

# PHYLOGENY OF THE CONJUGATING GREEN ALGAE (ZYGNEPHYCEAE) BASED ON *rbcL* SEQUENCES<sup>1</sup>

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Sequences of the gene encoding the large subunit of RUBISCO (*rbcL*) for 30 genera in the six currently recognized families of conjugating green algae (Desmidiaceae, Gonatozygaceae, Mesotaeniaceae, Peniaceae, and Zygnemataceae) were analyzed using maximum parsimony and maximum likelihood; bootstrap replications were performed as a measure of support for clades. Other Charophyceae *sensu* Mattox and Stewart and representative land plants were used as outgroups. All analyses supported the monophyly of the conjugating green algae. The Desmidiales, or placoderm desmids, constitute a monophyletic group, with moderate to strong support for the four component families of this assemblage (Closteriaceae, Desmidiaceae, Gonatozygaceae, and Peniaceae). The analyses showed that the two families of Zygnematales (Mesotaeniaceae, Zygnemataceae), which have plesiomorphic, unornamented and unsegmented cell walls, are not monophyletic. However, combined taxa of these two traditional families may constitute a monophyletic group. Partitioning the data by codon position revealed no significant differences across all positions or between partitions of positions one and two versus position three. The trees resulting from parsimony

analyses using first plus second positions versus third position differed only in topology of branches with poor bootstrap support. The tree derived from third positions only was more resolved than the tree derived from first and second positions. The *rbcL*-based phylogeny is largely congruent with published analyses of small subunit rDNA sequences for the Zygnematales. The molecular data do not support hypotheses of monophyly for groups of extant unicellular and filamentous or colonial desmid genera exhibiting a common cell shape. A trend is evident from simple omniradiate cell shapes to taxa with lobed cell and plastid shapes, which supports the hypothesis that chloroplast shape evolved generally from simple to complex. The data imply that multicellular placoderm desmids are monophyletic. Several anomalous placements of genera were found, including the saccoderm desmid *Roya* in the Gonatozygaceae and the zygnematacean *Entransia* in the Coleochaetales. The former is strongly supported, although the latter is not, and *Entransia*'s phylogenetic position warrants further study.

**Key index words:** Charophyceae; Desmidiales; green algal phylogeny; molecular systematics, *rbcL*, Zygnematales, Zygnemophyceae

**Abbreviations:** CI, consistency index; GTR, general time-reversible; ML, maximum likelihood; MP, maximum parsimony; *rbcL*, large subunit of RUBISCO; SSU, small subunit

<sup>1</sup> Received 9 June 1999. Accepted 16 April 2000.

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*I can find only one paper dealing with the question of the general evolution of Desmids . . . . Altogether it is a production testifying to the scant knowledge of the author with regard to the Desmidiaceae in general.*

West (1899)

*The classification of desmids is not altogether easy.*

West and Fritsch (1927)

*After 300 years of microscopy and, of late, intensive investigations, desmids continue to bewilder and intrigue.*

Prescott et al. (1972)

Organisms that have elicited such comments, ranging from derision by colleagues to sympathetic understanding to wonder, not surprisingly present interesting classification problems. The conjugating green algae (Orders Desmiales and Zygnematales) are so distinctive that they have occasionally been put in a class separate from other green algae (Fott 1971, Round 1971, Bold and Wynne 1985). The uniqueness of the group has been reinforced in a modern cladistic framework (Kenrick and Crane 1997). However, the relationships of the families and genera within the conjugating green algae have experienced a bewildering series of rearrangements (West 1904, West and Fritsch 1927, Transeau 1951, Randhawa 1959, Yamagishi 1963, Prescott et al. 1972, Mix 1973a, Hoshaw and McCourt 1988, Gerrath 1993). Moreover, the placement of the conjugating green algae among green algae and plants is still unclear, although they are considered one of several green algal groups monophyletic with land plants, with the entire group referred to as the Streptophyta (Mishler and Churchill 1985, Bremer 1985). Molecular and morphological analyses have supported the monophyly of the conjugating green algae and their relationship to other streptophytes (Hoshaw and McCourt 1988, Mishler et al. 1994, Surek et al. 1994, McCourt 1995, Melkonian and Surek 1995, Kranz and Huss 1996, Qiu and Palmer 1999) but have not explored phylogeny within the group in detail (Bhattacharya et al. 1994, McCourt et al. 1995, Park et al. 1996). The present paper uses DNA sequence data for *rbdL*, the chloroplast-encoded gene for the large subunit of the RUBISCO protein, in a broad sample of genera in all six families and the two orders of the conjugating green algae.

