

USING *rbcL* SEQUENCES TO TEST HYPOTHESES OF CHLOROPLAST AND THALLUS EVOLUTION IN CONJUGATING GREEN ALGAE (ZYGNEMATALES, CHAROPHYCEAE)¹

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ABSTRACT

Sequences for the Rubisco large subunit (*rbcL*) gene were used to test hypotheses about the evolution of chloroplast shape and thallus type in genera of two families of conjugating green algae (Zygnematales): the Mesotaeniaceae (saccoderm desmids, mostly unicellular) and the Zygnemataceae (strictly filamentous). Unicellular (*u*) and filamentous (*f*) genera exhibit a series of three similar chloroplast shapes: ribbonlike (e.g. *Spirotaenia* [*u*], *Spirogyra* [*f*], and *Sirogonium* [*f*]), laminate (e.g. *Mesotaenium* [*u*] and *Mougeotia* [*f*]), and twin-stellate (e.g. *Cylindrocystis* [*u*] and *Zygnema* [*f*]). Two conflicting phylogenetic hypotheses have been proposed: 1) families are polyphyletic constructs drawn from three lineages, each with unicellular and filamentous taxa characterized by a specific chloroplast shape; or 2) unicells form one monophyletic lineage (Mesotaeniaceae) and filaments form another (Zygnemataceae), with some chloroplast shapes independently derived. The *rbcL* data strongly refute hypothesis 2 (monophyly of the two traditional families) and support hypothesis 1 in part. Parsimony, maximum likelihood, and neighbor-joining analyses of the *rbcL* data strongly support monophyly of a clade containing taxa with ribbonlike chloroplasts and, to a lesser extent, monophyly of a second clade of the four genera with the other two chloroplast shapes. Two saccoderm genera (*Roya*, curved laminate chloroplasts; *Netrium*, "cucumber"-shaped chloroplasts) are not members of either of these clades, but they are included in a monophyletic Zygnematales.

Key index words: Charophyceae; desmids; green algal evolution; Mesotaeniaceae; molecular phylogeny; *rbcL*; Zygnemataceae; Zygnematales

Gene sequence data have been enthusiastically assimilated into systematics (Hillis and Moritz 1990), especially in studies of land plants (Soltis et al. 1992). In comparison, the green algae (sensu Bold and Wynne 1985), including taxa now considered as the sister groups of higher plants (Graham 1993, McCourt 1995), have been less intensively studied (Palmer 1985, Chapman and Buchheim 1991, McCourt 1995). The chloroplast-encoded gene for

the large subunit of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL*) has been extensively sampled in higher plants (Chase et al. 1993), and sequences from a number of green algal taxa have recently been added to the *rbcL* database (Daugbjerg et al. 1994, Manhart 1994, McCourt et al. 1996b). Although the utility of *rbcL* to decipher ancient branching patterns of diverse groups of plants has been questioned (Manhart 1994), this gene appears to be useful in analyzing the relationships of more circumscribed groups of green algae (Daugbjerg et al. 1994, McCourt et al. 1996b). We report here on the use of *rbcL* sequences to test existing hypotheses of phylogeny and character evolution within two families of the Zygnematales, an order near the base of a clade containing the green algal class Charophyceae and the embryophytes (Graham et al. 1991, Mishler et al. 1994, McCourt 1995, McCourt et al. 1996b).

The conjugating green algae make up a widespread group of freshwater algae that exhibit an intriguing array of thallus morphologies and chloroplast shapes (Fritsch 1935, Transeau 1951, Prescott et al. 1972, 1977, Brook 1981, Kadlubowska 1984, Hoshaw and McCourt 1988, Hoshaw et al. 1990). The group is characterized by the lack of flagellated cells and by sexual reproduction through the process of conjugation (Hoshaw et al. 1990). The approximately 55 genera in 6 families (Prescott et al. 1972, Mix 1972, 1975, Brook 1981, Hoshaw and McCourt 1988, Gerrath 1993) include unicellular, filamentous, and colonial forms. The most recent family-level classification based on ultrastructural features of cell-wall construction (Mix 1975) divides the order into three major groups: placoderm desmids (families Closteriaceae, Desmidiaceae, Peniaceae, and Gonatozygaceae), unicellular saccoderm desmids (family Mesotaeniaceae), and filamentous genera (family Zygnemataceae). Although some authors (Prescott et al. 1972) placed the saccoderm desmids in the same order as placoderm desmids (Desmidiales, i.e. desmids in the broad sense), the unsculptured, unsegmented walls and chloroplast forms of saccoderms are clearly similar to those of the Zygnemataceae, and these two families are now placed in the order Zygnematales (Mix 1975, Brook 1981, Gerrath 1993). Molecular phylogenetic analyses of small subunit (18S) ribosomal DNA support

