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CONCORDANCE OF MOLECULAR AND ULTRASTRUCTURAL DATA IN THE STUDY OF ZOOSPORIC CHLOROCOCCALEAN GREEN ALGAE[1]

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ABSTRACT

Alternative evolutionary hypotheses generated from features of vegetative cell morphology and motile cell ultrastructure were investigated using a molecular data set. Complete nuclear-encoded small subunit (18S) ribosomal RNA (rRNA) gene sequences were determined for six species (three each) of the chlorococcalean green algae "Neochloris" *and* Characium. *Based on motile cell ultrastructure, it was previously shown that both genera could be separated into three distinct groups possibly representing three separate orders and two classes of green algae. 18S rRNA gene sequences were also obtained for three additional taxa,* Dunaliella parva *Lerche,* Pediastrum duplex *Meyen, and* Friedmannia israelensis *Chantanachat and Bold. These organisms were selected because each, in turn, is a representative of one of the three ultrastructural groups into which the* Neochloris *and* Characium *species are separable. Phylogenetic analyses utilizing the molecular data fully support the ultrastructural findings, suggesting that the similar vegetative cell morphologies observed in these organisms have resulted from convergence.*

Key index words: Characium; *Chlorococcales; Chlorophyceae; Chlorophyta; flagellar apparatus;* Neochloris; *ribosomal RNA*

Systematists have traditionally relied on morphological characters in their attempts to reconstruct the evolutionary history of organisms. For green algae, the characters used have been relatively few in number and primarily involve vegetative cell morphology (e.g. cell shape, number of nuclei per cell, plastid shape, growth requirements; see Bold and Wynne 1985). Characters such as cell/colony shape have been shown to be misleading in some instances due to morphological plasticity (Trainor et al. 1971). Realizing the limitations of such characters, a number of workers over the past 20 years have focused their efforts on characters believed to be evolutionarily conserved, including ultrastructural features of the mitotic spindle, cytokinetic apparatus, and flagellar apparatus. These are assumed to be less environmentally influenced than are vegetative cell characteristics because they are involved in the vital functions of cell division, reproduction, and motility.

Ultrastructural studies have led to reconsideration of several morphologically based groups and resulted in the separation of the green algae into five classes (Pickett-Heaps 1975, Moestrup 1978, Stewart and Mattox 1978, Melkonian 1982, Floyd and O'Kelly 1984, Mattox and Stewart 1984, O'Kelly and Floyd 1984, Sluiman 1989). Through these studies it became apparent that many of the traditional groupings based on vegetative cell morphology contained genera that cut across the newly proposed class boundaries and that certain characters probably reflected parallel changes.

Two recent examples of the apparent discordance between vegetative morphological and ultrastructural classifications involve chlorococcalean genera that are vegetatively non-motile but produce motile zoospores. Watanabe and Floyd (1989) found that *Neochloris* species could be separated into three groups based on ultrastructural features of motile cells. The primary feature delimiting the three groups is the orientation of the basal bodies in the flagellar apparatus (FA), i.e. whether they are clockwise (CW), directly opposed (DO), or counterclockwise (CCW), when the cells are viewed "top-down." Watanabe and Floyd suggested that *Neochloris* species falling into the first two FA groups have affinities to the Chlorophyceae and that the species in the CCW group better belonged in another class, the Pleurastrophyceae (sensu Mattox and Stewart 1984). As in *Neochloris,* the genus *Characium* also appears to be paraphyletic, with the species falling into the same three ultrastructural groups as the *Neochloris* species (Floyd and Watanabe 1990).

Information that is independent of vegetative morphology and FA ultrastructure should help to determine which of the two character sets most accurately reflects the true phylogenetic relationships. Here we present a molecular data set obtained from complete DNA sequences of the 18S ribosomal RNA (rRNA) genes of representative species from each of the three ultrastructural groups into which *Neochloris* and *Characium* species can be separated. Because of the unavailability of appropriate sequences with which to compare those of the *Neochloris* and *Characium* species, we also obtained sequence data for three additional taxa, one from each of the three ultrastructural groups.

