristics of *Urostipulosphaera* except for the articulated stalks. Korshikov & Matvienko (1941) stained colonies with several dyes but did not find any septa. In cultured material, we have found articulated stalks in all *Urostipulosphaera* lineages genetically characterized 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 from material with species-specific cysts with a hook-like projection, differing from any other *Urostipulosphaera* species. *U. soniaca* was based on 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 from material lacking cysts, 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) listed one plastid in smaller cells, and 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 based on material with species-specific cysts clearly differing, 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 lack of 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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No potential conflict of interest was reported by the authors.

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Supplementary Information

The following supplementary material is accessible via the Supplementary Content tab on the article's online page at https://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, physicochemical water parameters (pH, conductivity, temperature) and the GenBank accession numbers for their ITS, *rbc*L 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 recognized 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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