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TABLE 1. Taxa of Zygnematales and outgroups used in the present analysis. Sources: UTEX = Culture Collection at the University of Texas at Austin, Texas; ARL = Algal Research Laboratory at University of Arizona, Tucson, Arizona (McCourt et al. 1986a) (DNA transferred to DePaul University, Chicago, Illinois). Material for published sequences is listed in Manhart (1994).

Taxon	Strain no.	Source	Genbank no.
Order Zygnematales			
<i>Cylindrocystis</i> sp.	1925	UTEX	U38695
<i>Mesotaenium caldariorum</i> (Lagerh.) Hansg.	41	UTEX	U38696
<i>Mougeotia</i> sp.	82-11	ARL	U38699
<i>Netrium digitus</i> (Ehr.) Itz. et Rothe	LB 561	UTEX	U38698
<i>Roya anglica</i> W. & G. S. West	934	UTEX	U38694
<i>Sirogonium melanosporum</i> (Randhawa) Transeau	700	ARL	L13484*
<i>Spirogyra maxima</i> (Hassall) Wittr.	LB 2495	UTEX	L11057*
<i>Spirotaenia condensata</i> Bréb.	1300	ARL	U38700
<i>Zygnema peliosporum</i> Wittr.	LB 45	UTEX	U38701
Outgroups			
<i>Chara connivens</i>	—	Manhart (1994)	L13476
<i>Nitella translucens</i>	—	Manhart (1994)	L13482
<i>Coleochaete orbicularis</i>	—	Manhart (1994)	L13477
<i>Marchantia polymorpha</i>	—	Ohyama et al. (1986)	X04465

* Sequences originally published in Manhart (1994).

monophyly of the entire group of conjugating chlorophytes and of placoderm desmids (Surek et al. 1993, Bhattacharya et al. 1994). The 18S data also suggest that the Mesotaeniaceae and Zygnemataceae may be polyphyletic (Surek et al. 1993, Bhattacharya et al. 1994).

Two general hypotheses have been proposed for the relationships of genera in the Mesotaeniaceae and the Zygnemataceae, one of which implies polyphyly of the two families (Hoshaw and McCourt 1988). One hypothesis (Fig. 1a) considers the group to consist of two clades, each conforming to a family of unicellular or filamentous forms (Prescott et al. 1972). In this scheme, the ancestral type was presumed to be a filament (West 1904). The second hypothesis (Fig. 1b) holds that the ancestral conjugating green alga was unicellular and that this ancestral stock gave rise to three lines of evolution, each characterized by a distinctive chloroplast shape (ribbonlike, stellate, and laminate) (Randhawa 1959, Yamagishi 1963). This second hypothesis places genera sharing a common chloroplast type into putative monophyletic groups (i.e. *Spirotaenia*, *Sirogonium*, and *Spirogyra* with ribbonlike chloroplasts; *Mesotaenium* and *Mougeotia* with laminate chloroplasts; and *Cylindrocystis* and *Zygnema* with twin stellate chloroplasts). Figure 1a is consistent with the current classification of two families; Figure 1b would require a dismantling of these families because they do not represent monophyletic groups (Theriot 1992).

A paucity of morphological characters has prevented resolution of these two conflicting hypotheses of character evolution (Hoshaw and McCourt 1988). Nor have molecular data from 18S rDNA been sampled for enough taxa to resolve the question (Surek et al. 1993, Bhattacharya et al. 1994). The molecular analysis using *rbcL* data presented here provides a test of these alternative hypotheses.

