Phylogenetic analysis of chloroplast small-subunit rRNA genes of the genus *Euglena* Ehrenberg

Rafał Milanowski, Bożena Zakryś and Jan Kwiatowski

Almost complete sequences of plastid SSU rDNA (16S rDNA) from 17 species belonging to the order Euglenales (sensu Németh, 1997; Shi et al., 1999) were determined and used to infer phylogenetic relationships between 10 species of *Euglena*, three of *Phacus*, and one of each of *Colacium*, *Lepocinclis*, *Strombomonas*, *Trachelomonas* and *Eutreptia*. The maximum-parsimony (MP), maximum-likelihood (ML) and distance analyses of the unambiguously aligned sequence fragments imply that the genus *Euglena* is not monophyletic. Parsimony and distance methods divide Euglenaceae into two sister groups. One comprises of representatives from the subgenera *Phacus*, *Lepocinclis* and *Discoglena* (sensu Zakrys, 1986), whereas the other includes members of *Euglena* and *Calliglena* subgenera (sensu Zakrys, 1986), intermixed with representatives of *Colacium*, *Strombomonas* and *Trachelomonas*. In all analyses subgenera *Euglena* – together with *Euglena polymorpha* (representative of the subgenus *Calliglena*) – and *Discoglena* – together with *Phacus* and *Lepocinclis* – form two well-defined clades. The data clearly indicate that a substantial revision of euglenoid systematics is very much required, nevertheless it must await while more information can be gathered, allowing resolution of outstanding relationships.

**Keywords:** chloroplast SSU rDNA, *Euglena*, *Calliglena*, *Discoglena*, molecular phylogeny

**INTRODUCTION**

The euglenoid flagellates are an ancient, distinct group of protists related to kinetoplastids (Triemer & Farmer, 1991; Cavalier-Smith, 1981, 1993; Corliss, 1994; Dawson & Walne, 1994; Kivic & Walne, 1983; Montegut-Felkner & Triemer, 1997; Linton et al., 1999) comprising green and colourless forms. The origin of the euglenoid chloroplast is not entirely certain. Most likely, it is monophyletic in nature and obtained by secondary symbiosis from green algae (Gibbs, 1978, 1981; Morden et al., 1992; Delwiche & Palmer, 1997).

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**Abbreviations:** ML, maximum-likelihood; MP, maximum-parsimony; 16S rDNA, chloroplast SSU rDNA.

The GenBank accession numbers for chloroplast SSU rDNA (16S rDNA) sequences reported in this papers are given in Methods.

The genus *Euglena* consists of organisms highly diversified with respect to cell architecture. Species included within the genus represent almost entire morphological monad diversity encountered within green euglenoids. Such an immense variety provoked many authors to construct numerous intrageneric classifications. Klebs (1883), for example, described five ‘types’ of *Euglena* (viridis, deses, oxyuris, spirogyra and acus); whereas Hansgirg (1892) described six ‘sections’ (Auteuglena, Platyglena, Oxyglena, Spirogyra, Acuglena and Pseudophacus). Other scholars have designated distinct groups [Lemmermann (1913) – four; Gojdics (1953) – eight; Pringsheim (1956) – five, ‘categories’ [Chu (1946) – four] or ‘subgenera’ [Zakryś (1986) – three] within the genus *Euglena*, with respect to the chloroplast structure. Other propositions have included the division of the genus into separate ‘groups’, according to the presence or absence of flagella and/or the type of cell movement. Thus, Elenkin (1924) proposed three ‘groups’, whereas Popova (1966) designated two ‘groups’ with 11 ‘sub-
groups’. Such classifications, based mainly on morphological characters, may reflect morphological convergence, rather than phylogenetic affiliations of considered taxa, as recent molecular studies suggest (Montegut-Felkner & Triemer, 1997; Linton et al., 1999, 2000; Preissfeld et al., 2000; Thompson et al., 1995).

We present here the phylogeny of 18 species of phototrophic euglenoids from the order Euglenales (Németh, 1977; Shi et al., 1999), which are classified into several genera: Colacium Ehrenberg, Euglena Ehrenberg, Euglenoideae, P. rbcL genes reported in this paper are: AF289238, AF289239, reported in this paper are: AF289238, AF289239, AF289240, AF289241, AF289242, AF289243, provided by cool-white fluorescent tubes.