With the acquisition of molecular data on these taxa, we were able to compare the evolutionary relationships suggested by three different character sets obtained from vegetative cell morphology, fla-

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TABLE 1. *Basal body orientation and source of chlorococcalean taxa examined.*

	B.B.O.[a]	Reference	Source[b]
"*Neochloris*"-like taxa[c]			
Ettlia minuta	CW	Watanabe and Floyd 1989	776
Neochloris aquatica	DO	Watanabe and Floyd 1989	138
Parietochloris pseudoalveolaris	CCW	Watanabe and Floyd 1989	975
"*Characium*"-like taxa			
C. vacuolatum	CW	Floyd and Watanabe 1990	2111
C. hindakii	DO	Floyd and Watanabe 1990	2098
C. perforatum	CCW	Floyd and Watanabe 1990	2104
Additional taxa in flagellar apparatus groups			
Dunaliella parva	CW	Chappell and Floyd (unpubl. obs.)	1983
Pediastrum duplex	DO	Wilcox and Floyd 1988	—
Friedmannia israelensis	CCW	Melkonian and Berns 1983	1181

[a] Basal body orientation. CW = clockwise, DO = directly opposite, CCW = counter-clockwise.
[b] Unialgal or axenic cultures were obtained from UTEX, the University of Texas Culture Collection (Starr and Zeikus 1987) except for *Pediastrum duplex*, which was collected from Lake Mendota, Wisconsin, by L.W.W.
[c] Nomenclature follows Deason et al. 1991.

gellar apparatus ultrastructure, and 18S rRNA sequence data.

MATERIALS AND METHODS

We sequenced the 18S rRNA genes from the "*Neochloris*" and *Characium* species and from three other green algae with known flagellar apparatus ultrastructure: *Dunaliella parva* Lerche, *Pediastrum duplex* Meyen, and *Friedmannia israelensis* Chantanachat and Bold (see Table 1).

We employed the 18S rDNA sequence from *Glycine max* (Eckenrode et al. 1985) as an outgroup taxon. An initial analysis (data not shown) incorporating the 18S sequence from the protist *Acanthamoeba* (Gunderson and Sogin 1986) was used to root the tree and to verify *G. max* as an outlier with respect to the algal sequences. Also, inclusion of unpublished sequences from the charophycean genera, *Coleochaete, Klebsormidium,* and *Chlorokybus,* yielded the same tree topology as when *Glycine* alone was employed as an outgroup taxon.

Growth of organisms and isolation of DNA. For most strains, the cells were grown in liquid medium (9:1; Starr and Zeikus 1987) or on 1.5% 9:1 agar plates. *Dunaliella* was maintained in F/2 (Guillard and Ryther 1962). DNA was extracted from either vegetative (non-motile) or motile cells. The vegetative cells were ground with liquid nitrogen and/or sterile sand in UNSET buffer (Garriga et al. 1984), followed by phenol : chloroform extraction and ethanol precipitation (Maniatis et al. 1982). Wall-less motile cells were lysed directly in UNSET, and DNA was extracted from the lysates.

DNA amplification, cloning, and sequencing. To obtain sufficient quantities of the 18S rRNA gene for analysis, total genomic DNA was used in PCR amplification. Oligonucleotide primers specific to conserved regions at the 5′ and 3′ ends were used to amplify the 18S rRNA gene. Products from multiple independent PCR amplifications were restricted for cloning and band-isolated. DNA was eluted from the gel using GENECLEAN (Bio. 101) and quantified. Insert DNA was ligated into prepared vectors (M13mp18 and -19), and the ligated vector was transformed into *Escherichia coli* strain DH5αF′ (M13 cloning manual; BRL). Clones containing an 18S rRNA gene insert were pooled, and single-stranded DNA was generated for dideoxy sequencing, which was performed according to the SEQUENASE v.2 protocol (U.S. Biochemical).

