SYSTEMATICS AND PHYLOGENY

Contemporary integrative taxonomy for sexually deprived protists: A case study of *Trachelomonas* (Euglenaceae) from western Ukraine

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Abstract As many other protist groups, euglenophytes are prone to false identification based solely on morphology because of a limited amount of morphological features and cryptic speciation. One of the supposedly completely asexual groups within the freshwater phototrophic representatives of euglenophytes is *Trachelomonas*, capable of forming an inorganic shell around its cell (i.e., the lorica). *The International Code of Nomenclature for algae, fungi, and plants* regulates the taxonomy not only of flowering plants, but explicitly also of phototrophic protists, and provides powerful tools to resolve various taxonomic challenges. To exemplify some of the problems and potential solutions, a number of *Trachelomonas* strains were collected from the muddy, lake-rich region of Dobrostany and cultivated under stable laboratory conditions. Being a type locality of 58 unclarified *Trachelomonas* names, this region in western Ukraine is of great taxonomic importance. Based on light and electron microscopy, and on RAxML and MrBayes phylogenetics using multiple loci and a representative taxon sample, a detailed description of investigated strains and their systematic placement is provided. Morphologically, the strains differed slightly but consistently in minute characters such as size, lorica shape and ornamentation. The presently most comprehensive molecular tree of the Euglenaceae indicated to the existence of at least five different species present in the newly investigated samples, although they were collected from localities in very close vicinity to each other and at the same date. Based on morphological comparisons with type illustrations of species validly described 100 or more years ago, biological material was used to epitypify three names of *Trachelomonas*, eternally linking morphology with reliable genetic information. This taxonomic application is one of the powerful methods to clarify ambiguous scientific names, which has particular importance in character-poor protists such as the euglenophytes.

Keywords asexual organism; epitypification; Euglenida; Euglenophyta; molecular phylogenetics; morphology; ribosomal RNA; type locality

Supporting Information may be found online in the Supporting Information section at the end of the article.

■ INTRODUCTION

Unambiguous scientific names are the prerequisite for proper identification and, therefore, any subsequent application of biological species. The taxonomy not only of flowering plants, but also of phototrophic protists, is regulated by the *International Code of Nomenclature for algae, fungi, and plants* (*ICN*; Turland & al., 2018). Unicellular organisms, which can only be identified under the microscope, are particularly prone to taxonomic confusion (De Clerck & al., 2013; Manoylov, 2014), and corresponding "standard" (i.e., type) material is often poorly preserved or entirely lost. Diverging methodological approaches have led to some deviations of phycological research within botany, which is also expressed by explicitly disregarding Rec. 46A of the *Code* (i.e., author abbreviations of scientific names) in a number of distinguished phycological journals. However, effective, sustainable and approved algal taxonomy can be assured only by a powerful federal organisation such as the International Association for Plant Taxonomy (IAPT) with its periodical *Taxon*.

The exemplary subject of this study to demonstrate a good practice to resolve taxonomic challenges in protists are euglenophytes. These represent an ecologically and economically important group of unicellular plankton organisms, as they include numerous primary producers but can cause massive algal blooms, resulting in fish culture losses (Kulczycka & al., 2018). *Trachelomonas* Ehrenb. comprises of not less than 1700 uncritically listed scientific names at the species level and below (Guiry & Guiry, 2017). Together with *Colacium* Ehrenb., *Cryptoglena* Ehrenb., *Discoplastis* Triemer, *Euglena* Ehrenb., *Euglenaformis* M.S.Bennett & Triemer, *Euglenaria* Karnkowska-Ishikawa & al., *Eutreptia* Perty, *Eutreptiella* A.G.Cunha, *Lepocinclis* Perty, nom. cons. (Senn in Briquet, 1930; Silva, 1960; Ross, 1966), *Monomorphina*

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Mereschk., *Phacus* Dujard., nom. cons. (Silva, 1960; Ross, 1966) and *Strombomonas* Deflandre, *Trachelomonas* represents an integral element of the freshwater Euglenophyceae as inferred from molecular phylogenetics (Kim & al., 2015; Bicudo & Menezes, 2016).

The striking apomorphy of all euglenophytes is the pellicle. It is a unique surface structure, comprised of a microtubular system, endoplasmic reticulum cisterns and a plasma membrane, connected with protein layers (Arnott & Walne, 1967). In addition, euglenophytes are capable of producing and secreting an additional mucilage layer (Dunlap & Walne, 1985). In some cases, such as *Strombomonas* and *Trachelomonas*, this extracellular matrix becomes thicker and gets impregnated with inorganic substances, forming an inorganic shell termed lorica (Pringsheim, 1953; Leedale, 1975). Chemical analyses show that the main inorganic compounds found in loricae of *Trachelomonas* are iron and manganese (Rino & Pereira, 1991), which are together with a great deal of other elements incorporated in the lorica via non-active physical processes (Pereira & al., 2003). Depending on its mineral composition, the colour of the lorica varies from translucent to brown or reddish-brown (Pringsheim, 1953).

Within Euglenaceae, state reconstruction indicates that a lorica is a synapomorphic feature shared by the sister groups *Strombomonas* and *Trachelomonas* (Karnkowska & al., 2015). *Trachelomonas* can be distinguished from *Strombomonas* based on lorica ontogeny: *Strombomonas* loricae namely always develop progressively from the anterior towards the posterior part of the cell, whereas the formation of *Trachelomonas* loricae takes place evenly across the entire cell (Brosnan & al., 2005). Additionally, *Strombomonas* loricae do not possess pores, whereas *Trachelomonas* loricae are always ornamented (i.e., possessing pores and/or spines) at least at one stage of their development (Brosnan & al., 2003). A diversity of spines and pores on the lorica surface, and their arrangement, have been thought to be of great taxonomic importance, not only at species but also on infraspecific levels of *Trachelomomas* (Rino & Pereira, 1988). However, environmental factors can alter lorica traits significantly (Nudelman & al., 2003), and its morphology also depends on the protoplast metaboly, as it starts to develop in later ontogenetic stages (Brosnan & al., 2005). Thus, such morphological characteristics should be treated with great caution when describing a certain species, as the classification based on lorica morphology alone is often inadequate and misleading (Pringsheim, 1953; Ciugulea & al., 2008).

Biodiversity assessment of euglenophytes and *Trachelomonas* is perplexing due to several reasons. Combining both phototrophic and heterotrophic representatives, this group is an example of the ambiregnal taxonomic treatment under both, the *ICN* (Turland & al., 2018) and the *International Code of Zoological Nomenclature* (*ICZN*; Ride, 2003). The diversity of biological phenomena further prevents us from an unambiguous definition of what a biological species actually is. Euglenophyte reproduction, and thus also of *Trachelomonas*, is considered to be almost entirely asexual (Leedale, 1967), and such organisms may be prone to greater mutability because of the missing regulatory system of crossing over. Nevertheless, asexual clones still possess a consistent set of traits, which are the result of natural selection, and in this manner resemble organisms with sexual reproduction (Gottschling, 2008). However, asexually propagating organisms such as cyanobacteria and bdelloid rotifers are still equally ranking in taxonomy with "regular" biological species.

During the past decades, molecular DNA sequence data have gained great importance for taxonomic delimitations in the microbial world including *Trachelomonas*. As in many other protist species, cryptic speciation is also observed here (Kim & al., 2013; Przybos & Tarcz, 2016), resulting in a number of morphologically indistinguishable species. In turn, it is also highly plausible that size variations and different ecological forms of the same species have been described as several distinct species due to their plasticity (Bicudo & Menezes, 2016). Thus, diversity of *Trachelomonas* has been continuously under- and overestimated at the same time.

Because of cryptic speciation, it is important to link DNA sequence data to a type specimen, but the original material of microscopic organisms consists in many (particularly older) cases of illustrations only. Since protists frequently lack diagnostic morphological features (Pawlowski & al., 2012), type specimens (including illustrations) cannot provide for unambiguous identification of certain species. As one is not able to connect a type illustration to a DNA sequence, the same problem arises, as if the type material is in poor condition. In such cases, it is recommended to designate an epitype via epitypification (Hyde & Zhang, 2008). This powerful tool has already been applied to solve taxonomic ambiguities in various protistan groups such as green algae (Demchenko & al., 2012) and dinophytes (Zinßmeister & al., 2011; John & al., 2014; Kretschmann & al., 2015, 2018a,b; Tillmann & al., 2017) but also in euglenophytes (Marin & al., 2003; Kosmala & al., 2005; Bennett & Triemer, 2012; Karnkowska-Ishikawa & al., 2012; Kim & Shin, 2014).

For all the above reasons, one is faced with big taxonomic confusion in euglenophytes (Brosnan & al., 2003, 2005). In this study, we provide a robust molecular phylogeny of Euglenaceae and clarify the taxonomy of three names within *Trachelomonas* (all of which have rarely been applied in the past: Starmach, 1983; Borisova & al., 2006). Fifty-eight taxa at the species level and below have been described from the lake district near Dobrostany west of Lviv (suppl. Table S1), making it a taxonomic hot spot of great importance. In the beginning of the 20th century, this lake district has comprised primarily of three small ponds, namely Wolicki pond in the North, centrally located Dobrostany pond and Białogórski pond in the South (Koczwara, 1915). Already available data of Euglenaceae are thus further enriched based on the material collected in western Ukraine, the most southern region ever affected by the maximum Scandinavian glaciations (Lindner & al., 2004). The utmost western part of the country is part of a so-called forest-steppe region, characterised by grey soils and covered with either steppe vegetation or mixed forests, dominated by oak (Borisova & al., 2006). Due

to massive human impact, the majority of water bodies are highly eutrophic or eutrophic-polytrophic (Konenko & al., 1965), representing suitable living conditions for euglenophytes. The region is also characterised by radioactive contamination of water bodies, last but not least because of the accident at the Chernobyl Nuclear Power Plant (Kuzmenko, 2000). Euglenophytes are resistant to moderate radiation (Margulis & al., 1990), and, therefore, we expected the investigated species to still be present on their type localities, from which they have been described as long as a century ago.

