Phylogenetic affinities of the Trentepohliales inferred from small-subunit rDNA

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Phylogenetic analyses of the nuclear-encoded small-subunit rDNA sequences from taxa representing all of the major lineages of green algae, including new sequences for the Trentepohliales, consistently indicated that the subaerial Trentepohliales are closely related to ulvophycean marine green algae, particularly to the siphonous and hemisiphonous orders. The presence of phragmoplast-type cytokinesis in the order Trentepohliales remains enigmatic, and it is interesting that this type of cell division is associated with terrestrial (subaerial) habits.

INTRODUCTION

Recent reviews (e.g. Chapman et al., 1999; Waters & Chapman, 1996) of analyses of the nuclear-encoded small-subunit (SSU) rDNA as well as the chloroplast-encoded large-subunit Rubisco gene (rbcL) provide support for the original suggestion, based on ultrastructural data, that there are two main lineages among the green plants (Pickett-Heaps & Marchand, 1972). One of the lineages comprises the charophycean algae and their descendants, the land plants (charophycean lineage sensu Pickett-Heaps & Marchant, 1972), forming a monophyletic group known as Streptophyta (sensu Cavalier-Smith, 1993). The charophycean algae include taxa from at least five orders: the Chlorokybales, Klebsormidiales, Zygnematales, Coleochaetales and Charales. The second lineage (chlorophycean algae sensu Pickett-Heaps & Marchant, 1972) consists exclusively of the remaining green algae (containing at least Chlamydomonas and its allies, the coccoid green algae, Dasyycladales, Cladophorales and Trebouxiophyceae), forming the monophyletic group Chlorophyta (sensu Cavalier-Smith, 1993; Friedl, 1997; Melkonian et al., 1995). The term ‘chlorophyte’ has often been used to denote all green algae; however, it should now be used only as an informal designation for green algae in the Chlorophyta [see Chapman et al. (1999) for a revisionary approach to the systematics of the green algae].

Conflicting hypotheses have been offered about the systematic position of the Trentepohliales among the several classes of green algae. The presence of multilayered structures in the flagellar apparatus and the demonstration of a phragmoplast-type cytokinesis in Cephaleuros parasiticus (Chapman & Henk, 1986) and Trentepohlia odorata (Chapman et al., 2001; Waters et al., 1998) suggest an affinity with the class Charophyceae. However, taxonomic features of the Trentepohliales such as the anticlockwise flagellar apparatus components can be cited as evidence for an affinity with the Ulvophyceae (Roberts, 1984). Based on biochemical, biophysical and physiological features, Raven (1987) classified the Trentepohliales among a third class, the Pleurostrophyceae. Moreover, it was noted that the Trentepohliales even share a rare ultrastructural feature (presumptive mating structures in the gametes) with members of a fourth class, the Chlorophyceae (Chapman & Henk, 1983, 1985). Therefore, the Trentepohliales exhibit some features associated with four of the five major classes of green algae in the system proposed by Mattox & Stewart (1984); however, the major discussions have been focused on ulvophycean versus charophycean affinities of this enigmatic order. Members of the Trentepohliales are most abundant in tropical and subtropical regions worldwide. A distinct assemblage of green algae characterized by special adaptations to subaerial habitats, the group includes vascular plant epiphytes, some of which are considered to be economically important, such as Cephaleuros virescens Kunze in Fries, which is a parasite on tea leaves (Chapman, 1984). The order remains incompletely characterized for phylogenetic purposes (O’Kelly & Floyd, 1990).

Where does the Trentepohliales belong in the various proposed classification schemes of green algal classes? What are the closest relatives of the Trentepohliales? In this study, we have addressed these questions by using phylogenetic analysis of newly obtained SSU rDNA sequences from several genera of Trentepohliales and representatives of the major classes and orders of green algae.

