Protist, Vol. 161, 603–619, October 2010
http://www.elsevier.de/protis
Published online date 22 April 2010

Protist

ORIGINAL PAPER

Reconstructing Euglenoid Evolutionary Relationships using Three Genes: Nuclear SSU and LSU, and Chloroplast SSU rDNA Sequences and the Description of *Euglenaria* gen. nov. (Euglenophyta)

Eric W. Linton[a,1], Anna Karnkowska-Ishikawa[c], Jong Im Kim[d], Woongghi Shin[d], Mathew S. Bennett[b], Jan Kwiatowski[c,e], Bożena Zakryś[c], and Richard E. Triemer[b]

[a]Central Michigan University, Department of Biology, Mt. Pleasant, MI 48859, USA
[b]Michigan State University, Department of Plant Biology, East Lansing, MI 48824, USA
[c]University of Warsaw, Department of Plant Systematics and Geography, Institute of Botany, Al. Ujazdowskie 4, 00-478 Warsaw, Poland
[d]Chungnam National University, Department of Biology, 220 Gung-Dong, Yuseong-Gu Daejeon 305-764, Republic of Korea
[e]University of California, Department of Ecology and Evolutionary Biology, 453 Steinhaus Hall, Irvine, CA 92697, USA

Submitted September 24, 2009; Accepted February 28, 2010
Monitoring Editor: Michael Melkonian

Using Maximum Likelihood and Bayesian analyses of three genes, nuclear SSU (nSSU) and LSU (nLSU) rDNA, and chloroplast SSU (cpSSU) rDNA, the relationships among 82 plastid-containing strains of euglenophytes were clarified. The resulting tree split into two major clades: clade one contained *Euglena*, *Trachelomonas*, *Strombomonas*, *Colacium*, *Monomorphina*, *Cryptoglena* and *Euglenaria*; clade two contained *Lepocinclis*, *Phacus* and *Discoplastis*. The majority of the members of *Euglena* were contained in clade A, but seven members were outside of this clade. *Euglena limnophila* grouped with, and was thus transferred to *Phacus*. *Euglena proxima* was a single taxon at the base of clade one and is unassociated with any subclade. Five members of *Euglena* grouped together within clade one and were transferred into the newly erected genus *Euglenaria*. The monophyly of the remaining genera was supported by Bayesian and Maximum Likelihood analyses. Combining datasets resolved the relationships among ten genera of photosynthetic euglenoids.

Key words: Bayesian inference; euglenophyta; LSU; maximum likelihood; SSU.

Introduction

Euglenoids are a group of protozoans that most commonly have two flagella, one or both may be emergent. Members live in salt, brackish or fresh

[1]Corresponding author; fax +1 11 989 772 3462.
e-mail eric.linton@cmich.edu (E.W. Linton).

doi:10.1016/j.protis.2010.02.002

water environments. Many are phagotrophic or osmotrophic, however, some euglenoids are also considered algae because they are photosynthetic, having acquired a chloroplast via secondary endosymbiosis of a eukaryotic green alga (Gibbs 1978; Ishida et al. 1997; Leander 2004; Martin et al. 1992; Nozaki 2005). Our research is focused on phylogenetic (evolutionary) relationships among the photosynthetic euglenoids with a single emergent flagellum, while members of the outgroup have two emergent flagella. Because of their long and intricate history, a brief review of euglenoid morphology and taxonomy is warranted. An in-depth review of euglenoid classification and molecular phylogenetics can be found in Triemer and Farmer (2007).

Since the genus *Euglena* was first described by Ehrenberg (1830a, 1830b), their classification by various authors has largely been based on morphological features such as: chloroplast type and distribution, flagellar length, shape and distribution of the storage product, and cell surface features. Therefore, an understanding of euglenoid morphological features is necessary for their proper classification into genera.

The unique surface feature of all euglenoids is the pellicle, a series of interlocking protein strips subtended by microtubules that lies below the plasma membrane. If the cell is rigid or capable of a slight bending motion, the pellicle strips can be arranged either longitudinally or helically. However, all cells capable of metaboly, a peristaltic wriggling motion (Triemer et al. 2006), have helically arranged pellicle strips. Underlying the pellicle strips of some euglenoids are membrane-bounded mucus secreting bodies known as mucocysts, sensu Mignot (1966). Mucocysts, when present, can be round or spindle-shaped, thus providing a diagnostic character for many species.

An exceedingly useful phylogenetic character of all photosynthetic euglenoids is the chloroplast. The chloroplast envelope consists of three membranes, indicating their secondary endosymbiotic origin, while the inner photosynthetic membranes (thylakoids) occur in stacks of three and lack the grana stacks found in land plants. The chloroplast can be absent, reduced to a colorless plastid in some secondary osmotrophic euglenoids, or contain chlorophyll and take on a variety of shapes, such as discoid, lenticular, lobed, spherical, ribbon-like or stellate. While the same chloroplast shape can be found in a variety of different euglenoid species, the shape for any particular species is specific and in some cases specific for the whole genus. The pyrenoid, a proteinaceous area within the chloroplast containing Rubisco, is associated with the formation of the storage products. In euglenoids this product is paramylon a beta 1,3-glucan storage compound. This structure, like the chloroplast, is often diagnostic of a species or an entire genus of euglenoids. When present, the pyrenoid may be capped by paramylon on one side (haplopyrenoids), both sides (diplopyrenoid), or lacking a paramylon cap (naked). Additional morphological features such as cell length, shape, degree of compression (flatness), presence and shape of the lorica (a protective extracellular matrix that surrounds cell) have also been used to define species and genera of euglenoids.

By 1849, taxonomists had erected six of the ten photosynthetic euglenoid genera analyzed in this paper. The first six genera were *Euglena* (Ehrenberg 1830a, 1830b, 1838), *Cryptoglena* (Ehrenberg 1831), *Trachelomonas* and *Colacium* (Ehrenberg 1833), *Phacus* (Dujardin 1841) and *Lepocinclis* (Perty 1849). The remaining four genera were separated from these original genera based on more detailed examinations of morphological features or on significant molecular differences. Using morphological differences *Monomorphina* was split off from *Lepocinclis* by Mereschkowsky (1877), but the description of the genus was not recognized in the English literature until recently (Marin et al. 2003). Differences in the structure of the lorica were used by Deflandre (1930) to erect *Strombomonas* from a subgroup of *Trachelomonas*. The use of molecular data, predominantly nuclear small subunit rDNA (nSSU), and modern phylogenetic methods (Maximum Likelihood and Bayesian analyses) ushered in a new era of euglenoid systematics.

In this new era, early molecular evidence noted that some genera (*Euglena, Phacus* and *Lepocinclis)* were not monophyletic (Linton et al. 1999, 2000; Milanowski et al. 2001; Müllner et al. 2001) which led to several taxonomic revisions. In 2003, Marin et al. resurrected *Monomorphina* and moved several former *Phacus* and a few *Lepocinclis* species into the genus thus making both genera monophyletic. In addition, several *Euglena* species that were nested with taxa from *Lepocinclis* were moved into this genus. *Strombomonas* was then dissolved back into *Trachelomonas*. More recently, Triemer et al. (2006) using increased taxon sampling and data from multiple genes, re-established *Trachelomonas* and *Strombomonas* as independent genera and erected the new genus *Discoplastis* to contain two species

formerly assigned to *Euglena*. Our study uses 82 taxa and an expanded dataset of three genes, two nuclear (nSSU and nLSU rDNA) and one plastid (cpSSU rDNA), to resolve relationships and define ten genera, nine previously described and one newly erected genus *Euglenaria*, containing five former members of *Euglena*.

Results

Phylogenetic Analysis

Eighty-two taxa representing 12 genera (10 ingroup and two outgroup) were analyzed using a combination of three genes. Two (nSSU and nLSU) were nuclear encoded and one (cpSSU) was encoded in the chloroplast or plastid (*Euglena longa*) for a total of 5161 bases used in the analysis (nSSU: 1740, nLSU: 2110 and cpSSU: 1311). Of the 246 sequences used in this study 92 were previously unpublished (nSSU: 12, nLSU: 22 and cpSSU: 58). Bayesian (single and partition model) and Maximum Likelihood (ML) analyses recovered trees of identical topology (Fig. 2), except for the unresolved position of *Euglena tristella*, see below. The tree shows only single model Bayesian support (pp) values then the ML support (bp) values. When the Bayesian support values differ between the single and partition models, the single model values are given in the text first, followed by the partition model values.

The backbone of this multi-gene tree was well supported by both Bayesian posterior probabilities (pp) and ML bootstrap numbers (bp). Within the ten ingroup genera ten separate clades (A–J), as well as a sister relationship of the single taxon *Euglena proxima* within the clades (A–G), were all well supported. Each genus formed a single major clade except for *Euglena* that formed two distinct well-supported clades (A and G) and the single taxon *Euglena proxima* making *Euglena* a polyphyletic genus (Fig. 2). Consequently, *Euglenaria* was erected to contain the taxa of clade G.

Analysis of each dataset individually (nSSU, nLSU and cpSSU) each resulted in less well-resolved and supported topologies (not shown) with separate clades for each genus, as in the combined tree. The nSSU tree was the most similar to the combined tree and differed only in placing the *Euglenaria* clade as sister to the *Euglena* clade (0.92 pp). However, the nLSU and cpSSU each supported the separation of *Euglenaria* from *Euglena*, as did a combined nSSU and nLSU dataset (not shown). The combined nSSU/nLSU dataset resulted in a well-supported (pp) tree of identical topology to the three gene tree presented in this study (Fig. 2).