Classification schemes of the group have been generally based on morphology and, more recently, ultrastructure (Transeau 1951, Prescott et al. 1972, Mix 1973a, Hoshaw and McCourt 1988, Gerrath 1993). Schemes based on cell wall and thallus structure (e.g. Prescott et al. 1972, 1975, 1981, Mix 1973a) have generally recognized three groups in the conjugating green algae. Two of these groups have been recognized as families, whose members exhibit smooth cell walls consisting of a single piece and lacking pores or other ornamentation (Transeau 1951, Prescott et al. 1972). One of these families, the Zygnemataceae (twelve genera; Transeau 1951, Kadlubowska 1972, 1984), comprises only filamentous taxa, whereas the other

family, the Mesotaeniaceae or saccoderm desmids (six to seven genera; Prescott et al. 1972, Gerrath 1993), comprises taxa of unicells or loosely joined cells. A third group, known as the placoderm desmids, exhibits the greatest range of morphological diversity. Cell walls in this group are segmented and have more elaborate wall ornamentation than the Zygnemataceae or Mesotaeniaceae. Some (Fritsch 1935, West and Fritsch 1927) placed placoderms (approximately 36 genera) along with the saccoderms in one family, the Desmidiaceae. But the generally accepted classification now recognizes saccoderms as a separate family, the Mesotaeniaceae, and moves some genera from the Desmidiaceae into three additional, smaller families: Closteriaceae (two genera), Gonatozygaceae (two genera), and Peniaceae (one genus) (Gerrath 1993). Kowwets and Coesel (1984) merged the two genera of the Gonatozygaceae into the Peniaceae.

Some authors have preferred to group all conjugating green algae within one order, the Zygnematales (Bold and Wynne 1985). However, placoderm desmids are often treated as members of the order Desmiales (Gerrath 1993), with the Zygnemataceae and Mesotaeniaceae classified separately in the Zygnematales. Whether one or two orders are used, the conjugating green algae are considered either members of a monophyletic group within the class Charophyceae (*sensu* Mattox and Stewart 1984) or, because the latter group is paraphyletic, as members of a separate class, the Zygnemophyceae (Kenrick and Crane 1997).

Sequence data for *rbdL* have been used in a wide variety of phylogenetic analyses of green plants and algae (Chase et al. 1993, Olmstead and Palmer 1994, Manhart 1994, Daughjerg et al. 1994, Nozaki et al. 1995, 1997a,b,c, 1999, Chapman et al. 1998). For this study, our analyses included *rbdL* sequences for 30 genera of conjugating green algae, including representatives of all six families along with 12 outgroup genera, to test hypotheses of relationships among genera, families, and orders. Preliminary results based on these *rbdL* data were cited as part of a review of molecular systematics of green algae (Chapman et al. 1998); the present study includes an additional ingroup genus (*Entransia*) and presents an expanded analysis of the sequence data.

## METHODS

*Specimens and culture methods.* Genera used in this study (Table 1) included (percentage of total genera in family is in parentheses): one Closteriaceae (50%), 14 Desmidiaceae (38%), two Gonatozygaceae (100%), five Mesotaeniaceae (71%), one Peniaceae (100%), and seven Zygnemataceae (58%). The data set comprises 19 new sequences of conjugating green algae plus 11 previously published sequences (McCourt et al. 1995, Park et al. 1996). Published *rbdL* sequences of 16 other streptophyte green algae and land plants were used as outgroups, specifically six genera (seven species) of Characeae, two genera (four species) of Coleochaetales, and five embryophytes (Pickett-Heaps 1975, Mishler and Churchill 1985, Graham 1993, Mishler et al. 1994, Melkonian and Surek 1995, McCourt 1995, Chapman et al. 1998, Qiu and Palmer 1999). An additional streptophyte green algal outgroup, *Klebsormidium* (Klebsormidiales) included initially was omitted from certain analyses for reasons discussed below.

