

PHYLOGENETIC RELATIONSHIPS OF FOUR CHAROPHYCEAN GREEN ALGAE INFERRED FROM COMPLETE NUCLEAR-ENCODED SMALL SUBUNIT rRNA GENE SEQUENCES¹

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Complete nuclear-encoded (18S) small subunit rRNA gene sequences were determined for four charophycean green algae, *Chlorokybus atmophyticus*, *Coleochaete orbicularis*, *Klebsormidium flaccidum*, and *Nitella* sp. *Chlorokybus atmophyticus* and *Coleochaete orbicularis* have been previously suggested to represent the most basal and most derived taxa within the charophytes, respectively. However, parsimony analysis of our 18S rDNA sequences along with a selection of other complete green algal and land plant 18S rDNA sequences yields a gene tree topology in which *Chlorokybus* is the most basal taxon, followed by the branching of *Coleochaete* and *Klebsormidium*. Two “sister” clades then diverge, one including *Nitella* and the land plants, and the second, members of the Chlorophyceae and Pleurostrophyceae. Despite producing slightly different gene tree topologies than those inferred from parsimony, distance analyses of the 18S rDNA sequences also do not indicate a strong affinity between the land plants and *Coleochaete*. Rather, *Klebsormidium* and *Coleochaete* are virtually equidistant from the land plant taxa. Other data are needed in order to assess the unexpected findings reported here, particularly the position of *Coleochaete*.

A preponderance of morphological, biochemical, and molecular data provide support for the green algae, specifically the class Charophyceae, being the sister group of the land plants (see Mattox and Stewart, 1984; Mishler and Churchill, 1985; Graham, Delwiche, and Mishler, 1991; Graham and Kaneko, 1991). Recent cladistic analyses based upon morphological, ultrastructural, biochemical, and other characters have suggested that *Chlorokybus* and *Coleochaete orbicularis* represent the most basal and most derived members of the Charophyceae, respectively (Mishler and Churchill, 1985; Graham, Delwiche, and Mishler, 1991).

Molecular data supporting the close relationship of the Charophyceae and the land plants include the discovery that group II introns found in plastid tRNA^{Ala} and tRNA^{Leu} genes of all land plants are present in certain charophycean taxa while lacking in other algae and eubacteria (Manhart and Palmer, 1990). Also, the plastid-derived *tufA* gene, which resides only in the nuclear genome of land plants, has been suggested to have made the transition from plastid to nucleus early in the charophycean lineage (Baldauf and Palmer, 1990; Baldauf, Manhart, and Palmer, 1990).

Analyses based on sequences of nuclear-encoded 5S, 18S, and 23S rRNA genes of charophycean algae have also suggested an affinity between the Charophyceae and land plants. There are limitations to the use of the 5S rRNA molecule for phylogenetic studies because of its small size (Steele et al., 1991). Nevertheless, results from this molecule are suggestive. Of the green algal taxa examined in an early study employing this gene, the charophyte *Nitella* was placed closest to land plants (Hori and Osawa, 1987). In a more extensive analysis, Devereux, Loeblich, and Fox (1990) found the charophycean genera, *Coleochaete*, *Klebsormidium*, *Nitella*, and *Spirogyra*, to

group with the land plants. Other analyses have reported partial 18S or partial 23S rRNA sequences which are informative for the question of land plant origins. An analysis using partial 18S and 23S rRNA sequences (Zechman et al., 1990) showed two charophycean taxa, *Klebsormidium flaccidum* and *Spirogyra maxima*, to belong to a clade terminating with *Equisetum* and *Glycine*. Kantz et al. (1990) used similar data to examine relationships of micromonadophycean (prasinophycean) and pleurostrophycean taxa. *Klebsormidium* was once again found to reside on a land plant-containing clade which branched off in the midst of micromonadophycean taxa.

In this paper, we present phylogenetic analyses of complete 18S rRNA gene sequences from *Chlorokybus atmophyticus*, *Coleochaete orbicularis*, *Klebsormidium flaccidum*, and *Nitella* sp. to further test the proposed relationship of the charophycean algae to the land plants and to the other green algae.

MATERIALS AND METHODS

Coleochaete orbicularis Pringsheim was generously provided by Dr. Linda E. Graham. *Chlorokybus atmophyticus* Geitler is the strain at one time maintained at the Culture Centre of Algae and Protozoa at Cambridge, England as #403/1. Because *Chlorokybus atmophyticus* is no longer available from this source, we have deposited it in the Culture Collection of Algae at the University of Texas at Austin (UTEX, LB2591) (Starr and Zeikus, 1987). *Klebsormidium flaccidum* (Kütz.) Silva, Mattox et Blackwell was obtained from UTEX (#2017), and *Nitella* sp. from the Carolina Biological Supply Company.