Overview

The phylogenetic relationships among the freshwater euglenoid genera *Euglena*, *Trachelomonas*,

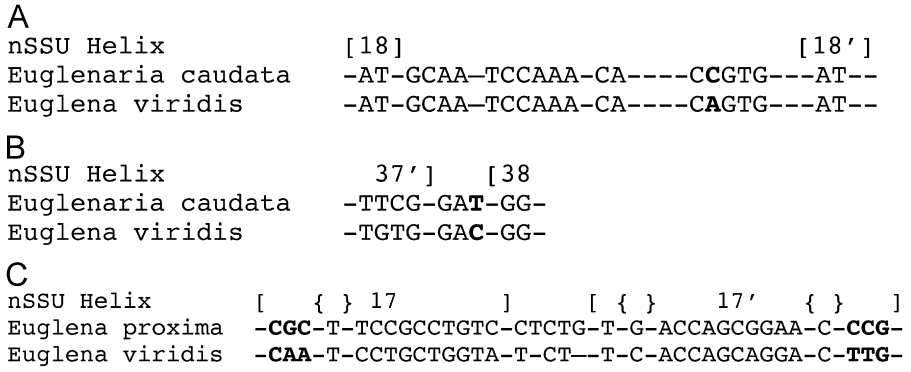

Figure 1. (**A**) Alignment of the nSSU sequences between helix 18 and 18′. *Euglenaria caudata* and *Euglena viridis* (representing all other euglenoids) are indicated with the base C among all members of the new genus *Euglenaria* and the base A among all other euglenoids used in this analysis, with the exception of *Eutreptia viridis* which has a G in this position (not shown). (**B**) Alignment of the nSSU sequences between helix 37 and 38. *Euglenaria caudata* and *Euglena viridis* (representing the genus *Euglena*) are indicated with the base T among all members of the new genus *Euglenaria* and the base A or C (not shown) among all members of the genus *Euglena* used in this analysis. The diagnostic bases are in bold. (**C**) Alignment of nSSU sequences of stem 17 between *Euglena proxima* and *Euglena viridis* (representing all other euglenoids). *Euglena proxima* had a unique CGC\CCG sequence while most other euglenoids had a CAA\TTG sequence. The bases in the stem of helix 17 are in bold.

Strombomonas, *Colacium*, *Monomorphina*, *Cryptoglena*, *Euglenaria*, *Lepocinclis*, *Phacus*, and *Discoplastis* are represented in Figure 2. The ingroup taxa were split into two main clades. Clade one was well supported by both Bayesian and ML analyses (pp 0.99 and 1.00, bp 84%) and contained eight genera: *Euglena*, *Trachelomonas*, *Strombomonas*, *Colacium*, *Monomorphina*, *Cryptoglena*, and *Euglenaria*, as well as the single taxon *Euglena proxima*. Clade two was well

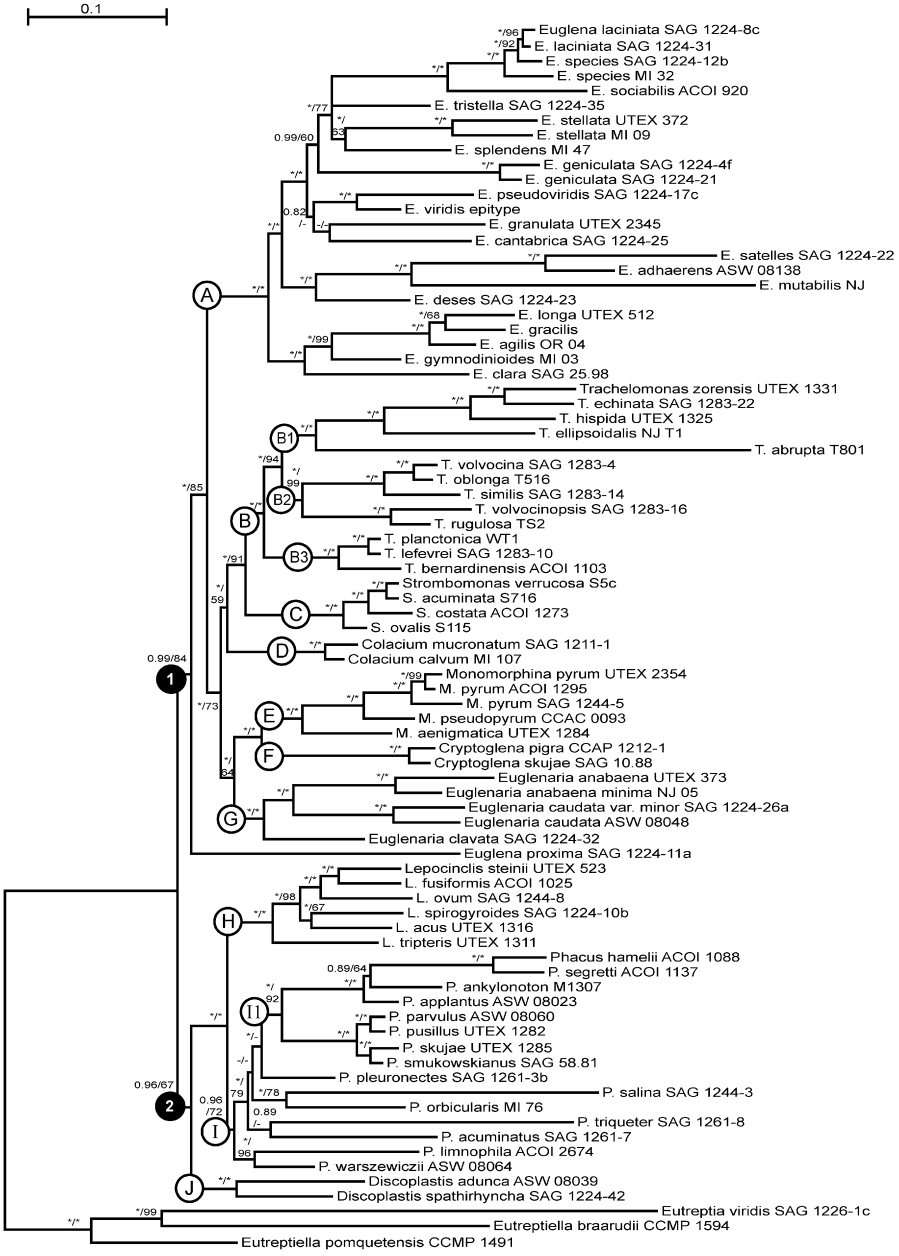

Figure 2. A rooted single model Bayesian (B) phylogenetic tree based on 5161 characters (nSSU, nLSU and cpSSU) obtained from 85 taxa. The Maximum Likelihood (ML) tree had identical topology. Numbers on the internodes represent Bayesian single model posterior probability (pp)/ML bootstrap support for the node. For pp values only those above 0.80 are shown. The ∗ indicates a pp=1.00 or bootstrap=100%. Each taxon name is followed by its SSU culture collection origin. The tree was outgroup rooted posteriorly with *Eutreptia* and *Eutreptiella* species. Scale bar represents number of substitutions/site. The clades that contained each genus are indicated by the capital letter at nodes of the tree.

supported (pp 0.96 and 0.99) by Bayesian, but weakly supported (bp 67%) by ML analysis, contained three genera: *Lepocinclis*, *Phacus*, and *Discoplastis*.

Clades A–G

Euglena: After the transfer of five taxa to the new genus *Euglenaria* (clade G), *Euglena* remains paraphyletic. *Euglena proxima* was well supported as an independent lineage from the crown *Euglena* clade A by Bayesian and ML analyses (pp 0.99 and 1.00, bp 84%) on its own branch. *Euglena proxima* lacked the diagnostic base C of *Euglenaria* and instead had the base A as found in all the remaining ingroup taxa. Still, *E. proxima* had a unique molecular signature in the nSSU at the base of stem 17 CGC\CCG where all but eight taxa on the tree had CAA\TTG (Fig. 1C). The eight taxa (seven ingroup and one outgroup) had various differences in this stem area, but none were like those found in *E. proxima*.

The crown clade A (pp 1.00, bp 100%) contained all of the remaining members of *Euglena* including the type species, *Euglena viridis*. Nearly all sister relationships within clade A were well supported by Bayesian posterior probabilities (0.96–1.00, 0.99–1.00) with the majority supported by 1.00. The exceptions were the position of *E. tristella* as sister to *E. splendens* weakly supported by ML (51%) but well supported by single Bayesian (0.96), but weakly supported as sister to *E. sociabilis* by partition Bayesian (0.61). Therefore, its final position is represented as an unresolved trichotomy. The position of *E. splendens* as sister to *E. stellata* was well supported by Bayesian but only weakly supported by ML (pp 1.00 and 0.99, bp 63%). Neither Bayesian nor ML fully supported the position of *E. cantabrica* and *E. granulata* as sisters (pp 0.65 and 0.83, bp $<$ 50%) or their sister relationships to the *E. viridis*, *E. pseudoviridis* clade (pp 0.82 and 0.85, bp $<$ 50%).

Trachelomonas/Strombomonas/Colacium: These sister clades (B, C and D) were connected by a short branch that was well supported by Bayesian analysis (pp 1.00 and 0.99), but only weakly supported by ML analysis (bp 59%). All members of these three clades produced profuse amounts of mucilage that was retained on the cell surface as either a lorica (*Trachelomonas* and *Strombomonas*) or a stalk (*Colacium*).

Relationships among members of *Trachelomonas*, a specious genus that formed various size and shaped loricas with spines and/or pores, were all well supported (pp 1.00, bp 94–100%) and contained within the single clade B (pp 1.00, bp 100%). Clade B divided into three well supported sister clades B1 (pp 1.00, bp 100%) B2 (pp 1.00, bp 99%) and B3 (pp 1.00, bp 100%), which differed based on the morphology of their loricas.

All *Strombomonas* were contained in clade C (pp 1.00, bp 100%) and all sister relationships among species were well supported (pp 1.00, bp 100%). The sister relationship between *Strombomonas* (clade C) and *Trachelomonas* (clade B) was well supported (pp 1.00, bp 91%) as well. *Colacium* contained six described species with two present in the analysis. These two members formed a single clade D (pp 1.00, bp 100%).

Monomorphina/Cryptoglena: The relationship between these two sister clades was well supported by both Bayesian and ML analysis (pp 1.00, bp 100%). Five members of *Monomorphina* were contained within clade E (pp 1.00, bp 100%) with strongly supported sister relationships (pp 1.00, bp 99–100%). The two members of *Cryptoglena* were contained within clade F (pp 1.00, bp 100%).