MATERIALS AND METHODS

Taxa employed in the present analysis are shown in Table 1. The taxa included zygnematalean genera of unicells and filaments with each of the three chloroplast shapes, as well as two additional saccoderm desmids (*Netrium*, with a "cucumber"-shaped chloroplast [Prescott et al. 1972], and *Roya*, with a curved laminate chloroplast). Filamentous taxa (*Mougeotia*, *Sirogonium*, *Spirogyra*, and *Zygnema*) included the four most common and species-rich genera in the Zygnemataceae (Transeau 1951, Hoshaw and McCourt 1988). Unicellular taxa (*Cylindrocystis*, *Mesotaenium*, *Netrium*, *Spirotaenia*, and *Roya*) included five of the seven currently recognized genera in the Mesotaeniaceae (Gerrath 1993). Outgroup taxa (Table 1) include sequences from green algae in the class Charophyceae (Manhart 1994) and the liverwort *Marchantia polymorpha* (Ohyama et al. 1986), which were chosen based on prior morphological and molecular analyses of the relationships of Charophyceae and embryophytes (Pickett-Heaps 1975, Mattox and Stewart 1984, Mishler and Churchill 1985, Graham et al. 1991, Graham 1993, Mishler et al. 1994, McCourt 1995, McCourt et al. 1996b).

Cultures of zygnematalean algae were obtained in unialgal condition from the Culture Collection of Algae at the University of Texas at Austin (UTEX) (Starr and Zeikus 1993) and from our own collections of Zygnematales (McCourt et al. 1986a). Published *rbcL* sequences were used for *Spirogyra* and *Sirogonium* (Manhart 1994). Cultures were maintained in unialgal condition in Woods Hole (WH) media, substituting HEPES for Tris buffer, or in soil-water medium (Nichols 1973). Some cultures were grown in double-strength media, which accelerated growth. Saccoderm desmids usually grew better in soil-water medium (Nichols 1973). Cultures were grown at 20°C under 40-W cool-white fluorescent lamps with an irradiance of 15 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ on a 18:6 h LD cycle. Harvested filaments or unicells were frozen in liquid nitrogen; DNA was isolated immediately or filaments were stored at -80°C for later extraction. Total DNA was isolated using a modified CTAB method (Doyle and Doyle 1987, Manhart et al. 1990). In most cases total DNA was purified on a cesium chloride gradient (Manhart et al. 1990), although amplification of *rbcL* from unpurified total DNA was also usually successful.

The *rbcL* gene was amplified using the 5' primer RH1 or a primer designed specifically for the Zygnematales based on sequences of *Spirogyra* and *Sirogonium*, and the 3' primer 1385R (Manhart 1994). Amplification was performed using one denaturation cycle at 94°C for 3 min, a primer annealing cycle at 48°C for 1 min, and an elongation cycle at 72°C for 2 min, followed

by 30 cycles of 94° C denaturation for 1 min, annealing at 48° C for 1 min, and elongation at 72° C for 1 min, and a final elongation cycle at 72° C for 5 min. PCR products were purified using a commercial kit (Geneclean II, BIO 101, La Jolla, California).

The PCR primers amplified a 1406-bp fragment of the gene (total length in green plants commonly 1428 bp [Manhart 1994]). Direct sequencing of purified PCR product was performed using a modified Sanger dideoxy method (Conti et al. 1993, Rodman et al. 1993). Sequencing primers were designed by G. Zurawski (DNAX Inc., Palo Alto, California), R. Olmstead (University of Colorado), J. Palmer (Indiana University), and J. Manhart (Texas A&M University). These and additional internal primers designed in our laboratory for charophycean *rbcL* sequences were obtained from Oligos Etc. (La Jolla, California). Primer sequences are available upon request from the authors. Commercial Sequenase (version 2.0) enzyme kits (U.S. Biochemical, Cleveland, Ohio) were used with forward and reverse internal primers to sequence the majority of both strands for template DNA.

Sequences were readily aligned with existing sequences for green algae and *Marchantia* (Manhart 1994) without any insertions or deletions. The 5' and 3' ends of the amplified fragment annealing to the PCR primers (first 26 and last 22 bp) were omitted from the analyses; in addition, bp 1381–1384 were poorly resolved across all taxa and were omitted from the analyses.

Parsimony analysis (MP, maximum parsimony) of sequences was performed using the branch-and-bound option in PAUP 3.1.1 (Swofford 1993). Two weighting criteria were employed: 1) equal weights (i.e. weight = 1) for all codon positions, with transitions and transversions weighted equally; and 2) the weighting criterion of Albert et al. (1993), which assigns relative weights based on codon position (2nd > 1st > 3rd) and type of change (transversions > transitions). The latter weighting scheme was used to lower the impact of potential homoplasy in third-position substitutions and transition-mutations that might be expected to bias the topology of parsimony results for these rather anciently diverged green algae (Hoshaw and McCourt 1988).

