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POLYPHYLY OF TETRASPORALEAN GREEN ALGAE INFERRED FROM NUCLEAR SMALL-SUBUNIT RIBOSOMAL DNA[1]

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ABSTRACT

Ultrastructural studies of tetrasporalean green algae have suggested the order is polyphyletic. These features, including the absolute orientation of the flagellar apparatus and the bi- versus quadriflagellated motile cell morphology, suggest that Chaetopeltis *as well as a number of others, may be ancestral to a group that includes* Tetraspora. *In this study, we examine the phylogenetic relationships of selected tetrasporalean taxa based on analysis of 18S ribosomal RNA gene sequences. Results show that the tetrasporalean taxa are polyphyletic. Biflagellated genera group with biflagellated volvocalean taxa, whereas the quadriflagellated species compose a distinct monophyletic clade not closely related to the biflagellated taxa. In addition, tetrasporalean taxa group with other chlorophycean algal species with similar flagellar apparatus absolute orientation, but the quadriflagellated Tetrasporales do not appear to be ancestral to the entire Chlorophyceae. These results are concordant with previous conclusions drawn from ultrastructural data and further confirm the utility of (small-subunit) ribosomal RNA gene sequences to discern green algal evolutionary relationships.*

Key index words: Chlorophyta evolution; SSU rRNA; Tetrasporales

Utilizing primarily ultrastructural features of the flagellar apparatus of motile cells, O'Kelly and Floyd (1984) proposed that *Tetraspora* may be more primitive than *Chlamydomonas.* They also proposed that *Chaetopeltis* is one of the ancestral chlorophycean green algae because body scales on the surface of, and proximal sheaths in, the zoospores of *Chaetopeltis* are quite similar to those known for members of the Ulvophyceae. These features, plus the nearly cruciate flagellar apparatus (i.e. directly opposed = DO), led O'Kelly and Floyd (1984) to propose that *Chaetopeltis* is an intermediate between algae with counterclockwise (CCW) absolute orientation (e.g. *Hafniomonas* and ulvophycean zoospores) and algae with clockwise (CW) absolute orientation (e.g. *Chlamydomonas* and zoospores of *Tetraspora*). Then, because *Tetraspora* produces naked gametes in gametangia similar to *Chaetopeltis,* they suggested that *Tetraspora* might have been derived from a *Chaetopeltis*-like alga and that the early *Chlamydomonas*-like organisms would have originated as a zoospore released from a *Tetraspora*-like alga. O'Kelly (1992) has reviewed these arguments with the same conclusions.

Recently, O'Kelly et al. (1994) examined the ultrastructure of motile cells of four other quadriflagellate chlorophycean algae. Zoospores of *Hormotilopsis gelatinosa, H. tetravacuolaris, Planophila terrestris,* and *Phyllogloea fimbriata* all had cruciate apparatuses, pyrenoids traversed by cytoplasmic channels, and scales present on the surfaces of the cells. Because these features are essentially identical to those of the *Chaetopeltis* zoospores, the authors created the new order Chaetopeltidales.

In this study, we examine the relationship of selected tetrasporalean taxa using small-subunit ribosomal DNA (SSU rDNA). This ubiquitous molecule has been widely used to examine the evolution of algae in a number of studies (e.g. Gunderson and Sogin 1986, Huss and Sogin 1990, Rausch et al. 1989, Bhattacharya et al. 1996). More recent studies assessing relationships of various chlorophycean algae with 18S rDNA data have clearly supported proposed relationships based primarily on ultrastructural features of flagellar apparatuses (Lewis et al. 1992, Wilcox et al. 1992, Friedl and Zeltner 1994, Friedl 1995). The present molecular work was conducted to test hypotheses suggested by the previous ultrastructural studies. The four primary hypotheses tested were: (1) the Tetrasporales is polyphyletic;

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quadriflagellate and biflagellate zoospore producing taxa will cluster as separate, coherent groups; (2) some biflagellate, zoospore-producing tetrasporalean taxa will cluster with taxa from the Volvocales; (3) taxa that produce quadriflagellate zoospores, including *Chaetopeltis,* which appears to possess the largest number of putative primitive flagellar apparatus features of any known chlorophycean organism, comprise the most ancient group within the Chlorophyceae; and (4) phylogenetic reconstruction would support an evolutionary history of absolute flagellar orientation as follows:

$$\text{CCW} \rightarrow \text{DO} \rightarrow \text{CW}.$$

MATERIALS AND METHODS

Tetrasporalean taxa included in this study were *Chaetopeltis orbicularis, Hormotilopsis gelatinosa, Hormotilopsis tetravacuolaris, Planophila terrestris, Gloeococcus maximus, Hormotila blennista, Paulschulzia pseudovolvox,* and *Tetraspora* sp. Algal cultures (Table 1) were obtained from UTEX—The Culture Collection of Algae at The University of Texas at Austin (Starr and Zeikus 1987) and were maintained in culture prior to DNA extraction in liquid media. Sequences of other taxa used in this study were determined previously in our laboratory or were obtained from GenBank.