Molecular methods—Methods employed for DNA isolation, cloning, and sequencing were essentially identical to those described previously (Lewis et al., 1992; Wilcox et al., 1992). To aid in lysing the cells of *Chlorokybus*, *Coleochaete*, and *Nitella*, material was gently ground with a mortar and pestle in extraction buffer. A french pressure cell was used to lyse *Klebsormidium* cells. PCR products

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representing the entire 18S rDNA sequence were cloned into M13 mp-18 and mp-19 sequencing vectors. Multiple M13 clones (five or more per orientation) were pooled and sequenced.

Sequence analysis—Sequences from the yeast *Saccharomyces cerevisiae* (Rubstov et al., 1980; Mankin, Skryabin, and Rubstov, 1986) and the amoeba *Acanthamoeba castellanii* (Gunderson and Sogin, 1986) were included in some analyses as outgroup taxa, since no complete sequences from any primitive green alga have yet been reported. As complete sequences for other green algal groups become available it will be important to reexamine the effect of our choice of outlier on the branching order. We wish to emphasize the preliminary nature of any analysis, given the small number of complete green algal 18S rDNA sequences that are currently available. Sources for other sequences are as follows: *Glycine max* (Eckenrode, Arnold, and Meagher, 1985); *Zamia pumila* (Nairn and Ferl, 1988); *Zea mays* (Messing et al., 1984); *Chlorella vulgaris* (Huss and Sogin, 1989); *Scenedesmus obliquus* and *Chlorella kessleri* (Huss and Sogin, 1990); *Characium hindakii*, *Characium perforatum*, *Dunaliella parva*, and *Friedmannia israelensis* (Lewis et al., 1992); *Hydrodictyon reticulatum* (Wilcox et al., 1992); and *Asteromonas gracilis* (this paper).

Two separate 18S rDNA sequence alignments were manually constructed using the program ESEE for the IBM PC (Cabot and Beckenbach, 1989). The first, which includes the *Saccharomyces* and *Acanthamoeba* sequences, contains a total of 1,543 unambiguously aligned positions which remain after excluding hypervariable regions. Of these, 223 are phylogenetically informative. The second, which omits the outgroup taxa, contains 1,638 positions with 230 informative sites. Note that the latter data set includes 48 “informative” sites from the first set that are no longer phylogenetically informative within the green algae, and includes 55 sites which can be aligned within the green algae, but which are ambiguous in homology when the outlier species are included. The second data set thus includes a considerably expanded group of informative sites specifically for the green algae. Data sets were subjected to parsimony analysis employing PAUP (Swofford, 1990). The data were also analyzed with distance methods. Distance analyses utilized the programs DNADIST (with Kimura correction), FITCH, and NEIGHBOR in the phylogenetic analysis package PHYLIP (Felsenstein, 1990).

Parsimony analyses were done using either weighted or unweighted data sets. It is not clear how one should weight different DNA sequence positions in any phylogenetic analysis, and many different weighting schemes can be proposed (see Williams and Fitch, 1990 for a review of many possible weighting schemes). In one analysis, all sites were equally weighted. In a second analysis, sites involved in base-pairing within stems of the secondary structure of the rRNA molecule were identified. Those sites that exhibited compensatory nucleotide substitutions at the complementary sites of the rRNA secondary structure in any pairwise comparison among the taxa in our analysis were down-weighted by one-half (see Lewis et al., 1992). Actual integer values used in PAUP for weighting sites in the parsimony analysis were 2 and 1.

To infer the shortest tree, a heuristic search with random addition of taxa (ten replicates) was used. Bootstrap analyses, consisting of 100 replicates, were also completed as were decay analyses (Mishler, Donoghue, and Albert, 1991).

Sequence availability—The new charophycean sequences have been deposited in GenBank and have been assigned the following accession numbers: *Chlorokybus atmophyticus*: M95612; *Coleochaete orbicularis*: M95611; *Klebsormidium flaccidum*: M95613; and *Nitella* sp.: M95615. The sequence for *Asteromonas gracilis*, also first reported here, has been deposited as M95614. The alignments used in the analyses are available from the authors.

RESULTS

Parsimony analysis—Analysis of the “first” data set that includes *Acanthamoeba* and *Saccharomyces* as outgroup taxa yielded a single most-parsimonious tree when all sites were weighted equally (Fig. 1). When base-paired positions exhibiting compensatory changes were down-weighted (see above), five trees resulted. The topology of the consensus tree determined from these five trees is indicated in Fig. 1. When differentially weighting sites, the ambiguity leading to multiple most-parsimonious trees resides among the chlorophycean and pleurostrophycean taxa. Tree lengths were 747 and 1,403 steps for the unweighted and weighted analyses, respectively.