To avoid potential problems associated with parsimony analyses involving long branches (Felsenstein 1978, Swofford and Olsen 1990), neighbor joining (NJ) and maximum likelihood (ML) analyses were performed using PHYLIP (Felsenstein 1992). Use of several methods, although each involves certain assumptions and biases (Swofford and Olsen 1990, Hillis et al. 1994), can reinforce conclusions regarding the strengths or weaknesses of certain aspects of tree topology.

To compare relative support for MP tree topologies, 1000 bootstrap replicates (Felsenstein 1985) and decay analyses (Mishler et al. 1991) were performed. Bootstrap analysis (1000 replicates) was also performed for the NJ analysis (Felsenstein 1992). To compare trees implied by alternative hypotheses of relationships, analyses were constrained (Swofford 1993) so that groups of taxa sharing chloroplast shape (three groups) or thallus type (two groups) were monophyletic. Generation of 10,000 random trees was performed to test for skewness (g_i) and phylogenetic signal in the data set (Hillis 1991).

RESULTS

The branch-and-bound search with equal weights for all positions yielded one most-parsimonious tree (Fig. 2) of 1308 steps (CI = 0.516; excluding uninformative characters 1184 steps, CI = 0.465). Of the 1354 characters sampled (*rbcL* sequence excluding primers), 398 were informative, 844 were constant, and 112 were variable but uninformative. Bootstrap (% of 1000 replicates) and decay values (+ number of steps) are also shown in Figure 2. The Zygnematales form a monophyletic sister group to

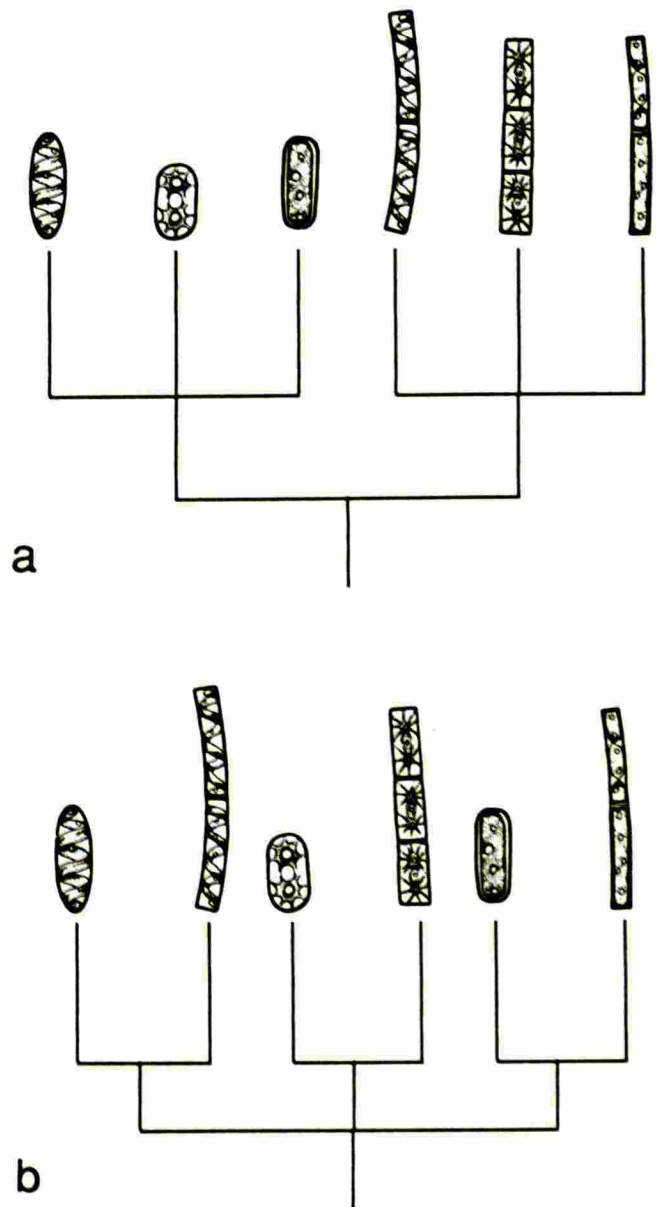


FIG. 1. Contrasting hypotheses of chloroplast evolution in the Zygnemataceae and Mesotaeniaceae. a) Hypothesis proposing that the two families are monophyletic, and that chloroplast shape has evolved convergently in several genera. b) Hypothesis that pairs of genera sharing chloroplast shapes are members of monophyletic groups and that thallus type evolved convergently in several genera.