Euglenaria: This newly erected genus contained five taxa previously in *Euglena* (clade A). Molecular analysis has shown that these taxa formed a strongly supported (pp 1.00, bp 100%) clade (G) separate from other *Euglena* species in clade A and the single taxon *Euglena proxima*. The sister relationships among the members of this new genus were equally well supported (pp 1.00, bp 100%). The relationship of *Euglenaria* (clade G) to *Monomorphina* (clade E) and *Cryptoglena* (clade F) was well supported by Bayesian (pp 1.00), but weakly supported by ML (bp 64%) analyses.

Clades H–J

Lepocinclis: *Lepocinclis* formed a single well supported clade H (pp 1.00, bp 100%). The sister relationships among the six species within this clade were all strongly supported (pp 1.00, bp 100%), except for the sister relationship between *L. spirogyroides* and *L. acus* that had only weak ML support (bp 67%). The sister relationship between *Lepocinclis* (clade H) and *Phacus* (clade I) was well supported (pp 1.00, bp 100%).

Phacus: Clade I was well supported by the single Bayesian model (pp 0.96) but had the weakest support of all the major clades (pp 0.82, bp 72%) from partition Bayesian and ML analyses. This clade contained 15 members of *Phacus*, which included the new member *P. limnophila*. The placement of

P. limnophila, was intriguing because of its spindle-shaped morphology, but was well supported (pp 1.00, bp 96%) as a sister to *P. warszewiczii* (cells triangular in apical view). Many of the branches in clade I were short with Bayesian and ML support ranging from no support (pp < 0.90, bp < 50%) to well supported (pp 1.00, bp 79%). The *Phacus* in clade I1 were strongly supported (pp 1.00, bp 92%) as a group, as were their sister relationships (pp 1.00, bp 100%), except for *P. ankylonoton* (pp 0.89 and 0.84, bp 64%).

Discoplastis: This genus diverges prior to the *Lepocinclis* and *Phacus* clades with strong Bayesian, but weak ML support (pp 0.96 and 0.99, bp 67%). The two members of this genus were well supported (pp 1.00, bp 100%) in clade J as sister taxa.

Taxonomic Revisions

Euglenaria Karnkowska, Linton et Kwiatowski, gen. nov.

Cellulae libere viventes, solitariae, cum uno flagello emergenti ubi natans, claviformes, fusiformes vel cylindrico-fusiformes, metabolicae, angustatae postice, gradatim decrescentes in caudam acutam; chloroplasti parietales, lobati, pyrenoidum singulum continentes; pilei paramylacei utrinque chloroplasto insidentes; mucocystae nullae.

Diagnosis

Cells free-living, solitary with one emergent flagellum when swimming; club-shaped, fusiform or cylindrically-fusiform, narrowing to the posterior and tapering into a pointed tail-piece; metabolic, display euglenoid movement; parietal, lobed chloroplasts, each with a single pyrenoid accompanied by bilateral paramylon caps located on both sides of the chloroplast (diplopyrenoid); mucocysts absent.

Type species:

Euglenaria caudata (Hübner) Karnkowska et Linton, comb. nov.

Basionym:

Euglena caudata 1886. Hübner, E.F.W. Euglenaceen-Flora von Stralsund. Program Realgymnasiums zu Stralsund. p. 13, Fig. 15.

Euglenaria caudata var. *minor* (Deflandre) Karnkowska et Linton, comb. nov.

Basonym:

Euglena caudata var. *minor* Deflandre, Bull. Soc. Bot. de France. 71: 1119, Fig. 7, 1924.

Euglenaria clavata (Skuja) Karnkowska et Linton, comb. nov.

Basionym:

Euglena clavata Skuja 1948. Taxonomie des phytoplantons einigen Seen in Uppsala, Schweden. Symbolae Botanicae Upsaliensis 9(3): 189, 190, pl. 22, Figs. 2–5.

Euglenaria anabaena (Mainx) Karnkowska et Linton, comb. nov.

Basionym:

Euglena anabaena 1926. Mainx, F. Einige neue Vertreter der Gattung *Euglena* Ehrenberg mit Unterstützung der Gesellschaft zur Förderung deutschen Wissenschaft, Kunst und Literatur in Böhmen. Arch. Protistenkd 54: 160-161, Fig. Dc.

Euglenaria anabaena var *minima* (Mainx) Karnkowska et Linton, comb. nov.

Basionym:

Euglena anabaena var *minima* 1927. Mainx, F. Beiträge zur Morphologie und Physiologie der Euglenen. I. Morphologische Beobachtung, Methode, und Erfolge der Reinkultur. II. Untersuchung über die Ernährungs- und Reizphysiologie. Arch. Protistenkd 60:334, pl. 10, Figs. 3, 4.

The name, *Euglenaria*, was derived from the genus *Euglena* and the Greek, "-aria" n. meaning like. Thus, these taxa were morphologically indistinguishable from members of *Euglena*, but were genetically distinct based on phylogenetic analysis. Moreover, in the nSSU alignment all members of *Euglenaria* had a C base, four bases upstream of helix 18′, where all other ingroup euglenoids and the two *Eutreptiella* species have an A, while *Eutreptia viridis* has a G (Fig. 1A). Furthermore, in the nSSU alignment all members of *Euglenaria* had a T, one base upstream of helix 38, while all members of *Euglena* had either an A or a C (Fig. 1B). The T base at this position was found in all other ingroup taxa except for *Phacus hamelii* and *P. segretti*, which had the C base. The base C at this position was also found in all three outgroup taxa suggesting that C is the primitive (plesiomorphic) state. Together, the C base upstream of helix 18′ and the T base upstream of helix 38 separate the *Euglenaria* from members of *Euglena* and the other ingroup euglenoids.

Phacus

The genus *Phacus* was created and diagnosed by Dujardin (1841) as rigid, free-swimming cells that were more or less flattened. Recently Marin et al. (2003) emended this diagnoses to more accurately define *Phacus* as a genus. However, due to *Lepocinclis salina* and *Euglena limnophila* being transferred into *Phacus* (see below), an emended diagnosis of the genus was again required.

Phacus Dujardin, Historie naturelle des Zoophytes-Infusoires. Paris. pp. 327, 334, 1841. Emend. Linton et Karnkowska

Phacus – Emended Diagnosis

Cells free-living, solitary with a single emergent flagellum when swimming; usually laterally compressed (rarely not compressed or triangular in apical view), spindle-shaped or ovoid; semi-rigid to rigid; numerous parietal disc-shaped chloroplasts of similar size without pyrenoids; paramylon grains dimorphic in size, large ring-shaped, rod-shaped or discoid paramylon grains. Colorless forms known.

Phacus salina (Fritsch) Linton et Karnkowska, comb. nov.

Basionym:

Lepocinclis salina 1914. Fritsch, F.E. Notes on British Flagellates, I-IV. The New Phytologist, vol. 13, Iss. 10. pg. 351, Fig. 3A, B.

Synonyms:

Lepocinclis texta var. *salina* (Fritch) Popova, Trudy Botanicheskogo Instituta Akademii Nauk SSSR. 7: 301, pl. 11 Fig. 18, 1951; *Euglena texta* var. *salina* (Fritch) Popova, Euglenovyje vodorosli. Opredelitel prosnovodnych vodoroslej SSSR, 7. [Euglenophyta. The Handbook of Freshwater Algae]. pg. 159, Fig. 59, 1955.

Phacus limnophila (Lemmermann) Linton et Karnkowska, comb. nov.

Basionym:

Euglena limnophila Lemmermann, Beiträge zur Kenntnis der Planktonalgen. II. Beschreibung neuer Formen. Beihefte Botan. Centralbl. 76: pg 152 and Lemmermann in Pascher's Süssw. – Fl., 2: 130, Fig. 205, 1913.

Phacus was distinguished from *Lepocinclis* based on phylogenetic analysis and molecular differences present in the nSSU alignment between the two genera. The base T was present one base downstream of helix 15 and two bases downstream of helix 23′ in all *Lepocinclis* whereas all *Phacus* had the base C and A, respectively.

Discussion

Euglena

Past studies of euglenoid phylogenies have used only nuclear encoded genes. Most often this was the nuclear SSU rDNA (Busse and Preisfeld 2002, 2003; Busse et al. 2003; Leander et al. 2001; Linton et al. 1999, 2000; Marin et al. 2003; Preisfeld et al. 2000, 2001; Von der Heyden et al. 2004) with one study (Talke and Preisfeld 2002) using the nuclear encoded flagellar PAR1 and PAR2 genes, the results from which agreed with previous nuclear SSU rDNA studies. The first non-nuclear gene study used chloroplast SSU rDNA sequence data from 17 photosynthetic species (Milanowski et al. 2001) and suggested polyphyly in *Euglena*, but the bootstrap support was weak.

More recent molecular studies have combined two genes. The first by Milanowski et al. (2006) combined nuclear and chloroplast SSU rDNA sequence data from 35 ingroup and 2 outgroup taxa to conclude, among other things, that *Euglena* was a polyphyletic genus with members present within four separate clades (A, B+C+D+, E and G) of their tree. Clade A contained most members of *Euglena* including the type specimen (*E. viridis*), B+C+D+ contained *Euglena anabaena,* E contained *E. proxima* while G contained *Euglena spathirhyncha* and *E. limnophila*. The second study by Triemer et al. (2006) combined nuclear SSU and partial LSU rDNA sequence data to establish a well resolved and supported phylogeny of Euglenales (sensu Leedale). Their analysis of 84 taxa supported 9 ingroup genera and established the new genus *Discoplastis* to contain *D. spathirhyncha* and *D. adunca*, two former members of *Euglena*. However, their study did not include *E. proxima* or *E. limnophila* as in Milanowski et al. (2006), but it did include *Euglena anabaena* and three sister taxa that formed an "*anabaena*" clade. This "*anabaena*" clade was supported (pp 0.92, Fig. 1 Triemer et al. 2006) as sister to the other members of *Euglena*. In summary, *Discoplastis* was erected to contain some former members of *Euglena* (Triemer et al. 2006) and establish the monophyly of the genus. Unfortunately, Milanowski et al. (2006) showed that at least three additional members of *Euglena* (*E. proxima*, *E. limnophila* and the "anabaena" clade) would require either the erection of a new genus or the transfer to an existing genus to fully establish the monophyly of *Euglena*. By combining a larger number of taxa and three genes, this study has resolved two of the three remaining taxonomic issues necessary to establish the monophyly of *Euglena*.