Regardless of weighting, the Charophyceae is indicated to be paraphyletic with *Chlorokybus* branching first, followed by a clade containing *Coleochaete* and *Klebsormidium*. The remaining taxa fall into two clades, one containing *Nitella* and the land plants and the other containing members of the Chlorophyceae and Pleurostrophyceae (Fig. 1). Note that *Nitella* grouped with the land plants in fewer than half the bootstrap replicates and that in a decay analysis, the strict consensus tree of trees of one additional step (eight in all) did not maintain *Nitella* on the land plant clade (see Fig. 1). Also, the pairing of *Coleochaete* and *Klebsormidium* is only weakly supported as seen in the results of both bootstrap and decay analysis (Fig. 1).

By excluding the two outgroup taxa, nearly 100 additional sequence positions are added to the alignment, of which 55 are phylogenetically informative. Parsimony analysis of this “second” data set results in a single tree of length 696 (unweighted) or 1,323 (weighted) (Fig. 2). Since we are dealing with analyses based on complete or nearly complete 18S rRNA sequences, the rooting of the tree remains problematic. In light of the preliminary nature of our analysis, we have chosen to root the tree inferred from this analysis with *Chlorokybus*, by analogy with the tree containing *Saccharomyces* and *Acanthamoeba*. With this as an assumption, we find that *Coleochaete* and *Klebsormidium* branch off in succession rather than forming a separate clade as in Fig. 1. The positions of *Friedmannia* and *Characium perforatum* are somewhat different than in Fig. 1, but are now in line with clustering that has been found in our earlier studies of the Chlorophyceae (Lewis et al., 1992). The second parsimony analysis continues to group *Nitella* with the land plant taxa. Unlike the first analysis, this clade is supported by

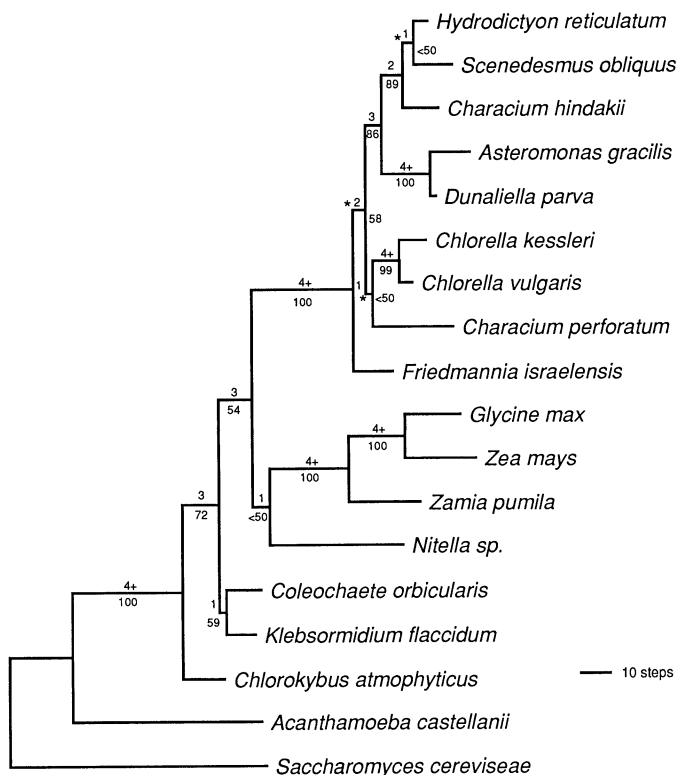


Fig. 1. Shortest tree resulting from heuristic search using data set with *Saccharomyces* and *Acanthamoeba* as outgroup taxa (all sites weighted equally). Numbers above each branch represent the number of additional steps required before branch "decays." Numbers below branches reflect result of bootstrap analysis. Branches indicated by asterisks are not supported in the five trees inferred when base-paired sites exhibiting compensatory substitutions are down-weighted. Consistency index (CI) values = 0.705 (all sites equally weighted) and 0.715 (differentially weighted).

bootstrap analysis in more than 75% of the replicates and does not decay until three additional steps longer are considered (Fig. 2). Once again, the branching order inferred for *Coleochaete* and *Klebsormidium* is only weakly supported by both bootstrap and decay analysis.