Data analysis. Sequences were aligned manually with published sequences using the program ESEE for the IBM-PC (Cabot and Beckenbach 1989). Regions not clearly alignable for all taxa, corresponding to positions 494–500, 688, 1356–1377, and 1693–1722 of the *Glycine max* sequence (Eckenrode et al. 1985), were excluded from the analyses. The total number of aligned nucleotide sites (including insertions/deletions) compared for all species in the study was 1713.

Three group I introns (Michel and Westhof 1990) were found in the 18S rRNA genes of two of the taxa included in this analysis. Their locations with respect to the *Glycine max* sequence are between positions 567 and 568 (*Neochloris aquatica* Starr), 1172 and 1173 (*Dunaliella parva*), and 1781 and 1782 (*D. parva*). Further characterization of the introns will be published elsewhere.

Pairwise nucleotide distance (for all sites) was estimated using the program DNADIST and PHYLIP, with the Kimura correction (Felsenstein 1990), and relationships between taxa were then determined from the nucleotide distances using the FITCH tree-building program of PHYLIP (Fitch and Margoliash 1967, Felsenstein 1990).

One hundred thirty-six nucleotide sites representing all phylogenetically informative positions in the data set were analyzed by a parsimony approach using PAUP (Swofford 1990). Because rRNA has a specific secondary structure that may cause non-independence of sites, compensatory changes in stems were down-weighted by one half (see Swofford and Olsen 1990). The branch and bound procedure of PAUP was used to find the shortest tree. The reliability of the resulting clades was then tested by a bootstrap analysis (heuristic search using MULPARS, TBR branch swapping, random stepwise addition, 50% majority rule, 100 replications) and by a "decay" study, where trees of progressively longer lengths are examined in order to assess how easily branches collapse (Mishler et al. 1991). The consistency index (CI; Kluge and Farris 1969) was also calculated.

Sequence availability. The sequences reported here have been deposited in GENBANK. Accession numbers are *Neochloris aquatica*: M62861; *Friedmannia israelensis*: M62995; *Ettlia* (*Neochloris*) *minuta* (Arce and Bold) Komárek: M62996; *Pediastrum duplex*: M62997; *Dunaliella parva*: M62998; *Characium perforatum* Lee et Bold: M62999; *Characium hindakii* Lee et Bold: M63000; *Characium vacuolatum* Lee et Bold: M63001; and *Parietochloris* (*Neochloris*) *pseudoalveolaris* (Deason and Bold) Watanabe et Floyd: M63002. The sequence alignment used in our analyses is available upon request.

RESULTS

Distance analysis. Pairwise distances among the taxa that had been previously included in the genus *Neochloris* (*Neochloris aquatica, Ettlia minuta,* and *Parietochloris pseudoalveolaris*) ranged from 0.058 to 0.074;

TABLE 2. *Matrix of all pairwise distances calculated in PHYLIP using the Kimura model of nucleotide substitutions in DNADIST.*

	Gm	*Dp*	*Em*	*Na*	*Pd*	*Cp*	*Ch*	*Cv*	*Pp*
Glycine	—								
Dunaliella	0.114	—							
Ettlia	0.130	0.051	—						
Neochloris	0.116	0.043	0.067	—					
Pediastrum	0.114	0.044	0.066	0.028	—				
C. perforatum	0.128	0.067	0.082	0.071	0.069	—			
C. hindakii	0.115	0.045	0.072	0.024	0.030	0.065	—		
C. vacuolatum	0.132	0.035	0.035	0.056	0.060	0.077	0.062	—	
Parietochloris	0.120	0.051	0.074	0.058	0.055	0.053	0.055	0.068	—
Friedmannia	0.113	0.049	0.073	0.054	0.049	0.057	0.053	0.065	0.044

the range among the *Characium* species was from 0.062 to 0.077 (Table 2). When taxa are grouped according to flagellar apparatus ultrastructure (CW, DO, or CCW; see Table 1) the among-group distances (ave. = 0.061) were always greater than the within-group distances (ave. within CW = 0.040, within DO = 0.027, within CCW = 0.057). In fact, the two most similar taxa were *Characium hindakii* and *Neochloris aquatica* (D = 0.024), which share the DO basal body configuration.