The GenBank/EMBL/DDBJ accession numbers for the SSU rDNA sequences of Cephaleuros parasiticus, Cephaleuros virescens, Physcomitrium monile, Trentepohlia arborum, Trentepohlia aurea, Trentepohlia dialepta, Trentepohlia sp. and Phycopeltis sp. are respectively AY052563–AY052570.
METHODS

Taxa. Trentepohlialean samples used in this study were obtained from a variety of sources. Some samples were collected in the field and preserved using silica gel. Other samples came from trentepohlialean cultures maintained at Louisiana State University (LSU) and some were purchased from the algal culture collection of The University of Texas at Austin (UTEX) and the Culture Collection of Algae and Protozoa (CCAP), UK. In all cases, the unialgal cultures were grown in liquid and/or agarized M2H media (Nichols, 1973) under continuous cool white fluorescent light. Trentepohlialean taxa included in this study were *Cephaleuros parasiticus* Karsten (unialgal culture isolate LSU BR52; isolated on the LSU campus), *Cephaleuros virescens* Kunze in Fries (unialgal culture isolate LSU BR50; LSU campus), *Trentepohlia* sp. (unialgal culture UTEX 1227; Cape Cod, MA, USA), *Trentepohlia dialepta* (Nylander) Hariot (unialgal culture CCAP 483/2; Kampala, Uganda), *Physollinion monile* (De Wildeman) Printz (unialgal culture collection LSU; unknown location), *Trentepohlia aurea* (L.) Marius (unialgal culture UTEX LB429; Abersystwyth, UK), *Trentepohlia arborum* (Agardh) Hariot (silica gel-preserved sample; greenhouse, University of São Paulo campus, São Paulo, Brazil) and *Phycopeltis* sp. (silica gel-preserved sample; Galway, Ireland).

DNA extraction. Total cellular DNA was extracted from trentepohlialean samples as follows. Aliquots of unialgal cultures were rinsed with 0.5 M Tris/HCl, pH 7.2. Cells were disrupted by grinding in liquid nitrogen with a chilled mortar and pestle. Samples were then treated as described in the DNaseasy Plant Mini kit (Qiagen) handbook for DNA isolation from plant tissue. The nucleic acid pellet was dissolved in sterile distilled water. DNA samples were stored at −20 °C. The integrity of DNA preparations was checked by electrophoresis in agarose gels stained with ethidium bromide.

PCR amplification and sequencing. DNA was amplified by PCR of the nuclear-encoded SSU rRNA gene using primers designed originally for both green algae and land plants (Hamby et al., 1988). These primers were designed based on conserved regions from the SSU rRNA and have been shown to amplify rRNA genes from other green algae, bryophytes and lower vascular plants (Chapman & Buchheim, 1991). Template DNA was amplified in 0.2 ml thin-walled PCR tubes (DOT Scientific) in a total reaction volume of 50 μl. Each amplification reaction consisted of 2 μl template DNA, 2 μl of two flanking primers, 5 μl MgCl2 (25 mM), 5 μl Thermophilic DNA polymerase 10× buffer (Promega), 4 μl 1 mM deoxynucleotide mixture (containing dATP, TTP, dCTP and dGTP), 0.4 μl Taq DNA polymerase in storage buffer B (Promega) and sterile water. Negative controls without DNA template were included. Amplification cycles were controlled in a GeneAmp PCR System 2400 (Perkin-Elmer) thermocycler with an initial denaturation step at 95 °C for 3 min followed by 30 cycles of 1 min at 95 °C, primer annealing at 52 °C for 1 min and extension at 72 °C for 2 min. Amplified products and a standard 1 kb DNA ladder (Life Technologies) were visualized by electrophoresis on 0.5% agarose gels stained with ethidium bromide for correct length, yield and purity. DNA products were purified with Millipore UFC3 TTK 00 (30000 NMWL) filter units with an Eppendorf 5415 C table-top microcentrifuge. Purified DNA amplification products were sequenced with internal SSU rRNA primers using the protocol of the DNA sequencing kit (Big-Dye Terminator cycle sequencing ready reaction ABI PRISM; Perkin Elmer Applied Biosystems) in an ABI 377 PRISM automated sequencer. DNA sequences were captured as text as well as colour-coded electropherograms. DNA products were purified with Millipore UFC3 TTK 00 (30 000 NMWL) filter units with an Eppendorf 5415 C table-top microcentrifuge. Purified DNA amplification products were sequenced with internal SSU rRNA primers using the protocol of the DNA sequencing kit (Big-Dye Terminator cycle sequencing ready reaction ABI PRISM; Perkin Elmer Applied Biosystems) in an ABI 377 PRISM automated sequencer. DNA sequences were captured as text as well as colour-coded electropherograms.