This study has shown that other than most members having a spindle shaped body, no derived (synapomorphic) morphological or molecular character capable of uniting members of *Euglena* has yet been identified. However, some important characteristics of this genus were identified. The chloroplasts show more morphological diversity than any other genus, ranging from absent to ribbon-like to lenticular to lobed or discoid with pyrenoids present in the chloroplast of some species but absent in others. Moreover the mucocysts, mucus secreting bodies below the pellicle strips, show great morphological diversity.

They may be absent or present, spherical or spindle in shape (Kosmala et al. 2009).

Because of the morphological diversity in *Euglena*, the species *E. proxima* (with chloroplasts that are discoid and lack pyrenoids) cannot be separated from *Euglena* (clade A) nor joined to the *Euglenaria* (clade G), because it lacks the diagnostic nSSU bases (see diagnosis above). At this time *E. proxima* must remain as a single unassociated taxon until additional data (sequence and/or taxa) cause it to either clade with an existing genus or other taxa clade with it allowing the diagnosis of a new genus.

Regrettably, despite the erection of *Euglenaria* (above) and the transfer of *Euglena limnophila* to *Phacus* the genus *Euglena* remains paraphyletic. Clade A contains the type *Euglena viridis* and thus retains the name *Euglena*.

Trachelomonas/*Strombomonas*/*Colacium*

A great deal of debate has occurred recently (Brosnan et al. 2003, 2005; Ciugulea et al. 2008; Marin et al. 2003; Moreira et al. 2001; Müllner et al. 2001; Nudelman et al. 2003; Triemer et al. 2006) about the existence of *Strombomonas* (clade C) and whether to dissolve it back into *Trachelomonas* (clade B). While all of these molecular studies supported the monophyly of the loricate forming genera (Brosnan et al. 2003; Marin et al. 2003; Moreira et al. 2001; Müllner et al. 2001; Nudelman et al. 2003) most failed to sustain *Trachelomonas* and *Strombomonas* as distinct genera (significant pp or bp support). All of these studies suffered from limited taxa sampling, a weakness recognized by many of the authors (Brosnan et al. 2003; Moreira et al. 2001; Müllner et al. 2001; Nudelman et al. 2003). Fortunately, the two most recent molecular studies (Ciugulea et al. 2008; Triemer et al. 2006) used a larger number of taxa and two genes (nuclear SSU/LSU rDNA), which showed significant support for maintaining these as distinct genera.

The morphological diversity of the lorica has been the chief diagnostic character essential for genus and species identification within these two genera. Our analysis has shown *Trachelomonas* to be composed of three clades (B1, B2 and B3) that can be distinguished morphologically by the lorica and chloroplast. Species with spiny loricas, and large disc-shaped plastids with diplopyrenoids are contained in clade B1, except for *T. abrupta* with inner projecting haplopyrenoids. Clade B2 contained species that are small and round with two to five or more chloroplasts, five being the most common, all with inner projecting haplopyrenoids. Clade B3 contained species that were oblong in shape with irregular surface ornamentation and up to ten chloroplasts, all with inner projecting haplopyrenoids. Since all taxa in clades B2, B3 and *T. abrupta*, the basal member of B1, contain inner projecting haplopyrenoids; this would be the plesiomorphic state. These results are in agreement with the five *Trachelomonas* clades of Ciugulea et al. ((2008) A–E, Fig. 1).

The four members of *Strombomonas* formed a single clade (C) that was well supported and distinct from its sister *Trachelomonas* (clade B) in our study. The lorica lacks the distinctive spines and pores associated with the loricas of *Trachelomonas*. Although, our study contained only four taxa compared to the eight of Ciugulea et al. ((2008) F–H, Fig. 1) with three taxa from their clade F and one from their clade H (*S. ovalis*), our results are congruent with theirs. We expect future work involving molecular and morphological data to enable a much-needed revision of the current *Trachelomonas* and *Strombomonas* taxonomy based on lorica morphology alone.

The two members of *Colacium* anchored the profuse mucilage producing genera in our study. Although *Colacium* species do not produce loricas they do produce copious amounts of mucilage from their anterior end. This mucilage is used for attachment to a substrate and subsequent stalk, and in some species, colony formation (Rosowski and Kugrens 1973; Rosowski and Willey 1977). Moreover, like most *Strombomonas* and the basal members of *Trachelomonas*, all species of *Colacium* possess inner projecting haplopyrenoids. Our results are in agreement with recent molecular studies (Ciugulea et al. 2008; Triemer et al. 2006) that support *Colacium* as the basal clade to copious mucilage producing genera. Notably, this is the first study to support the anchor position of *Colacium* by ML analysis, although the bootstrap support was weak (59%).

Monomorphina/*Cryptoglena*

Five members representing three species of *Monomorphina* were used in our analyses. All members of *Monomorphina* are diagnosed sensu Kosmala et al. (2007) as being ovoid or slightly oblate and rigid; a single parietal, spherical chloroplast that is often perforated, 2–4 prominent lateral paramylon plates between the pellicle and the chloroplast with *M. pyrum* as the epitype. Furthermore, based on molecular and morphological evidence Kosmala et al. (2007) synonymized

11 species of *Monomorphina* as *M*. *pyrum* and erected one new species *M*. *pseudopyrum* for the culture CCAC 0093 (originally misidentified as *M*. *reeuwykiana* by Marin et al. 2003). Our study supports their changes and we have followed their recommendations; *M*. *inconspicuus* (ACOI 1295) and *M*. *ovata* (SAG 1244-5) are now considered as *M*. *pyrum*. Additionally, our study supports *Monomorphina* (clade E) as being monophyletic and sister to *Cryptoglena* (clade F).

The members of *Cryptoglena* are similar to *Monomorphina*; cells ovoid, rigid with a single parietal chloroplast and lateral, shield-shaped paramylon grains, pellicle lacking strip reduction. However, these genera have been shown in previous molecular studies (Ciugulea et al. 2008; Kosmala et al. 2007; Marin et al. 2003; Milanowski et al. 2006; Triemer et al. 2006) to be closely related, but distinct genera. Additionally, based on the amended diagnosis (Kosmala et al. 2007) *Cryptoglena* differ by lacking the strong pellicular ridges found in *Monomorphina*, possessing a median longitudinal furrow and having a chloroplast in the form of an open cylinder (C-shaped) lacking pyrenoids. These traits distinguish *Cryptoglena* from *Monomorphina*. Our study supports the sister relationship between these genera and the sister relationship to the newly erected genus *Euglenaria*.

Euglenaria

Members of this genus were formerly placed in the genus *Euglena* and were morphologically indistinguishable from members of that genus. This position was supported by molecular analyses, such as Marin et al. (2003) and Triemer et al. (2006) as well. However, molecular analysis by Milanowski et al. (2001, 2006) suggested that *Euglena anabaena* was not a member of *Euglena*. But these studies had only one member of what is now the *Euglenaria* clade (G) *Euglena anabaena*, so no reassignment of the taxon could have been made. Our study included five taxa in clade G, which has allowed an evaluation of both the morphological and molecular evidence. Morphologically uniting features of *Euglenaria* are the parietal lobed chloroplast with diplopyrenoids and absence of mucocysts. However, these morphological features do not differentiation *Euglenaria* from *Euglena*. Instead, the molecular nSSU characters are the diagnostic characters that separate *Euglenaria* from *Euglena*. While all *Euglenaria* have a C base upstream of helix 18′ and a T base upstream of helix 38, all *Euglena* have an A base upstream of helix 18′ and a C or A base upstream of helix 38. Furthermore, both Bayesian and ML analyses supported the separation of these five taxa from *Euglena* and their placement as sister to the *Cryptoglena* and *Monomorphina* clades. Therefore, there is strong evidence and support for the erection of the new genus *Euglenaria* and the transfer of five former members of *Euglena* into it.

Lepocinclis/*Phacus*

Six members of *Lepocinclis* and 15 members of *Phacus* were included in our study. Cells are rigid or semi-ridged, sometimes exhibiting a slight bending or twisting motion, but none are metabolic as defined by Triemer et al. (2006). The cells contain numerous small discoid chloroplast that lack pyrenoids. This diagnosis could be applied equally to both *Phacus* and *Lepocinclis*. The major distinguishing characteristic was that all members of *Phacus* were laterally compressed or flattened, while all members of *Lepocinclis* were not flattened in cross section. However, our study has resulted in the transfer of *Lepocinclis salina* and *Euglena limnophila* into the genus *Phacus* requiring the diagnosis to be emended to include cells that are spindle-shaped and ovoid. Therefore, being flattened is no longer a defining characteristic separating *Phacus* from *Lepocinclis*.

What evidence is there for continuing this separation and not dissolving *Lepocinclis* (Perty 1849) into *Phacus* (Dujardin 1841)? Neither this nor any previous molecular phylogenetic study has shown merging between these two genera; all have shown them to be separate, well supported clades. In addition, we have defined molecular synapomorphies (shared derived) that distinguish these genera from each other. In the nSSU alignments *Lepocinclis* species have the nucleotide T one base downstream of helix 15 and two bases downstream of helix 23′. At these same positions in *Phacus* the nucleotides C and A are present, respectively. We therefore recognize these as two closely related sister clades and recommend maintaining them as separate distinct genera.

Discoplastis

Erected by Triemer et al. (2006), the two members of *Discoplastis* contain numerous small discoid chloroplasts lacking pyrenoids and cells that are not flattened, characteristics found in the closely

related *Lepocinclis* and *Phacus* genera. However, *Discoplastis* is easily distinguished from the other two genera by being strongly metabolic, a characteristic not found in either *Lepocinclis* or *Phacus*. Our phylogenetic analysis of the molecular differences among these genera supports this genus and its basal position to the *Lepocinclis* and *Phacus* genera.