TABLE 1. Species sampled in this study.<sup>a,b</sup>

Taxon	Source	Culture no.	GenBank
<b>Desmidiaceae and Zygnematales</b>			
<i>Arthrodesmus</i> (= <i>Staurodesmus</i> ) sp.	UTEX	LB 2508	AF203491
<i>Closterium acerosum</i> (Schrank) Ehr.	UTEX	LB 1075	AF203492
<i>Cosmarium botrytis</i> Menegh.	UTEX	301	AF203493
<i>Cosmoecium perissum</i> Roy et Bisset	UTEX	2447	AF203494
<i>Cylindrocapsa</i> sp.	UTEX	1925	U38695
<i>Desmidium</i> sp.	UTEX	LB 612	AF203495
<i>Entransia fimbriata</i> Hughes	UTEX	LB 2353	AF203496
<i>Euastrum pectinatum</i> Bréb.	UTEX	LB 1748	AF203497
<i>Genicularia spirotaenia</i> (De Bary) De Bary	SVCK	329	U71439
<i>Gonatozygon monotaenium</i> De Bary	UTEX	LB 1253	U71438
<i>Groenbladia undulata</i> (Nordstedt) Förster	SVCK	440	AF203498
<i>Hyalotheca dissiliens</i> (Smith) Bréb.	UTEX	LB 476	AF203499
<i>Mesotaenium caldariorum</i> (Lagerh.) Hansg.	UTEX	41	U38696
<i>Micrasterias rotata</i> Ralfs	UTEX	LB 1941	AF203500
<i>Mougeotia</i> sp.	ARL	82-11	U38699
<i>Netrium digitus</i> (Ehr.) Itz. et Rothe	UTEX	LB 561	U38698
<i>Onychonema</i> sp.	UTEX	LB 832	AF203501
<i>Penium margaritaceum</i> (Ehr.) Bréb.	UTEX	LB 600	AF203502
<i>Pleurotaenium trabecula</i> (Ehr.) Näg.	UTEX	489	AF203503
<i>Roya angelica</i> G.S. West	UTEX	LB 934	U38694
<i>Sirogonium melanosporum</i> (Randhawa) Transeau	ARL	700	L13484
<i>Sphaeroszoma</i> sp.	UTCC	284	AF203504
<i>Spirogyra maxima</i> (Hassall) Wittrock	UTEX	LB 2495	L11057
<i>Spirotaenia condensata</i> Bréb.	ARL	1300	U38700
<i>Spondylium pulchellum</i> Archer in Pritchard	CCAP	680/1	AF203505
<i>Staurostrum pinque</i> Teiling	UTEX	LB 1606	AF203506
<i>Xanthidium subhastiferum</i> W. West	CCAP	690/1	AF203507
<i>Zygnema peliosporum</i> Witr.	UTEX	LB 45	U38701
<i>Zygnemopsis</i> sp.	CCAP	699/1	AF203508
<i>Zygogonium tunetanum</i> Gauthier-Lievre	UTCC	136	AF203509
<b>Charales</b>			
<i>Chara connivens</i>	Manhart (1994)		L13476
<i>Lamprothamnium macropogon</i>	McCourt et al. (1996)		U27534
<i>Lychnothamnus barbatus</i>	McCourt et al. (1996)		U27533
<i>Nitella translucens</i>	Manhart (1994)		L13482
<i>Nitellopsis obtusa</i>	McCourt et al. (1996)		U27530
<i>Tolypella nidifica</i>	McCourt et al. (1996)		U27531
<i>Tolypella prolifera</i>	McCourt et al. (1996)		U27532
<b>Coleochaetales</b>			
<i>Chaetosphaeridium globosum</i>	Delwiche (personal communication)		Unpublished
<i>Chaetosphaeridium ovalis</i>	Delwiche (personal communication)		Unpublished
<i>Coleochaete irregularis</i>	Delwiche (personal communication)		Unpublished
<i>Coleochaete orbicularis</i>	Manhart (1994)		L13477
<b>Klebsormidiales</b>			
<i>Klebsormidium flaccidum</i>	Manhart (1994)		L13478
<b>Embryophytes</b>			
<i>Marchantia polymorpha</i>	Ohyama et al. (1986)		X04465
<i>Ophioglossum engelmannii</i>	Manhart (1994)		L11058
<i>Oryza sativa</i>	Nishizawa and Hirai (1987)		D00207
<i>Pseudotsuga menziesii</i>	Hipkins et al. (1990)		X52937
<i>Psilotum nudum</i>	Manhart (1994)		L11059

<sup>a</sup> Names used are those of strains in cultures from collections indicated. Sequences for Zygnematales and Desmidiaceae are new except for those with a literature reference listed in the Source column. For authors, sources, and vouchers of outgroups refer to original papers cited.

