Phylogenetic position and inter-relationships of the osmotrophic euglenids based on SSU rDNA data, with emphasis on the Rhabdomonadales (Euglenozoa)

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In order to reconstruct the evolution of euglenid flagellates, euglenozoan SSU rDNA data have been used to investigate phylogenetic relationships with a focus on osmotrophic taxa and especially on the Rhabdomonadales. The dataset consisting of the SSU rDNAs of osmotrophic, phagotrophic and phototrophic taxa was used in parsimony, maximum-likelihood and distance analyses. Five genera make up the Rhabdomonadales, all of them osmotrophic: Gyropaigne, Menoidium, Parmidium, Rhabdomonas and Rhabdospira. According to our analyses they form a strongly supported monophyletic assemblage which is characterized by a low sequence divergence compared to the euglenids in general. Closest relatives are the members of the osmotrophic genus Distigma. All primary osmotrophic species constitute a larger monophyletic group with the phototrophic euglenids and the phagotroph Peranema trichophorum. The combination of three rhabdomonadalian species Rhabdomonas gibba, Rhabdomonas spiralis and Rhabdospira spiralis with nearly identical SSU rDNA sequences is strongly recommended. The phagotroph Petalomonas cantuscygni branches at the bottom of the euglenid subtree with significantly weaker support. The inter-relationship of the three distinct euglenozoan taxa (euglenids, kinetoplastids and diplonemids) could not be convincingly resolved by this study.

Keywords: euglenids, molecular phylogeny, osmotrophs, Rhabdomonadales, SSU rDNA

INTRODUCTION

According to morphological resemblance (Cavalier-Smith, 1981; Kivic & Walne, 1984) and to molecular data (Montegut-Felkner & Triemer, 1997; Linton et al., 1999, 2000; Maslov et al., 1999; Preisfeld et al., 2000) euglenids and kinetoplastids have been placed in a supertaxon Euglenozoa (Cavalier-Smith, 1981). This was extended to include the genus Diplonema (Cavalier-Smith, 1993; Montegut-Felkner & Triemer, 1994, 1996) and Postgaardi (Simpson, 1997). The Euglenozoa form a monophyletic group of flagellated cells whose systematic position still remains unclear. Based on SSU rRNA analyses they branch at the bottom of the Eukaryotes (Sogin et al., 1986, 1989; Vossbrinck et al., 1987; Sogin & Silberman, 1998). However, Morin (2000) considers the early divergence of the Euglenozoa in a eukaryotic SSU rDNA tree as a long branch attraction due to an accelerated rate of evolution. Incongruencies between SSU rDNA based trees and trees based on protein-coding genes like translation elongation factors 1α and 2 (Hashimoto et al., 1997), α- and β-tubulin (Keeling, 1998) show that the phylogenetic position of the Euglenozoa among the eukaryotes is still controversial.

The euglenids are characterized by a pellicle structure built by an epiplasmic layer of proteins which underlies the plasma membrane. The pellicle structure is...
stabilized by microtubules as cytoskeletal elements (Kivic & Walne, 1984). Euglenids undergo a closed mitosis with intranuclear spindle, and retain an intact nuclear envelope and nucleolus during division (Triemer & Farmer, 1991). It is thought that a phagotrophic euglenid has engulfed a green alga or its chloroplast and developed a plastid with three surrounding membranes to create the phototrophic euglenids (Gibbs, 1978). The uptake of eukaryotic prey has to meet the requirements of a complex ingestion apparatus like the one in *Peranema trichophorum*. The flagella of the euglenids can either be emergent or one can be reduced or completely absent, as in *Petalomonas cantuscygni* (Farmer & Triemer, 1988). Emergent flagella of euglenids have a paraxodal rod (PAR) and non-tubular mastigonemes (Willey et al., 1988). The carbohydrate storage is paramylon, a β-1,3-glucan (Kiss et al., 1987).