The number of unique (nonhomoplasious) character state changes was determined for each branch in the tree inferred from the second data set (see Fig. 2). Note that three unique characters support the *Nitella*/land plant clade. Examination of individual informative sequence positions showed that no character uniquely groups *Coleochaete* with the higher plants. Also of note are the comparatively short branch lengths of *Coleochaete*, *Klebsormidium*, and *Chlorokybus*, as well as the smaller number of autapomorphies in these taxa, on average, than are found among the land plant taxa and *Nitella*.

A user-defined tree (Fig. 3) was also examined. This tree has a branching order conforming to the topologies inferred for morphological characters in cladistic analyses by Mishler and Churchill (1985) and Graham, Delwiche, and Mishler (1991). This topology adds an additional 17 steps, compared to the most parsimonious tree.

Distance analysis—The distance matrix in Table 1 was used to construct trees using both FITCH and NEIGHBOR. The FITCH tree (Fig. 4) shows the land plants

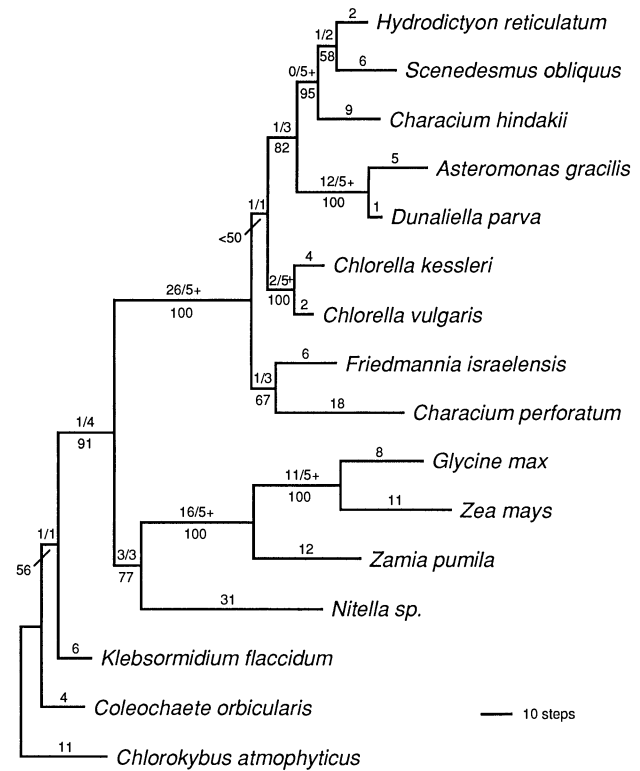


Fig. 2. Single most-parsimonious tree found in heuristic search using data set excluding outgroup taxa. Bootstrap values are as in Fig. 1. Total length of tree is 696 steps (unweighted) and 1,323 (weighted). The first number above each branch specifies the number of unique steps defining the clade to the right or the number of autapomorphies, when on terminal branches. The second number shows the decay index. CI = 0.717 (equal weighting) and 0.725 (differential weighting).

branching nearest the outgroup taxa, with *Nitella* and the other charophycean taxa branching next. The NEIGHBOR tree (not shown) differs by showing *Nitella* branching first, followed by the land plants and then the remaining charophycean organisms. Note that the branches supporting the land plant, *Nitella*, and *Coleochaete*/*Klebsormidium*/*Chlorokybus* assemblage, are very short for both distance analyses (Fig. 4).

DISCUSSION

Of the green algae for which complete 18S rDNA sequences are available, the charophycean taxa show the greatest affinity to the land plants. Parsimony analysis suggests that *Chlorokybus* occupies the basal-most position of the charophycean taxa. This agrees with earlier cladistic analyses based on other lines of evidence (Mishler and Churchill, 1985; Bremer et al., 1987; Graham, Delwiche, and Mishler, 1991). However, the relative positions of the remaining charophycean taxa on the 18S rRNA gene trees are different than those presented earlier, where the Klebsormidiales are the next most-basal group to the Chlorokybales, followed by, in succession, the Zygnematales, *Chaetosphaeridium*, Charales, and *Coleochaete* (Mishler and Churchill, 1985; Bremer et al., 1987; Graham, Delwiche, and Mishler, 1991).