The tree obtained from the distance matrix is shown in Figure 1, illustrating that the groups inferred from FA orientation coincide precisely with those obtained with the 18S rRNA gene sequences. The members of *Characium* and "*Neochloris*" are found to group with similar flagellar apparatus forms and not with forms traditionally classified into the same genus. The presence/absence of zoospore cell walls distinguishes the CW from the DO and CCW groups, while the character of vegetative cell nuclear condition sets apart the multinucleate DO group from the uninucleate CW and CCW groups (Fig. 1). Thus, neither of these characters by itself is capable of resolving all three FA groups (see later). As predicted by FA ultrastructure, *Dunaliella* groups with the other CW organisms, while *Pediastrum duplex* and *Friedmannia israelensis* group with the DO and CCW taxa, respectively.

Although branch lengths leading to the three groups are short, the distance tree suggests that the CW and DO taxa share a more recent common ancestor than does either with CCW taxa.

Parsimony analysis. The most parsimonious molecular tree (Fig. 2) has the same topology as the distance tree, with the three clades corresponding exactly with the flagellar apparatus groups. Flagellar orientation groups are well supported, with 99%, 87%, and 91% bootstrap values for the CW, DO, and CCW clades, respectively. The parsimony tree also suggests that taxa with CW and DO flagellar apparatus orientations share a more recent common ancestor than either does with the CCW taxa. Once again, this must be viewed with caution because trees two steps longer do not maintain this relationship and the bootstrap value for this branch is 58% (see Fig. 2).