<sup>b</sup> ARL, Algal Research Laboratory at the University of Arizona (vouchers Academy of Natural Sciences); CCAP, Culture Collection of Algae and Protozoa, Ambleside, Cumbria; SVCK, Sammlung von Conjugaten-Kulturen der Universität Hamburg; UTCC, University of Toronto Culture Collection, Ontario; UTEX, Culture Collection of Algae at the University of Texas, Austin, TX.

Algae were obtained in unialgal condition from culture collections (Starr and Zeikus 1993, Mix 1973b, Engels and Mix 1980) or from isolations of clones from our own collections. Most cultures were grown in double-strength Woods Hole media (Nichols 1973), substituting Hepes for Tris buffer. Saccoderm desmids generally grew best in soil-water medium (Nichols 1973). Cultures were grown in glass culture vessels ranging from 200 mL to 20 L at constant 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 an 18:6 hour light/dark cycle.

**DNA extraction and purification.** Wet thalli were blotted dry on paper towels and frozen in liquid nitrogen before grinding

in a cold mortar and pestle. Total DNA was extracted from powdered frozen thalli using a modified cetyltrimethylammonium bromide (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 gene amplifications were usually successful without this purification step.

**Gene amplification and sequencing.** The *rbcL* gene was amplified with the PCR using one of two forward primers starting at bp 1: (RH1: ATGTCACCAACAGAACTAAAGC; or Z1: ATGTCACCAACAGAACTAAAGCAAGT), and reverse primer 1385R (GGAAAGAAATTAATTTGAATT) (Manhart 1994). These primers amplified a 1407 bp fragment (1354 bp excluding primers, ap-

TABLE 2. Kishino-Hasegawa test results for trees with different placements of *Klebsormidium* as sister to: (1) all other *Charophyceae*; (2) *Closterium*; and (3) *Mougeotia*. Tests compare trees 1 and 3 with the best tree (2).

Tree	−ln L	Diff −ln L	SD (diff)	t	<i>P</i> <sup>a</sup>
1	18401.03716	7.372743	8.79493	0.8383	0.4020
2	18393.66461	(best)			
3	18425.26302	31.59828	14.50837	2.1779	0.0296 <sup>b</sup>

<sup>a</sup> Probability of getting a more extreme t-value under the null hypothesis of no difference between the two trees (two-tailed test).

<sup>b</sup> Significant at *P* < 0.05.

proximately 95% of the *rbdL* gene); the 3' terminus downstream of the reverse primer was omitted because it is too variable in these algae for efficient primer design. PCR products were purified using GENECLAN (BIO 101, La Jolla, CA) or Wizard® PCR Preps (Promega Corp., Madison, WI). Sequencing of purified PCR product was performed using a modified Sanger dideoxy method with commercial Sequenase (Version 2.0) enzyme kits (U.S. Biochemical, Cleveland, OH) (McCourt et al. 1995, 1996). Sequencing primer data were provided by R. Olmstead (University of Washington), J. Palmer (Indiana University), and J. Manhart (Texas A&M University). Both strands of the PCR product were sequenced for the majority of the DNA templates. Those portions not sequenced in both directions were sequenced in overlapping fragments for one strand. Most sequences were confirmed by sequencing the same template DNA on a Perkin Elmer Applied Biosystems 377 automated sequencer (Perkin Elmer Biosystems, Foster City, CA).

**Phylogenetic analysis.** Sequences were aligned with those from existing green plant sequences (Manhart 1994) and no insertions or deletions were detected in any sequences. Primer regions (positions 1–28 and 1,385–1,407) were omitted from analysis. Three base pairs immediately upstream of the 3' primer were usually unreadable and also omitted.

The primary data set analyzed consisted of all the conjugating green algal genera and outgroups listed in Table 1. Maximum parsimony (MP) and maximum likelihood (ML) analyses of sequences were performed using PAUP\* (version 4.0d64) with permission of the author (Swofford 1998). Heuristic MP searches of molecular data sets were performed, with equal weights, tree bisection-reconnection (TBR), swap on all minimal trees (MULPARS), and steepest descent options in effect. The step-wise random addition option (10 replicate heuristic searches with a random shuffling of taxon input order) was used to avoid local optima or islands of most-parsimonious trees (Maddison 1991). To compare relative support for branches, 1000 bootstrap (Felsenstein 1985) replications were performed (without step-wise random addition or steepest descent options in effect). Taxon deletion experiments were performed in which sets of genera corresponding to traditional families of conjugating green algae were deleted singly or in combination from MP heuristic analyses. To compare the MP tree with the tree implied by traditional classification schemes, backbone constraints were imposed in which genera of traditional families (Transeau 1951, Mix 1973a) were constrained as monophyletic (although genera were unconstrained in their placements within the families). To assess the possibility that codon position influences optimal tree topology, MP analyses were performed using first plus second positions, or third positions only. In addition, a character partition homogeneity test (Swofford 1998) was also performed using a partition for each of the three codon positions, and for a partition of position 1 plus position 2 versus position 3.