DNA extractions, PCR amplifications, and sequencing. Algal culturing, DNA extractions, and PCR amplification conditions were performed as previously reported in Booton et al. (1997). 18S rRNA genes were amplified in two overlapping halves. The 5′ portion was amplified using primers CRN5 and 1262. These are forward and reverse primers respectively. The 3′ section of the gene was amplified using primers 892C and SSU2. All primers used in PCR amplification and subsequent 18S rDNA sequencing reactions are shown in Table 2. The PCR reactions were carried out as follows: denaturation at 94°–97° C for 1 min, annealing at 50°–53° C for 1.5 min, and extension at 72° C for 3 min; 35 cycles. Reactions were performed in a Perkin-Elmer (Norwalk, Connecticut) Cetus TC1 thermocycler. Ten microliters of PCR products were electrophoresed on a 1% agarose gel to determine size and approximate quantity before sequencing. Separate PCR reactions from the same individual that produced a band of the correct size were pooled and used directly for subsequent sequencing reactions. Sequencing primers were γ^{32}P-labeled (ICN Inc., Irvine, California) and 3 μL of PCR product was sequenced directly by double-stranded cycle sequencing using a commercial kit following manufacturer's instructions (Gibco BRL dsDNA Cycle Sequencing System, Gibco BRL, Inc., Gaithersburg, Maryland).

Phylogenetic reconstruction and data analysis. The primary sequences of *Chaetopeltis orbicularis, Hormotilopsis gelatinosa, H. tetravacuolaris, Planophila terrestris, Gloeococcus maximus, Hormotila blennista, Paulschulzia pseudovolvox,* and *Tetraspora* sp. were aligned with other 18S rRNA gene sequences examined in this study using the sequence alignment program Eyeball Sequence Editor for the PC (ESEE, Cabot and Beckenbach 1989). The proposed secondary structure of the SSU ribosomal RNA (rRNA) was used to determine the homology of sites within the alignment. The final alignment contained a charophycean taxon, *Nitella* sp., as an outgroup in subsequent analyses and contained 1784 bases (Wilcox et al. 1993). This corresponds to sites 24–1764 of the *C. reinhardtii* sequence (Gunderson et al. 1987) Two regions (sites 1348–1371 and 1679–1700 of the published *C. reinhardtii* sequence) were removed from the alignment before the data were used in subsequent analyses because unambiguous alignment of these regions was not possible across all taxa aligned. The remaining 1718 sites were used in subsequent phylogenetic analyses. Sequence alignment for the SSU rDNA is available at http://www.biosci.ohio-state.edu/~pfuerst.

Cladistic analysis was done using the phylogenetic analysis package PAUP 3.0 (Phylogenetic Analysis Using Parsimony, Swofford 1990). Maximum parsimony analysis was performed using a heuristic search algorithm, using the random addition of taxa option, repeated 10 times. Strict consensus and majority rule consensus trees were produced from the three most parsimonious trees produced. The three maximum parsimony trees produced in PAUP were examined for alternative topologies, as described below in MacClade 3.04 (Maddison and Maddison 1992).

Phenetic analyses were done using the program MEGA (Molecular Evolutionary Genetic Analysis, Kumar et al. 1993). Ambiguous sites identified above were removed prior to distance calculation. Corrected sequence divergences were quantified using

TABLE 1. *Taxonomic authorities and GenBank references for species whose ribosomal sequences were utilized in this study.*