Sequence alignment and data analysis. Sequence data were converted using ReadSeq with the Pearson/FASTA output format available at http://bimas.dct.nih.gov/molbio/readseq/ (Bioinformatics & Molecular Analysis Section, NIH, USA). Converted sequences were submitted for automatic multiple alignment to MultAlin, a multiple sequence alignment program available at http://prodes.toulouse.inra.fr/multalin/multalin.html (Laboratoire du Génétique Cellulaire, Institut National de la Recherche Agronomique, Toulouse, France) (Corpet, 1988). The SSU sequences were manually adjusted using by SeqPup version 0.6 (ftp://jubio.bio.indiana.edu/ mollbio/seqpup/). All analyses were performed using a G4 Macintosh computer (Apple). Regions in the data matrix that could not be unambiguously aligned were excluded from the analyses. A total of 100 green plants and two outgroup taxa (glaucoectophytes) were used to construct a general data matrix. A subset with 1729 positions was used as input for distance and maximum-parsimony analyses of 55 taxa of Viridiplantae with two glaucocystophyte taxa as the outgroup to position the Trentepohliales among the Viridiplantae. A more restricted subset from the large matrix containing 34 taxa of ulvophycean algae, including representatives of all the orders, was used as input for distance, maximum-parsimony and maximum-likelihood analyses to evaluate the position of the Trentepohliales within the ulvophycean algae. Distance analyses were performed with the PAUP* 4.0 package (Swofford, 2000). The two-parameter model of Kimura (1980) was used to generate distance matrices, which were converted to phylogenetic trees with the neighbour-joining method (Saitou & Nei, 1987). Maximum-parsimony analyses were performed in PAUP* 4.0 with the heuristic search option with a branch-swapping algorithm (tree bisection-reconnection) and random sequence addition (1000 replicates). For maximum-likelihood analyses, PAUP* 4.0 was used. Bootstrap analyses were performed in PAUP* 4.0 to assess the stability/support of nodes with 1000–5000 replications for maximum-parsimony and neighbour-joining analyses.

RESULTS AND DISCUSSION

The phylogenetic position of the Trentepohliales

In order to elucidate the overall position of the Trentepohliales in a nuclear-encoded SSU rDNA phylogeny of the Viridiplantae, a global analysis was performed with a subset of 57 taxa (Fig. 1) and 1729 (equally weighted) aligned characters. Two glaucoectophytes (*Cyanoptyle gloeocystis* and *Glaucocystis nostochinearum*) were included as the outgroup. Fifty-five green plants were included as representatives of the Viridiplantae, from the streptophyte lineage (Charophyceae and Embryophyta), as well as from the chlorophyte lineage. Both the distance and maximum-parsimony analyses positioned the trentepohlialean taxa unequivocally within the chlorophytan lineage, the Chlorophyta. The Chlorophyta lineage forms a monophyletic group (bootstrap support 97–82%). The present results based on nuclear-encoded SSU rDNA confirm previous reports from both SSU rDNA data and chloroplast-encoded Rubisco large subunit data on the existence of two lineages of Viridiplantae. The land plants within the Streptophyte clade diverged from the Chlorophyceae, as expected from previous reports.