A ML analysis was also performed. Following the method described by Swofford et al. (1996), likelihood scores for the optimal trees obtained in the MP heuristic search were calculated using a series of likelihood models incorporated into PAUP\*

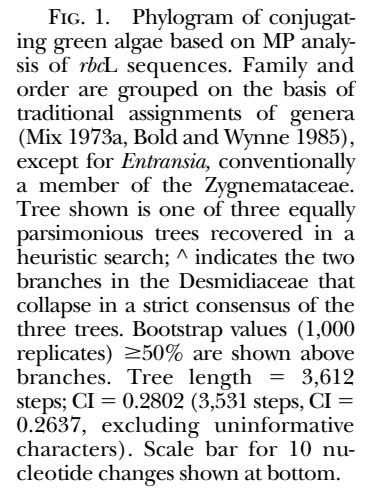
and then compared using the log likelihood ratio test. The model providing the best fit of the data while invoking the fewest parameters was used to fix parameters used in a subsequent heuristic ML search. For the *rbdL* data, the best model was the general time-reversible model (GTR, equals REV of Yang 1994a) where the proportion of invariant sites was estimated (Hasegawa et al. 1985), and rates for variable sites were drawn from a gamma distribution with shape parameter  $\alpha$  ( $\Gamma$ , Yang 1994b). This model is denoted GTR + I +  $\Gamma$  (Gu et al. 1995). A total of 100 ML bootstrap replications were performed (Felsenstein 1985). A ML search was performed using the same traditional family constraints described above. In addition, the likelihood score was calculated for a topology constrained to have monophyletic traditional families but with an unresolved topology.

## RESULTS

Initially *Klebsormidium* was included in the taxon set as a member of the outgroup genera; however, this genus, in contrast with others, exhibited the most unstable topological placement in the resulting trees. The MP analysis (Fig. 1) joined *Klebsormidium* with *Mougeotia* (Chapman et al. 1998), and ML analysis placed *Klebsormidium* in a sister relationship with *Closterium* (Fig. 2). Because the branch for *Klebsormidium* might be attracting long branches from the ingroup (Felsenstein 1978), we performed a series of Kishino-Hasegawa tests comparing the MP and ML topologies with alternate placements of *Klebsormidium* within the *Charophyceae sensu* Mattox and Stewart (1984). We calculated likelihood scores for three trees with different placements of *Klebsormidium* (indicated by boldface numbers next to branches in Fig. 2): (1) tree with *Klebsormidium* sister to all other *Charophyceae* in data set (i.e. the “expected tree” if *Klebsormidium* is primitive); (2) tree with *Klebsormidium* sister to *Closterium* (ML tree); and (3) tree with *Klebsormidium* sister to *Mougeotia* (MP tree). The results of pairwise K-H tests (Table 2) show that trees 1 and 2 and trees 1 and 3 are not significantly different from each other, whereas the best ML tree (2) is significantly better than tree 3. The tree with *Klebsormidium* sister to *Mougeotia* is significantly worse than other placements, but the other arrangements of *Klebsormidium* are equally likely under the constraints of the ML model. From these results, we conclude that the *rbdL* sequence for *Klebsormidium* introduces significant (i.e. misleading) homoplasy into the data set and therefore we excluded *Klebsormidium* from subsequent analyses.

Optimal trees found in the heuristic MP (Fig. 1) and ML (Fig. 2) analyses of the primary data set excluding *Klebsormidium* had very similar branching topologies, particularly for clades receiving moderate (60–75%) to strong (>75%) bootstrap support. Comparing support for branches in a pairwise fashion for each analysis showed values were higher for the ML tree than for the MP tree for most, but not all, branches. ML and MP bootstrap percentages are listed, respectively, within parentheses in the discussion that follows.