Species	SSU reference (or UTEX culture no.)[a]	GenBank accession no.
Ankistrodesmus stipitatus (= *A. falcatus* var. *stipitatus*) (Chod.) Lemm	Huss and Sogin 1990	X56100
Asteromonas gracilis Artari (= *Stephanoptera gracilis*) Artari	Wilcox et al. 1993	M95614
Chaetopeltis orbicularis Berthold	(422) this study	U83125
Characium hindakii Lee & Bold	Lewis et al. 1992	M63000
Chlamydomonas reinhardtii Dang	Gunderson et al. 1987	M32703
Chlamydopodium vacuolatum (Korschikoff) Ettl et Komárek	Lewis et al. 1992	M63001
Chlorella fusca Shihara & Krauss	Huss and Sogin 1990	X56104
Chlorella kessleri Fott & Nováková	Huss and Sogin 1990	X56105
Chlorella minutissima Fott & Nováková	Huss and Sogin 1990	X56102
Ettlia minuta (Ance et Bold) Komárek	Lewis et al. 1992	M62996
Dunaliella salina (Dunal) Teod.	Lewis et al. 1992	M84320
Fusochloris perforata (Lee & Bold) Floyd, Watanabe, and Deason	Lewis et al. 1992	M62999
Gloeotilopsis planctonica Iyengar & Phillipose	Friedl and Zeltner 1994	Z28970
Gloeococcus maximus (Mainx) Fott & Novakova	(166) this study	U83122
Hormotila blennista Trainor & Hilton	(1239) this study	U83123
Hormotilopsis gelatinosa Trainor & Bold	(104) this study	U83126
Hormotilopsis tetravacuolaris Trainor & Bold	(946) this study	U83124
Hydrodictyon reticulatum (L.) Lagerh.	Wilcox et al. 1992a	M74497
Myrmecia israelensis (Chantanachat et Bold) Friedl	Lewis et al. 1992	M62995
Neochloris aquatica Starr	Lewis et al. 1992	M62861
Nephroselmis olivacea Stein	Steinkötter et al. 1994	X74754
Nitella sp. Kütz	Wilcox et al. 1993	M95615
Paulschulzia pseudovolvox Skuja	(167) this study	U83120
Pediastrum duplex Meyen	Lewis et al. 1992	M62997
Planophila terrestris Groove & Hofstetter	(1709) this study	U83127
Protoderma sarcinoidea (Grover & Bold) Tupa	Bhattacharya et al. 1996	Z47998
Pseudoscourfieldia marina (Throndsen) Kütz.	Steinkötter et al. 1994	X75565
Scenedesmus obliquus (Turp.) Kütz	Huss and Sogin 1990	X56103
Tetraspora sp. Link	(234) this study	U83121
Volvox carteri Iyengar	Rausch et al. 1989	X53904

[a] UTEX, The culture collection of the algae at the University of Texas at Austin (Starr and Zeikus 1987).

TABLE 2. *18S Ribosomal DNA primers used in amplification and sequencing.*

Primer name	Primer sequence (all primers shown in 5′ → 3′ orientation)	Primer size	Melting temp. (°C)	Orientation
CRN5	tggttgatcctgccagtag	19-mer	48.0	Forward
170	gcatgtattagctctaga	18-mer	33.7	Reverse
373	aggctccctctccggaatc	19-mer	54.5	Reverse
373C	gattccggagagggagcct	19-mer	54.5	Forward
570	gctattggagctggaattac	20-mer	46.0	Reverse
570C	gtaattccagctccaatagc	20-mer	46.0	Forward
892	ccaagaatttcacctctgac	20-mer	46.0	Reverse
892C	gtcagaggtgaaattcttgg	20-mer	46.0	Forward
1137	gtgcccttccgtcaat	16-mer	45.1	Reverse
PCR2	gaaacttaaaggaattga	18-mer	38.7	Forward
1262	gaacggccatgcaccac	17-mer	52.4	Reverse
1262C	gtggtgcatggccgttctta	20-mer	55.7	Forward
1200	gggcatcacagacctg	16-mer	42.9	Reverse
1200C	caggtctgtgatgccc	16-mer	42.9	Forward
1/F	cacaccgcccgtcg	14-mer	49.3	Forward
SSU2	ccgcggccgcggatcctgatccctccgcaggttcac	36-mer	86.2	Reverse

the Kimura two-parameter model (Kimura 1980), which takes into account multiple substitution events at a given site. Distances derived by this method were then used to produce phylogenetic trees using the neighbor-joining algorithm. Bootstrapping of the data as an indicator of the robustness of the trees produced was also performed (1000 resamplings).