Our study was the first euglenoid phylogeny to use three genes, two nuclear and one chloroplast to establish relationships among freshwater photosynthetic euglenoids. This study showed strong support by both Bayesian and ML analysis for 10 major ingroup clades with 10 corresponding genera and the single unallied taxon *Euglena proxima*. Our analysis resulted in the transfer of two taxa (*Lepocinclis salina* and *Euglena limnophila*) to the genus *Phacus* and the concomitant emended diagnosis of *Phacus* to contain cells that were not flattened. This represents the first change in the defining morphological characteristic, cell flattened, of *Phacus* since Dujardin established it in 1841. The polyphyletic genus *Euglena* was moved closer towards monophyly by the erection and transfer of five taxa to the new genus *Euglenaria* along with the transfer of *Euglena limnophila* to the genus *Phacus*. Unfortunately the unallied, but strongly supported, position of *Euglena proxima* remains and consequently maintains *Euglena* as a paraphyletic genus.

Methods

Cultures: Table 1 lists the 82 taxa with sources and GenBank accession numbers used in this study. Alternative name(s) in culture collections or GenBank for the taxa used in this study, due to misidentification or taxonomic changes, are also given in Table 1. All taxa were obtained from culture collections or were collected from small ponds in New Jersey (NJ) and Michigan (MI), USA. Cultures NJ and MI used in this study, which are not available from public culture collections, will be made available upon request. Ponds were sampled with a plankton net (mesh size, 20 μm) and euglenoid cells isolated by a Pasteur capillary pipette. Cells were brought into unialgal culture and checked periodically for eukaryotic contaminants. All isolates were grown in modified AF-6 medium (Watanabe and Hiroki 1997) and/or soil–water medium (medium 3c, Schlösser 1994). Cultures were maintained at 20–22 °C under conditions of a 14:10 light:dark cycle with approximately 30 μmol photons m^{-2} s^{-1} provided from cool white fluorescent tubes.

Specimen identification: Identifications of cultures were confirmed microscopically when received using a Zeiss Axioskop 2 Plus microscope (Carl Zeiss Inc., Hallbergmoos, Germany) or a Nikon Eclipse E-600 microscope (Nikon, Tokyo, Japan) both equipped with differential interference contrast (DIC) optics. Images were captured with an AxioCam HRC (Hallbergmoos) or Nikon DX-1200 photomicrographic system attached to the microscope, respectively. A representative species of each clade in the context of their phylogenetic relationships is shown in Figure 3.

DNA extraction, amplification, sequencing, and sequence alignment: DNA extraction, PCR amplification procedures, purification, sequencing, and sequence alignment were conducted as described by Brosnan et al. (2003). Total genomic DNA was isolated from cultures with the DNeasy Tissue Kit (Qiagen Co., Valencia, CA, USA; Cat #69504) using the animal tissues protocol. The nSSU, nLSU and cpSSU rDNA sequences were amplified in 20-μL reactions. Amplification was performed using; GeneAmp PCR System 9700 (Perkin Elmer Co., Norwalk, CT, USA), MJ Research PTC-0200 DNA Engine Gradient Thermo Cycler (MJ Research Inc., Waltham, MA, USA), Mastercycler Personal and Mastercycler Gradient (Eppendorf AG, Hamburg, Germany), see Table 2 for PCR programs used. The PCR products were sized on 1% agarose gels and then purified using the MiniElute Gel Extraction Kit (Qiagen Co.; Cat #28606) or QIAEXII Gel Extraction Kit (Qiagen Co.; Cat#20051) according to the manufacturer's protocol. The purified template was sequenced with internal primers for conserved regions (nSSU, Linton et al. 2000 and Table 3; nLSU, Brosnan et al. 2003; cpSSU Table 3) using either an ABI 377, 3700, 3730 or 3730*xl* dye terminator sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were assembled into contigs by the SeqMan program from the LASERGENE package (DnaStar, Madison, WI, USA) or using the Contig Assembly Program (CAP2) in the Genetic Data Environment (GDE 2.2) program (Smith et al. 1994). The sequences for the nSSU, nLSU and cpSSU rDNAs were aligned by eye using the secondary structure of *Euglena gracilis* Klebs as a guide (Kjer 1995; Wuyts et al. 2001) in GDE 2.2. The conserved areas of the genes were readily alignable across taxa and were used for phylogenetic analyses. Areas that could not be unambiguously aligned were excluded from analyses. All three gene sequences used in this study were derived from the same culture when possible (Table 1). When not possible due to culturing or sequencing difficulties alternative sources were used, the taxon identities were confirmed microscopically and by sequence comparisons among the first 700 bases of the nSSU sequences.

Phylogenetic analysis: The three genes resulted in a combined dataset of 5161 bases: 1740 from nSSU rDNA, 2110 from nLSU rDNA and 1311 from cpSSU rDNA for analysis. All analyses were done from within MacGDE 2.3. The Bayesian Information Criterion (BIC) analysis using MODELTEST 3.7 (Posada and Crandall 1998) was used to determine the best model for each dataset (nSSU, nLSU and cpSSU) for use in partition Bayesian analysis while a single model based on the BIC was determined for the combined data for use in Bayesian and Maximum Likelihood (ML) analysis. Trees were rooted using *Eutreptia viridis, Eutreptiella braarudii* and *Eutreptiella pomquetensis* as outgroup taxa. The three gene data set and tree (Fig. 2) have been deposited in TreeBASE, study accession number is S2655 and the matrix accession number is M5103.

Bayesian analyses: The analyses were performed in Mr. Bayes 3.1.2 (Ronquist and Huelsenbeck 2003) using a single model for the data or with an individual model for each of the three data partitions. Parameters for the single model were as follows (GTR+I+Γ): Prset revmatpr=dirichlet (1.49, 3.86, 1.63, 0.65, 5.78, 1.00) statefreqpr=dirichlet (0.23, 0.24, 0.29, 0.23) shapepr=exponential (0.79) pinvarpr=fixed (0.30). Parameters for the partitioned datasets were as follows (GTR+I+Γ): nSSU

Table 1. Taxa used with culture collection and GenBank accession codes.

	Culture for sequencing [a]			GenBank accession number		
Taxon	nSSU	nLSU	cpSSU	nSSU	nLSU	cpSSU
Colacium calvum Stein	MI 107	MI 107	MI 107	**EF999907**	**EF999910**	**EU624035**
C. mucronatum Bourrelly *et* Chadefaud	SAG 1211-1 (*C. sideropus*)	SAG 1211-1 (*C. sideropus*)	SAG 1211-1 (*C. sideropus*)	AJ532441	**EF999906**	**EU221482**
Cryptoglena pigra Ehrenberg	CCAP 1212/1	CCAP 1212/1	CCAP 1212/1	AJ532437	DQ140101	**EU221483**
C. skujae Marin *et* Melkonian	SAG 10.88 (*Phacus agilis*)	SAG 10.88 (*Phacus agilis*)	SAG 10.88 (*Phacus agilis*)	AY014998	AY130236	**EU221484**
Discoplastis adunca (Schiller) Triemer	ASW 08039 (*Euglena* cf. *adunca*)	ASW 08039 (*Euglena* cf. *adunca*)	ASW 08095 (*Euglena* cf. *adunca*)	AJ532453	DQ140102	**EU750704**
D. spathirhyncha (Skuja) Triemer	SAG 1224-42 (*Euglena spathirhyncha*)	SAG 1224-42 (*Euglena spathirhyncha*)	SAG 1224-42 (*Euglena spathirhyncha*)	AJ532454	DQ140100	AY626060
Euglena adhaerens Matvienko	ASW 08138	ASW 08138	ASW 08138	**EU750713**	**FJ486278**	**EU750706**
E. agilis Carter	OR 04	OR 04	OR 04	AF115279	**FJ377538**	**EU624033**
E. cantabrica Pringsheim	SAG 1224-25	SAG 1224-25	SAG 1224-25	AJ532412	AY523020	AY626047
E. clara Skuja	SAG 25.98	SAG 25.98	SAG 25.98	AJ532423	DQ140114	**EU750707**
E. deses Ehrenberg	SAG 1224-23 (*E. limnophila*)	SAG 1224-23 (*E. limnophila*)	SAG 1224-23 (*E. limnophila*)	AJ532407	DQ140122	**EU750709**
E. geniculata Dujardin	SAG 1224-4f	SAG 1224-4f	SAG 1224-4f	AY070248	**EU624022**	AY070252
E. geniculata Dujardin	SAG 1224-21 (*E. viridis*)	SAG 1224-21 (*E. viridis*)	SAG 1224-4b	AY523034	AY523027	AF289241
E. gracilis Klebs	SAG 1224-5/15	UCLA variety	SAG 1224-5/15	M12677	X53361	X12890
E. granulata (Klebs) Schmitz	UTEX 2345 (*E. sanguinea*)	UTEX 2345 (*E. sanguinea*)	UTEX 2345 (*E. sanguinea*)	AJ532422	DQ140099	**EU370511**
E. gymnodinioides Zakryś	MI 03	MI 03	MI 03	DQ140148	DQ140105	**EU221486**
E. laciniata Pringsheim	SAG 1224-8c (*E. granulata*)	SAG 1224-8c (*E. granulata*)	SAG 1224-8c (*E. granulata*)	AJ532421	AY523021	**EU221488**
E. laciniata Pringsheim	SAG 1224-31	SAG 1224-31	SAG 1224-31	AJ532420	AY523022	**EU221487**
E. longa (Pringsheim) Marin *et* Melkonian	UTEX 512 (*Astasia longa*)	UTEX 512 (*Astasia longa*)	CCAP 1204/17a (*Astasia longa*)	AF112871	AY130223	AJ294725
E. mutabilis Schmitz	NJ	NJ	Sandy	AY523038	DQ140124	**EU221489**
E. proxima Dangeard	SAG 1224-11a	SAG 1224-11a	SAG 1224-11a	**EU624027**	**EU624017**	**FJ374877**
E. pseudoviridis Chadefaud	SAG 1224-17c (*E. viridis*)	SAG 1224-17c (*E. viridis*)	SAG 1224-17c (*E. viridis*)	AY523037	DQ140125	**EU370498**
E. satelles Braslavska-Spectorova	SAG1224-22 (*E. deses*)	SAG1224-22 (*E. deses*)	SAG1224-22 (*E. deses*)	AJ532406	**EU624016**	**EU373477**
E. sociabilis Dangeard	ACOI 920	ACOI 920	ACOI 920	**EU750715**	**EU624024**	**EU750710**
E. species	MI 32	MI 32	MI 32	DQ140158	DQ140123	**EU221490**
E. species	SAG 1224-12b (*E. sociabilis*)	SAG 1224-12b (*E. sociabilis*)	SAG 1224-12b (*E. sociabilis*)	DQ140149	DQ140106	**EU221491**
E. splendens Dangeard	MI 47	MI 47	MI 47	DQ140150	DQ140107	**EU221492**
E. stellata Mainx	MI 09	MI 09	MI 09	AY523032	AY523024	**EU221493**
E. stellata Mainx	UTEX 372	UTEX 372	SAG 1224-14	AF150936	AY130229	AF289244
E. tristella Chu	NJ	NJ	SAG 1224-35	DQ140151	DQ140109	AF289246