**DNA isolation, amplification and sequencing.** The total DNA was isolated from 20–30 mg centrifuged cells, using DNeasy Tissue Kit (Qiagen), according to the manufacturer’s protocol (with proteinase K addition). PCR cycle conditions were individually tailored to amplify overlapping fragments of the 16S rRNA gene from a single species, using different combinations of forward and reverse primers listed in Table 1. A 50 µl reaction mixture contained 1 U Taq polymerase (MBI Fermentas), 0.2 mM dNTPs, 2.5 mM MgCl₂, 10 pmol each primer, reaction buffer (MBI Fermentas) and 10–50 ng DNA. The PCR protocol consisted of 5 min of denaturation at 95 °C, followed by seven initial cycles comprising 1 min at 95 °C, 2 min at 44–58 °C and 0.5–1 min at 72 °C, then by 30 cycles comprising 0.5 min at 95 °C, 0.5 min at 54–64 °C and 0.5–1 min at 72 °C. The final extension step was performed for 7 min at 72 °C. PCR products were purified either by precipitation with 98% ethanol and 0.3 M potassium acetate, or by electrophoresis and excision of a predominant band, followed by purification with QIAEXII Gel Extraction Kit (Qiagen). PCR products were sequenced from both strands by cycle sequencing using BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems) and primers from Table 1. The readings from ABI Prism 377 DNA sequenator, after removal of primer sequences, were assembled into ‘contigs’ by the SeqMan program of the LASERGENE package (DnaStar) and checked manually for consistency.

**Table 1. Primers used for PCR amplification and sequencing of euglenoid 16S rRNA genes**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position of 3’ end</th>
<th>Sequence (5’–3’)</th>
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<tbody>
<tr>
<td>16SF</td>
<td>39</td>
<td>TTGATCCTGGCTCAGGATGAAACGCT</td>
</tr>
<tr>
<td>16S223F</td>
<td>242</td>
<td>ATGAGCTTGACATCGTATTAG</td>
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<td>16S379R</td>
<td>381</td>
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</tr>
<tr>
<td>16S647F</td>
<td>664</td>
<td>ATTTCCAGTGGTACCGGTG</td>
</tr>
<tr>
<td>16S781R</td>
<td>781</td>
<td>ACTTAGATCCATAGTTACG</td>
</tr>
<tr>
<td>16S834R</td>
<td>828</td>
<td>AGGCGGACACCTAAGCGGT</td>
</tr>
<tr>
<td>16S1180R</td>
<td>1170</td>
<td>TGAGCAGCTGGTGCGCCAG</td>
</tr>
<tr>
<td>16SR</td>
<td>1470</td>
<td>CAAGGAGTGGATTCAGGCGCACTT</td>
</tr>
</tbody>
</table>

F denotes forward primers, R–reverse. Position of 3’ end refers to 16S rDNA of *Euglena gracilis* (GenBank no. X12890).
Phylogenetic analysis of euglenoid 16S rDNA

Sequence alignment and phylogenetic analysis. The additional sequence of *Euglena gracilis* 'Z' (X12890) was retrieved from GenBank. Alignment of sequences was obtained using the CLUSTAL W 1.8 program (Thompson et al., 1994) with default options, manually checked and edited according to the secondary structure of *Euglena gracilis* (Van de Peer et al., 1999). Several regions, which could not be unambiguously aligned, were omitted from analyses. All nucleotides were treated as independent and unordered, multistate characters of equal weight. The alignment of 18 euglenoid sequences used for analysis is available on-line at http://ulmus.bot.uw.edu.pl/~jmkwiato/align.html, or upon request from the corresponding author. Neighbour-joining trees (Saitou & Nei, 1987) were obtained with the MEGA program, version 1.0 (Kumar et al., 1993). Jukes–Cantor (Jukes & Cantor, 1969), Kimura two-parameter (Kimura, 1980), and Tamura–Nei (Tamura & Nei, 1993) models with equal or varying rates among sites were used to calculate distances between sequences. Gamma distribution shape parameters from 0·5 to 1·0 were tested. Maximum-parsimony (MP) and maximum-likelihood (ML) analyses and the nucleotide homogeneity test were performed by PAUP*, version 4·0b4a for Microsoft Windows (Swofford, 1998). Heuristic Search option with MULPARS, tree-bisection-reconnection (TBR) branch swapping, ACCTRAN optimization, and random addition with 100 replicates was used to find the best tree. For ML analyses, the model of nucleotide substitution of Rodriguez et al. (1990) was applied, in addition to the models used for distance analyses. Bootstrap support of specific nodes (Felsenstein, 1985) was estimated with 1000 replications (100 for ML analysis) and default options, as implemented in MEGA and PAUP*. Decay indices (Bremer, 1988, 1994) were calculated by the SPLIT program (Salisbury, 2000). The sequence of *Eutreptia viridis*, member of Eutreptiaceae, was used to root the trees. There is evidence, both molecular (Linton et al., 1999, 2000; Preisfeld et al., 2000; Thompson et al., 1995) and morphological (Bourelly, 1970; Németh, 1997; Shi et al., 1999), that Eutreptiaceae are indeed an outgroup with respect to Euglenaceae. Trees were drawn by TreeView, version 1·6·1 for Microsoft Windows (Page, 1996).