Coleochaete and *Klebsormidium* have similar 18S rDNA

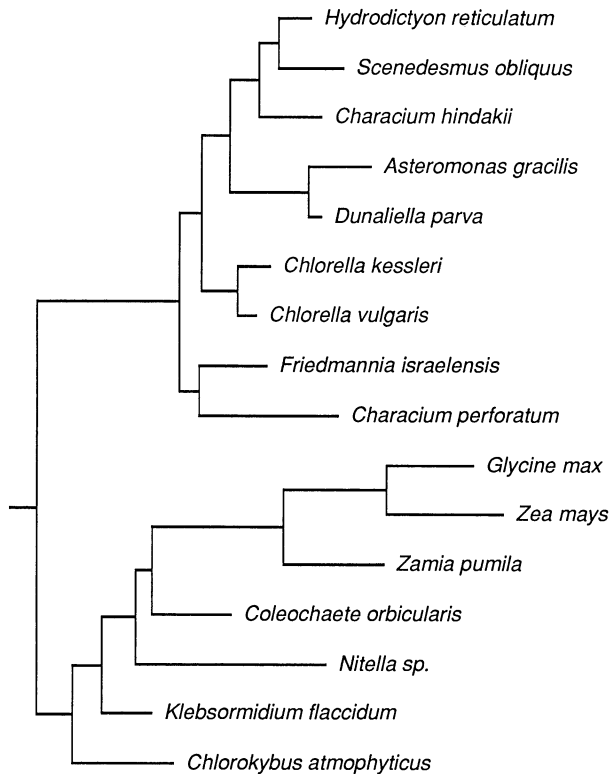
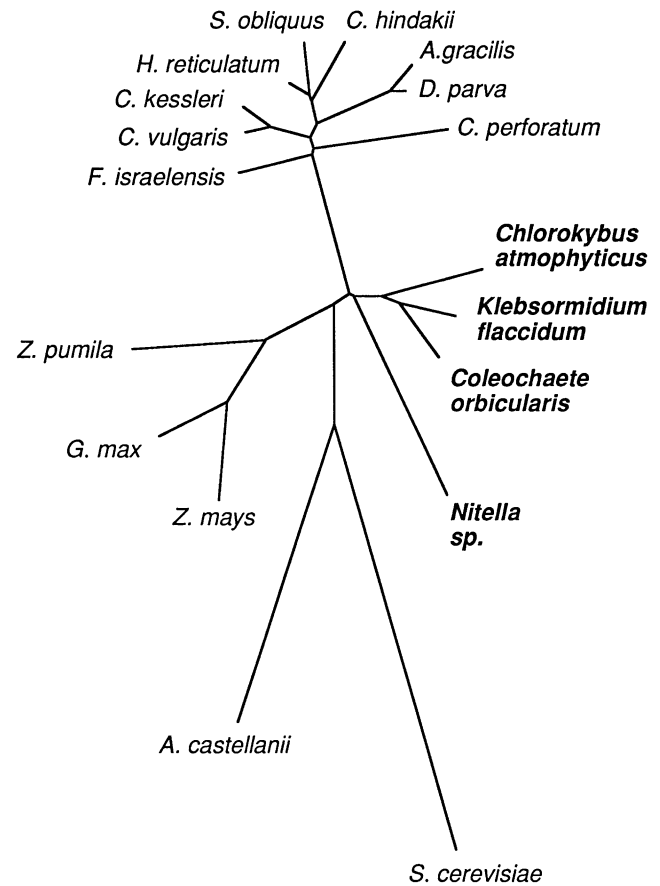


Fig. 3. User-defined tree generated in PAUP reflecting the topology inferred by Mishler and Churchill (1985) and Graham, Delwiche, and Mishler (1991), and the chlorophyte tree structure determined by Lewis et al. (1992). Tree lengths and CI values for this topology are 713/0.700 (equally weighted) and 1,356/0.707 (differentially weighted).

sequences, which bring them together in both parsimony and distance trees. Based on distance values, they are virtually equidistant from all other taxa examined (see Table 1). Parsimony analyses of the 18S rRNA gene sequences suggest that *Nitella* shares a more recent common ancestor with the land plants than does *Coleochaete*. These results are surprising since *C. orbicularis* appears to possess more land plantlike features than any other charophycean algae, including other species of *Coleochaete*. *Coleochaete orbicularis* has been represented as a sister taxon to the land plants (embryophytes) in cladistic analyses by Mishler and Churchill (1985) and Graham, Delwiche, and Mishler (1991). Parsimony analysis of the 18S rDNA sequence data instead positions the land plants plus *Nitella* as a sister group to one consisting of chlorophycean and pleurostrophycean taxa (see below). Forcing the 18S rDNA sequence data to yield a topology conforming to that inferred by Mishler and Churchill (1985) and Graham, Delwiche, and Mishler (1991) requires 17 additional steps in our parsimony analysis (see Fig. 3).

Our analysis is in agreement with earlier studies of partial nuclear 18S and 26S rRNA sequences (Chapman and Buchheim, 1991). They show a tree (their fig. 14) in which *Chara* and *Nitella* spp. are placed closer to the land plants than are *Coleochaete* and *Klebsormidium*. Chapman and Buchheim's (1991) report included the filamentous *Coleochaete nitellarum*. The parenchymatous *C. orbicularis* examined here would appear to be quite closely



1.0% distance

Fig. 4. FITCH tree inferred using the pairwise distance matrix in Table 1.

related to this species, judging by the relative position of *Klebsormidium flaccidum* in each study.