the other Charophyceae (Fig. 2). The tree includes three clades characterized by a particular chloroplast shape: ribbonlike (*Spirotaenia*, *Sirogonium*, *Spirogyra*), stellate (*Cylindrocystis*, *Zygnema*), and laminate (*Mesotaenium*, *Mougeotia*). The clades with stellate and laminate chloroplasts are sister taxa. Within the ribbonlike clade, the filamentous genera are sister taxa, with *Spirotaenia* basal to this branch. *Roya* and *Netrium* are sister taxa on a clade sister to the group with ribbonlike chloroplasts.

Bootstrap and decay values for the equal-weights parsimony analysis provided strong support for the

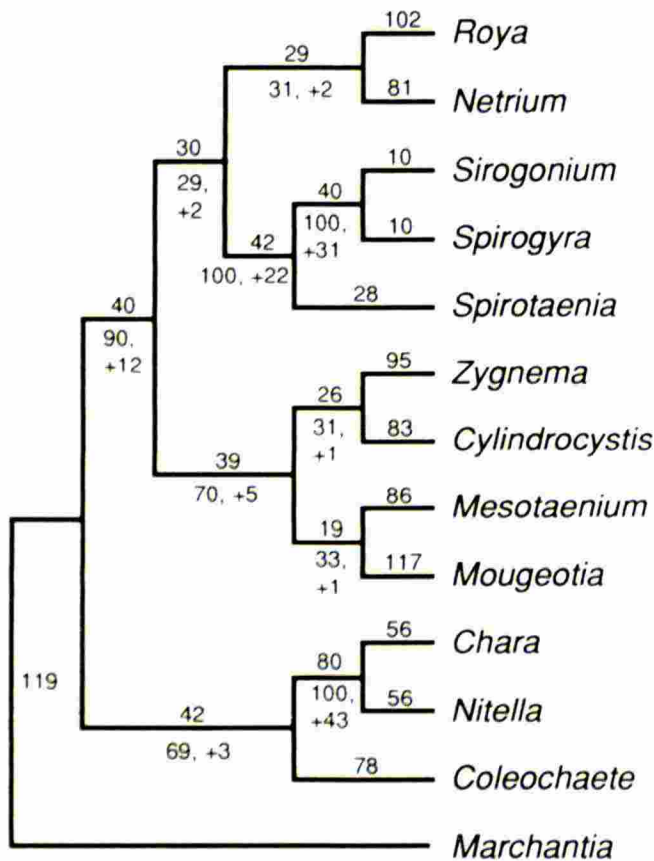


FIG. 2. Most-parsimonious tree obtained with branch-and-bound search in PAUP 3.1.1 (Swofford 1993) using the equal weighting criteria (all changes weighted 1, regardless of codon position or type of change [transition or transversion]). Number of steps (all characters included) above branches; bootstrap (% of 1000 replicates) and decay values (+ number of steps) below branches.

clade with ribbonlike chloroplasts and for the Zygnematales as a whole (Fig. 2). Moderate support was found for a clade combining taxa with laminate and stellate chloroplasts, however, within this laminate-stellate clade, pairings of taxa that shared a chloroplast type were weakly supported.

Constraining the equal-weights search of trees to those in which filaments formed one monophyletic group and unicells (including *Roya* and *Netrium*) formed another produced one most-parsimonious tree of 1370 steps (CI = 0.493; excluding uninformative characters 1246 steps, CI = 0.442), 62 steps longer than the tree found in the unconstrained search. Constraining the search according to chloroplast type produced the same tree as in Figure 2. The distribution of tree lengths for 10,000 randomly generated trees was significantly left-skewed ($g_1 = -0.953972$), which indicated that there is significant phylogenetic signal in the data set (Hillis 1991).