RESULTS AND DISCUSSION

Cladistic analysis of the 18S rDNA data resulted in three most parsimonious trees, consisting of 1111 steps (CI = 0.568, HI = 0.432). These trees differed in the following respects. Tree one placed the quadriflagellated Tetrasporales as a monophyletic group, which diverged prior to the remainder of the chlorophycean taxa examined. In the remaining two trees the quadriflagellated tetrasporalean group was a sister clade to the Sphaeropleales (Deason et al. 1991). In tree two, the four taxa from the Treboux-iophyceae (Friedl and Zeltner 1994) were not found as a monophyletic group, but instead had a topology where the lineage leading to two *Chlorella* taxa diverged prior to a group containing *Myrmecia* and *Fusochloris*. In tree three, the topology had the Trebouxiophyceae as a monophyletic clade, whereas the quadriflagellated Tetrasporales were a sister group to the Sphaeropleales. The remaining taxa had the same branching order in all three trees. A majority rule consensus tree of these three most parsimonious trees was constructed and is presented in Figure 1. Numbers at the nodes in this tree represent the percentage of trees that had this branching order in the three most parsimonious trees.

A neighbor-joining tree with the same topology as the majority rule consensus tree is presented in Figure 2. A key feature of this topology is the high bootstrap support for the polyphyly of the Tetrasporales. The quadriflagellated Tetrasporales comprise a monophyletic clade with 100% bootstrap support, whereas the paraphyletic biflagellated Tetrasporales topology is also supported by a 99% bootstrap. Also of note within both topologies is the lack of support for monophyly of the two quadriflagellated species of the genus *Hormotilopsis*. The *Hormotilopsis tetravacuolaris* lineage has accumulated more substitutions since its divergence from the lineages leading to the other three taxa in this group, and this may account for this branching order. An alternative branching order, which forces the two *Hormotilopsis* taxa together as a monophyletic group, requires an additional ten steps—clearly not close to the most parsimonious tree. Future resolution of this apparent discrepancy in *Hormotilopsis* branching order may require additional sequence information from taxa in this genus.

The first hypothesis tested concerns the putative polyphyly of tetrasporalean taxa; in fact, our results consistently support polyphyly. Using both phenetic and cladistic gene tree reconstruction, tetrasporalean taxa are grouped according to zoospore flagellar number, and not as a monophyletic order. To explore alternative trees, we examined the three most parsimonious trees in MacClade. In each case, an alternative tree that clusters the two types of tetrasporalean taxa together as a monophyletic group would require an additional 56 steps—clearly not close to the most parsimonious reconstructions. In addition, there was strong bootstrap support of a paraphyletic topology of Tetrasporales in the neighbor-joining reconstruction. Therefore, we conclude that the Tetrasporales are in fact a polyphyletic order as hypothesized. Furthermore, this conclusion lends further support to the proposal of O'Kelly et al. (1994) regarding the creation of a new order: the Chaetopeltidales.

Our second hypothesis stated that some biflagellate zoospore-producing tetrasporalean taxa would cluster with taxa from the Volvocales. This hypothesis is supported by the observation that in addition to being polyphyletic as an order, the biflagellated Tetrasporales do not form a monophyletic group, but rather are found in a clade that harbors the biflagellated *Chlamydomonas reinhardtii*. This group