RESULTS

Almost complete chloroplast 16S rRNA genes were sequenced from 17 species. The sequences, excluding PCR primers, varied in length from 1386 bp in *Lepocinclis*, to 1430 bp in *Eutreptia*. Several regions, constituting 139 positions, which could not be unambiguously aligned, were omitted from analyses. The resulting matrix of 18 sequences, including *Euglena gracilis* sequence obtained from GenBank, had 1350 of total characters, 745 of which were constant. A total of 203 variable characters were parsimony-uninformative, and 402 were parsimony-informative. When gaps were treated as a fifth base, the number of constant, variable uninformative and informative characters was 730, 215 and 405, respectively. Removal of the ambiguous positions substantially improved the homogeneity of base frequencies across taxa [$\chi^2 = 24·80$ (df = 51), $P = 0·9993$].

Fig. 1. shows the distance tree obtained by the Neighbor-joining method using the Jukes–Cantor model of nucleotide substitutions (Jukes & Cantor, 1969). *Eutreptia*, not included in Euglenaceae, and known to diverge first (Bourelly, 1970; Németh, 1997; Linton et al., 1999; Shi et al., 1999; Preisfeld et al., 2000; Thompson et al., 1995), was used to root the tree. The main feature of this tree is its division into two well-defined sister groups, both supported by high bootstrap values; 91 and 86%, for the upper and lower clades, respectively. The upper branch consists of representatives of two *Euglena* subgenera (sensu Zakryś): *Euglena* (E) and *Calliglena* (C), intermixed with species of three other euglenoid genera: *Colacium*, *Strombomonas* and *Trachelomonas*. None of the affiliations between genera are well supported. Only grouping the species of *Euglena* subgenus together with *Euglena polymorpha* (C) (74%), and that of two other *Calliglena* species (*Euglena gracilis* and *Euglena agilis*) with each other (99%), gain substantial support.

[Fig. 1. The neighbour-joining tree of euglenoid 16S rDNA sequences based on Jukes–Cantor distances. Bootstrap values higher than 90% (1000 replications) are shown at the nodes. Subgenera: *Euglena*, *Calliglena*, *Discoglena*.]

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Fig. 2. The strict consensus tree of two most parsimonious trees obtained when gaps are treated as ‘missing’. The numbers at nodes indicate the percentage of bootstrap support better than 50% (1000 replications) and decay indices greater than 1 (preceded by d). X denotes the branches collapsed in strict consensus of seven trees obtained when gaps were treated as a fifth character state. The lower branch consists of early diverging species of *Phacus* and a very well-supported cluster (100%), consisting of *Euglena* subgenus *Discoglena* (D) grouped together with *Lepocinclis*—another genus of Euglenaceae. However, the obtained topology makes *Discoglena* subgenus paraphyletic. Applying Kimura two-parameter (Kimura, 1980) and Tamura-Nei (1993) models of sequence evolution, with rates equal or varying among sites, has not influenced the topology of the tree in any substantial way. The branching order within weakly supported groups was unstable, but the clades with bootstrap values marked in Fig. 1 were always preserved.