■ MATERIALS AND METHODS

Sampling and cultivation. — During a field trip, water tow samples were collected with a plankton net (with pores' diameter 20 µm) at several different localities – in Poland on 10 September 2012, and in Ukraine on 15 and 16 September 2012. Single, motile, greenish through brownish cells were isolated using a glass micropipette under a CKX41 inverted microscope (Olympus; Hamburg, Germany). To avoid contaminations, the isolated cells were rinsed at least three times in WC-Medium, prior to cultivation (Guillard & Lorenzen, 1972). Single or few cells were then transferred in six-well microplates (Zefa; Munich, Germany), containing 5 ml of modified WC-Medium (Guillard & Lorenzen, 1972) and 1 ml of soil extract (McFadden & Melkonian, 1986). Plates were stored in a climate chamber Percival I-36VL (CLF Plant Climatics; Emersacker, Germany) at 18°C, at a light intensity of 80 µmol photons $m^{-2}s^{-1}$ and under a 12 : 12 h light : dark photoperiod.

To maintain the established strains, they were supplied with fresh medium every four weeks by being transferred into a new well. Prior to adding 2 ml of fresh medium to the strain, the same volume of the old medium was discarded using a syringe equipped with a disposable sterile filter and a pore size of 0.2 µm (Sartorius; Göttingen, Germany). In order to avoid further contamination with possible bacteria and fungi, medium transfer was done under a clean bench (ET 130/H, Ehret; Emmendingen, Germany). Once dense material of *Trachelomonas* strains was obtained, a part of the grown strain was transferred into a new 1.5 ml collection Eppendorf tube (Eppendorf; Hamburg, Germany), centrifuged and stored in the freezer at −20°C for DNA isolation.

Light and electron microscopy. — For morphological analysis, 80 cells of every strain were separated and individually measured (length and width) with a CKX41 inverted microscope (Olympus) equipped with a DP73 digital camera (Olympus). Measurements were represented by mean values and standard errors and were statistically evaluated by one-way analysis of variance (ANOVA), using SigmaPlot v.11.0 (Systat Software; San Jose, California, U.S.A.). Significant differences between mean values were determined using the post-hoc Dunn's test (Dunn, 1961). Only mature cells exhibiting lorica were measured, to minimise the measuring errors as much as possible.

For Scanning Electron Microscopy (SEM), cultivated material was fixed in 4% OsO_4 (1 : 1 ratio, resulting in a final concentration of 2% OsO_4) for at least 1 h. The cells were then filtered onto an Omnipore-membrane filter (5 µm; Merck; Darmstadt, Germany), which was placed in a Swinnex filter holder (Merck, Millipore; Darmstadt, Germany). The preparation liquids were exchanged with the aid of a plastic syringe connected to the above-mentioned filter holder. Fixed cells were rinsed four times; first in sodium cacodylate buffer for 15 min and then three times in distilled water (the first time for 5 min, second time for 15 min and third time for 30 min). Next step included subsequent dehydration in a graded acetone series of 15 min in 10%, 30%, 50%, 70% and 90% acetone, followed by three additional dehydration steps in 100% acetone (the first time for 5 min and two times for 30 min). The membrane filters were critical-point dried in liquid CO_2 and afterwards glued to a SEM-stub with double-adhesive carbon disks. Additionally, small drops of Planocarbon N650 (Plano; Wetzlar, Germany) were applied on the edges of the filter to improve the electrical contact between the filter and the stub. The filter was coated with platinum (BAL-TEC SCD 050 sputter coater; Schalksmühle, Germany) for 4 min and examined in a LEO 435VP SEM (LEO Electron Microscopy; Cambridge, U.K.).

For epitype preparation, 1 ml of the corresponding strain was taken and centrifuged in a 1.5 ml Eppendorf-tube at a maximum speed of 200g (Z320, Hermile; Gosheim, Germany) for 30 min to make the sample denser. The cells were then fixed in 2.5% glutaraldehyde in 75 mM NaCacodylate-buffer ($C_2H_6AsNaO_2 \cdot 3H_2O$) containing 2 mM $MgCl_2$ for at least 1 h. Afterwards, the cells were stained in 0.5% astra blue and 2% tartaric acid dissolved in WC-Medium for 30 min. After staining, a cleaning step in WC-Medium and dehydration in a graded ethanol series (30%, 50%, 70% and 90%, followed by two changes to 100%; each time for 15 min) was performed. A second staining with 0.1% (ethanol-based) eosin was carried out during the incubation in 90% ethanol for 30 min. Ethanol-based Technovit 7100 (Heraeus; Wehrheim, Germany) was used for embedding, following the manufacturer's instructions. For the final preparation, 40 µl aliquots of the Technovit mixture including the embedded samples were transferred to three microscopic glass slides and were enclosed by a cover slide.

Molecular phylogenetics. — Genomic DNA was extracted from fresh material using the Nucleo Spin Plant II Kit (Machery-Nagel; Düren, Germany). Two nuclear regions of the rRNA were amplified, using primers and following the standard protocols (Hansen & al., 2000; Kim & al., 2010). The taxon sample included all Euglenaceae taxa (excluding *Euglena* itself, see Discussion), whose sequences were available for at least two out of three nuclear loci (or parts of loci; Appendix 1). The only exception is *Trachelomonas* cf. *volvocinopsis* Svirenko (only SSU), which was used as a reference sequence (based on a BLAST search: Altschul & al., 1990) for the strain GeoM*521 established in this study. The sequences were separately aligned, using MAFFT v.7.304 (Katoh & Standley, 2013; freely available at http://mafft.cbrc.jp/alignment/software/) under default settings, and concatenated afterwards (Appendix S1).

Phylogenetic analyses were carried out with maximum likelihood (ML) and Bayesian approaches, as described in detail

previously (Gottschling & al., 2012), using the resources available from the CIPRES Science Gateway (Miller & al., 2010). The Bayesian analysis was performed using MrBayes v.3.2.6 (Ronquist & al., 2012; freely available at http://mrbayes.sourceforge.net/download.php) under the GTR + Γ substitution model and the random-addition-sequence method with 10 replicates. Two independent analyses of four chains (one cold and three heated) with 15 million cycles were run, sampled every 1000th cycle, with an appropriate burn-in (10%) as inferred from the evaluation of the trace files using Tracer v.1.6 (freely available at http://tree.bio.ed.ac.uk/software/tracer/). For the ML calculation, the MPI version of RAxML v.8.0.24 (Stamatakis, 2014; freely available at http://www.exelixis-lab.org/) was applied by using the GTR + Γ substitution model. To determine the best-fitted ML tree, 10 tree searches from distinct random-stepwise-addition-sequence maximum parsimony starting trees were executed and 1000 non-parametric bootstrap replicates were performed. Statistical support values (LBS: ML bootstrap support, BPP: Bayesian posterior probabilities) were drawn on the resulting, best-scoring tree. The phylogenetic tree was rooted posteriorly with *Cryptoglena* and *Monomorphina*.

■ RESULTS

General morphological observations. — All six investigated strains differed morphologically between each other based on lorica shape and ornamentation. Another morphological trait that was taken into consideration is size, since some strains were significantly larger than others, considering length and width (Figs. 1, 2, suppl. Tables S2, S3). Generally, the motile stage was predominant in all investigated strains not showing a considerable contamination, but immotile stages were more frequent in heavily contaminated sub-strains. Cell replication and lorica formation were enhanced with an addition of a soil extract. Dividing motile cells formed big cell clumps, which were either moving extremely slowly or were lying on the bottom of the cultivation plates.

Three developmental stages were observed in the life-history of a strain, namely small and transparent cells, bigger and partially transparent cells and non-transparent brown cells, which did not differ greatly in size in comparison to the partially transparent cells. For clearer terminology, the cells were given terms "immature cells" (Figs. 3A,E,I, 4M,Q,U), "mature naked cells" (Figs. 3B,F,J, 4N,R,V) and "mature loricate cells" (Figs. 3C,G,K, 4O,S,W); empty loricae are depicted in the fourth column of every strain illustration (Figs. 3D,H,L, 4P,T, X). These terms are not used in the strains' descriptions, as they present an artificial classification and as the description itself is built on more than one stage of life-history.

Regardless of the ontogenetic stage, the majority of the cells possessed a clearly distinctive eye spot, which was located always at the anterior end of the cell, near the flagellar apparatus. This structure also served as an anchor point to orientate the cells into the same direction. Length of the visible flagellum varied greatly between strains. Moreover, the cells were densely filled with granules of varying sizes and chloroplasts, which were hard to distinguish from the rest of granular structures, in mature loricate cells.

Strain descriptions. — *GeoM 520* (Figs. 3A–D, 5A–F). – Cell colour yellowish to greenish-brown; lorica ellipsoid, 19.88 (±1.66) µm long, 16.01 (±1.19) µm wide, the surface finely punctate, with uniformly and somewhat densely covered short, conical spines; apical pore without a collar or with a short, slightly raised margin; flagellum at least twice the length of the cell; chloroplasts up to 5, plate-like, green, including paramylon cups accompanying the diplopyrenoids; eye spot orange, visible in most cells, except in mature loricate cells.