As predicted by Mattox & Stewart (1984), the monophyletic Chlorophyta taxa (*Mamiella* sp., *Mantonella* spp., *Micromonas pusilla*, *Pycnococcus provasoli*, *Pseudosouillardia marina*, *Nephroselmis olivacea*, *Tetraselmis striata* and *Scheffelia dubia*) are not a natural or monophyletic group but rather a series of basal divergences forming a
This paraphyletic group is also known as Prasinophyceae, and the use of this term has been recommended by Sym & Pienaar (1993). Similar results supporting the paraphyletic nature of this group have been reported (Nakayama et al., 1998; Fawley et al., 1999). Our results for the clade formed by Pseudoscourfieldia marina and Pycnococcus provasolii (bootstrap support 100/100%) confirm the recent study by Fawley et al. (1999) using SSU rDNA sequence data in grouping these two taxa in one family, Pycnococcaceae. Furthermore, recent rDNA and actin analysis of Mesostigma (not shown in Fig. 2) has placed this taxon in the charophycean lineage (Melkonian et al., 1995; Bhattacharya et al., 1998), thus indicating polyphyly for the algae we call the prasinophytes. Therefore, it may be time to end the use of 'Prasinophyceae' or 'Micromonadophyceae', and maybe even the very convenient term 'prasinophytes'. However, in the absence of new names for the seven distinct lineages, the term 'prasinophytes' will be used in this report. In general, the prasinophytes are considered as the modern representatives...
of the earliest green algae (Graham & Wilcox, 2000) and, despite the fact that they are not a monophyletic group, their basal position is well supported.

Three classes of chlorophytes

The representatives of the ulvophycean taxa in this study consistently form a monophyletic group (bootstrap support 80/53 %) that includes the taxa of the order Trentepohliales. This clade is the sister group of the remaining green algae, the Chlorophyceae and the Pleurastrophyceae. There is no strong support for the recognition of the monophyletic nature of the Chlorophyceae and the Pleurastrophyceae sensu Mattox & Stewart. The Pleurastrophyceae of Mattox & Stewart has been shown to be a polyphyletic group (Friedl & Zeltner, 1994). Friedl (1995) erected a new class name, and Stewart has been shown to be a polyphyletic group (Friedl & Zeltner, 1994). Friedl (1995) erected a new class name, and members of the Microthamniales [sensu Melkonian (1982, 1990) or Pleurastrales sensu Mattox & Stewart (1984)] based on rDNA sequence comparisons (Friedl, 1995, 1997). In our analyses, the Trebouxiophyceae form a clade with moderate support (64–79 %).

The Chlorophyceae analysed in the present study formed a well-supported clade (97–91 %) and comprise two distinct monophyletic lineages defined by ultrastructural details of the flagellar apparatus; one group (Chlamydomonas reinhardtii, Volvox carterii, Gongrostra papuasia, Botryococcus braunii, Protosiphon botryoides and Dunaliella salina) with a clockwise basal body configuration (CW group, Fig. 1) and the other group (Ankistrodesmus stipitatus, Scenedesmus obliquus, Neochloris aquatica, Pediasstrum duplex and Hydrodictyon reticulatum) with directly opposed basal bodies (DO group, Fig. 1) (Lewis et al., 1992; Nakayama et al., 1996).

In the present study, the ulvophycean taxa formed a monophyletic group in all analyses. Distance methods and maximum-parsimony analysis consistently positioned the taxa of the Trentepohliales within this ulvophycean clade. The monophyly of the Trentepohliales (bootstrap support 100/100 %) is not surprising, since some features such as the sporangium-associated apparatus and the flagellar apparatus are unique to this order. An affinity with the Pleurastrophyceae (Trebouxiophyceae), as suggested by Raven (1987), is not supported. An ulvophycean affinity for the Trentepohliales has been expressed before (Roberts, 1984) based on ultrastructural features of the flagellar apparatus. The ulvophycean and trentepohlialean taxa share an anticlockwise basal body configuration as well as an alternation of generations. Based on preliminary partial nuclear-encoded SSU rRNA sequence data, Zechman et al. (1990) also related the Trentepohliales to the ulvophycean clade.