Recently published molecular data with the SSU rDNA (Montegut-Felkner & Triemer, 1997; Linton et al., 1999, 2000; Preisfeld et al., 2000) support the monophyly of the Euglenozoa and of the sister clades kinetoplastids and euglenids. Maslov et al. (1999) who analysed two *Diplonema* SSU rDNA sequences and one COI sequence of *Diplonema papillatum* could not resolve the inter-relationships within the Euglenozoa.

The phototrophic species of the Euglenales, including the secondary osmotrophs *Astasia longa* and *Kawkinea quadrantha*, and the phototrophic Eutreptiales form a monophyletic assemblage with the phagotrophic taxon *Peranema trichophorum* (Heteromastigata) as next relative (Montegut-Felkner & Triemer, 1997; Linton et al., 1999, 2000; Preisfeld et al., 2000). At the molecular level, little is known about the phagotrophic and the osmotrophic euglenids. The lack of structures visible by light microscopy, such as plastids and ingestion devices, has led to confusing and inconsistent systematic positions and relations within the osmotrophs in general and the Rhabdomonadales (Bourrelly & Georges, 1951; Cann, 1986; Christen, 1958, 1963; Huber-Pestalozzi, 1955; Leedale, 1967; Leedale & Hibberd, 1974; Pringsheim, 1936, 1942, 1963; Skuja, 1939, 1948, 1956). In this study we present the results of SSU rDNA data analysis with a focus on the position and inter-relationship of the Rhabdomonadales, and reflect on possible evolutionary pathways.

**METHODS**

**Culture conditions.** Cultures were obtained from the Sammlung von Algenkulturen der Universität Göttingen (SAG), Germany, and The Culture Collection of Algae and Protozoa (CCAP), UK. *Menodinium bicucullatum* Pringsheim (SAG 1247-1), *Menodinium cultrellus* Pringsheim (SAG 1247-2), *Menodinium intermedium* Pringsheim nom. nud. (SAG 1247-3), *Parvidium scutatum* (Skuja) Christen (SAG 232.80), *Parvidium circulare* Christen (SAG 230.80), *Rhabdomonas spiralis* Pringsheim (SAG 233.80), *Rhabdomonas costata* (Korshikov) Pringsheim (SAG 236.80), *Rhabdomonas gibba* (Skuja) Pringsheim nom. nud. (SAG 1271-2), *R. incurva* (Fresenius) Klebs (SAG 235.80), *Rhabdomonas intermedia* Christen (SAG 238.80) and *Rhabdospora spiralis* Pringsheim (CCAP 1271/5), were grown in soil water medium 3c at 20 °C in the dark (accession numbers are shown on the figures). *Eutreptia viridis* Perty (SAG 1226-1c) AF157312 was cultivated under a 14:10 h light:dark photoperiod in brackish water medium 6 and *Lepocinclis ovum* (Ehrenberg) Lemmermann (SAG 1244-8) AF110419 in medium 3b (all media: Schlösser, 1994).

**DNA isolation, amplification and sequencing.** Isolation of DNA, amplification and sequencing of SSU rDNA was performed as described elsewhere (Preisfeld et al., 2000). Briefly, oligonucleotides specific to conserved regions of eukaryotic SSU rDNA were used as primers for PCR with REDTaq DNA polymerase (Sigma) as reaction enzyme. The SSU rDNA gene was purified by E.Z.N.A. Cycle Pure Kit (Peqlab) and subsequently cloned in Escherichia coli Top 10F' using TOPO TA Cloning Kit (Invitrogen) following manufacturers’ instructions. Isolation of plasmids was performed with E.Z.N.A. Plasmid Miniprep Kit II (Peqlab) following manufacturers’ instructions. Plasmids were sequenced via primer walking using both strands of the SSU rDNA (Ready Reaction Kit (Peqlab) and subsequently cloned in *Escherichia coli* Top 10F’ using TOPO TA Cloning Kit (Invitrogen) following manufacturers’ instructions. Isolation of plasmids was performed with E.Z.N.A. Plasmid Miniprep Kit II (Peqlab).