*GeoM*524* (Figs. 3E–H, 6A). – Cell colour yellowish to brownish, greenish only during division; lorica spherical through ellipsoid, 24.85 (±3.71) µm long, 21.45 (±2.83) µm wide, the surface densely, deeply and somewhat uniformly reticulate without spines (reticulation could be a result of an uncompleted lorica formation); apical pore without a collar (in SEM images) and with a distinctive collar (in some LM images); flagellum length variable; chloroplasts up to 5, plate-like, green, including paramylon cups accompanying the diplopyrenoids; eye spot orange to orange reddish, visible in most cells, except in mature loricate cells.

GeoM 526 (Figs. 3I–L, 6B). – Cell colour yellow-greenish to greenish-brown; lorica broadly ellipsoid, 24.23 (±2.45) µm long, 19.53 (±1.79) µm wide, the surface punctate; apical pore with a distinctive diagonally ended collar (seen only in naked cells, but not in loricate ones); flagellum at least twice as long as the cell; chloroplasts up to 5, plate-like, green, including paramylon cups accompanying the diplopyrenoids; eye spot bright-orange to orange-dark reddish, visible in most cells, regardless of cell maturity.

GeoM 527 (Figs. 4M–P, 5G–L). – Cell colour dark greenish to dark greenish-brown; lorica very widely ellipsoid through almost spherical, 19.98 (±2.19) µm long, 16.08 (±1.78) µm wide, the surface irregularly and sparsely punctate; apical pore without a collar; flagellum at least three times as long as the cell, at least in younger cell stages; chloroplasts up to 5, plate-like, yellowish-green including paramylon cups accompanying the diplopyrenoids; eye spot red to reddish, visible in all cells, regardless of cell maturity.

GeoM 529 (Figs. 4Q–T, 6C). Cell colour bright greenish, greenish through dark greenish-brownish; lorica ellipsoid, 24.46 (±2.27) µm long, 20.79 (±2.24) µm wide, the surface uniformly, densely and deeply reticulate, covered sparsely with sharp spines; apical pore without collar or with short, slightly raised margin; flagellum at least twice as long as the cell; chloroplasts up to 5, plate-like, green, including paramylon cups accompanying the diplopyrenoids (hard to observe, since the granular structures in the mature naked cells have almost the same colour as the chloroplasts); eye spot big, orange to dark-reddish, visible in all cells, except in mature loricate cells.

GeoM 540 (Figs. 4U–X, 5M–R). Cell colour yellow-brownish to almost grey-brownish; lorica ellipsoid, 18.79 (±1.76) µm long, 15.57 (±1.50) µm wide, the surface uniformly and densely reticulate; apical pore without collar or

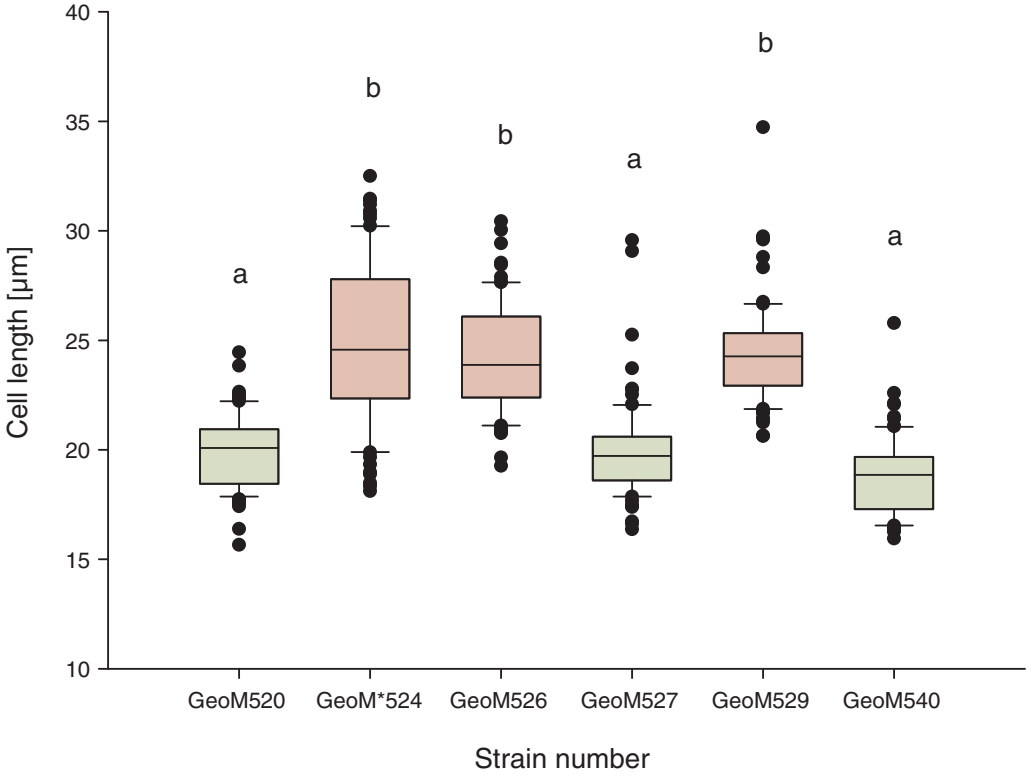

Fig. 1. Box plot display of cell length of investigated mature naked cells and mature loricate cells of *Trachelomonas* strains. Statistically significant clusters are indicated with letters a or b and green or red colour, corresponding to the phylogenetic tree (see Fig. 7).

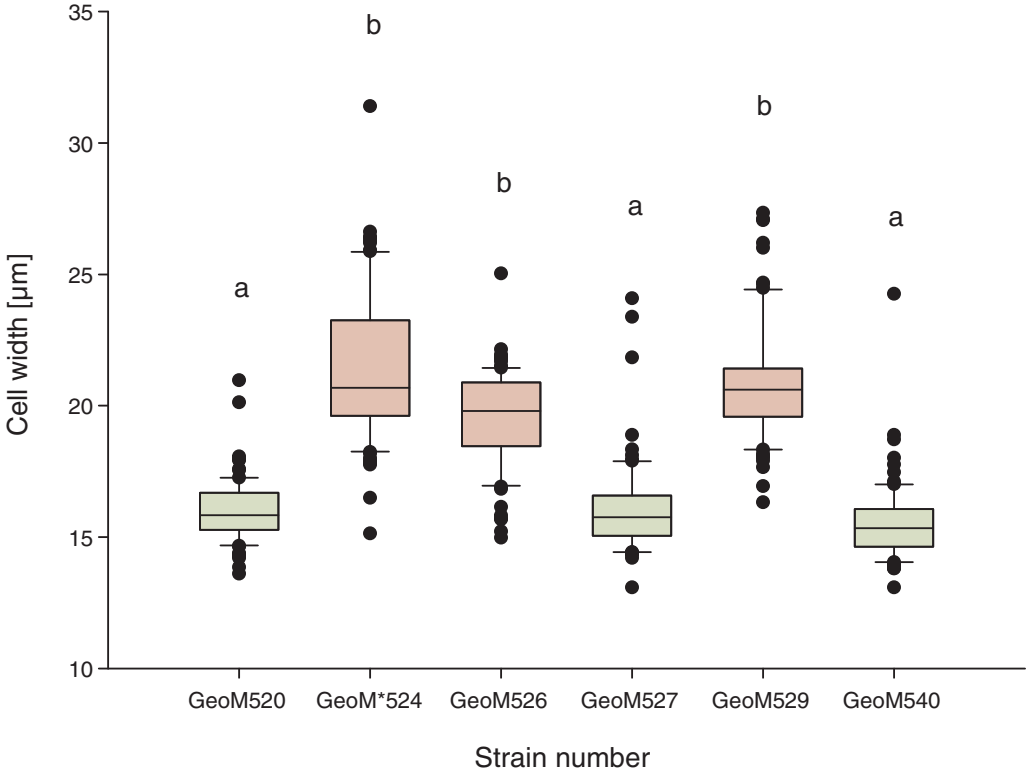

Fig. 2. Box plot display of cell width of investigated mature naked cells and mature loricate cells of *Trachelomonas* strains. Statistically significant clusters are indicated with letters a or b and green or red colour, corresponding to the phylogenetic tree (see Fig. 7).