A second source of support for the findings presented here is provided by the analysis of the 5S rRNA molecule by Devereux, Loeblich, and Fox (1990) where *Coleochaete scutata* de Brébisson and *Klebsormidium* are paired, and *Nitella* is grouped with the land plants. However, the small size and subsequent relatively low information content of this molecule suggest that caution must be exercised when interpreting phylogenetic relationships obtained from 5S rRNA sequences (Steele et al., 1991). Nevertheless, the available data from the 5S, 18S, and 26S rRNA molecules are basically in agreement and consistent with our findings.

Our parsimony analysis suggests an unusual positioning of the land plants vs. the chlorophytes and pleurostrophytes. We find them to form two sister groups, suggesting that both share a charophycean ancestry. An earlier split is generally assumed, with unicellular flagellates giving rise to the major green algal classes (e.g., Pickett-Heaps and Marchant, 1972; O'Kelly and Floyd, 1984; Mishler and Churchill, 1985; Sluiman, 1985; Graham, Delwiche, and Mishler, 1991). This observation must be viewed with great caution, however, because it depends on assumptions concerning the root of the tree, which is a problem because of the lack of complete 18S rRNA sequences from

TABLE 1. Pairwise distance values generated using DNADIST of PHYLIP employing the Kimura two parameter correction.

	Ag	Dp	Ch	Hr	So	Ck	Cv	Fi	Cp	Co	N	Cu	Kf	Zp	Gm	Zm	Ac
<i>A. gracilis</i>	—																
<i>D. parva</i>	0.011	—															
<i>C. hindakii</i>	0.039	0.029	—														
<i>H. reticulatum</i>	0.032	0.025	0.016	—													
<i>S. obliquus</i>	0.039	0.030	0.021	0.014	—												
<i>C. kessleri</i>	0.040	0.033	0.030	0.024	0.027	—											
<i>C. vulgaris</i>	0.039	0.031	0.028	0.021	0.031	0.010	—										
<i>F. israelensis</i>	0.040	0.031	0.032	0.026	0.027	0.027	0.027	—									
<i>C. perforatum</i>	0.053	0.044	0.039	0.038	0.040	0.037	0.032	0.034	—								
<i>C. orbicularis</i>	0.069	0.065	0.065	0.053	0.060	0.058	0.056	0.054	0.067	—							
<i>Nitella</i> sp.	0.083	0.083	0.088	0.080	0.085	0.079	0.076	0.075	0.089	0.055	—						
<i>C. atmophyticus</i>	0.072	0.066	0.070	0.064	0.066	0.063	0.064	0.060	0.075	0.027	0.062	—					
<i>K. flaccidum</i>	0.071	0.065	0.065	0.056	0.061	0.060	0.058	0.056	0.064	0.017	0.053	0.030	—				
<i>Z. pumila</i>	0.090	0.087	0.086	0.079	0.085	0.080	0.076	0.080	0.092	0.052	0.077	0.061	0.053	—			
<i>G. max</i>	0.087	0.083	0.085	0.085	0.085	0.082	0.079	0.076	0.092	0.057	0.080	0.063	0.058	0.047	—		
<i>Z. mayis</i>	0.089	0.086	0.086	0.083	0.088	0.083	0.081	0.079	0.098	0.065	0.084	0.074	0.066	0.049	0.029	—	
<i>A. castellanii</i>	0.122	0.123	0.128	0.125	0.132	0.126	0.125	0.123	0.136	0.098	0.106	0.091	0.100	0.111	0.107	0.114	—
<i>S. cerevisiae</i>	0.142	0.139	0.147	0.140	0.141	0.137	0.137	0.141	0.149	0.126	0.143	0.121	0.127	0.143	0.141	0.150	0.134

appropriate primitive green algal groups. The inclusion of 18S rDNA sequences from other relevant taxa, including prasinophytes and additional charophytes, might yield an altered rooting and topology, particularly with respect to the relationship between the land plant and Chlorophyceae/Pleurostrophyceae clades. It would appear unlikely, however, that additional 18S rRNA gene sequences would significantly alter the relative positions of *Coleochaete* and *Klebsormidium* with respect to the land plants.