**Sequence alignment.** Euglenid sequences were aligned to available sequences of euglenozoan and outgroup organisms. A preliminary alignment was performed using CLUSTAL X (Thompson et al., 1997), with gap opening penalty 10, gap-extension penalty 0.5, and DNA weight matrix IUB (Preisfeld et al., 2000). It was necessary to correct the alignments manually by means of secondary structure based on the models of *Euglena gracilis*, Petalol...
monas cantuscygni, Peranema trichophorum, Khawkinea quartana, Bodo caudatus, Trypanosoma brucei and Saccharomyces cerevisiae SSU rRNA obtained from the rRNA web server (Van de Peer et al., 2000) with the help of GeneDoc (Nicholas & Nicholas, 1997). Non-homologous regions were excluded prior to final analyses. Because of the unusually long genes of the osmotrophs and the high sequence divergence within euglenozoan taxa, several alignments had to be produced for different aims of tree inferences.

**Phylogenetic analysis of SSU rDNA data.** Maximum-parsimony and maximum-likelihood analyses were performed using PAUP* (version 4.0b4a; Swofford, 1998) with a heuristic search (gaps are treated as missing; addseq = random; nreps, parsimony = 25; nreps, likelihood = 5). Distance analysis and neighbour-joining tree construction were performed with TREECON 1.3b (Van de Peer & De Wachter, 1994).

Decay support (Bremer, 1994) for monophyletic groups found in parsimony analyses was determined with PAUP* using the clade constraint method described by Morgan (1997). Non-parametrical bootstrapping was performed, generating 1000 pseudoreplicates, to obtain a statistical measure for tree robustness (Felsenstein, 1985). The sequence divergence was calculated by dividing the observed number of differences by the number of aligned sequence positions.

**RESULTS**

**General tree topology and position of the Rhabdomonadales**

In order to gain information on the general grouping of euglenid taxa, and especially on the position of the taxon Rhabdomonadales, the euglenozoan dataset, in which all published and newly sequenced euglenid SSU rDNA sequences are included, has been analysed using maximum-parsimony, maximum-likelihood and distance methods. The resulting tree is drawn schematically to facilitate the recognition of all major monophyletic assemblages (Fig. 1). The alignment used to infer this tree had to be reduced drastically to 984 unambiguously homologous positions according to secondary structures. The Rhabdomonadales form a well-supported monophyletic assemblage with a close relationship to members of the genus Distigma which represents a paraphyletic taxon. The resulting clade comprises all members of the ancestrally osmotrophic euglenids, which form a monophyletic group with the phagotrophic Peranema trichophorum (Heteronematales) and the monophyletic phototrophs. The support for the sister group relationship of Peranema trichophorum and the phototrophs could not be confirmed by maximum-parsimony analysis with the result that the sister group of the osmotrophs cannot be identified unambiguously. Within the phototrophic euglenids, the Euglenales form a well-supported clade the bootstrap support for which increases to 93% when the highly derived sequence of Euglena mutabilis is omitted. The phototrophic Eutreptiales branch at the bottom of the phototrophic clade, their monophyly being weakly supported. The phagotroph Petalonomas cantuscygni, a member of the Sphenomonadales, is located at the base of the euglenids. This species is separated by a long branch from all other euglenids included in our analyses. The two Diplonema species form a monophyletic clade within the euglenozoan radiation. The relationship between euglenids, diplomonads and kinetoplastids could not be unambiguously resolved. Either diplomonads and euglenids form a monophyletic group (maximum-likelihood analysis) or diplomonads cluster together with kinetoplastids (maximum-parsimony and distance analyses). The kinetoplastids comprising bodonids and trypanosomatids form a well-supported monophyletic group which is separated by a long branch from the diplomonads and euglenids.

**Sequence divergence**

A possible explanation for the weak support of some of the internal nodes can be found in high pairwise sequence divergences (Fig. 2) derived from an uncorrected distance matrix. There is a high genetic diversity of the euglenid SSU rDNA compared to the kinetoplastids. The Diplonema sequences are very similar to each other but very different when compared with the euglenids and the kinetoplastids, reflecting their unstable position. The Rhabdomonadales form a homogeneous taxon with low sequence divergences. Adding the other osmotrophic genus Distigma to the Rhabdomonadales results in a higher sequence divergence comparable to the phototrophs.