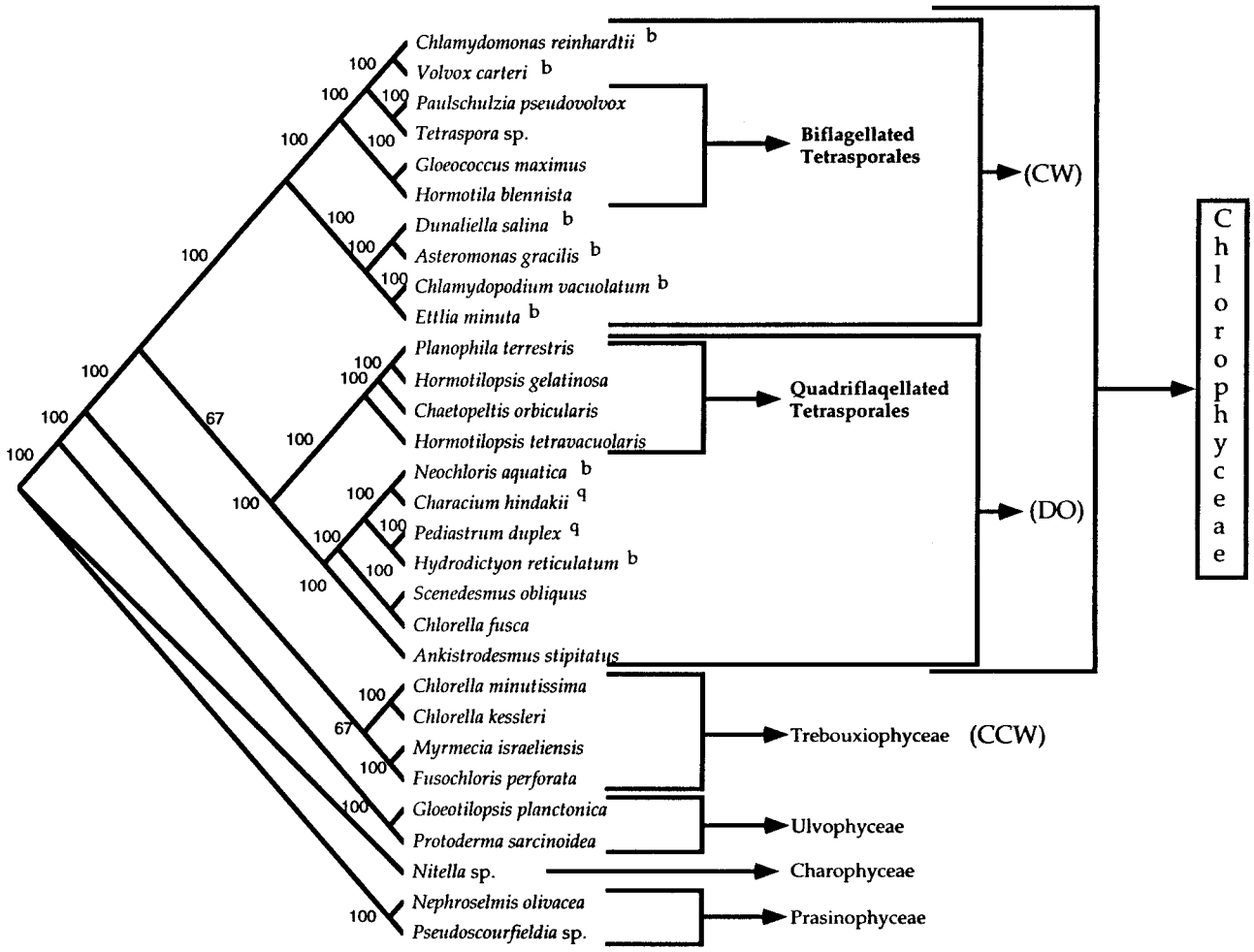

FIG. 1. Tetrasporalean 18S rRNA majority rule consensus tree. Numbers at nodes represent the percentage this topology is supported in the three maximum parsimony trees (see text). Absolute flagellar orientation key: CCW (counterclockwise), DO (directly opposed), and CW (clockwise). Bi (biflagellated vegetative cells or zoospores), Quad (quadriflagellated vegetative cells or zoospores). b, Those taxa with biflagellated vegetative cell or zoospores; q, those taxa with quadriflagellated vegetative cells or zoospores.

also contains *Volvox carteri*. Within this group, both reconstructions support the hypothesis of O'Kelly and Floyd (1984) that a lineage leading to the extant *Tetraspora* is primitive to a lineage leading to the extant *Chlamydomonas reinhardtii*. Last, in both reconstruction methodologies, the biflagellated tetrasporaleans, together with *V. carteri* and *C. reinhardtii,* are a sister group to a chlorophycean clade containing *Asteromonas gracilis,* a probable *Chlamydomonas* relative (Floyd 1978) and *Chlamydopodium vacuolatum* (see Floyd et al. 1993 for probable relationships of this alga). This relationship is found in all three of the most parsimonious trees and is supported in the bootstrapped neighbor-joining tree.

Our third hypothesis proposed that quadriflagellated taxa and those with putative primitive flagellar apparatus structures, such as *Chaetopeltis,* would comprise the most ancient group within the Chlorophyceae. To address this hypothesis, we examined the results obtained from the second group under examination: members of the Tetrasporales with quadriflagellated zoospores. Results from both reconstructions establishes that this group is monophyletic but is not closely related to the biflagellated Tetrasporales, as discussed above. The quadriflagellate tetrasporaleans are a sister group of the Sphaeropleales (Deason et al. 1991) in the neighbor-joining gene tree reconstruction with high bootstrap support. This topology is also observed in the consensus maximum parsimony tree. This entire clade (Sphaeropleales and quadriflagellated Tetrasporales) is a sister group to the remaining chlorophycean taxa examined, including the biflagellated Tetrasporales. This result fails to support our third hypothesis that the quadriflagellated lineage is an ancient one within all of the Chlorophyceae. Instead, the results suggest that extant quadriflagellated Tetrasporales represent an early branch within a large clade of chlorophycean species with a directly opposed flagellar apparatus orientation.