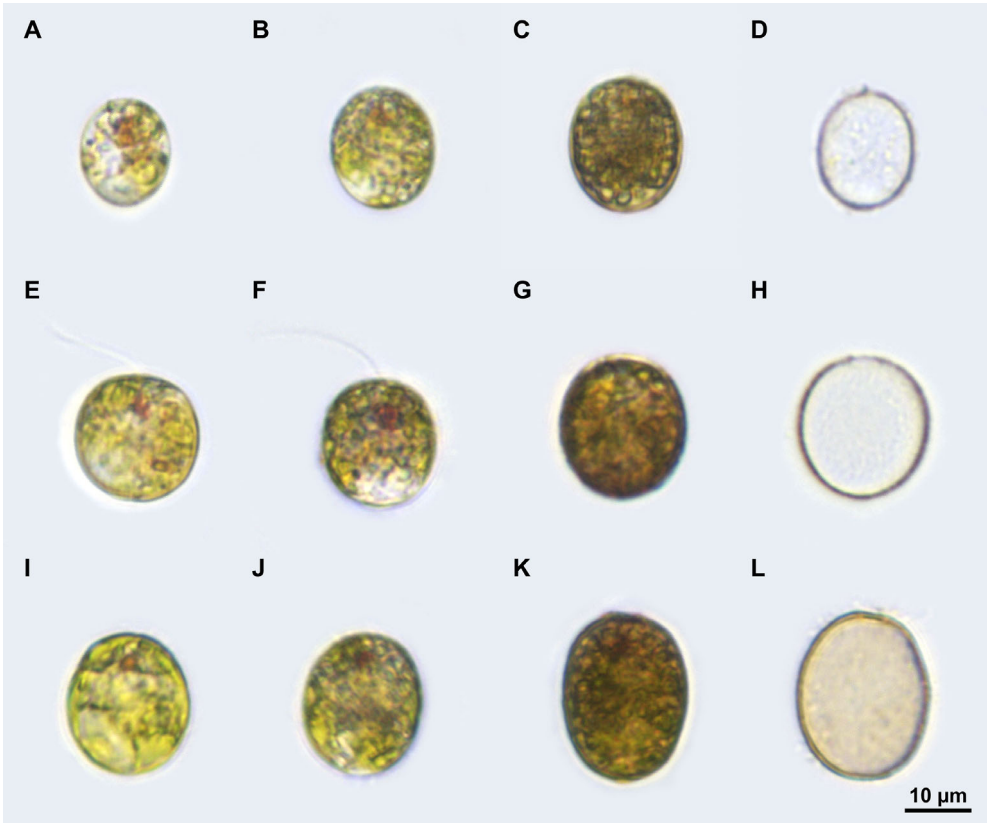

Fig. 3. Different ontogenetic stages and empty loricae of selected *Trachelomonas* strains (LM; all at the same scale). **A–D,** Young immature cell, mature naked cell, mature loricate cell and empty lorica of *Trachelomonas hispida* var. *volicensis* GeoM 520; **E–H,** Young immature cell, mature naked cell, mature loricate cell and empty lorica of *Trachelomonas* sp. GeoM*524; **I–L,** Young immature cell, mature naked cell, mature loricate cell and empty lorica of *Trachelomonas* sp. GeoM 526.

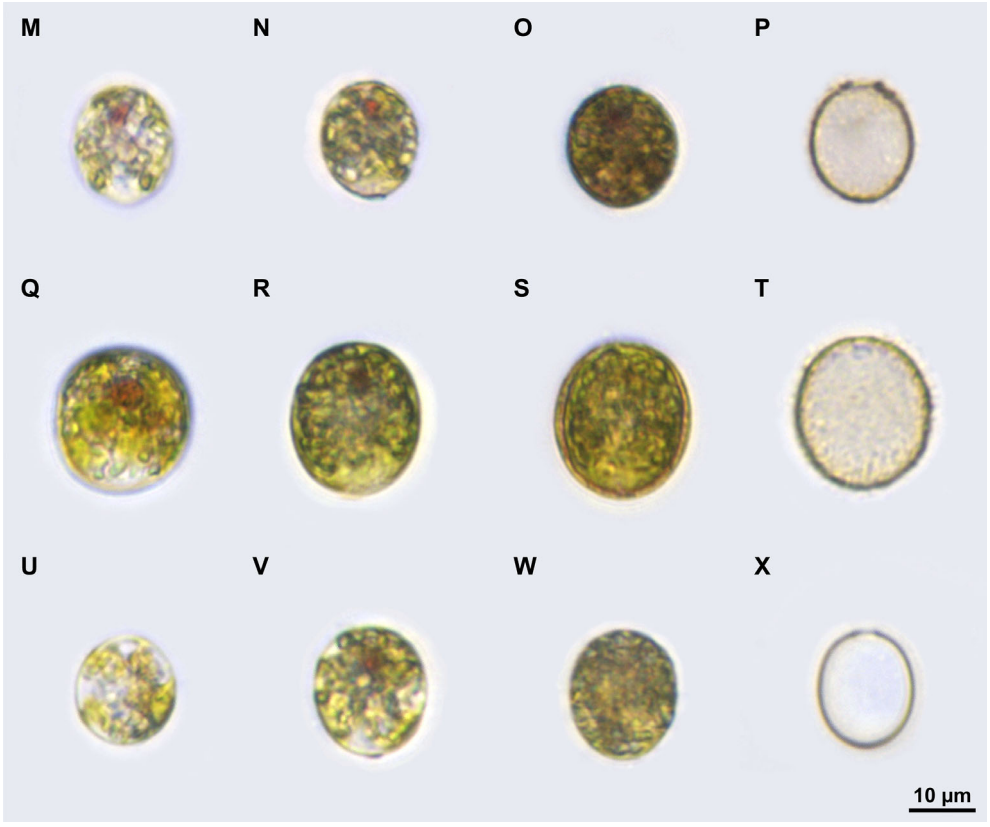

Fig. 4. Different ontogenetic stages and empty loricae of selected *Trachelomonas* strains (LM; all at the same scale). **M–P,** Young immature cell, mature naked cell, mature loricate cell and empty lorica of *Trachelomonas teres* var. *minor* GeoM 527; **Q–T,** Young immature cell, mature naked cell, mature loricate cell and empty lorica of *Trachelomonas hispida* var. *irregularis* GeoM 529; **U–X,** Young immature cell, mature naked cell, mature loricate cell and empty lorica of *Trachelomonas teres* var. *granulata* GeoM 540.

with short, slightly raised margin (seen only in mature naked cells under SEM); flagellum at least twice as long as the cell; chloroplasts up to 5, plate-like, green, including paramylon cups accompanying the diplopyrenoids; eye spot orange through reddish, visible in most cells.

Molecular phylogenetics. — Newly obtained sequences were deposited in the NCBI GenBank under MK894259–MK894271. The alignment comprised 4150 parsimony-informative positions, and the proportion of gaps and ambiguous characters was 57.12% (for additional alignment traits, see suppl. Table S3). Our dataset provided a well-resolved backbone phylogeny of a representative taxon sample of selected photosynthetic euglenacean clades (Fig. 7). Maximum likelihood and Bayesian analyses recovered phylogenetic trees with congruent topologies. Five monophyletic groups were recovered: *Colacium* (100 LBS, 1.00 BPP), *Cryptoglena* (100 LBS, 1.00 BPP), *Monomorphina* (100 LBS, 1.00 BPP), *Strombomonas* (100 LBS, 1.00 BPP) and *Trachelomonas* (89 LBS, 1.00 BPP). Both loricate taxa *Strombomonas* and *Trachelomonas* constituted sister groups (97 LBS, 1.00 BPP).

Trachelomonas segregated into a number of well-supported lineages at high taxonomic level. As such lineages could not be correlated with non-DNA sequence traits, they are informally indicated with Greek letters (Fig. 7). The type of *Trachelomonas*, *T. volvocina* (Ehrenb.) Ehrenb., is nested with clade α (99 LBS, 1.00 BPP), which also one of our strains

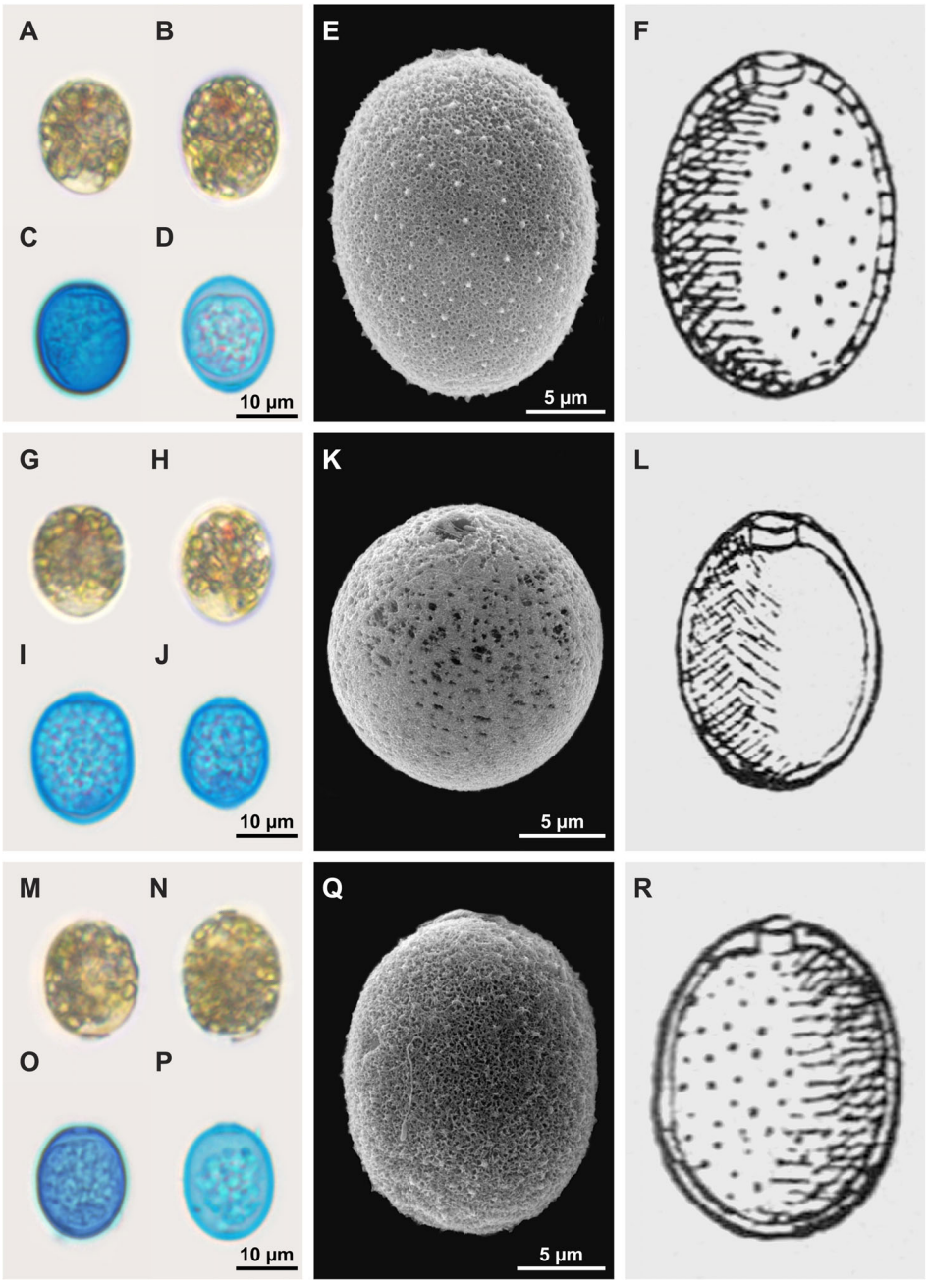

Fig. 5. LM, epitype, SEM and protologue images of mature *Trachelomonas* cells from selected strains. **A–F,** Mature loricate cells **(A & B),** epitype images **(C & D),** SEM image **(E)** and protologue **(F)** of *Trachelomonas hispida* var. *volicensis* GeoM 520; **G–L,** Mature loricate cells **(G & H),** epitype images **(I & J),** SEM image **(K)** and protologue **(L)** of *Trachelomonas teres* var. *minor* GeoM 527; **M–R,** Mature loricate cells **(M & N),** epitype images **(O & P),** SEM image **(Q)** and protologue **(R)** of *Trachelomonas teres* var. *granulata* GeoM 540.