**Tree inference of the osmotrophic euglenids**

After the general position of the Rhabdomonadales within the euglenids was resolved, it became obvious that the chosen alignment gave no proper resolution of the relationship within the clade under study. Therefore a second alignment comprising only the osmotrophic taxa was produced to include more homologous positions. The aim was to find the basal members of the Rhabdomonadales to get more information about the relationships within this clade. Distigma proteus and Distigma curvatum, the closest relatives in the previous analyses (Fig. 2), were used to root a maximum-parsimony tree (Fig. 3). The genus Parmidium forms a highly supported monophyletic clade that is the sister group to the remaining taxa of the Rhabdomonadales, leading to a non-resolved more weakly supported cluster of the genera Rhabdomonas, Menoidium and Gyropaigne.

**Inter-relationship of the Rhabdomonadales**

The following analyses were based on an alignment with only the Rhabdomonadales sequences. Numerous positions which can only be homologized within this clade could be interpreted as molecular autopomorphies of this homogeneous taxon. The omission of the Distigma sequences allowed the inclusion of additional 600 general and 65 parsimony informative characters and offered a well-supported resolution of
Fig. 1. Euglenozoan maximum-likelihood tree (Hasegawa et al., 1985) of SSU rDNA. Bootstrap values for maximum-parsimony analysis above; for neighbour-joining (Kimura, 1980) below branches. Consensus of 1000 bootstrap replicates. General tree topology was independent of the evolutionary model used.

Fig. 2. Sequence divergences as mean values and standard deviation of taxa joined in the analysis. Same dataset as in Fig. 1.

all Rhabdomonadales (Fig. 4). Parmidium as root within this clade is supported by high bootstrap values and decay indices. At first sight it is evident that Rhabdomonas is a paraphyletic taxon. Rhabdomonas intermedia branches next to the genus Parmidium at the bottom of the Rhabdomonadales, giving rise to two sister clades. One includes Rhabdomonas costata, Rhabdomonas incurva (type species) and Gyropaigne lefevrei. The other one forms a larger monophylum with Rhabdospira spiralis, Rhabdomonas gibba and
Phylogeny of euglenid osmotrophs

Fig. 3. Maximum-parsimony tree of SSU rDNA of the primary osmotrophic taxa of euglenids. Most parsimonious tree: 791 steps, 1224 characters, 751 constant and 227 informative characters. Numbers above branches, bootstrap values; numbers below branches, decay indices. Consensus of 1000 bootstrap replicates.

Fig. 4. Maximum-likelihood tree (Hasegawa et al., 1985) of SSU rDNA of the Rhabdomonadales. Maximum-parsimony (843 steps, 1805 characters, 1232 constant and 291 informative characters), bootstraps above branches. Distance bootstraps below branches. Consensus of 1000 bootstrap replicates.

Rhabdomonas spiralis, as the sister clade of the genus Menoidium. Although only 1173 nucleotides of M. intermedii have been sequenced, the monophyly of this clade can be considered to be well supported with a bootstrap value of 100 and a decay index of 12. When M. intermedium was excluded from the analysis, the decay index for the monophyly of the genus Menoidium increased to 52 (data not shown), but the decay indices of all other nodes remained stable. It is important to mention that the sequences of Rhabdospira spiralis, Rhabdomonas spiralis and Rhabdospira gibba are identical with less than 1% difference in their base pairs, a very unusual phenomenon in this highly divergent group of protists.

How do molecular and morphological data fit together?