Multiple heuristic searches for the most parsimonious topology, with different modes of sequence addition, produced two equally parsimonious trees of 1715 steps, when gaps were treated as ‘missing’. Fig. 2 shows a strict consensus of these trees with bootstrap values greater than 50% and decay indices indicating stability of particular nodes. When gaps were treated as a fifth base, seven equally parsimonious trees of 1756 steps were produced. The strict consensus tree obtained from them is different from the one depicted in Fig. 2 in that the internal branches marked by an ‘X’ have collapsed. Again, two main divisions within euglenoid species, observed on the distance tree of Fig. 1, are visible, although without strong bootstrap and decay support. The same clades of [{Euglena subgenus}+ {Euglena polymorpha} (C)] and [{Euglena gracilis} (C) + {Euglena agilis} (C)] in the upper branch; and (Discoglena + Lepocinclis) in the lower one, are also present. However, all three *Euglena* subgenera are paraphyletic in the parsimony trees.

Topologies of the trees obtained by ML analyses highly depended on the model of nucleotide substitution applied. When the Jukes–Cantor and Felsenstein (Felsenstein, 1981) model was used, the topology obtained was generally consistent with the trees from Figs 1 and 2. The tree with the best score (Ln likelihood = −9175.4), shown in Fig. 3, was produced when the model of Rodriguez et al. (Rodriguez et al., 1990; Yang, 1994a, b) was used, with estimated values for the following parameters: base frequencies (A = 0.2826, C = 0.1728, G = 0.2619, T = 0.2827), instantaneous rates of substitutions (AC = 1.288, AG =

Fig. 3. The maximum-likelihood tree obtained under general-time-reversible model, with unequal rates of substitutions among sites and parameters estimated by ML. Bootstrap values higher than 50% (100 replications) are given at the nodes.
Table 2. Kishino–Hasegawa test of different tree topologies under the MP criterion

<table>
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<tr>
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<th>ln L difference</th>
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<th>P</th>
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<td>3</td>
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</tr>
<tr>
<td>4</td>
<td>91754</td>
<td>(best)</td>
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Table 3. Kishino–Hasegawa test of different tree topologies under the MP criterion

<table>
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<th>P</th>
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<td>1720</td>
<td>5</td>
<td>4.58225</td>
<td>0.2754</td>
</tr>
<tr>
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<td>20</td>
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</tr>
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<td>1811</td>
<td>96</td>
<td>19.42815</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

DISCUSSION

The phylogeny obtained in this as well as other molecular studies (Montegut-Felkner & Triemer, 1997; Linton et al., 1999, 2000; Preisfeld et al., 2000; Thompson et al., 1995) is not consistent with the present classifications based on morphological, particularly non-chloroplast characters such as the shape and rigidity of the cell, the presence of the flagellum, the nature of its movement, or the ability to form palmella.

One of the authors, Zakryš (1986), proposed a system of intrageneric Euglena classification in which the following are treated as the main criteria in the classification of the Euglena species: (i) the position of chloroplasts in the cell (axial or parietal); (ii) their number, size, shape; (iii) and presence or absence of pyrenoids. We also believe that the paramylon grain features are correlated with the characters mentioned above. In most of the species with small, parietal, disc-shaped and numerous chloroplasts without pyrenoids, the paramylon grains are dimorphic. Those of the one type are scarce (one, two or a few per cell), large rod-like or ring-shaped, while those of the other one are more numerous and small (Zakryš unpublished observations). Two of the chloroplast features (the presence or absence of pyrenoids and the position of chloroplasts in the cell) are assumed to be fundamental for this classification, which has divided the genus Euglena into three subgenera: Euglena, Calliglena and Discoglena. All species belonging to the subgenus Euglena have axial chloroplasts with pyrenoids. In the subgenus Calliglena, chloroplasts are partially or entirely parietal, and also have pyrenoids. In the subgenus Discoglena, all chloroplasts are parietal, and do not have pyrenoids. The evidence for the concept of three subgenera (Euglena, Calliglena and Discoglena) comes from the phenetic analysis of the representative of Rodriguez et al. (1990), with the above-mentioned parameter values used to obtain the ML tree in Fig. 3. None of the trees were significantly worse than the one in Fig. 3, except the trees 6, 7 and 8, in which either one of the well-established clades (Phacus, Lepocinclis, Discoglena) or (Euglena subgenus, Euglena polymorpha) was deprived of its early diverging member. Qualitatively identical results were obtained, when other models of the evolutionary change of sequences (Felsenstein, 1981; Hasegawa et al., 1985), with or without estimation of various parameter values, were applied under the ML criterion (not shown). Table 3 shows the results of the comparisons under MP criterion. Again, the results are similar, supporting the notion that the (Phacus, Lepocinclis, Discoglena) and (Euglena subgenus + Euglena polymorpha) clades are well established and that the monophyletic nature of the Euglena subgenus could not be excluded. Similar results were obtained when trees from Figs 1 and 2 were altered in the same way as the one from Fig. 3 (not shown).
set of 58 taxa by different distance methods, describing 28 characters with 129 states (Batko & Zakrys, 1995).