(GeoM*521) was assigned to. However, this strain represented a phylogenetically isolated branch shared with strain M1422 (determined as *T.* cf. *volvocinopsis*). All other our strains were nested within clade β (100 LBS, 1.00 BPP), which included both significantly small and large representatives. Strains GeoM*524, GeoM 526 and GeoM 529 clustered together with other large species such as *T. hispida* (Perty) F.Stein, *T. pertyi* E.G.Pringsh. and *T. variabilis* Kam.P.Singh, while strains GeoM 520, GeoM 527 and GeoM 540 build a clade together with small-celled *T. echinata* A.M.Cunha (100 LBS, 1.00 BPP). Notably, strains GeoM 520 and GeoM 540 (and also SAG 1283-22, determined as *T. echinata*) shared identical SSU and LSU sequences, but possessed different morphological features (see above).

■ DISCUSSION

Molecular phylogenetics and at least a few correlations to morphology. — The study of the euglenophytes in general and *Trachelomonas* in particular is challenging because of various biological phenomena such as elusive diagnostic traits, intraspecific modifications because of environmental influence, cryptic speciation and last but not least due to the absence of sexual reproduction (Leedale, 1967; Kim & al., 2013; Przybos & Tarcz, 2016; Wołowski & al., 2016). Subsequently, depicting driving forces and dynamics of the molecular evolution in *Trachelomonas* is extremely problematic, causing an extensive taxonomic mess still persisting within numerous protist lineages. In such cases, an integrative approach considering morphological and molecular data combined (as applied in the present study) is advised.

The necessary prerequisite to infer evolutionary dynamics is a well-supported phylogenetic tree, and the concatenation of loci generally improves the reconstructions (not only in euglenophytes: Brosnan & al., 2003, Kim & al., 2015, but also in other protist groups such as the dinophytes: Gottschling & al., 2012, 2020; Tillmann & al., 2014; Kretschmann & al., 2018a,b). This approach reveals statistically highly supported topologies, which is due to a proportionally high share of parsimony-informative sites (suppl. Table S4). Anyhow, a persistent limitation hindering phylogenetic reconstructions in the microbial world is the taxon sample, being biased towards phototrophic and therefore easily cultivatable taxa. Only a single (of few) heterotrophic presenter is included into the phylogenetic analysis, namely *Trachelomonas reticulata* G.A.Klebs, whose phototrophy is believed to have been lost secondarily (Ciugulea & al., 2008). We do not know yet how many times phototrophy has been lost in *Trachelomonas* (or Euglenaceae in general), but corresponding species (groups) are presumably small and nested within their phototrophic relatives. Thus, our analysis still illustrates relationships based on a representative taxon sample of *Trachelomonas* (Kim & al., 2015; Bicudo & Menezes, 2016).

Four *Trachelomonas* subclades are recovered in our analysis, in comparison to five in a previous study (Ciugulea & al., 2008). There is no obligatory criterion for subclade delimitation due to the complex biology of *Trachelomonas*. Our choice to delimitate individual subclades within the phylogeny is based on branch length and bootstrap support, and not on differences in lorica morphology as in Ciugulea & al. (2008). Our reasoning is that lorica morphology is evidently more influenced by the environment than the phylogenetic signal, making the latter one a more reliable source for subclade delimitation. As *Trachelomonas*, *Euglena* also includes phototrophic and heterotrophic presenters as well (Marin & al., 2003; Triemer & al., 2006). Including *Euglena* to the taxon sample resulted in the same topology as shown in Fig. 7, but with lower statistical support of the backbone. *Euglena* is set on a clade exhibiting long branches (phylogenetic tree not shown), which is indicative for strong rate heterogeneity perturbing phylogenetic reconstructions in general. Moreover, *Euglena* possesses a high number of autapomorphic sequence positions, which are impossible to align reliably with the rest of euglenophytes' genetic features.

Based on our well-resolved phylogenetic tree of Euglenaceae, an unexpected phylogenetic signal has been recovered between the DNA tree and cell size. Due to plasticity, size

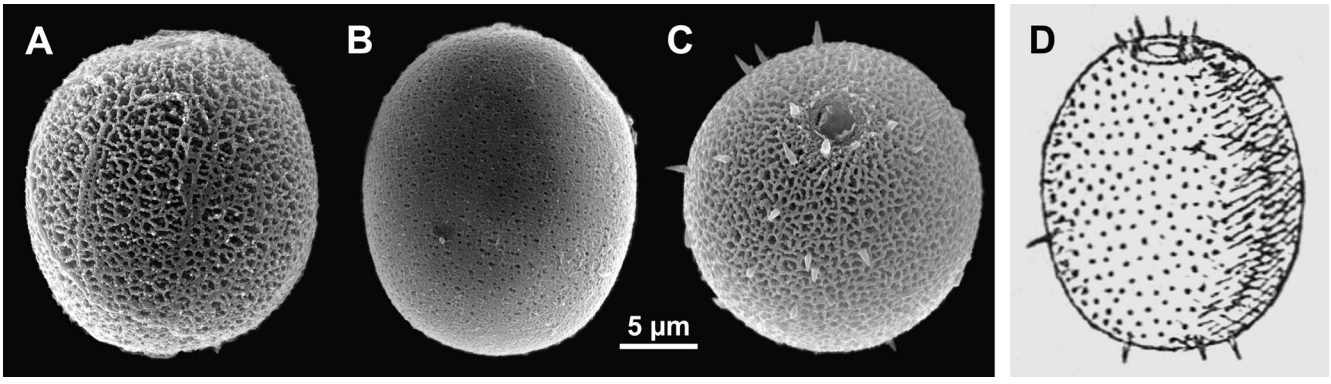

Fig. 6. Loricae showing different shape and ornamentation (SEM; all at the same scale). **A,** Lorica of *Trachelomonas* sp.GeoM*524; **B,** Lorica of *Trachelomonas* sp. GeoM 526; **C,** Lorica of *Trachelomonas hispida* var. *irregularis* GeoM 529; **D,** Protologue of *Trachelomonas hispida* var. *irregularis*.