In an attempt to combine our molecular data with morphological characters, the autapomorphies of major clades in the Rhabdomonadales have been mapped onto a bootstrapped maximum-parsimony tree of the Rhabdomonadales (Fig. 5). Our molecular data correspond well with morphological data. The monophyly of the Rhabdomonadales can be justified by the scroll, a structure which is built by a sheath of microtubules and amorphous material surrounding the canal (Leedale & Hibberd, 1974; Cann, 1986). Ultrastructural investigations show that the scroll is not present in other euglenid organisms and especially not in Distigma species (Yamaguchi & Anderson, 1994; Angeler, 1999). Beyond that, they can be identified easily by the fusion of pellicular ridges and grooves, and the reduction of the ventral flagellum. Parmidium can be separated from Rhabdomonas, Menoidium and Gyropaigne by the deep indentations of the cell body. The development of prominent keels can be found in one monophyletic group that includes Gyropaigne lefevrei. The other clade comprises organisms with flattened cell bodies and diverges as a group in which the posterior end is twisted. Rhabdomonas gibba, Rhabdomonas spiralis and Rhabdospira spiralis are almost identical in their SSU rDNA sequences and likewise in their morphology. The lineage of Menoidium includes extremely flattened cells with elongated anterior and posterior ends (Leedale, 1967, and references therein).

DISCUSSION

Phylogenetic position of major Euglenozoa clades

Phylogenetic analyses of SSU rDNA sequences of the euglenids are consistent with the existing hypotheses concerning their evolution: phagotrophs arose prior to phototrophs (Montegut-Felkner & Triemer, 1997; Linton et al., 1999, 2000; Preisfeld et al., 2000). Phototrophs evolved in a single event after an uptake of eukaryotic phototroph prey by a phagotrophic Peranema-like organism, followed by the evolution of plastids (Gibbs, 1978).

The phototrophs form a highly supported clade comprising two groups: firstly, the members of the
well-founded clade Euglenales that share the synapomorphy of the reduced ventral flagellum, and secondly, the green Eutreptiales with two or more emergent flagella which we interpret as a plesiomorphic character. The molecular support for the Eutreptiales is correspondingly weak. In contrast to the Euglenales, which include osmotrophic taxa whose plastids have been lost (K hawkinea quartana, Astasia longa), no evidence for the former possession of plastids could be found for the osmotrophic Eutreptiales. Whereas the osmotrophic members of the Euglenales are placed within the same clade as the phototrophic Euglenales, molecular (Montegut-Felkner & Triemer, 1997; Linton et al., 1999, 2000) and morphological (Leedale, 1967; Dawson & Walne, 1994) data separate the osmotrophic members of the Eutreptiales from the phototrophic Eutreptiales (Preisfeld et al., 2000). The occurrence of the phototrophic members of the Eutreptiales in a clade together with all phototrophic euglenids and the divergence of the osmotrophic Eutreptiales in a major osmotrophic clade contradicts the validity of a taxon Eutreptiales comprising both phototrophs and osmotrophs.

The overall tree is heavily influenced by an unbalanced taxon sampling. Only two sequences from phototrophic euglenids could be included in this analysis. The phylogenetic position of Petalomonas cantuscygni and Peranema trichophorum has therefore to be regarded with caution. This may also be the reason for the weak resolution at the base of the euglenozoan tree. We can confirm that the Euglenozoa comprise three major clades (euglenids, kinetoplastids and diplonemids), but the inter-relationship is not resolvable with the present taxon sampling.

**Phylogenetic position of the Rhabdomonadales and taxonomic implications**

Until now the only primary osmotrophic taxa whose SSU rDNA sequences had been studied (Preisfeld et al., 2000) were two Distigma species (Eutreptiales) and Gyropaigue (Rhabdomonadales). With the addition of 11 taxa of the Rhabdomonadales, an intensive study of this clade gives strong support for the monophyly in a major clade of osmotrophs with Distigma as next relatives. The homogeneity of the rhabdomonadal SSU rDNA can also be corroborated by a comparison of their sequence divergence relative to those of other euglenids. The latter and the high support for the clade Rhabdomonadales in all tree inferences sustain the validity of this taxon that was erected by Leedale (1967) based on morphological characters.