A branch-and-bound search using the Albert et al. (1993) weighting criterion yielded a tree similar to that of the equal-weights tree in that the ribbonlike and laminate-stellate clades are still present, and *Roya* and *Netrium* still form a sister clade to the rib-

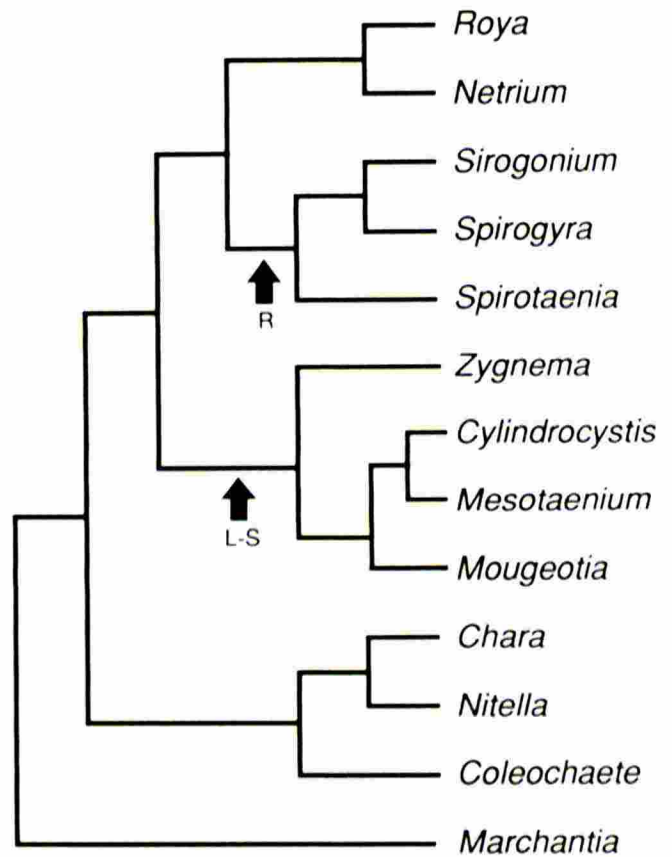


FIG. 3. Most-parsimonious tree obtained with a branch-and-bound search in PAUP 3.1.1 (Swofford 1993) using the Albert et al. (1993) weighting criteria (weights assigned according to codon position and type of change [transition or transversion]). The ribbonlike and laminate-stellate clades found in the equal-weights analysis are indicated with arrows.

bonlike clade (Fig. 3). Topology differed from that of the equal-weights tree within the laminate-stellate clade; the two unicells are sister taxa, and *Zygnema* is basal to the other three genera.

In the two distance analyses, the positions of *Roya* and *Netrium* change within the Zygnematales, and arrangement of genera within the laminate-stellate clade is different from the parsimony analyses in both NJ and ML trees (Figs. 4, 5). Nevertheless, the two major clades of genera with ribbonlike and laminate-stellate chloroplasts are found.

DISCUSSION

The *rbcL* analysis implies that the two traditional families Mesotaeniaceae and Zygnemataceae are polyphyletic groupings of taxa with homoplasious thallus type (i.e. filaments do not form a monophyletic group, nor do unicells). Instead, the molecular data provide support for the hypothesis that chloroplast shape is synapomorphic in at least one group of unicells and filaments. Randhawa (1959) placed *Mesotaenium* (unicellular [u]) ancestral to three lineages: one leading to *Mougeotia* (filamentous [f]) and other filamentous genera with platelike chloroplast,