The 18S rDNA sequence data presented here yield slightly different topologies depending upon which tree-building algorithm is employed. We are inclined to favor the branching order obtained from parsimony, primarily because of the unexpected placement of land plants in the distance analysis (see Fig. 4). The reasons for discrepancies among methods are not altogether clear, but may be related to rate differences between taxa (Felsenstein, 1988). Examination of both distance and parsimony trees suggests that the terminal branches leading to *Coleochaete*, *Klebsormidium*, and *Chlorokybus* are generally shorter than those leading to other taxa, suggesting that these taxa may be evolving more slowly. Relative rate tests (data not shown) indicate, for example, that the rate of nucleotide substitution for the 18S rRNA gene in *Coleochaete* and *Klebsormidium* is approximately 2.5 times slower than that in *Characium hindakii*. All forms of phylogenetic analysis are subject to potential errors in reconstruction when evolutionary rates differ significantly between taxa (Li et al., 1987; Felsenstein, 1988).

The reasons for the incongruence of phylogenetic trees based on ultrastructural, biochemical, developmental, and some molecular characters, such as presence/absence of introns, and trees constructed from nuclear rRNA gene sequence data, remain unclear. The 18S rDNA sequences represent an independent data set that needs to be evaluated in light of other available data. It is a data set that supports a charophycean origin of the land plants, but also one that appears inconsistent with hypotheses that position *Coleochaete orbicularis* as the charophycean taxon sharing the most recent common ancestor with the higher plants. The results suggest that all current data, including molecular data such as 18S rDNA sequences, and the available morphological, biochemical, and life history evidence should be reevaluated to examine where inconsistencies may be arising, and if the different data can be reconciled. Clearly, additional data are needed, particularly from other genes, in order to better assess an accurate picture of charophycean phylogeny.

LITERATURE CITED

- BALDAUF, S. L., AND J. D. PALMER. 1990. Evolutionary transfer of the chloroplast *tufA* gene to the nucleus. *Nature* 344: 262–265.
- , J. R. MANHART, AND J. D. PALMER. 1990. Different fates of the chloroplast *tufA* gene following its transfer to the nucleus in green algae. *Proceedings of the National Academy of Sciences* 87: 5317–5321.
- BREMER, K., C. J. HUMPHRIES, B. D. MISHLER, AND S. P. CHURCHILL. 1987. On cladistic relationships in green plants. *Taxon* 36: 339–349.
- CABOT, E. L., AND A. T. BECKENBACH. 1989. Simultaneous editing of multiple nucleic acid and protein sequences with ESEE. *Computer Applications in the Biosciences* 5: 233–234.