Table 1. (*continued*)

	Culture for sequencing [a]			GenBank accession number		
Taxon	nSSU	nLSU	cpSSU	nSSU	nLSU	cpSSU
E. viridis epitype Ehrenberg	ATCC PRA110	ATCC PRA110	ATCC PRA110	AY523036	AY523031	**EU221494**
Euglenaria anabaena (Mainx) Karnkowska et Linton	UTEX 373	UTEX 373	SAG 1224-15b	AF242548	DQ140115	AF289240
E. anabaena var. *minima* (Mainx) Karnkowska et Linton	NJ 05	NJ 05	NJ 05	DQ140155	DQ140119	**EU221485**
E. caudata (Hübner) Karnkowska et Linton	ASW 08048	ASW 08048	ASW 08048	**EU750714**	**EU624010**	**EU750698**
E. caudata var. *minor* (Deflandre) Karnkowska et Linton	SAG 1224-26a (*E. caudata*)	SAG 1224-26a (*E. caudata*)	SAG 1224-26a (*E. caudata*)	DQ140147	DQ140103	**FJ374877**
E. clavata (Skuja) Karnkowska et Linton	SAG 1224-32 (*Euglena polymorpha*)	SAG 1224-32 (*Euglena polymorpha*)	NJ CP	AJ532436	DQ140104	**EU750708**
Eutreptia viridis Perty	SAG 1226-1c	SAG 1226-1c	SAG 1226-1c	AJ532395	DQ140108	AF289247
Eutreptiella braarudii Throndsen	CCMP 1594	CCMP 1594	CCMP 1594	DQ249879	**EU624026**	DQ249873
Eutreptiella pomquetensis (McLachlan, Seguel *et* Fritz) Marin *et* Melkonian	CCMP 1491	CCMP 1491	CCMP 1491	AJ532398	**EU624012**	**EU750699**
Lepocinclis acus var. *major* (Müller) Marin *et* Melkonian	UTEX 1316 (*Euglena acus* var. *major*)	UTEX 1316 (*Euglena acus* var. *major*)	SAG 1224-1b (*Euglena acus*)	AF152104	AY130226	**EU221495**
L. fusiformis (Carter) Lemmermann	ACOI 1025	ACOI 1025	ACOI 1025	AY935697	**EU624011**	AF289249
L. ovum (Ehrenberg) Minkevich	SAG 1244-8	SAG 1244-8	SAG 1244-8	AF110419	AY130235	AY221726
L. spirogyroides Marin *et* Melkonian	SAG 1224-10b (*Euglena oxyuris*)	SAG 1224-10b (*Euglena oxyuris*)	SAG 1224-10b (*Euglena oxyuris*)	AJ532464	AY130814	AF289243 (*Euglena spirogyra*)
L. steinii Lemmermann	UTEX 523 (*L. buetschlii*)	UTEX 523 (*L. buetschlii*)	UTEX 523 (*L. buetschlii*)	AF096993	AY130815	**EU221496**
L. tripteris (Dujardin) Marin *et* Melkonian	UTEX 1311 (*Euglena tripteris*)	UTEX 1311 (*Euglena tripteris*)	SAG 1224-16	AF445459	AY130230	**EU221497**
Monomorphina aenigmatica (Dreżepolski) Nudelman *et* Triemer	UTEX 1284 (*Phacus megalopsis*)	UTEX 1284 (*Phacus megalopsis*)	CCAP 1261/9 (*Phacus aenigmaticus*)	AF190814	DQ140117	AY626054 (*Monomorphina striata*)
M. pseudopyrum Kosmala *et* al.	CCAC 0093 (*M. reeuwykiana*)	CCAC 0093 (*M. reeuwykiana*)	CCAC 0093 (*M. reeuwykiana*)	AJ532433	**EF999909**	**EU750700**
M. pyrum (Ehrenberg) Marin *et* Melkonian	SAG 1244-5 (*Lepocinclis ovata*)	SAG 1244-5 (*Lepocinclis ovata*)	SAG 1244-5 (*Lepocinclis ovata*)	AF061338	AY130234	**EU221500**
M. pyrum (Ehrenberg) Marin *et* Melkonian	ACOI 1295 (*Phacus inconspicuus*)	ACOI 1295 (*Phacus inconspicuus*)	ACOI 1295 (*Phacus inconspicuus*)	DQ140129	DQ140111	**EU221499**
M. pyrum (Ehrenberg) Marin *et* Melkonian	UTEX 2354 (*Phacus pyrum*)	UTEX 2354 (*Phacus pyrum*)	UTEX 2354 (*Phacus pyrum*)	AF112874	AY130238	**EU624034**
Phacus acuminatus Stokes	SAG 1261-7 (*P. brachykentron*)	UTEX 1317 (*P. brachykentron*)	SAG 1261-7 (*P. brachykentron*)	AJ532481	AY130820	**EU221501**
P. ankylonoton Pochmann	CCAC 0043 (*P. ranula*)	CCAC 0043 (*P. ranula*)	CCAC 0043 (*P. ranula*)	AJ532484	**EU624020**	**EU624036**
P. applanatus Pochmann	ASW 08023	ASW 08023	ASW 08023	**EU624031**	**EU624015**	**EU750701**

P. hamelii Allorge *et* Lefèvre	ACOI 1088	ACOI 1088	ACOI 1088	DQ397673	**EU624019**	**EU221502**
P. limnophila (Lemmermann) Linton et Karnkowska	ACOI 1026 (*Euglena limnophila*)	ACOI 1026 (*Euglena limnophila*)	ACOI 1026 (*Euglena limnophila*)	DQ249877	**EU624025**	AY626056
P. orbicularis Hübner	AICB 502	MI 76	AICB 502	AY935698	**EU624018**	AY626057
P. parvulus Klebs	ASW 08060	ASW 08060	ACOI 1093 (*P. pusillus*)	AF283314	DQ140127	**EU221503**
P. pleuronectes (Müller) Dujardin	SAG 1261-3b	SAG 1261-3b	SAG 1261-3b	AJ532475	AY130824	AF289251
P. pusillus Lemmermann	UTEX 1282	UTEX 1282	UTEX 1282	AF190815	AY130237	**EU750702**
P. salina (Fritsch) Linton et Karnkowska	SAG 1244-3 (*Lepocinclis salina*)	SAG 1244-3 (*Lepocinclis salina*)	SAG 1244-3 (*Lepocinclis salina*)	**EU624028**	**EU624023**	AY221727
P. segretii Allorge *et* Lefèvre	ACOI 1337	ACOI 1337	ACOI 1337	**EU624030**	**EU624014**	**EU221504**
P. skujae Skvortzov	UTEX 1285 (*P. caudata*)	UTEX 1285 (*P. caudata*)	UTEX 1285 (*P. caudata*)	AF181968	AY130823	**EU750711**
P. smulkowskianus (Zakryś) Kusber	SAG 58.81 (*P. similis*)	SAG 58.81 (*P. similis*)	ACOI 1226 (*P. similis*)	AJ532467	AY130239	AY626059
P. triqueter (Ehrenberg) Dujardin	SAG 1261-8	SAG 1261-8	SAG 1261-8	AJ532485	**EU624013**	**EU221505**
P. warszewiczii Drežepolski	ASW 08064	ASW 08064	ASW 08064	**EU624032**	**EU624021**	**EU750703**
Strombomonas acuminata (Schmarda) Deflandre	NJ S716	NJ S716	SAG 1280-1 (*T. conspersa*)	**EU624029**	AY359914	**EU221506**
S. costata Deflandre	ACOI 1273	ACOI 1273	ACOI 2992	DQ140152	AY359915	AF289253
S. ovalis (Playfair) Deflandre	NJ S115	NJ S115	NJ S115	DQ140133	AY359919	**EU750712**
S. verrucosa (von Daday) Deflandre	NJ S5c	NJ S5c	ACOI 2476 (*S. acuminata*)	**EF999896**	AY359911	AY626051
Trachelomonas abrupta (Swirenko) Deflandre	NJ T801	NJ T801	NJ T801	DQ140134	AY359941	**EU221507**
T. bernardinensis Vischer	ACOI 1103	ACOI 1103	ACOI 1103	**EF999908**	AY359950	**EU221509**
T. echinata Singh	SAG 1283-22	SAG 1283-22	SAG 1283-22	AY015001	AY130242	**EU221510**
T. ellipsoidalis Singh	NJ ST1	NJ ST1	NJ ST1	DQ140135	AY359935	**EU221511**
T. hispida (Perty) Deflandre	UTEX 1325 (*T. oblonga* var. *punctata*)	UTEX 1326	UTEX 539 (*T. hispida var. coronata*)	AF445462	AY130817	**EU221513**
T. lefevrei Deflandre	SAG 1283-10	SAG 1283-10	SAG 1283-10	DQ140136	AY359949	**EU221514**
T. oblonga Lemmerman	NJ T516	NJ T516	NJ T516	DQ140137	AY359947	**EU221515**
T. planctonica Swirenko	NJ WT1	NJ WT1	NJ WT1	DQ140138	AY359954	**EU221516**
T. rugulosa Stein	NJ TS2	NJ TS2	NJ TS2	DQ140140	AY359942	**EU221517**
T. similis Stokes	SAG 1283-14	SAG 1283-14	SAG 1283-14	DQ140142	AY359948	AY626055
T. volvocina Ehrenberg	SAG 1283-4	UTEX 1327	UTEX 1327	AJ532451	AY359953	**EU221519**
T. volvocinopsis Svirenko	SAG 1283-16	SAG 1283-16	SAG 1283-16	DQ140144	AY359944	**EU221520**
T. zorensis Lefèvre	UTEX 1331	UTEX 1331	UTEX 1331	DQ140145	AY359952	**EU221521**

New or updated sequences are indicated in bold type.