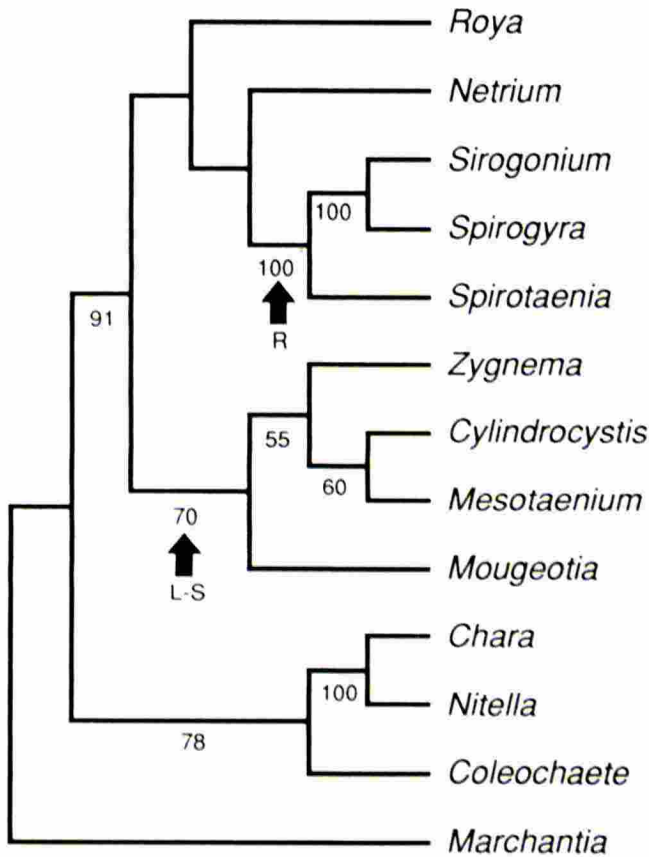


FIG. 4. Tree obtained by neighbor-joining (NJ) analysis using PHYLIP 3.4 (Felsenstein 1992). Bootstrap values (% of 1000 replicates) are shown below branches. The ribbonlike and laminate-stellate clades found in the equal-weights analysis are indicated with arrows.

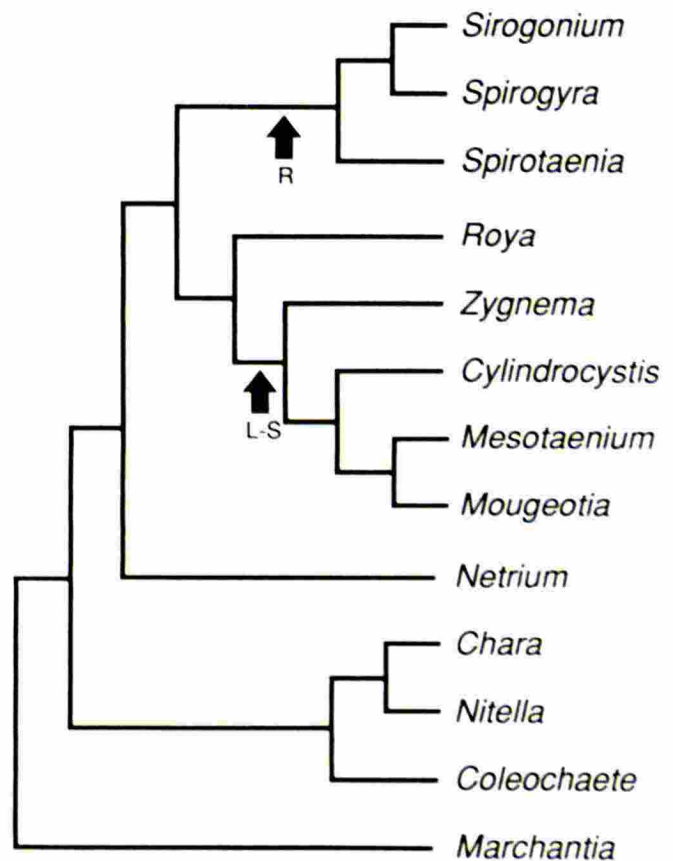


FIG. 5. Tree obtained by maximum likelihood (ML) analysis using PHYLIP 3.4 (Felsenstein 1992). The ribbonlike and laminate-stellate clades found in the equal-weights analysis are indicated with arrows.

a second with *Cylandrocystis* (u) basal leading to *Zygnema* (f) and other genera with stellate chloroplasts, and a third with *Spirotaenia* basal leading to *Spirogyra* and *Sirogonium* with ribbonlike chloroplasts. The *rbcl* data strongly support the latter group and, in the equal-weights parsimony analysis, there is some weak support for the other two clades as well. Overall, the various weighting schemes and methods of analysis support the existence of two major clades: one with ribbonlike chloroplasts and one with laminate and stellate chloroplasts. Within the latter clade the arrangement of taxa is variable between methods (Figs. 2–5) and no firm conclusions on topology can be drawn.

The placement of *Netrium* and *Royia* is also uncertain based on *rbcl* data alone. Yamagishi (1963) postulated that the *Netrium* diverged from the *Cylandrocystis*-*Zygnema* lineage and gave rise to the placoderm desmids. *Royia* has been considered closely related to the placoderm genus *Closterium* (West 1904), but recent treatments (Mix 1975) place *Royia* with the saccoderm. In terms of chloroplast structure, *Royia* (lamine with pyrenoids) might be expected to fall in the laminate-stellate clade. The chloroplasts of *Netrium* are "cucumber"-shaped and occur in one or two pairs (Prescott et al. 1972). The

instability of the placement of these two genera indicates that, although neither is part of the two major clades, their phylogenetic positions are uncertain.