Previous studies on chlorophycean algae have

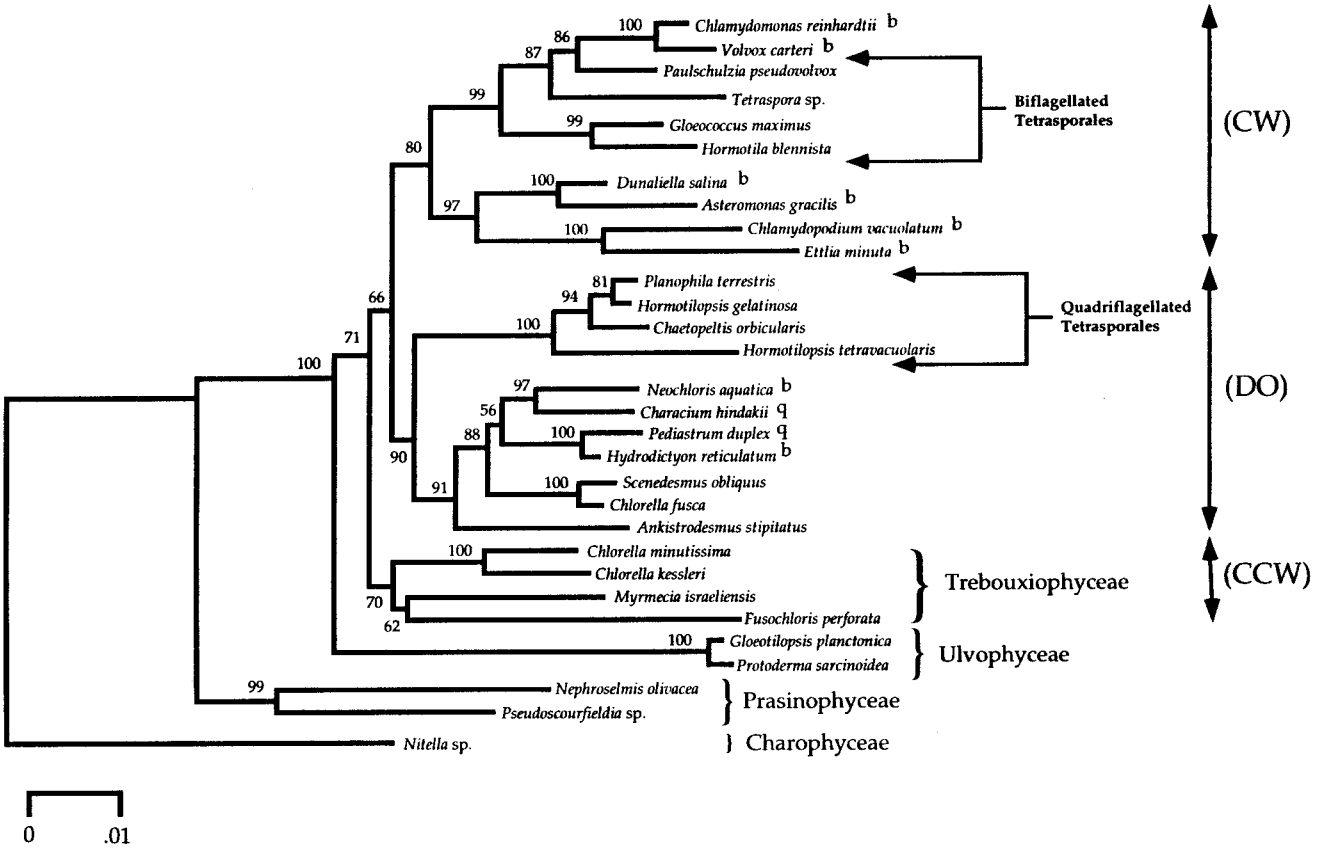

FIG. 2. Tetrasporalean 18S rRNA neighbor-joining tree. Numbers at nodes are bootstrap frequency values (1000 resamplings). Distance bar is at lower left and represents the corrected number of nucleotide substitutions/site. Absolute flagellar orientation key: CCW (counterclockwise), DO (directly opposed), and CW (clockwise). Bi (biflagellated vegetative cells or zoospores), Quad (quadriflagellated vegetative cells or zoospores). b, Those taxa with biflagellated vegetative cell or zoospores; q, those taxa with quadriflagellated vegetative cells or zoospores.