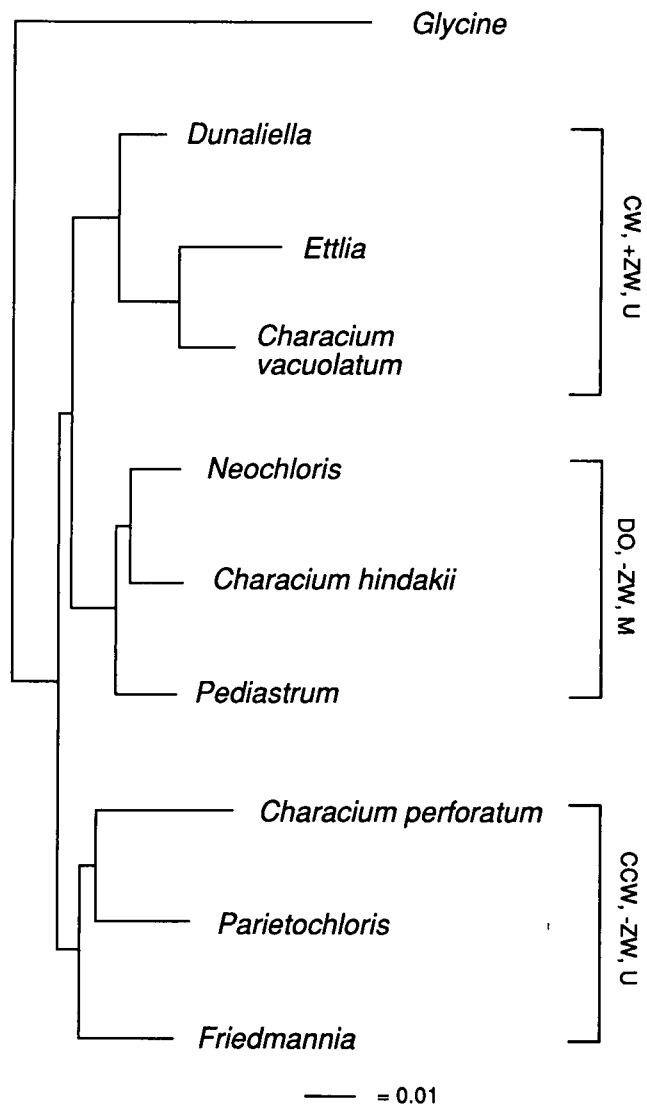

FIG. 1. Distance tree generated from 18S rRNA gene sequence data showing three distinct groups. Scale indicates fixed mutations per sequence position. Brackets indicate FA orientation (CW, DO, or CCW), presence or absence of zoospore cell walls (ZW), and whether vegetative cells are uninucleate (U) or multinucleate (M).

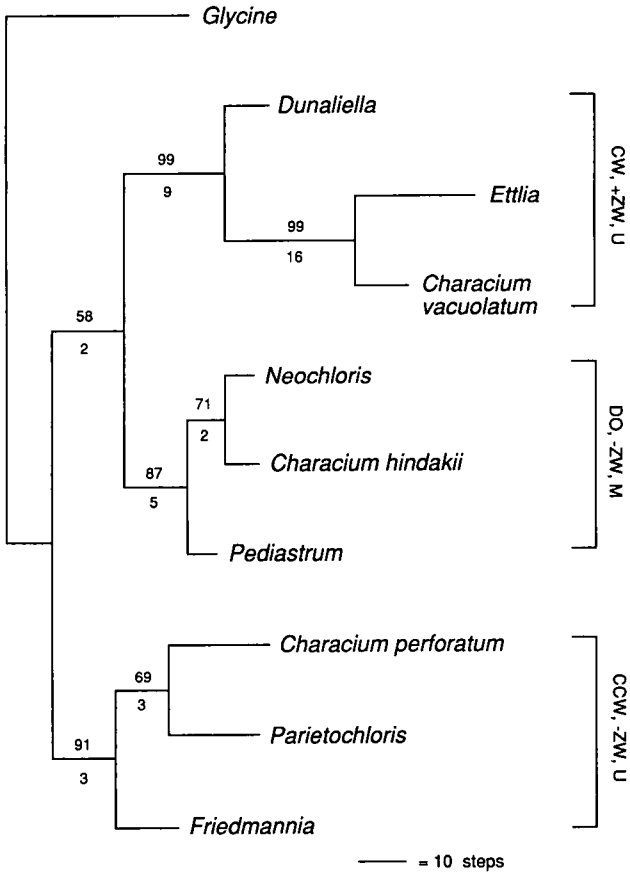

FIG. 2. Most parsimonious tree generated from the 136 informative sites using the branch and bound option in PAUP. Tree length = 272 steps and CI = 0.61. Brackets are as in Figure 1. Branch lengths correspond to the number of apomorphies supporting each. Bootstrap values are shown above, and decay indices below, each branch. The decay index refers to the number of additional steps (beyond the length of the most parsimonious tree) at which point the branch collapses when semi-strict consensus trees of progressively longer branch and bound trees are examined.

DISCUSSION

For the taxa examined here, flagellar apparatus ultrastructure, specifically basal body orientation, is a better predictor of phylogenetic relationships than are the features of vegetative cell morphology used in traditional classification schemes, since an independent molecular data set consisting of 18S rRNA gene sequences yields precisely the same groups delimited by FA ultrastructure. These groups, taxonomically, are thought to represent different classes and/or orders within the green algae.