Bootstrap and decay analyses for all weighting schemes and methods of analysis provide strong or moderate support for the ribbonlike and laminate-stellate clades (Figs. 2–5). The weakly supported branches in all analyses generally involved the same genera. Thus, the various weighting schemes and methods all support the same aspects of phylogeny within this group. Differences between the results of the various methods do not demonstrate the superiority of any given method because all methods were equally ineffectual in resolving certain branches. Constraining the equal-weights parsimony search so that genera in the traditional families Mesotaeniaceae and Zygnemataceae were maintained in two separate clades produced a tree much longer than the unconstrained search. Therefore, even though some chloroplast-type clades are not strongly supported by the *rbcl* data, these results clearly refute the monophyly of the traditional families Mesotaeniaceae and Zygnemataceae.

Chloroplast shape is a synapomorphy for the *Spirogyra*-*Sirogonium*-*Spirotaenia* clade, but the coiling of

the plastid varies among the genera. *Spirotaenia* is described as twisting to the left, whereas *Spirogyra* twists to the right (Fig. 1) (Fritsch 1935). *Sirogonium* plastids are either straight or weakly coiled (Transeau 1951). Thus, direction or lack of coiling differentiate genera, but the *rbcL* data suggest that the ribbonlike shape is a shared derived character state. Hoshaw and Hilton (1966) pointed out similarities in chloroplast development among *Spirotaenia*, *Sirogonium*, and *Spirogyra* and suggested that these three genera were closely related. Additional studies of chloroplast ontogeny may reveal whether similarities in chloroplast shape in other genera are due to superficial appearance or are truly homologous.

Other molecular phylogenetic studies are generally congruent with the results presented here and suggest that the Mesotaeniaceae and Zygnemataceae may be polyphyletic. Surek et al. (1993) analyzed small subunit (18S) rDNA sequences from green algae and found that *Mesotaenium* and *Mougeotia* were sister taxa, although their sample included only two genera of Desmidiaceae (*Genicularia* and *Staurastrum*). A related study of 18S rDNA sequences and an included intron (Bhattacharya et al. 1994) that sampled nine Zygnematales and Desmidiaceae suggested that *Mesotaenium* (unicell with laminate chloroplasts) is more closely related to *Zygnemopsis* (filament with twin stellate chloroplasts) than to *Mougeotia* (filament with laminate chloroplasts). This result suggested that neither chloroplast shape nor thallus type defines monophyletic groups. Taxa with ribbonlike chloroplasts were not included in their study. Additional sampling of *rbcL* and 18S rDNA sequences from other genera in the Zygnematales will further test the monophyly of families within this order and hypotheses of chloroplast evolution.

The results of this analysis do not clearly designate a primitive thallus type (filament or unicell) for the Zygnematales (West 1904, Prescott et al. 1972). Yamagishi (1963) proposed that *Mesotaenium* was basal to the group, based on *a priori* assumptions that the ancestor of the Zygnematales was a unicell and contained a flat, relatively undifferentiated chloroplast. Assigning ancestor status to an extant genus is difficult (Theriot 1992), but based on *rbcL* data *Mesotaenium* appears to be a relatively derived genus. West (1904) argued forcefully that the ancestral conjugate was a filament that gave rise to unicellular forms through fragmentation. The charophycean green algae related to the Zygnematales contain unicells, filaments, and branching thalli (Bold and Wynne 1985, Graham 1993), and the uncertain state of knowledge regarding the branching order of these groups (McCourt 1995) makes character-state polarization of thallus type very difficult.

Some morphological data conflict with the groups of unicells and filaments found in these results. The diploid zygospore produced by conjugation of filamentous Zygnemataceae undergoes meiosis and

three nuclei degenerate, which results in a single surviving germling. Zygospores of genera in the Mesotaeniaceae produce two or four germlings (Hoshaw and Hilton 1966, Bold and Wynne 1985). If the relationships supported by the *rbcL* data are correct, the single or multiple germling character state (or possibly both states) may be homoplasious, and there may be some functional constraint upon germling number imposed by the filamentous or unicellular conditions.

In summary, the *rbcL* strongly suggest that the Mesotaeniaceae and Zygnemataceae are polyphyletic assemblages and that thallus type (unicell or filament) does not diagnose two monophyletic families. The molecular data support the hypotheses that the ribbonlike chloroplast shape is a synapomorphy of genera sharing ribbonlike chloroplasts and that genera with the other two chloroplast shapes (laminate and stellate) together form a monophyletic group.

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