One question arising from these results is how to explain the presence of the phragmoplast in Cephalheuros (Chapman & Henk, 1986) and Trentepohlia (Chapman et al., 2001). The presence of a phragmoplast-type cytokinesis is well documented in some charophycean algae and is the typical mode of cytokinesis in land plants. The presence of a phragmoplast-type cytokinesis in the chlorophycean lineage raises the question of the ‘homology’ of this process: is it possible that the phragmoplast evolved more than once? It is difficult to understand how a highly sophisticated complex cytological process involving many genes evolved in two different lineages. However, the genus Koliella provides another example of very similar forms of cell division occurring in both major lineages of green algae. The general similarities in cytokinesis and karyokinesis in Koliella and Klebsormidium suggested a close phylogenetic affinity (Lokhorst & Star, 1998), but molecular analyses (Katana et al., 2001) indicate that the Koliella species belong to the Trebouxiophyceae in the chlorophycean lineage. Thus, the similarities in cytokinesis and karyokinesis are homoplasious. Explanations of the enigmatic presence of a phragmoplast in the Trentepohliales in the ulvophycean clade will come from thorough ultrastructural and immunological analyses of the evolution of the phragmoplast in the basal lineages of the streptophytes as well as in the Trentepohliales.

Relationships within the Ulvophyceae

In order to elucidate the precise position of the Trentepohliales within the nuclear-encoded SSU rDNA phylogeny of the ulvophycean green algae, a subset of 38 taxa from the complete data matrix was analysed with 1714 (equally weighted) characters (Fig. 2). Two trebouxiophycean algae (Myrmecia israeliensis and Trebouxia impressa) and two chlorophycean algae (Pediasstrum duplex and Scenedesmus obliquus) were included as the outgroup taxa. Thirty-four algal taxa were included in the ingroup, representing most of the major orders of the ulvophytes.

In all phylogenetic analyses, including neighbour-joining, parsimony and likelihood methods, the Trentepohliales formed a monophyletic group that was well supported by bootstrap analysis in the neighbour-joining method (100 %) and maximum-parsimony method (100 %) as well as the maximum-likelihood approach (Figs 2 and 3). In the distance and parsimony analyses, the Trentepohliales emerged as a sister group to the clade containing the Siphonocladales/Cladophorales complex and Dasycladales, both of which contain representatives mainly from the marine environment. However, in the maximum-likelihood analysis, the Trentepohliales are resolved as a sister clade only to the Dasycladales.

Previous concepts of the Ulvophyceae

In the scheme of Mattox & Stewart (1984), the Ulvophyceae were defined in terms of ultrastructural features that make them a group distinct from the Chlorophyceae and Charophyceae. In the same paper, ulvophycean algae were considered more ‘advanced’, since their vegetative state is non-motile and non-flagellated and is presumably derived from scaly green flagellates. O’Kelly & Floyd (1984)
presented a more detailed study of the Ulvophyceae, defining them in terms of ultrastructural, reproductive and biochemical features. The scheme recognized five orders: Ulotrichales, Ulvales, Siphonocladales (including Cladochlorales), Dasycladales and Caulerpales. In this study, the Ulvales and Ulotricales were considered primitive orders of the Ulvophyceae and the Siphonocladales, Dasycladales and Caulerpales more advanced orders. The authors also implied that the Siphonocladales and Dasycladales are sister groups. This arrangement of orders, as well the relationships of the orders in the Ulvophyceae, was based on (i) the absence of quadriflagellated motile cells or modification of the flagellar apparatus, (ii) development of basal body orientation perpendicular to the long axis of the cell during forward swimming, (iii) loss of the isomorphic life history, (iv) an increasing complexity in the zoosporangial and gametangial structure and development and (v) structural and chemical composition of the cell walls. O’Kelly & Floyd (1984), although recognizing that trentepohlialean motile cells are consistent with their definition of the class Ulvophyceae, decided to exclude the order Trentepohliales from the Ulvophyceae because of the presence of multilayered structures associated with two of the four rootlets and plasmodesmata in the cross walls between vegetative cells.