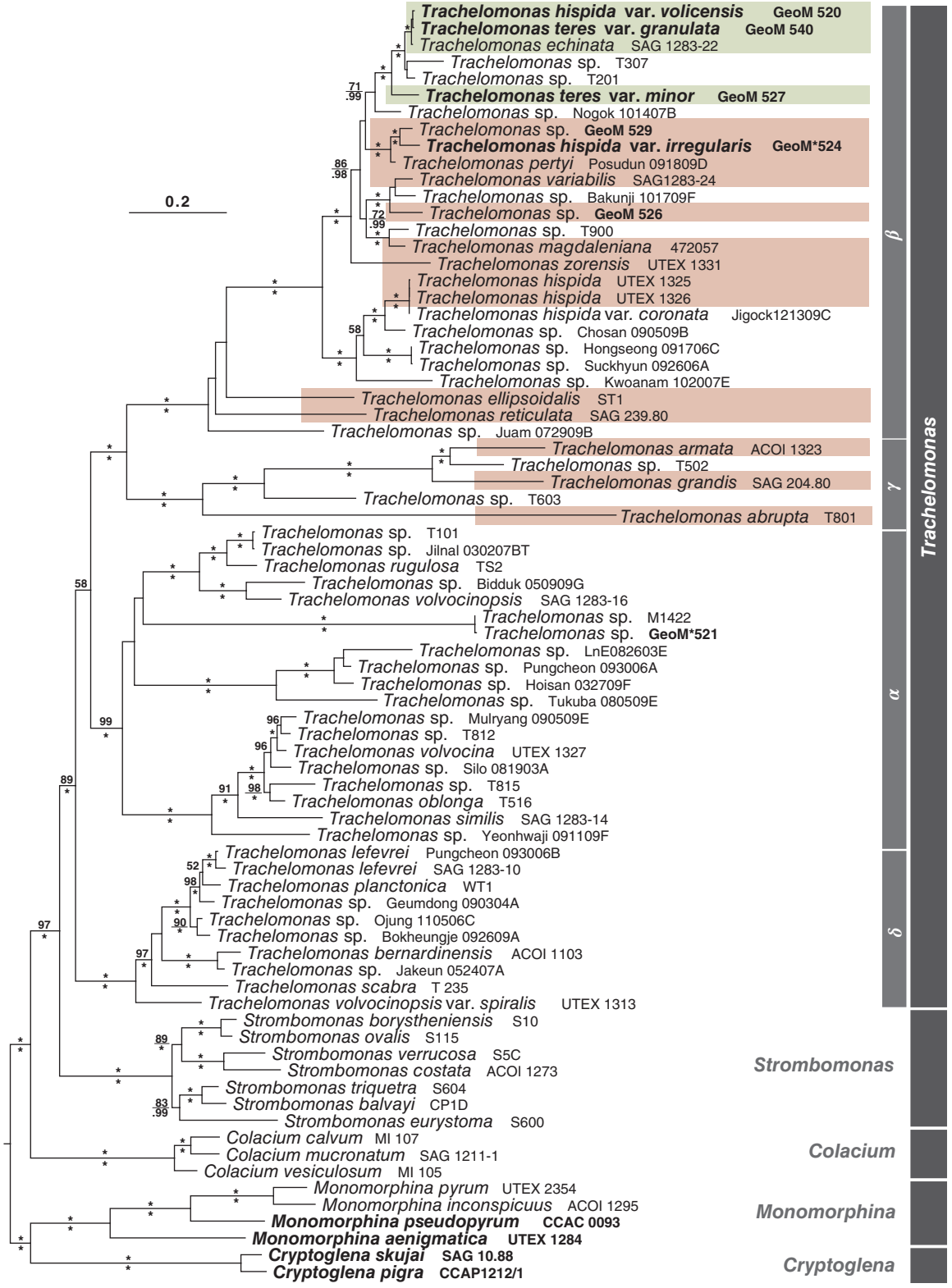

Fig. 7. Maximum likelihood-phylogenetic tree of Euglenaceae, derived from concatenated 18S rRNA and partial 28S rRNA loci. Major clades are indicated, and strains of newly obtained sequences are pointed out in bold font. Epitypified taxa (or those corresponding to reference material) are marked in bold. Branch lengths are drawn to scale, with the scale bar indicating the number of nt substitutions per site. The numbers on the branches show statistical support values (above: ML bootstrap values, values <50 are not shown; below: Bayesian posterior probabilities, values <.90 are not shown). Asterisks indicate maximal support. Significantly smaller taxa are shaded in green and significantly larger taxa in red. Well-supported lineages that could not be correlated with non-DNA sequence traits are informally indicated with Greek letters.

does not usually play an important role in taxon delimitation in protists, but three (out of six investigated) strains forming significantly smaller cells, cluster together (shaded in light green in Fig. 7). As inferred from outgroup comparison, smaller size appears of an apomorphic nature within *Trachelomonas*. It should be pointed out, however, that cell biometry is reliable only for the strains investigated in this study. Assessments of other material for which sequence data are available are inferred from the names introduced in the original literature (Swirenko, 1914; Koczwara, 1915; Dreżepolski, 1923, 1925; Skvortzov, 1925; Deflandre, 1926). In addition, the considerable amount of biometric data is missing, as most of the *Trachelomonas* accessions (as well as those of other Euglenaceae) included in the phylogenetic analysis are missing their names.

Towards clarified taxonomy in *Trachelomonas*. — Presenting a polyphyletic group, algae are extremely diverse in terms of their biology. This heterogeneity could be at least partially controlled via a robust taxonomy. However, researchers are not obliged to follow the *ICN* regulations, which further hinders us from understanding of the fundamental issues in phycological research (as for example in our case of *Trachelomonas*). Because of the complex biology, loricate euglenophytes represent also a taxonomically very challenging organismal group. Merely, 18% of all described species are currently accepted (Guiry & Guiry, 2017), but the unadjusted synonymy includes likewise many historical names dating back to 100 years ago or more. Essentially, all scientific names in the microbial world dating prior to the DNA era are ambiguous in the sense of *ICN* Art. 9.9, as original material mostly comprises of illustrations (frequently of a single cell) with no DNA sequence information. It is therefore of even greater importance to link the DNA sequence of newly collected strains to original material, since individual species in cryptic complexes cannot be distinguished in any other way. For all the above reasons, any application of historical names is particularly unreliable in *Trachelomonas*, and thus each clarified name is an important step towards a stable taxonomy (Romeikat & al., 2019).

Fifty-eight unclarified *Trachelomonas* names described from either Dobrostany lakes or from nearby vicinity of Lviv were compiled from the literature in the course of our study (suppl. Table S1). Of such names, approximately half correspond to large taxa (i.e., more than 20 µm in length), but we could not assign any of our larger strains to them due to incompatibilities in shape and ornamentation of the lorica. Excluding criteria for the smaller taxa (i.e., maximally 20 µm in length) have been specialised structures such as a distinct collar and extreme spherical or cylindric shapes, all absent in our investigated strains. Consequently, we apply *ICN* Art. 9.9. while epitypifying the remaining three such historical names, namely *Trachelomonas hispida* var. *volicensis* Drezep., *Trachelomonas teres* var. *granulata* Drezep. and *Trachelomonas teres* var. *minor* Drezep. (see Taxonomy). In no single case, contradictions to the corresponding protologues have been found based on the present integrative study of the material collected at Ukrainian type localities. A fourth name can be assigned to the collected material (namely *T. hispida* var. *irregularis* Drezep.: Fig. 6C,D), but the strain did not originate from the corresponding type locality, and we thus refrain from its epitypification. However, the DNA sequence information assigned to the taxon can be regarded as reference until the final clarification of the name.

Sequences of strain GeoM 527 (corresponding to *T. teres* var. *minor*) are distinct from any other GenBank record, but we refrain from elevating the taxon to species level as long as the identity of *Trachelomonas teres* Maskell from New Zealand is unclarified. Surprisingly, DNA sequence data alone are not always discriminative for species identification in *Trachelomonas*. The two strains GeoM 520 (corresponding to *T. hispida* var. *volicensis*) and GeoM 540 (corresponding to *T. teres* var. *granulata*) share identical SSU and partial LSU sequences. However, both strains possess distinct lorica morphologies, which considerably alter from each other, despite insignificant size differences (Figs. 1, 2, 3A–E, 4U–X, 5A–F, M–R, suppl. Tables S2, S3). Both of the strains have been cultivated in the same medium, during the same time, under the same conditions and have been added soil extract at the same time; therefore, it is unlikely that the morphological differences are of ecological and/or ontogenetic nature, a cultivation artefact or expression of intraspecific variability. We refrain from taxonomic conclusions here as well (i.e., elevation to species rank or synonymisation under, for example, *Trachelomonas echinata* A.M.Cunha) because we cannot irrefutably conclude on its taxonomic status at this moment in time. Those two taxa may present a similar dinophyte "species pair" as brackish *Apocalathium malmogiense* (G.Sjöstedt) Craveiro & al. and freshwater *Apocalathium aciculiferum* (Lemmerm.) Craveiro & al., which are molecularly indistinguishable, yet possess morphological and ecological differences (Gottschling & al., 2005; Kremp & al., 2005; Annenkova & al., 2015).

Considerable effort has been made in the past years to clarify the complex taxonomy of euglenophytes (e.g., Shin & Triemer, 2004; Kosmala & al., 2005, 2007; Łukomska-Kowalczyk & al., 2015). However, there have been several cases in which Art. 7.11 of the *ICN* was violated ["… designation of a type is achieved only …, if the typification statement includes the phrase 'designated here' (hic designatus) or an equivalent"], and subsequently, all such typfications should thus remain neglected (though effectively published from the nomenclatural point of view). Moreover, many typification statements do not provide locality information, which is an indication that those authors doubt its relevance. Thus, it seems like they are supporters of the "everything is everywhere" hypothesis (Finlay, 2002), which has been challenged numerous times in the past and is nowadays rather outdated (Bass & Boenigk, 2011; Bates & al., 2013; Žerdoner Čalasan & al., 2019). We are certain – and this is exemplified by the approach pled in the present study – that taxa, for example, from Ukraine or Germany, should not (readily) be epitypified with material collected in far distant places, such as in England, Portugal or even in the U.S.

The questionable taxonomic approaches are also illustrated by, for example, the typifications of *Phacus longicauda* var. *torta* Lemmerm.: Łukomska-Kowalczyk & al. (2015) chose an illustration as lectotype that was published 16 years after the protologue (Lemmermann, 1910; Skuja, 1926) and is thus not original material in the sense of the *ICN* (Art. 9.4). Lemmermann (1910) referred to an image of Stein (1878), which Łukomska-Kowalczyk & al. (2015) chose for typification purposes of another taxon, namely *Phacus helikoides* Pochm. More thought should be given to the taxonomy of euglenophytes, and our study might stimulate a discussion about more appropriate ways to disentangle the existing historical complexities. The fact that numerous cases of taxonomic activity are not in accordance with the rules of the *ICN* provides also an opportunity, frankly speaking, to revise former, inaccurate decisions regarding typifications in euglenophytes.

In conclusion, three historical names of *Trachelomonas* are taxonomically clarified, and meaningful in-depth studies of such epitypified taxa are from this point onward possible. Based on our taxonomic decisions, we are also able to take intraspecific variability of lorica traits into consideration, confirming a much higher morphological variability than previously thought (Wołowski & al., 2016). This is once more a strong argument for epitypification particularly for the microbial world, as the names clarified here have been rarely applied in the past (Starmach, 1983; Borisova & al., 2006). This study furthermore gives an example of successful taxonomic clarification within protists, putting them side-by-side with macroorganismic groups, in which taxonomy does not represent such a topical issue. Anyhow, we live in an era, where the newly produced amount of data is getting out of hand, and scientific publications in favour of discarding all long-forgotten names, which could cause taxonomic instability (Smith & al., 2016), are a matter of great concern. Nevertheless, not only that authors of such papers simply ignore the fact that taxonomy of different groups of organisms is facing different obstacles; with such a radical approach, they are sabotaging their own research by discarding the work of taxonomists of previous centuries, not realising that their work could be treated in the same way after a period of time. Despite being time consuming, epitypification (if done properly) is still one of the most powerful methods to clarify ambiguous scientific names, which is of particular importance especially in character-poor protists such as the euglenophytes. Integrating both morphological and phylogenetic methods, taking type locality into consideration and carrying out epitypification, this study represents a considerable step forward towards stable and reliable taxonomy also in protists. As epitypification is only available under the *ICN*, we furthermore urge fellow researchers to carry out any kind of taxonomic treatments of ambiregnal taxa under the *ICN*.