- CHAPMAN, R. L., AND M. A. BUCHHEIM. 1991. Ribosomal RNA gene sequences: analysis and significance in the phylogeny and taxonomy of green algae. *Critical Reviews in Plant Sciences* 10: 343–368.
- DEVEREUX, R., A. R. LOEBLICH III, AND G. E. FOX. 1990. Higher plant origins and the phylogeny of green algae. *Journal of Molecular Evolution* 31: 18–24.
- ECKENRODE, V. K., J. ARNOLD, AND R. B. MEAGHER. 1985. Comparison of the nucleotide sequence of soybean 18S rRNA with the sequences of other small-subunit rRNAs. *Journal of Molecular Evolution* 21: 259–269.
- FELSENSTEIN, J. 1988. Phylogenies from molecular sequences: inference and reliability. *Annual Review of Genetics* 22: 521–565.
- . 1990. PHYLIP manual version 3.3. University Herbarium, University of California, Berkeley, CA.
- GRAHAM, L. E., C. F. DELWICHE, AND B. D. MISHLER. 1991. Phylogenetic connections between the 'green algae' and the 'bryophytes'. *Advances in Bryology* 4: 213–244.
- , AND Y. KANEKO. 1991. Subcellular structures of relevance to the origin of land plants (embryophytes) from green algae. *Critical Reviews in Plant Sciences* 10: 323–342.
- GUNDERSON, J. H., AND M. L. SOGIN. 1986. Length variation in eukaryotic rRNAs: small subunit rRNAs from the protists *Acanthamoeba castellanii* and *Euglena gracilis*. *Gene* 44: 63–70.
- HORI, H., AND S. OSAWA. 1987. Origin and evolution of organisms as deduced from 5S rRNA ribosomal RNA sequences. *Molecular Biology and Evolution* 4: 445–472.
- HUSS, V. A. R., AND M. L. SOGIN. 1989. Primary structure of the *Chlorella vulgaris* small-subunit ribosomal RNA coding region. *Nucleic Acids Research* 17: 1255.
- , AND ———. 1990. Phylogenetic position of some *Chlorella* species within the Chlorococcales based upon complete small-subunit ribosomal RNA sequences. *Journal of Molecular Evolution* 31: 432–442.
- KANTZ, T. S., E. C. THERIOT, E. A. ZIMMER, AND R. L. CHAPMAN. 1990. The Pleurastrophyceae and Micromonadophyceae: a cladistic analysis of nuclear rRNA sequence data. *Journal of Phycology* 26: 711–721.
- LEWIS, L. A., L. W. WILCOX, P. A. FUERST, AND G. L. FLOYD. 1992. Concordance of molecular and ultrastructural data in the study of zoosporic chlorococcalean green algae. *Journal of Phycology* 28: 375–380.
- LI, W.-H., K. H. WOLFE, J. SOURDIS, AND P. M. SHARP. 1987. Reconstruction of phylogenetic trees and estimation of divergence times under nonconstant rates of evolution. *Cold Spring Harbor Symposium on Quantitative Biology* 52: 847–856.
- MANHART, J. R., AND J. D. PALMER. 1990. The gain of two chloroplast tRNA introns marks the green algal ancestors of land plants. *Nature* 345: 268–270.
- MANKIN, A. S., K. G. SKRYABIN, AND P. M. RUBSTOV. 1986. Identification of ten additional nucleotides in the primary structure of yeast 18S rRNA. *Gene* 44: 143.
- MATTOX, K. R., AND K. D. STEWART. 1984. Classification of the green algae: a concept based on comparative cytology. In D. E. G. Irvine and D. M. John [eds.], *The systematics of the green algae*, 29–72. Academic Press, London.
- MESSING, J., J. CARLSON, G. HAGEN, I. RUBENSTEIN, AND A. OLESON. 1984. Cloning and sequencing of the ribosomal RNA genes in maize: the 17S region. *DNA* 3: 31–40.
- MISHLER, B. D., AND S. P. CHURCHILL. 1985. Transition to a land flora: phylogenetic relationships of the green algae and bryophytes. *Cladistics* 1: 305–328.
- , M. J. DONOGHUE, AND V. A. ALBERT. 1991. The decay index as a measure of relative robustness within a cladogram (Abstract). Willi Henning Society Meeting, Toronto, Ontario.
- NAIRN, C. J., AND R. J. FERL. 1988. The complete nucleotide sequence of the small-subunit ribosomal RNA coding region for the cycad *Zamia pumila*: phylogenetic implications. *Journal of Molecular Evolution* 27: 133–141.
- O'KELLY, C. J., AND G. L. FLOYD. 1984. Flagellar apparatus absolute orientations and the phylogeny of the green algae. *BioSystems* 16: 227–251.
- PICKETT-HEAPS, J. D., AND H. J. MARCHANT. 1972. The phylogeny of the green algae: a new proposal. *Cytobios* 6: 255–264.
- RUBSTOV, P. M., M. M. MUSAKHANOV, V. M. ZAHARYEV, A. S. KREYEV, K. G. SKRYABIN, AND A. A. BAYEV. 1980. The structure of the yeast ribosomal RNA genes. I. The complete nucleotide sequence of the 18S ribosomal RNA gene from *Saccharomyces cerevisiae*. *Nucleic Acids Research* 8: 5779–5794.
- SLUIMAN, H. J. 1985. A cladistic evaluation of the lower and higher green plants (Viridiplantae). *Plant Systematics and Evolution* 149: 217–232.
- STARR, R. C., AND J. A. ZEIKUS. 1987. UTEX—the culture collection of algae at the University of Texas at Austin. *Journal of Phycology* 23: 1–47 (Supplement).
- STEELE, K. P., K. E. HOLSINGER, R. K. JANSEN, AND D. W. TAYLOR. 1991. Assessing the reliability of 5S rRNA sequence data for phylogenetic analysis in green plants. *Molecular Biology and Evolution* 8: 240–248.
- SWOFFORD, D. L. 1990. PAUP: phylogenetic analysis using parsimony version 3.0. Illinois Natural History Survey, University of Illinois, Champaign, IL.
- WILCOX, L. W., L. A. LEWIS, P. A. FUERST, AND G. L. FLOYD. 1992. Assessing the relationships of autosporic and zoosporic chlorococcalean green algae with 18S rDNA sequence data. *Journal of Phycology* 28: 381–386.
- WILLIAMS, P. L., AND W. M. FITCH. 1990. Phylogeny determination using dynamically weighted parsimony method. In R. F. Doolittle [ed.], *Molecular evolution: computer analysis of protein and nucleic acid sequences*, 615–626. Academic Press, San Diego, CA.
- ZECHMAN, F. W., E. C. THERIOT, E. A. ZIMMER, AND R. L. CHAPMAN. 1990. Phylogeny of the Ulvophyceae (Chlorophyta): cladistic analysis of nuclear-encoded rRNA sequence data. *Journal of Phycology* 26: 700–710.