Our analysis of 16S rDNA, as well as that of 18S rDNA (Linton et al., 1999, 2000), suggest a clear-cut separation of taxa included in the subgenus Discoglena from the rest of the Euglena species, which together with the genera Phacus and Lepocinclis form a well-defined clade. However, the Discoglena subgenus is represented here by only two species, is clearly paraphyletic. Both Phacus and Lepocinclis have the same type of photosynthetic apparatus as organisms classified as Discoglena. All have numerous, small, disc-shaped, parietal chloroplasts without pyrenoids, often with large paramylon grains. These large pararnylon grains distinguish them from the other Euglena species, as well as from Strombomonas, Trachelomonas, Colacium and Eutrepta. Eutrepta is considered to have primitive chloroplast features (Dawson & Walne, 1991); not parietal, small and numerous chloroplasts devoid of pyrenoids – which are present in the cytoplasm – and small paramylon grains. The only character states that differentiate Phacus and Lepocinclis from the subgenus Discoglena are the rigidity and flatness of their cells. However, considering molecular data, such features may not be taxonomically significant. There has been continuous discussion concerning the taxonomic position of Lepocinclis and Phacus. For example, Euglena tripteris was first described by Dujardin (1841) as Phacus tripteris, later moved by Klebs (1883) to the genus Euglena, but now, according to 16S rDNA data, seem to be associated with Phacus again. Euglena texta (Duj.) Hübner, is still considered by some authors as Lepocinclis texta Lemmermann (Huber-Pestalozzi, 1955; Tell & Conforti, 1986; Compere, 1989; Németh, 1997; Dillard, 2000), but by others as Euglena texta (Asaul, 1975; Zakryś, 1986; Zakryś & Walne, 1994; Kim et al., 1998; Shi et al., 1999). One possibility to alleviate this situation would be to return Phacus and Lepocinclis to the Euglena genus (Linton et al., 2000) and classify as Discoglena those species which cluster with it, thus making the subgenus monophyletic. Alternatively, if Discoglena proves to be nested entirely within a wider Phacus or Lepocinclis clade it should be renamed accordingly.

When Eutrepta is used as an outgroup in distance and parsimony analyses, a sister group to the (Phacus, Lepocinclis, Discoglena) clade consisting of genera: Colacium, Strombomonas and Trachelomonas and subgenus Calliglena and Euglena, of the genus Euglena is present (Figs 1 and 2). Most, if not all, species of these taxa have pyrenoids in chloroplasts (Asaul, 1975; Popova & Safonowa, 1976), which may not always be very visible under light microscope, e.g. in the subgenus Euglena, Colacium vesiculosum, Trachelomonas vovocinopsis and Euglena proxima. This uncertainty was resolved by electron microscopic studies (Dragos et al., 1979; Péterfi et al., 1979; Zakryś & Walne, 1998; Brown, Zakryś & Farmer, unpublished data). Similar findings may be expected in Strombomonas costata, since it has relatively large chloroplasts, similar in appearance to those having pyrenoids (Huber-Pestalozzi, 1955; Popova, 1966; Németh, 1997; Shi et al., 1999). However, in the cells of the strains of Colacium and Strombomonas maintained in our laboratory, pyrenoids are not visible under a light microscope.