Based upon vegetative morphology, taxa at one time included in *Neochloris* and those in *Characium* have been considered to be members of the order Chlorococcales, class Chlorophyceae (Bold and Wynne 1985). Our analysis shows that the rRNA genes of some species within *Neochloris* or *Characium* are less similar to one another than they are to genes from taxa in other genera within the same FA group. Therefore, both the molecular and ultrastructural studies suggest that the similar vegetative morphologies observed in these taxa are a result of parallel evolution. As stated earlier, both genera were divided into three groups, on the basis of FA features (Watanabe and Floyd 1989, Floyd and Watanabe 1990). The taxa having basal bodies arranged in a CW orientation, *Characium vacuolatum* and *Ettlia minuta* (syn. *Neochloris minuta*), were considered to belong to the Chlorophyceae. *Characium hindakii* and *Neochloris aquatica,* with DO basal bodies, were also thought to be part of the Chlorophyceae but were suggested to belong to a separate order. Affinities were noted with both hydrodictyacean and sphaeroplealean taxa, based on details of the flagellar apparatus (Watanabe and Floyd 1989, Floyd and Watanabe 1990). *Characium perforatum* and *Parietochloris pseudoalveolaris* (syn. *Neochloris pseudoalveolaris*) were suggested to belong to the class Pleurastrophyceae, whose members also possess CCW basal bodies.

While information on the FA has been helpful in elucidating relationships among motile cell-producing taxa at one time considered part of the Chlorococcales, it is of course unavailable for those chlorococcalean taxa that reproduce solely by autospores or that produce motile cells only rarely. However, molecular data, now available for both zoosporic and autosporic "chlorococcalean" taxa, would appear to provide an ideal means by which their relationships may be assessed. We have attempted to do so by analyzing published 18S rDNA sequences from autosporic taxa along with our sequence data on zoosporic taxa (see Wilcox et al. 1992).

Although a formal revision of *Characium,* incorporating FA information, has not yet been completed, Watanabe and Floyd (1989) did treat *Neochloris.* They proposed the name *Chlorococcopsis* for the CW species and *Parietochloris* (in the Pleurastrophyceae) for CCW taxa, while maintaining *Neochloris* for the DO species. At about the same time, Komárek (1989) transferred *Neochloris* species with uninucleate vegetative cells into a new genus, *Ettlia,* and kept the multinucleate taxa in *Neochloris.* Watanabe and Floyd also considered nuclear condition in their study and found that the multinucleate species all fell into the DO group. Thus, this feature, although not yielding results that conflict with the FA findings, does not resolve the vegetatively uninucleate species into two groups. Deason et al. (1991) have attempted to reconcile minor conflicts and overlaps in the treatments of Komárek (1989) and Watanabe and Floyd (1989). We have used here the names proposed in Deason et al. (1991) for the former *Neochloris* species: *Ettlia, Neochloris,* and *Parietochloris,* which refer to the CW, DO, and CCW taxa, respectively. These authors have also provided their view of the suprageneric classification of the taxa formerly included in *Neochloris.* While the molecular findings agree with their reclassification, sequence data for additional taxa need to be gathered in order to rigorously test its validity. For example, Deason

et al. (1991) placed the DO (*Neochloris*) taxa within the Sphaeropleales. However, despite having directly opposed basal bodies, a number of differences exist among the FAs of *Neochloris* and *Sphaeroplea* and *Atractomorpha* (Cácares and Robinson 1980, Hoffman 1984, Buchheim and Hoffman 1986). Sequence data from these organisms would be helpful in determining whether or not this placement is valid.

Regarding the classification of the *Characium* species, the presence/absence of a zoospore cell wall was used to separate species into two groups (Ettl and Komárek 1982, Ettl and Gärtner 1988). Those possessing walls were moved into *Chlamydopodium.* The use of presence/absence of zoospore cell walls as a taxonomic character does not conflict with FA or molecular findings but is by itself unable to separate the "naked" species into the two groups found using sequence and FA data.