shown concordance between the ultrastructural characteristics of flagellar apparatus ''absolute orientation'' and molecular data (Lewis et al. 1992, Wilcox et al. 1992, Mishler et al. 1994). That is, taxa with like flagellar apparatus orientation cluster as monophyletic clades using 18S rRNA gene sequence analysis. However, although similar types grouped together in these studies, the evolutionary order and polarity of types was not resolved. Trebouxiophycean taxa (see Friedl 1995) have a counterclockwise (CCW) orientation of the motile cell flagellar apparatus, which O'Kelly and Floyd (1984) suggested as the primitive condition. A proposed intermediate form, directly opposed (DO), and a derived form, clockwise (CW), are known. So as first proposed by O'Kelly and Floyd, our fourth hypothesis was to restate that the evolution of flagellar apparatus orientation was: CCW → DO → CW. Both maximum parsimony and distance reconstruction results from this study show that the species examined here do group according to their flagellar orientation type. However, neither reconstruction supports the CCW → DO → CW polarity. Instead, the two derived states (DO and CW) comprise paraphyletic groups, as has been observed in previous studies (Mishler et al. 1994, Lewis 1997). Within the directly opposed group, the lineage leading to the quadriflagellated Tetrasporales appears to have diverged prior to the remaining directly opposed groups. This supports the primitive state of this group only within the directly opposed clade, and not with respect to the remainder of the chlorophycean species, including *Chlamydomonas reinhardtii.* The failure of these results to support our third (ancient ancestry of the *Chaetopeltis* lineage) or fourth (CCW → DO → CW) hypothesis leads to a similar conclusion. Apparently, primitive CCW taxa gave rise to two distinct derived lineages, consisting of taxa with DO and CW orientations. That is, it does not appear that DO taxa represent an intermediate state between CCW and CW species, but rather an independently evolving lineage.

In summary, both distance and parsimony analyses support the hypothesis that the tetrasporalean lineage is polyphyletic and that the number of zoospore flagella is an indicator of phylogenetic relationships. As hypothesized, the biflagellated Tetrasporales cluster with the biflagellated *Chlamydomonas.* Also, the quadriflagellated Tetrasporales do not appear to be a primitive lineage of the entire Chlo-

rophyceae, but for those taxa examined, do appear to be an early lineage of taxa with directly opposed basal body ultrastructure. Last, orientation of the flagellar apparatus and flagellar number appears to be indicative of evolutionary relatedness. Our results do not support the hypothesized polarity in the order of evolution of the absolute orientation of the flagellar apparatus but do agree with previous conclusions (Mishler et al. 1994, Friedl 1995, Bhattacharya et al. 1996, Lewis 1997) regarding these structures.

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Bhattacharya, D., Friedl, T. & Damberger, S. 1996. Nuclear-encoded rDNA group I introns: origin and phylogenetic relationships of insertion site lineages in the green algae. *Mol. Biol. Evol.* 13(7):978–89.

Booton, G. C., Floyd, G. L. & Fuerst, P. A. 1997. Origins and affinities of the filamentous green algal orders Chaetophorales and Oedogoniales based on small subunit ribosomal RNA gene sequences. *J. Phycol.* 34:312–318.

Cabot, E. L. & Beckenbach, A. T. 1989. Simultaneous editing of multiple nucleic acid and protein sequences with ESEE. *Comput. Appl. Biosci.* 5:233–4.

Deason, T. R., Silva, P. C., Watanabe, S. & Floyd, G. L. 1991. Taxonomic status of the species of the green algal genus *Neochloris. Pl. Syst. Evol.* 177:213–19.

Floyd, G. L. 1978. Mitosis and cytokinesis in *Asteromonas,* a walless green alga. *J. Phycol.* 14:440–5.

Floyd, G. L., Watanabe, S. & Deason, T. R. 1993. Comparative ultrastructure of the zoospores of eight species of *Characium* (Chlorophyta). *Arch. Protistenkd.* 143:63–73.

Friedl, T. 1995. Inferring taxonomic positions and testing genus level assignments in coccoid green lichen algae: a phylogenetic analysis of 18S ribosomal RNA sequences from *Dictyochloris reticulata* and from members of the genus *Myrmecia* (Chlorophyta, Trebouxiophyceae Cl. Nov.) *J. Phycol.* 31:632–9.