■ TAXONOMY

Trachelomonas hispida var. ***volicensis*** Drezep. in Kosmos (Lvov) 50: 216, t. 1, fig. 35. 1925 – **Lectotype (designated here):** [illustration] in Kosmos (Lvov) 50: t. 1, fig. 35. 1925! [showing a non-fossil individual from Republic of Ukraine. Lviv; exact locality and collecting date unknown: *R. Drežepolski s.n.*] – **Epitype (designated here):** Republic of Ukraine. Lviv, Yavoriv, Dobrostany, 15 Sep 2012 [non-fossil]: *M. Gottschling, N.H. Filipowicz & C. Zinßmeister P57* [J. Kretschmann GeoM 520] (KW No. KW-A-221!; isoepitypes: B barcode B 40 0043801!, M barcodes M-0299991! & M-0299992!).

The nomenclatural act has been registered in PhycoBank under http://phycobank.org/100580.

Other original elements. – Non-fossil specimens from Lviv and Wolicki pond (Ukraine), without exact dates (January, March), collected by R. Drežepolski, none preserved.

Note. – Average length, width and shape of strain GeoM 520 correspond to the protologue data. Additionally, absence of collar and overall punctated surface, which is more pronounced at the anterior part of the cell (seen in SEM images), is also in correspondence with the protologue.

Trachelomonas teres var. ***granulata*** Drezep., nom. corr. (*ICN* Art. 60.1.), in Kosmos (Lvov) 50: 223, t. 1, fig. [32]. 1925 – **Lectotype (designated here):** [illustration] in Kosmos (Lvov) 50: t. 1, fig. [32]. 1925! [not numbered on the plate; showing a non-fossil individual from Republic of Ukraine; Lviv, Yavoriv, Dobrostany, Jul (without year): *R. Drežepolski s.n.*] – **Epitype (designated here):** Republic of Ukraine. Lviv, Yavoriv, Dobrostany, 15 Sep 2012 [non-fossil]: *M. Gottschling, N.H. Filipowicz & C. Zinßmeister P56* [J. Kretschmann GeoM 540] (KW No. KW-A-224!; isoepitypes: B barcode B 40 0043804!, M barcodes M-0299987! & M-0299988!).

The nomenclatural act has been registered in PhycoBank under http://phycobank.org/100568.

Note. – Average length and width of the strain GeoM 540 more or less correspond to the protologue data. Cell shape and overall ornamentation (seen in LM and SEM images) are in correspondence with the protologue. Additionally, thickened edge on the flagellar pore region (seen in LM images) corresponds to the original description. Finally, samples were collected in summer, which also correlates with the original description.

Trachelomonas teres var. ***minor*** Drezep. in Kosmos (Lvov) 50: 223, t. 1, fig. 26. 1925 – **Lectotype (designated here):** [illustration] in Kosmos (Lvov) 50: t. 1, fig. 26. 1925! [showing a non-fossil individual; exact locality and collecting date unknown: *R. Drežepolski s.n.*] – **Epitype (designated here):** Republic of Ukraine. Lviv, Lubień Mały, 15 Sep 2012 [non-fossil]: *C. Zinßmeister, N.H. Filipowicz & M. Gottschling P39* [J. Kretschmann GeoM 527] (KW No. KW-A-222!; isoepitypes: B barcodes B 40 0043802! & B 40 0043803!, KW No. KW-A-223!, M barcodes M-0299989! & M-0299990!).

The nomenclatural act has been registered in PhycoBank under http://phycobank.org/100569.

Other original elements. – Non-fossil specimens from Dobrostany and Lviv (Ukraine), Łuków (Poland) and Tuhanowicze (Belarus), without exact date (May, Jul, Sep, Nov), collected by R. Drežepolski, none preserved.

Note. – Average length, width and shape of the strain GeoM 527 correspond to the protologue data. Double-husked pyrenoids are not seen in all the cells, yet the subclade within strain GeoM 527 clustered is known to possess diplopyrenoids (Ciugulea & al., 2008). Additionally, absence of collar (seen in SEM and LM images) and overall smooth surface (seen in LM images) are also in correspondence with the protologue. Finally, samples were collected in September, which also correlates with the original description.

■ AUTHOR CONTRIBUTIONS

AZC: morphological study, molecular phylogenetics, led the writing; JK: strain establishment, morphological study; MG: scientific concept, field work, taxonomy; all authors critically read and approved the written text. — AZC, https://orcid.org/0000-0003-2081-2076; MG, https://orcid.org/0000-0002-4381-8051

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Appendix 1. Voucher list for the multilocus-based alignment of a representative taxon set of selected photosynthetic euglenoid clades.

Taxon, strain number, locality, collector plus collection number, GenBank accession numbers for 18S rRNA and partial 28S rRNA loci. The latter locus occasionally under two different GenBank accession numbers.

Colacium calvum F.Stein, MI 107, locality unknown, collector and coll. number unknown, EF999907, EF999910; ***Colacium mucronatum*** Bourr. & Chadef., SAG 1211-1, United Kingdom, England, Cambridge, epizoic on Daphnia, *E.G. Pringsheim, s.n.*, AJ532441, EF999906; ***Colacium vesiculosum*** Ehrenb., MI 105, locality unknown, collector and coll. number unknown, EF999905, EF999912; ***Cryptoglena pigra*** Ehrenb., CCAP1212/1, United Kingdom, England,

Appendix 1. Continued.