[a]Strains labeled, ACOI, Coimbra Collection of Algae, Coimbra, Portugal; ASW, Algenkultur-Sammlung an der Universität Wien, Vienna, Austria; ATCC, American Type Culture collection, Manassa, VA, USA; CCAC, Culture collection of Algae at the University of Cologne, Germany; CCAP, Culture collection of Algae and Protozoa, Scotland; CCMP, Culture collection of Marine Phytoplankton, USA; MI, Michigan isolate Triemer lab, USA; NJ, New Jersey isolate Triemer lab, USA; SAG, Sammlung von Algenkulturen Pflanzenphysiologisches Institut der Universität Göttingen, Germany; UTEX Culture Center of Algae, Austin, TX, USA.

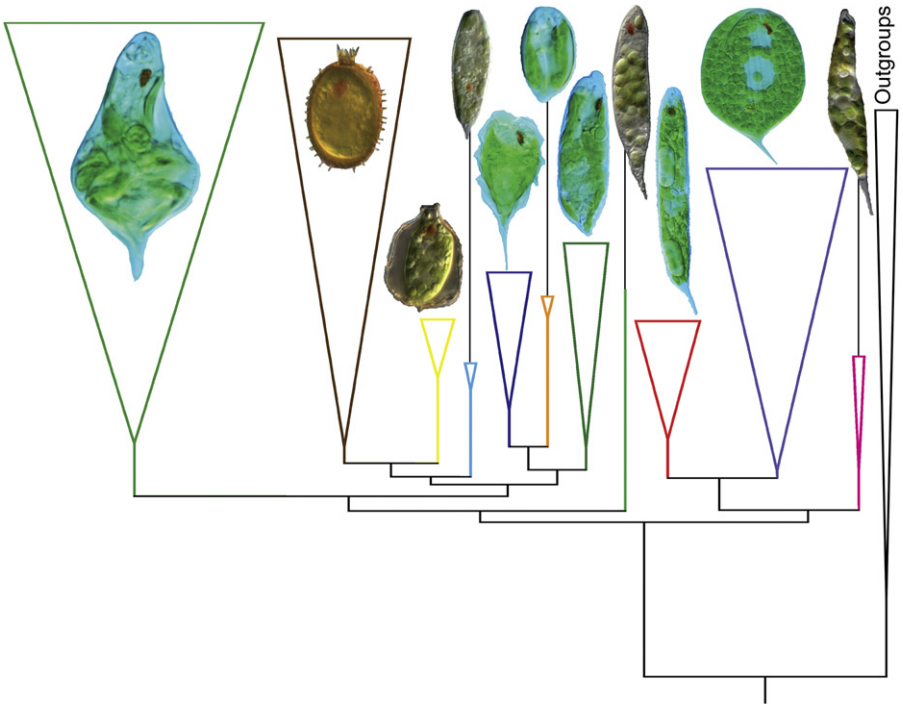

Figure 3. A rooted Bayesian phylogenetic tree, as in Figure 2 but with each of the major clades collapsed (indicated by a capital letter) and a representative taxon from that clade pictured (not to scale). Names of pictured taxa from left to right; *Euglena viridis*, *Trachelomonas hispida*, *Strombomonas verrucosa*, *Colacium mucronatum*, *Monomorphina pyrum*, *Cryptoglena skujae*, *Euglenaria anabaena*, *Euglena proxima*, *Lepocinclis spirogyroides*, *Phacus orbicularis*, *Discoplastis spathirhyncha*. The tree was outgroup rooted posteriorly with *Eutreptia* and *Eutreptiella* species (not shown).

Table 2. PCR reaction parameters.

General PCR parameters 96 °C 2 min, 35 cycles (95 °C 30 s, 37 °C – 56 °C 30 s, 72 °C 1 min) 72 °C 6 min, and a 4 °C hold
PCR parameters for cpSSUF/cpSSUR and cpSSU647F/cpSSUR 95 °C 5 min, 37 cycles (95 °C 1 min, 44 °C 2 min, 72 °C 2 min – 7 cycles and 95 °C 30 s, 54 °C 30 s, 72 °C 1 min – 30 cycles) 72 °C 10 min, and a 7 °C hold
PCR parameters for cpSSUF/cpSSU854R 95° C 5 min, 37 cycles (95° C 1 min, 48° C 2 min, 72° C 2 min – 7 cycles and 95° C 30 s, 56° C 30 s, 72° C 1 min – 30 cycles) 72° C 10 min, and a 7° C hold
PCR parameters for nSSUF/nSSU557R 95 °C 5 min, 39 cycles (95 °C 1 min, 48 °C 90 s, 72 °C 1 min – 7 cycles and 95 °C 30 s, 56 °C 30 s, 72 °C 45 s – 32 cycles) 72 °C 10 min, and a 7 °C hold
PCR parameters for nSSU382F/nSSU1263R 95 °C 5 min, 39 cycles (95 °C 1 min, 40 °C 90 s, 72 °C 90 s – 7 cycles and 95 °C 30 s, 49 °C 30 s, 72 °C 90 s – 32 cycles) 72 °C 10 min, and a 7 °C hold
PCR parameters for nSSU1154F/nSSUR 95 °C 5 min, 37 cycles (95 °C 1 min, 54 °C 1 min, 72 °C 45 s – 7 cycles and 95 °C 30 s, 59 °C 30 s, 72 °C 45 s – 32 cycles) 72 °C 10 min, and a 7 °C hold

Table 3. New primers used in this study.

Primer	Sequence (5'–3')
cpSSU-F	TTG ATC CTG GCT CAG GAT GAA CGC T
cpSSU-647F	ATT TCC AGT GTA GCG GTG
cpSSU-854R	AGG CGG GAC ACT TAA CGC GTT
cpSSU-R	CAA GGA GGT GAT CCA GCC GCA CCT T
nSSU-F	CAG TGG GTC TGT GAA TGG CTC C
nSSU-557R	TTA CCG CAG CTG CTG GC
nSSU-570F	GTG CCA GCA GCT GCG GT
nSSU-1141F	GAA ACT TAA AGG AAT TG
nSSU-1154F	GGA ATT GAC GGA ATG GCA CC
nSSU-1263R	GAG CGG CCA TGC ACC AC
nSSU-R	CGA CGG GCG GTG TGT ACA AGT

F=Forward Primer, R=Reverse Primer.

revmatpr=dirichlet (1.37, 3.16, 1.45, 0.59, 4.53, 1.00) statefreqpr=dirichlet (0.21, 0.29, 0.28, 0.22) shapepr=exponential (0.73) pinvarpr=fixed (0.28); nLSU revmatpr=dirichlet (1.70, 3.69, 1.80, 0.62, 6.23, 1.00) statefreqpr=dirichlet (0.21, 0.26, 0.33, 0.21) shapepr=exponential (0.82) pinvarpr=fixed (0.33); cpSSU revmatpr=dirichlet (1.00, 5.18, 1.00, 1.00, 8.85, 1.00) statefreqp=dirichlet (0.33, 0.13, 0.20, 0.34) shapepr=exponential (0.59) pinvarp r=fixed (0.24). The analysis used four Markov chains (4,000,000 generations per chain), with trees saved every 100 generations discarding the first 8,000 trees. A majority-rule consensus tree was created from the remaining 32,001 trees. Convergence among these trees was confirmed via the sump command.

Maximum likelihood analyses: The analysis was performed with PAUP* 4.0b10 for Macintosh OS X (Swofford 2002) using the following parameters (GTR+I+Γ): Base=(0.2275 0.2439 0.2943) Nst=6 Rmat=(1.4872 3.8611 1.6340 0.6481 5.7788) Rates=gamma Shape=0.7907 Pinvar=0.3016 Shape=0.7907 NCat=4 RepRate=Mean Initbrlen=Rogers STartvals=ParsApprox RECon=Marginal. The single ML tree was obtained by a heuristic search using random stepwise addition with 5 replicates, TBR branch swapping and MULTREES on. The 100 ML bootstrap repetitions were obtained by a Heuristic search using Random Stepwise addition with two replicates. The 100 non-parametric bootstraps were done as four simultaneous independent searches of 25 bootstraps, each run on a separate CPU. The trees were aggregated and weighted according to the number of trees found in each bootstrap replicate, so that all replicates had equal weight, a 50% majority rule consensus bootstrap tree was obtained.