Friedl, T. & Zeltner, C. 1994. Assessing the relationships of some coccoid green lichen algae and the Microthamniales (Chlorophyta) with 18S ribosomal gene sequence comparisons. *J. Phycol.* 30:500–6.

Gunderson, J. H., Elwood, H., Ingold, A., Kindle, K. & Sogin, M. L. 1987. Phylogenetic relationships between chlorophytes, chrysophytes, and oomycetes. *Proc. Natl. Acad. Sci. USA* 84: 5823–7.

Gunderson, J. H. & Sogin, M. L. 1986. Length variation in eukaryotic rRNAs: small subunit rRNAs from the protists *Acanthamoeba castellanii* and *Euglena gracilis. Gene* 44:63–70.

Huss, V. A. & Sogin, M. L. 1990. Phylogenetic position of some *Chlorella* species within the Chlorococcales based upon complete small-subunit ribosomal RNA sequences. *J. Mol. Evol.* 31:432–42.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16:111–20.

Kumar, S., Tamura, K. & Nei, M. 1993. *MEGA—Molecular Evolutionary Genetic Analysis,* Ver. 1.02. The Pennsylvania State University, State College.

Lewis, L. A. 1997. Diversity and phylogenetic placement of *Bracteacoccus* Tereg (Chlorophyceae, Chlorophyta) based on 18S ribosomal RNA gene sequence data. *J. Phycol.* 33:279–85.

Lewis, L. A., Wilcox, L. W., Fuerst, P. A. & Floyd, G. L. 1992. Concordance of molecular and ultrastructural data in the study of zoosporic chlorococcalean green algae. *J. Phycol.* 28: 375–80.

Maddison, W. P. & Maddison, D. R. 1992. *MacClade: Analysis of Phylogeny and Character Evolution,* Ver. 3.0. Sinauer Associates, Sunderland, Massachusetts.

Mishler, B. D., Lewis, L. A., Buchheim, M. A., Renzaglia, K. S., Garbary, D. J., Delwiche, C. F., Zechman, F. W., Kantz, T. S. & Chapman, R. S. 1994. Phylogenetic relationships of the ''green algae'' and ''bryophytes.'' *Ann. Missouri Bot. Gard.* 81: 451–83

O'Kelly, C. J. 1992. Flagellar apparatus architecture and the phylogeny of ''green'' algae: chlorophytes, euglenoids, glaucophytes. *In* Menzel, D. [Ed.] *The Cytoskeleton of the Algae.* CRC Press, London, pp. 315–45.

O'Kelly, C. J. & Floyd, G. L. 1984. Flagellar apparatus orientations and the phylogeny of the green algae. *BioSystems* 16: 227–51.

O'Kelly, C. J., Watanabe, S. & Floyd, G. L. 1994. Ultrastructure and phylogenetic relationships of Chaetopeltidales Ord. Nov. (Chlorophyta, Chlorophyceae). *J. Phycol.* 30:118–28.

Rausch, H., Larson, N. & Schmitt, R. 1989. Phylogenetic relationships of the green alga *Volvox carteri* deduced from small-subunit ribosomal RNA comparisons. *J. Mol. Evol.* 29:255–65.

Starr, R. C. & Zeikus, J. A. 1987. UTEX—the culture collection of algae at the University of Texas at Austin. *J. Phycol.* 29: (Suppl.) 1–47.

Steinkötter, J., Bhattacharya, D., Semmelroth, I., Bibeau, C. & Melkonian, M. 1994. Prasinophytes form independent lineages within the chlorophyta: evidence from ribosomal RNA sequence comparisons. *J. Phycol.* 30:340–5.

Swofford, D. L. 1990. *PAUP—Phylogenetic Analysis Using Parsimony,* Ver. 3.0L. Illinois Natural History Survey, University of Illinois, Champaign.

Wilcox, L. W., Fuerst, P. A. & Floyd, G. L. 1993. Phylogenetic relationships of four charophycean green algae inferred from complete nuclear-encoded small subunit rRNA gene sequences. *Am. J. Bot.* 80:1028–33.

Wilcox, L. W., Lewis, L. A., Fuerst, P. A. & Floyd, G. L. 1992. Assessing the relationships of autosporic and zoosporic chlorococcalean green algae with 18S rDNA sequence data. *J. Phycol.* 28:381–6.