Cambridge, Jesus Ditch, collector and coll. number unknown, AJ532437, DQ140101; ***Cryptoglena skujae*** B.Marin & Melkonian, SAG 10.88, Austria, Neusiedler See at Rust, *E. Kusel-Fetzmann, s.n.*, AY014998, AY130236; ***Monomorphina aenigmatica*** (Drezep.) Nudelman & Triemer, UTEX 1284, United Kingdom, England, Lyme Regis, *M.R. Droop, s.n.*, AF190814, DQ140117; ***Monomorphina inconspicuus*** (Deflandre) B.Marin & Melkonian, ACOI 1295, Portugal, Castelo Branco, Penha Garcia, *L. Santos, s.n.*, DQ140129, DQ140111; ***Monomorphina pseudopyrum*** Kosmala, Milanowski, Brzóska, Pękala, Kwitowski & Zakryś, CCAC 0093, The Netherlands, Ameland (West), (Nassau) Entenkooi, *Klingberg, M., s.n.*, AJ532433, EF999909; ***Monomorphina pyrum*** (Ehrenb.) Mereschk., UTEX 2354, locality unknown, collector and coll. number unknown, AF112874, AY130238; ***Strombomonas balvayi*** Bourr. & Couté, CP1D, locality unknown, collector and coll. number unknown, EF999897, AY359916, KT304970; ***Strombomonas borystheniensis*** (Y.V.Roll) T.G.Popova, S10, locality unknown, collector and coll. number unknown, DQ140131, AY359920, KT304971; ***Strombomonas costata*** Deflandre, ACOI 1273, Portugal, Quiaios, Lagoa das Braças, *M. F. Santos, s.n.*, DQ140152, AY359915, KT304972; ***Strombomonas eurystoma*** (F.Stein) T.G.Popova, S600, locality unknown, collector and coll. number unknown, DQ140132, AY359918, KT304974; ***Strombomonas ovalis*** (Playfair) Deflandre, S115, locality unknown, collector and coll. number unknown, DQ140133, AY359919, KT304975; ***Strombomonas triquetra*** (Playfair) Deflandre, S604, locality unknown, collector and coll. number unknown, DQ140153, AY359917, KT304978; ***Strombomonas verrucosa*** (Daday) Deflandre, S5C, locality unknown, collector and coll. number unknown, AF445461, AY359911, KT304979; ***Trachelomonas abrupta*** Svirenko, T 801, USA, collector and coll. number unknown, DQ140134, AY359941, KT304980; ***Trachelomonas armata*** (Ehrenb.) F.Stein, ACOI 1323, Portugal, Casal Novo do Rio, canal, *M.F. Santos, s.n.*, EF999904, KT304981; ***Trachelomonas bernardinensis*** Vischer, ACOI 1103, Portugal, Castelo Branco, Penamacor, *L. Santos, s.n.*, EF999908, AY359950, KT304982; ***Trachelomonas echinata*** A.M.Cunha, SAG 1283-22, United States, Indiana, Griffy Lake at Bloomington, *R.C. Starr, s.n.*, AY015001, AY130242, KT304985; ***Trachelomonas ellipsoidalis*** Kam.P.Singh, ST1, locality unknown, collector and coll. number unknown, DQ140135, AY359935; ***Trachelomonas grandis*** Kam.P.Singh, SAG 204.80, United States, Tennessee, quarry in Nashville, *K.P. Singh (reisolated by E.G. Pringsheim), s.n.*, AJ532446, AY359936; ***Trachelomonas hispida*** (Perty) F.Stein, UTEX 1325, United Kingdom, England, Cambridge, Landbeach, *E.G. Pringsheim, s.n.*, AF445462, AY130817; ***Trachelomonas hispida*** (Perty) F.Stein, UTEX 1326, United Kingdom, England, Debden, *E.G. Pringsheim, s.n.*, AF090377, AY130817; ***Trachelomonas hispida*** var. ***coronata*** Lemmerm., Jigock 121309C, Republic of Korea, Jigock, collector and coll. number unknown, KT304850, KT304989; ***Trachelomonas hispida*** var. ***volicensis*** Drezep., GeoM 520 (≡ CCCM7069, CCAC 6780 B), Republic of Ukraine, Lviv, Yavoriv, Dobrostany, 15 Sep 2012: *M. Gottschling, N.H. Filipowicz & C. Zinßmeister P56* [J. Kretschmann GeoM 520] (epitype: KW No. KW-A-224!; isoepitypes: B barcode B 40 0043804!, M barcodes M-0299987! & M-0299988!), MK894269*, MK894263*; ***Trachelomonas lefevrei*** Deflandre, Pungcheon 093006B, Republic of Korea, Pungcheon, collector and coll. number unknown, KT304852, KT304993; ***Trachelomonas lefevrei*** Deflandre, SAG 1283-10, United Kingdom, England, Shelford/Cambridge, *E.G. Pringsheim, s.n.*, DQ140136, AY359949; ***Trachelomonas magdaleniana*** Deflandre, 472057, Argentina, collector and coll. number unknown, EF999903, EF999911; ***Trachelomonas oblonga*** Lemmerm, T 516, locality unknown, collector and coll. number unknown, DQ140137, AY359947, KT304994; ***Trachelomonas pertyi*** E.G.Pringsh., Posudun 091809D, Republic of Korea, Posudun, collector and coll. number unknown, KT304853, KT304995; ***Trachelomonas planctonica*** Svirenko, WT1, locality unknown, collector and coll. number unknown, DQ140138, AY359954, KT304996; ***Trachelomonas reticulata*** G.A.Klebs, SAG 239.80, Germany, Göttingen, mud from a road puddle, *O. Pringsheim, s.n.*, DQ140139, AY359934; ***Trachelomonas rugulosa*** F.Stein, TS2, locality unknown, collector and coll. number unknown, DQ140140, AY359942, KT304997; ***Trachelomonas scabra*** Playfair, T 235, United States, collector and coll. number unknown, DQ140141, AY359951; ***Trachelomonas similis*** A.Stokes, SAG 1283-14, United Kingdom, England, pond near Debden, *E.G. Pringsheim, s.n.*, DQ140142, AY359948, KT304999; ***Trachelomonas* sp.**, Bakunji 101709F, Republic of Korea, Bakunji, collector and coll. number unknown, KT304858, KT305004; ***Trachelomonas* sp.**, Bidduk 050909G, Republic of Korea, Bidduk, collector and coll. number unknown, KT304859, KT305005; ***Trachelomonas* sp.**, Bokheungje 092609A, Republic of Korea, Bokheungje, collector and coll. number unknown, KT304860, KT305006; ***Trachelomonas* sp.**, Chosan 090509B, Republic of Korea, Chosan, collector and coll. number unknown, KT304861, KT305007; ***Trachelomonas* sp.**, GeoM*521, Republic of Ukraine, Lviv, Zhovkva, Dublyany, 16 Sep 2012: *N.H. Filipowicz, C. Zinßmeister & M. Gottschling, P58* [J. Kretschmann GeoM*521], MK894271*; ***Trachelomonas* sp.**, GeoM*524, Republic of Ukraine, Lviv, Lubień Mały, 15 Sep 2012: *C. Zinßmeister, N.H. Filipowicz & M. Gottschling, P38* [J. Kretschmann GeoM*524], MK894265*, MK894259*; ***Trachelomonas* sp.**, GeoM 526, Republic of Poland, Włocławek, Chodecz, Chodeckie lake, 10 Sep 2012: *C. Zinßmeister, N.H. Filipowicz & M. Gottschling, P1* [J. Kretschmann GeoM 526], MK894267*, MK894261*; ***Trachelomonas* sp.**, GeoM 529 (≡ CCCM7132), Republic of Ukraine, Lviv, Lubień Mały, 15 Sep 2012: *C. Zinßmeister, N.H. Filipowicz & M. Gottschling, P37* [J. Kretschmann GeoM 529], MK894266*, MK894260*; ***Trachelomonas* sp.**, Geumdong 090304A, Republic of Korea, Geumdong, collector and coll. number unknown, KT304865, KT305011; ***Trachelomonas* sp.**, Hoisan 032709F, Republic of Korea, Hoisan, collector and coll. number unknown, KT304866, KT305012; ***Trachelomonas* sp.**, Hongseong 091706C, Republic of Korea, Hongseong, collector and coll. number unknown, KT304867, KT305013; ***Trachelomonas* sp.**, Jakeun 052407A, Republic of Korea, Jakeun, collector and coll. number unknown, KT304868, KT305014; ***Trachelomonas* sp.**, Jilnal 030207BT, Republic of Korea, Jilnal, collector and coll. number unknown, KT304870, KT305016; ***Trachelomonas* sp.**, Juam 072909B, Republic of Korea, Juam, collector and coll. number unknown, KT304871, KT305017; ***Trachelomonas* sp.**, Kwoanam 102007E, Republic of Korea, Kwoanam, collector and coll. number unknown, KT304872, KT305018; ***Trachelomonas* sp.**, LnE082603E, locality unknown, collector and coll. number unknown, KT304864, KT305010; ***Trachelomonas* sp.**, Mulryang 090509E, Republic of Korea, Mulryang, collector and coll. number unknown, KT304873, KT305019; ***Trachelomonas* sp.**, Nogok 101407B, Republic of Korea, Nogok, collector and coll. number unknown, KT304875, KT305021; ***Trachelomonas* sp.**, Ojung 110506C, Republic of Korea, Ojung, collector and coll. number unknown, KT304876, KT305022; ***Trachelomonas* sp.**, Pungcheon 093006A, Republic of Korea, Pungcheon, collector and coll. number unknown, KT304877, KT305023; ***Trachelomonas* sp.**, Silo081903A, United States, collector and coll. number unknown, KT304880, KT305026; ***Trachelomonas* sp.**, Suckhyun 092606A, Republic of Korea, Suckhyun, collector and coll. number unknown, KT304885, KT305031; ***Trachelomonas* sp.**, T 101, locality unknown, collector and coll. number unknown, EF999898, AY359943; ***Trachelomonas* sp.**, T 201, locality unknown, collector and coll. number unknown, EF999899, AY359922; ***Trachelomonas* sp.**, T 307, locality unknown, collector and coll. number unknown, EF999900, AY359923; ***Trachelomonas* sp.**, T 502, locality unknown, collector and coll. number unknown, EF999901, AY359939; ***Trachelomonas* sp.**, T 603, locality unknown, collector and coll. number unknown, DQ140143, AY359940; ***Trachelomonas* sp.**, T 812, United States, collector and coll. number unknown, KT304886, AY359945; ***Trachelomonas* sp.**, T 815, locality unknown, collector and coll. number unknown, EF999902, AY359946; ***Trachelomonas* sp.**, T 900, United States, collector and coll. number unknown, KT304887, AY359927; ***Trachelomonas* sp.**, Tukuba 080509E, Japan, Tukuba, collector and coll. number unknown, KT304888, KT305034; ***Trachelomonas* sp.**, Yeonhwaji 091109F, Republic of Korea, Yeonhwaji, collector and coll. number unknown, KT304890, KT305036; ***Trachelomonas teres*** var. ***granulata*** Drezep., GeoM 540 (≡ CCAC 6782 B), Republic of Ukraine, Lviv, Yavoriv, Dobrostany, 15 Sep 2012: *M. Gottschling, N.H. Filipowicz & C. Zinßmeister, P56* [J. Kretschmann GeoM 540] (epitype: KW No. KW-A-224!; isoepitypes: B barcode B 40 0043804!, M barcodes M-0299987! & M-0299988!), MK894270*, MK894264*; ***Trachelomonas teres*** var. ***minor*** Drezep., GeoM 527 (≡ CCCM7129, CCAC 6781 B), Republic of Ukraine, Lviv, Lubień Mały, 15 Sep 2012: C. Zinßmeister, N.H. Filipowicz & M. Gottschling, P39 [J. Kretschmann GeoM 527] (epitype: KW No. KW-A-222!; isoepitypes: B barcodes B 40 0043802! & B 40 0043803!, KW No. KW-A-223!, M barcodes M-0299989! & M-0299990!), MK894268*, MK894262*; ***Trachelomonas variabilis*** Kam.P.Singh, SAG 1283-24, United States, Tennessee, Nashville, pond in greenhouse of Vanderbilt University, *E.G. Pringsheim, s.n.*, KT304891, KT305038; ***Trachelomonas volvocina*** (Ehrenb.) Ehrenb., UTEX 1327, United Kingdom, England, Debden, *E.G. Pringsheim, s.n.*, KT304892, AY359953; ***Trachelomonas*** cf. ***volvocinopsis*** Svirenko, M1422, Germany, Muenster castle, small waterfall, *U. Powalowski, s.n.*, AJ532452; ***Trachelomonas volvocinopsis*** Svirenko, SAG 1283-16, United Kingdom, England, Trumpington/Cambridge, *E.G. Pringsheim, s.n.*, DQ140144, AY359944, KT305040; ***Trachelomonas volvocinopsis*** var. ***spiralis*** E.G.Pringsh., UTEX 1313, locality unknown, collector and coll. number unknown, AY015004, AY130816; ***Trachelomonas zorensis*** Deflandre, UTEX 1331, locality unknown, collector and coll. number unknown, DQ140145, AY359952, KT305037.