Acknowledgements

The authors wish to acknowledge the financial support provided by the National Science Foundation PEET program (Partnership for Enhanced Expertise in Taxonomy, grant no. DEB 4-21348) and by the Ministry of Science and Higher Education grant no. N303 010 32/0552. We thank Prof. Tomasz Majewski, Warsaw, Poland, for providing a Latin diagnose for *Euglenaria gen. nov.*

References

Brosnan S, Brown PJ, Farmer MA, Triemer RE (2005) Morphological separation of the euglenoid genera *Trachelomonas* and *Strombomonas* based on lorica development and posterior strip reduction. J Phycol **41**: 590–605

Brosnan S, Shin W, Kjer KM, Triemer RE (2003) Phylogeny of the photosynthetic euglenophytes inferred from the nuclear SSU and partial LSU rDNA. Int J Syst Evol Microbiol **53**:1175–1186

Busse I, Preisfeld A (2002) Phylogenetic position of *Rhynchopus* sp. and *Diplonema ambulator* as indicated by analyses of euglenozoan small subunit ribosomal DNA. Gene **284**: 83–91

Busse I, Preisfeld A (2003) Systematics of primary osmotrophic euglenids: a molecular approach to the phylogeny of *Distigma* and *Astasia* (Euglenozoa). Int J Syst Evol Microbiol **53**:617–624

Busse I, Patterson DJ, Preisfeld A (2003) Phylogeny of phagotrophic euglenids ("Euglenozoa"): a molecular approach based on culture material and environmental samples. J Phycol **39**:828–836

Ciugulea I, Nudelman MA, Brosnan S, Triemer RE (2008) Phylogeny of the euglenoid loricate genera *Trachelomonas* and *Strombomonas* (Euglenophyta) inferred from nuclear SSU and LSU rDNA. J Phycol **44**:406–418

Dujardin F (1841) In: Histoire naturelle des Zoophytes. Infusoires Roret, Paris

Ehrenberg CG (1830a) Neue Beobachtungen über blutartige Erscheinungen in Ägypten, Arabien und Sibirien, nebst einer Übersicht und Kritik der früher bekannten. Pogg Annalen der Physik Chemie **18**:477–514

Ehrenberg CG (1830b) Beiträge zur Kenntnis der Organisation der Infusorien und ihrer geographischen Verbreitung, besonders in Sibirien. Abh Königl Akad Wissensch Berlin:1–88

Ehrenberg CG (1831) Über die Entwickelung und Lebensdauer der Infusionsthiere; nebst ferneren Beiträgen zu einer Vergleichung ihrer organischen Systeme. Abh Königl Akad Wissensch Berlin **1832**:1–154

Ehrenberg CG (1833) Dritter Beitrag zur Erkenntnis grosser Organisation in der Richtung des kleinsten Raumes. Abh Königl Akad Wissensch Berlin:145–336

Ehrenberg CG (1838) Die Infusionsthierchen als vollkommene Organismen. Ein Blick in das tiefere organische Leben der Natur. Nebst einem Atlas von vierundsechzig colorirten Kupfertafeln, gezeichnet vom Verfasser. Leipzig Verlag von Leopold Voss.

Fritsch FE (1914) Notes on British Flagellates, I-IV. New Phytol **vol 13** (Iss 10): 341–351

Gibbs S (1978) The chloroplast of *Euglena* may have evolved from symbiotic green algae. Can J Bot **56**:2883–2889

Hübner EFW (1886) Euglenaceen-Flora von Stralsund. Program Realgymnasiums zu Stralsund, pp 1–20

Ishida K, Cao Y, Hasegawa M, Okada N, Hara Y (1997) The origin of chlorarachniophyte plastids, as inferred from phylogenetic comparisons of amino acid sequences of EF-Tu. J Mol Evol **45**:682–687

Kjer KM (1995) Use of rRNA secondary structure in phylogenetic studies to identify homologous positions: an example of alignment and data presentation from the frogs. Mol Phylogenet Evol **4**:314–330

Kosmala S, Karnkowska-Ishikawa A, Milanowski R, Kwiatowski J, Zakryś B (2009) Phylogeny and systematics of species from the genus *Euglena* (Euglenaceae) with axial, stellate chloroplasts based on morphological and molecular data – new taxa, emended diagnoses and epitypifications. J Phycol **45**:464–481

Kosmala S, Milanowski R, Brzóska K, Pekala M, Kwiatowski J, Zakryś B (2007) Phylogeny and systematics of the genus *Monomorphina* (Euglenaceae) based on morphological and molecular data. J Phycol **43**:171–185

Leander BS, Triemer RE, Farmer MA (2001) Character evolution in heterotrophic euglenoids. Eur J Protistol **37**: 337–356

Leander B (2004) Did trypanosomatid parasites have photosynthetic ancestors? Trends Microbiol **12**:251–258

Linton E, Hittner D, Lewandowski CL, Auld T, Triemer RE (1999) A molecular study of euglenoid phylogeny using small subunit rDNA. J Eukaryot Microbiol **46**:217–223

Linton EW, Nudelman MA, Conforti V, Triemer RE (2000) A molecular analysis of the euglenophytes using SSU rDNA. J Phycol **36**:740–746

Mainx F (1926) Einige neue Vertreter der Gattung *Euglena* Ehrenberg mit Unterstützung der Gesellschaft zur Förderung deutschen Wissenschaft. Kunst und Literatur in Böhmen. Arch Protistenkd **54**:150–160

Mainx F (1927) Beiträge zur Morphologie und Physiologie der Euglenen. I. Morphologische Beobachtung, Methode, und Erfolge der Reinkultur. II. Untersuchung über die Ernährungs- und Reizphysiologie. Arch Protistenkd **60**:305–414

Marin B, Palm A, Klingberg M, Melkonian M (2003) Phylogeny and taxonomic revision of plastid-containing Euglenophytes based on SSU rDNA sequence comparisons and synapomorphic signatures in the SSU rRNA secondary structure. Protist **154**:99–145

Martin W, Sommerville CC, Loiseaux de Goer S (1992) Molecular phylogenies of plastid origins and algal evolution. J Mol Evol **35**:385–403

Mereschkowsky KS (1877) Etjudy nad prostejsimi zivotnymi severa Rossii. Trudy S-Peterburgsk Obshch Estestvoisp **8**:1–299

Milanowski R, Zakryś B, Kwiatowski J (2001) Phylogenetic analysis of chloroplast small-subunit rRNA genes of the genus *Euglena* Ehrenberg. Int J Syst Evol Microbiol **51**:773–781

Milanowski R, Kosmala S, Zakryś B, Kwiatowski J (2006) Phylogeny of photosynthetic euglenophytes based on combined chloroplast and cytoplasmic SSU rDNA sequence analysis. J Phycol **42**:721–730

Mignot JP (1966) Structure et ultrastructure de quelques euglénomonadines. Protistologia **2**:51–140

Moreira D, Lopez-Garcia P, Rodriguez-Valera F (2001) New insights into the phylogenetic position of diplonemids: G+C content bias, differences of evolutionary rate and a new environmental sequence. Int J Syst Evol Microbiol **51**:2211–2219

Müllner AN, Angler DG, Samuel R, Linton EW, Triemer RE (2001) Phylogenetic analysis of phagotrophic, phototrophic, and osmotrophic euglenoids by using the nuclear 18S rDNA sequence. Int J Syst Evol Microbiol **51**:783–791

Nozaki H (2005) A new scenario of plastid evolution: plastid primary endosymbiosis before the divergence of the "Plantae," emended. J Plant Res **118**:247–255

Nudelman MA, Rossi MS, Conforti V, Triemer RE (2003) Phylogeny of the Euglenophyceae based on small subunit rDNA sequences: taxonomic implications. J Phycol **39**:226–235

Perty M (1849) Über verticale Verbreitung mikroskopischer Lebensformen. Mitt Naturforsch Ges Bern:17–45

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. Bioinformatics **14**:817–818

Preisfeld A, Berger S, Busse I, Liller S, Ruppel HG (2000) Phylogenetic analyses of various euglenoid taxa (Euglenozoa) based on 18S rDNA sequence data. J Phycol **36**:220–226

Preisfeld A, Busse I, Klingberg M, Talke S, Ruppel HG (2001) Phylogenetic position and inter-relationships of the osmotrophic euglenids based on SSU rDNA data, with emphasis on the Rhabdomonadales (Euglenozoa). Int J Syst Evol Microbiol **51**:751–758

Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics **19**:1572–1574

Rosowski JR, Kugrens P (1973) Observations on the euglenoid *Colacium* with special reference to the formation and morphology of attachment material. J Phycol **9**:370–383

Rosowski JR, Willey RL (1977) Development of mucilaginous surfaces in euglenoids. I. Stalk morphology of *Colacium mucronatum*. J Phycol **13**:16–21

Schlösser UG (1994) SAG Sammlung von Algenkulturen at the University of Göttingen. Catalogue of strains of 1994. Bot Acta **107**:113–187

Skuja H (1948) Taxonomie des Phytoplanktons Einiger Seen in Uppland Schweden. Symb Bot Ups **9**:1–399

Smith SW, Overbeek R, Woese CR, Gilbert W, Gillevet PM (1994) The genetic data environment: an expandable GUI for multiple sequence analysis. Comput Appl Biosci **10**:671–675

Swofford DL (2002) In: PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.0b10. Sinauer Associates, Sunderland, MA

Talke S, Preisfeld A (2002) Molecular evolution of euglenozoan paraxonemal rod genes par1 and par2 coincides with phylogenetic reconstruction based on small subunit rDNA data. J Phycol **38**:995–1003

Triemer R, Linton E, Shin W, Nudelman M, Brosnan S, Monfils A, Bennett M (2006) Phylogeny of the Euglenales based upon combined SSU and LSU rDNA sequence comparisons and description of *Discoplastis* gen. nov. (Euglenophyta). J Phycol **42**:731–740

Triemer RE, Farmer MA (2007) A Decade of Euglenoid Molecular Phylogenetics. In Brodie J, Lewis J (eds) Unraveling the Algae: The Past, Present and Future of Algal Systematics. Systematics Association Series. CRC Press, Boca Raton, FL, pp 315–329

Von der Heyden S, Chao EE, Vickerman K, Cavalier-Smith T (2004) Ribosomal RNA phylogeny of bodonid and diplonemid flagellates and the evolution of Euglenozoa. J Eukaryot Microbiol **51**:402–416

Watanabe MM, Hiroki M (1997) In: NIES – Collection List of Strains, 5th ed. National Institute for Environmental Studies, Tsukuba, 127 pp

Wuyts J, De Rijk P, Van de Peer Y, Winkelman T, DeWachter R (2001) The European large subunit ribosomal RNA database. Nucleic Acids Res **29**:175–177