Relationships within flagellar apparatus groups. Basal body orientation and the other FA characters examined by Watanabe and Floyd (1989) and Floyd and Watanabe (1990), while separating the coccoid taxa into three distinct groups, are unable to elucidate within-FA group relationships. The 18S rDNA sequence data do suggest finer level relationships, however, and provide a framework by which other morphological or ultrastructural characters can be evaluated. For example, among closely related taxa, features of vegetative morphology may be important. Based on the sequence data, the two coccoid CW taxa, *Ettlia* and *Characium vacuolatum,* group apart from the vegetatively motile *Dunaliella.* Similarly, among the DO taxa, the unicellular *Neochloris aquatica* and *Characium hindakii* appear to be more closely related to one another than is either to the coenobial *Pediastrum.* Among the CCW taxa examined here, the coccoids *Parietochloris* and *Characium perforatum* group separately from the sarcinoid *Friedmannia.*

Relationship between flagellar apparatus groups. It has been hypothesized that among organisms with more or less cruciate FAs, the CW and DO configurations represent sister groups within the Chlorophyceae (Mattox and Stewart 1984) and are derived compared to the CCW orientation present in the Ulvophyceae and Pleurastrophyceae (Mattox and Stewart 1984, O'Kelly and Floyd 1984). Both distance and parsimony analyses of the molecular data agree with this view in that the CW and DO taxa share a more recent common ancestor, while the CCW clade falls outside of that branch, nearer to the root (see Figs. 1, 2). However, the DO/CW branch is the least well-supported, as indicated by its short length on the distance tree and by both bootstrap and decay analysis of the parsimony tree.

A rapid radiation within each of the FA/18S rDNA groups subsequent to their divergence might account for the problematic branching order of the three groups. If this were the case, one would expect to find comparatively few characters that distinguish the groups. Furthermore, given the relatively long time period since the groups diverged, an accumulation of multiple substitutions at particular sequence positions would decrease the number of true informative sites relative to the number of misleading characters. It may therefore be difficult to find characters, molecular or otherwise, with the capacity to more satisfactorily elucidate the branching pattern. One would have more confidence that the branching pattern seen here is meaningful if sequence data from other molecules were to yield similar results.

Examination of 18S sequences from additional key taxa, ulvophycean, chlorophycean, and pleurastrophycean, might break up long branch lengths and provide additional resolution. Some partial 18S and 26S rRNA sequences are available for a number of such organisms (Kantz et al. 1990, Zechman et al. 1990). The finding by Kantz et al. (1990) that the Pleurastrophyceae is a sister group to the Chlorophyceae is in agreement with our results, providing some additional support for the branching pattern we have shown. The incorporation of representative partial 18S rRNA sequences reported by Kantz et al. (1990) and Zechman et al. (1990) into our analyses does not seem to provide further information either substantiating or contradicting the topology we present here. This is primarily because the two data sets overlap by only about 500 bp, once regions of questionable alignment are excluded, with the number of informative sites being reduced by approximately one-half. Consequently, the resolution of the combined data is greatly reduced. Analysis of this combined data set by parsimony, for example, leads to numerous most-parsimonious trees (typically from 10 to 30, depending on which and how many taxa are included), compared to a single tree obtained in the analysis of the complete rDNA sequences. In addition, consideration of the strict consensus trees determined from the equally parsimonious trees in the combined data analysis shows that nearly all of the clades collapse to tri- or polychotomies.

In conclusion, ultrastructural studies, especially those of the flagellar apparatus, have aided our understanding of the evolution of green algae. They have indicated parallel morphological evolution of vegetative features among some of the major orders of green algae. We have shown here that molecular data provide the same results, thereby substantiating earlier ultrastructurally based hypotheses of green algal evolution. Other recent studies employing molecular techniques have also demonstrated that the possession of a similar vegetative cell morphology does not necessarily indicate a close historical relationship [e.g. in *Chlorella* (Huss and Sogin 1990) and *Chlamydomonas* (Buchheim et al. 1990)]. Because the characters comprising the FA and molecular data sets result from different, independent sets of genes,

which are clearly not under the same evolutionary constraints, concordance of the data sets gives us confidence that the inferred patterns of divergence have phylogenetic significance.

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