Generally, the molecular analyses performed with the SSU rRNA gene in this study are in accordance with previous morphological and taxonomic investigations. The taxon Rhabdomonadales was erected by Leedale (1967) as an order with five genera: Gyropaigue, Menoidium, Rhabdomonas, Rhabdospira and Par midium. He pointed out that all these taxa have one emergent flagellum which is flexible during locomotion throughout its length and stiff in stationary cells; have rigid pellicles; have osmotrophic nutrition; lack an ingestion apparatus; lack plastids, stigma and paraxial swellings. The scroll which surrounds the canal can be considered as autapomorphy of this clade (Leedale & Hibberd, 1974). It is exclusively reported from species of this taxon (Leedale, 1967; Leedale & Hibberd, 1974). Distigma, the next relative to the Rhabdomonadales, has two emergent flagella and a flexible...
pellicle. The reduction of one flagellum and a rigid pellicle accompanied by the fusion of the pellicle strips to a nearly continuous epiplasmic layer can be interpreted as autapomorphies of the Rhabdomonadales, but arose convergently at several points (i.e. Euglenales) in the evolution of the Euglenozoa (Leedale, 1978).

Further analysis, restricted to the osmotrophs and using an alignment that allows the inclusion of more informative characters, suggested that the root of the Rhabdomonadales is probably the genus Parmidium. Christen (1963) already suggested that this genus could have given rise to the other genera of the Rhabdomonadales according to the rounded cell shape of Parmidium that allows all cell-shape modifications having taken place in the other genera of the order. The monophyletic genus Parmidium can be distinguished morphologically by the ventral indentation and a shield-like cell body.

The genus Rhabdomonas and taxonomic considerations

The validity of the genus Rhabdomonas has been questioned since its erection by Fresenius (1858). Skuja never accepted this taxon because he saw many transitions between Rhabdomonas and Menoidium (Skuja, 1948). Our analyses suggest that Rhabdomonas cannot be considered as a monophyletic genus because it branches on several nodes of the trees. The most basal branch is Rhabdomonas intermedia. Another group consists of Rhabdospira spiralis, Rhabdomonas spiralis and Rhabdomonas gibba. The sequences of these taxa differ by less than 1%. The taxonomic descriptions that can be found for these osmotrophs are confusing: Rhabdospira spiralis Pringsheim, which was treated as a valid taxon by Leedale (1967), seems never to have been described properly. This, and other references about Rhabdospira, quote Pringsheim (1963) who only suggested the new genus Rhabdospira, but never described it following the rules of any taxonomic Code. Something similar happened to Rhabdomonas gibba (Skuja) Pringsheim. No reference for a valid description could be found. This species was described as Menoidium gibbum Skuja (1939) and Pringsheim probably renamed this strain as Rhabdomonas gibba in the collection without presenting a formal description. Rhabdomonas spiralis was described by Pringsheim (1942). He pointed out the strong resemblance to Menoidium gibbum Skuja with a small difference in the degree of flattening of the cell body. Christen (1963) suggested to transfer Rhabdomonas spiralis to the genus Menoidium. According to taxonomic Codes (International Code of Zoological Nomenclature and International Code of Botanical Nomenclature) the first species name given is the one with priority. With this information and the results of the molecular analyses we recommend to label Rhabdomonas gibba (SAG 1271-2) and Rhabdospira spiralis (CCAP 1271/5) in the appropriate culture collections as Menoidium gibbum Skuja (1939), because no valid descriptions of these taxa exist. Menoidium gibbum would then be the sister taxon to the clade of Menoidium bibacillatum, Menoidium cultellus and Menoidium intermedium nom. nud. This genus is characterized by a strongly flattened cell body and elongated posterior ends, which in some cases can be slightly twisted.

The genus Rhabdomonas appears as another paraphyletic group in this analysis. Rhabdomonas costata and the type species Rhabdomonas incurva can be found intermingled with Gyropaigne lefevrei. Gyropaigne can be distinguished from the genus Rhabdomonas because it has a number of pronounced helical keels (Christen, 1960). There is a range of forms from none to slight to pronounced keels, for instance the species Rhabdomonas torta (Christen, 1963). As only one species of Gyropaigne has been analysed in this study, no changes in the taxonomy are recommended.

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