Sluiman (1989) considered the Ulvophyceae to be represented by eight orders: Ulotrichales, Ctenocladales, Trentepohliales, Pleurastrales, Acrosiphonales, Cladophorales, Bryopsidales and Dasycladales. His system was based mainly on ultrastructural features of the flagellar apparatus, de-emphasizing the importance of cell division at the

Fig. 2. Phylogeny of the Ulvophyceae based on nuclear-encoded SSU rDNA sequence analyses by distance and maximum-parsimony methods. This phylogenetic tree corresponds to the bootstrap analysis (parsimony) of 38 taxa and 1714 aligned characters. Tree rooted with two outgroup sets: two chlorophycean taxa (Pediastrum duplex and Scenedesmus obliquus) and two trebouxiophycean algae (Myrmecia israeliensis and Trebouxia impressa). *, Different topology in the distance analysis. Bootstrap values of distance (number before the slash) and maximum-parsimony (number after the slash) analysis using an identical dataset are indicated (only values \(>50\%\) were recorded).
ordinal level. It is important to mention that this was the first paper to assign the order Trentepohliales formally to the Ulvophyceae. Mattox & Stewart (1984) mentioned the order Trentepohliales in the Ulvophyceae by referring to the ultrastructural studies of Roberts (1984), and O’Kelly & Floyd (1984) did not mention the order Trentepohliales in their Ulvophycean scheme. However, Sluiman’s concept of the orders in the class Ulvophyceae, acknowledging the ulvophycean nature of the order Trentepohliales, implied that some features such as cell division may be homoplasious (independently derived), particularly the phragmoplast-type cytokinesis in the overall scheme of the chlorophytes.

**Current concepts**

Results from the present study support two groups within the ulvophycean algae. The first group is represented by the orders Ulotrichales (Gloeotilopsis planctonica, Parotoderma sarcoidea, Pseudodendronium basiliense, Ulothrix zonata, Monostroma grevillii, Urosora penicillusformis and Acrosiphonia sp.) and Ulvales (Ulva rigida and Enteromorpha intestinalis). The second monophyletic group of ulvophytes contains the order Trentepohliales and the siphonous and hemisiphonous ulvophycean algae (bootstrap support of 100 and 90%). Within this group, the order Trentepohliales either is a sister clade to the Siphonocladales/Cladophorales complex (represented by Chamaedoris penicillum, Cladophoropsis membranacea, Siphonoclados tropicus, Ernodesmis verticillata, Chaetomorpha sp., Cladophora rupestris, Microdictyon boergeseni, Cladophora coelothrix, Cladophora vagabunda and Valonia utricularis) and the Dasycladales (distance and parsimony methods) or belongs to a more derived clade (maximum-likelihood analyses), with the sister group formed only by Dasycladales (Acetabularia major, Acularia schenkl, Polyphysa parvula, Batophora occidentalis, Chlorocladus australasicus, Cymopolia...
versus ‘advanced’ orders.

ulvophytes, that is, the Ulvales

non-monophyly for Ulvophyceae. The two groups of

the Pleurastrales (pleurastrophycean algae

and/or character sampling. Our results do not support the

were susceptible to rearrangement by changes in taxon

separated by the Pleurastrophyceae and Chlorophyceae.

In conclusion, our study provides robust support to

position the order Trentepohliales within the ulvophycean

algae in the chlorophyтан lineage. Our results clearly

indicate that the ‘advanced’ marine taxa (orders Siphono-

oclades/Cladophorales complex and Dasycladales)

are the most closely related to the trentepohlialean algae.

Our analyses provide only moderate support for the monophyly

of the class Ulvophyceae. The evolutionary implications of

these results for the origin of the Trentepohliales indicate that they presumably diverged from an ulvophycean-like

macroscopic filamentous marine ancestor. The presence of the

phragmoplast-type cytokinesis in the order Trentepohliales remains enigmatic, but, if such a system evolved in the

freshwater Streptophyta lineage, there is no reason why an almost identical system could not have evolved in the

marine portion of the Chlorophyta lineage. Finally, it is interesting that, in both lineages, phragmoplast-mediated
cytokinesis is associated with terrestrial (subaerial) habits.

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