In some ML analyses, particularly those using more complex models of sequence evolution (Fig. 3) the ‘pyrenoid’ clade does not exist, but its species diverge successively from the base of the tree. However, the clade consisting of Euglena subgenus and Euglena polymorpha (C), present on both distance (Fig. 1) and parsimony (Fig. 2) trees, is even much better supported (bootstrap value 99%). It remains to be seen whether the subgenus Euglena, with axially located chloroplasts, constitutes a natural assemblage. Its representatives (Euglena viridis, Euglena stellata, Euglena tristella and Euglena geniculata) are always grouped closely together in 16S rDNA, and form a clade in 18S rDNA trees, although only two species (Euglena viridis, Euglena stellata) are included in the latter study (Linton et al., 2000). Even though they are paraphyletic in most analyses of 16S rDNA, the monophyly of the subgenus Euglena cannot be ruled out. If they do form a clade, they are not a sister group with respect to the Calliglena subgenus, with chloroplasts located differently, and whose one representative, Euglena polymorpha is closely associated with them. The rest of the Calliglena subgenus, as well as Colacium, Strombomonas and Trachelomonas are outside of the Euglena subgenus clade, but their relationships could not be established with great confidence, except for the close affiliation of two Calliglena species Euglena agilis and Euglena gracilis. Our analysis does not seem to agree with the analysis of rbcL gene (Thompson et al., 1995) in one instance. Euglena gracilis is more closely associated with Euglena geniculata than with Euglena pisciformis, which apparently is synonymous with Euglena agilis (Zakryś & Kucharski, 1996; Zakryś et al., 1996; Zakryś, 1997a, b). All other relationships between Calliglena and subgenus Euglena species are not well resolved in the rbcL gene analysis (Thompson et al., 1995).

Subgenus Calliglena is the most genetically diversified of the three Euglena subgenera, at least with respect to species sampled here and in the study of 18S rDNA (Linton et al., 2000), whereas Euglena anabaena is a sister group to the rest of Euglenales. In some of our ML analyses, Euglena anabaena also constitutes a sister lineage to the rest of Euglenales, comprising additionally of the Colacium, Strombomonas and Trachelomonas species, whose chloroplasts are similar to those of Calliglena. It is therefore likely, that the chloroplast features of all these taxa represent plesiomorphic character states. They differ from Eutrepta only in that they have pyrenoids in chloroplasts, not in the cytoplasm. On the other hand, some of the features of the photosynthetic apparatus of the two remaining subgenera are likely to be autapomorphies. In the case...
of Discoglena, as well as Phacus and Lepocinclis, they are the complete lack of pyrenoids in the cell, the disc-like shape of the parietal located chloroplasts, and the dimorphic appearance of the paramylon grains (see above). In the case of the Euglenid subgenus, the few large, axially located chloroplasts (only one, two or three per cell), seem to be an autapomorphy.

Several interesting questions arise from, or are left unresolved by, this study. One is whether lorica (the cell envelope composed of mineral-impregnated mucilage) is a shared-derived character in Trachelomonas and Strombomonas. On the 16S rDNA trees, these species are not closer together than they are to any other member of the ‘pyrenoid’ clade. Therefore, before resolving the pattern of their relative divergence, no conclusion can be reached about the emergence (and possible loss) of this feature. Colacium is a sedentary species, but it is clustered together with free swimming members of Euglena, Strombomonas and Trachelomonas in parsimony and distance trees. Some authors have included the genus Colacium in the family Euglenaceae (Pringsheim, 1956; Németh, 1997; Popova, 1966; Shi et al., 1999). Others, however, have regarded it as a member of the family Colaciaceae (Popova & Sazonova, 1976; Compere, 1989), or even have placed it in a separate order Colaciidae (Bourrelly, 1970; Tell & Conforti, 1986), opposite the order Euglenidae, consisting of all the rest of the green and colourless euglenoids. Genera Colacium, Lepocinclis, Strombomonas and Trachelomonas are represented here by single species. They may be poly phylectic and/or paraphyletic, as suggested by 18S rDNA data, with respect to Lepocinclis and Phacus (Linton et al., 2000). Resolving all of these issues requires greater amount of sequence data on more taxa.

Conclusions

Analysis of 16S rDNA confirms that the genus Euglena is not monophyletic.

Euglena species classified as Discoglena are firmly associated with the genera Lepocinclis and Phacus, with which they share essential features of chloroplasts. Different schemes are possible to render these three taxa monophyletic.

The question of whether the subgenus Euglena, with unique, axially located chloroplasts forms a clade, could not be convincingly resolved, but they are closely related and their monophyly is not contradicted by 16S rDNA data.

Species of the Calliglena subgenus, which share chloroplast features with Colacium, Strombomonas and Trachelomonas, are the most genetically diversified among the Euglena species and may be among the first diverging lineages of Euglenales. Therefore, they are not likely to be a natural group.

The molecular data obtained so far, beg for the taxonomic revision of the order Euglenales, and of all the genera included therein. Yet, due to many still unresolved relations, more sequence data are needed before the gruelling task of constructing a natural and consistent system of taxonomical hierarchy can be achieved. We believe that, for the time being, even minor changes in classification may cause more harm than good.

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