Monophyly of the conjugating green algae *in toto* is strongly supported (95%, 83%). Within the conjugating green algae, monophyly of the Desmidiaceae (plus



The Zygnematales *sensu* Brook (1981) and Gerrath (1993) comprises two families, the Mesotaeniaceae and Zygnemataceae. The monophyly of both families is strongly refuted by well-supported clades on the MP and ML trees. The saccoderm desmids in particular

The ML analysis suggests that most of the traditional zygnematacean and mesotaeniacean genera are arranged in two sister groups, each of which is strongly supported: (a) *Sirogonium*, *Spirogyra*, and *Spirotaenia* in one clade; and (b) *Cylindrocystis*, *Mesotaenium*, *Mougeotia*, *Zygnema*, *Zygnemopsis* and *Zygogonium* in a second.

**Deleting taxa and codon position effects.** A series of taxon deletion experiments using MP heuristic searches (10 random taxon additions, steepest descent, and MULPARS on) revealed that the topology in Fig. 1 is robust

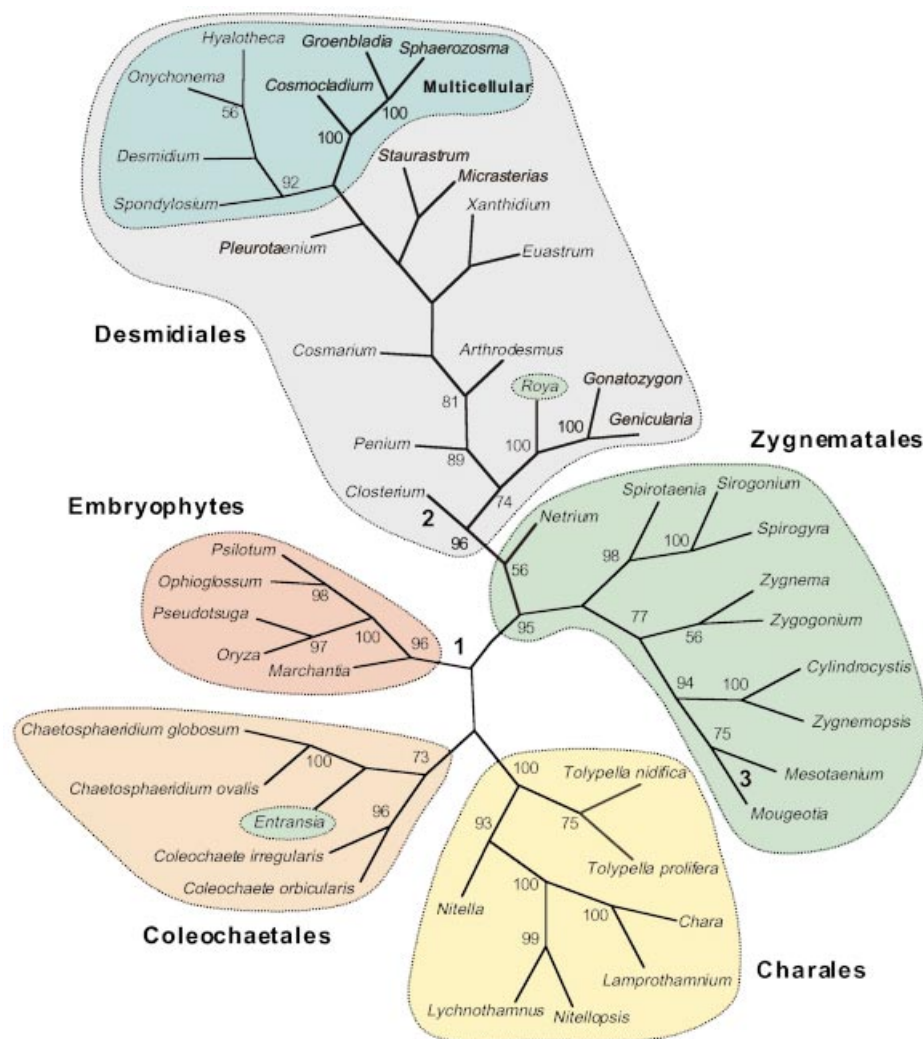


FIG. 2. Unrooted network showing phylogenetic relationships of the conjugating green algae based on a ML analysis of *rbcL* sequences ( $-\ln = 17634.66154$ ). Embryophytes and major clades of charophycean algae are enclosed by lines; also indicated are families of Desmidiaceae and a clade of multicellular (filamentous or colonial) placoderm desmids. Bootstrap values (100 replications)  $\geq 50\%$  are shown adjacent to branches. Numbers 1, 2, and 3 refer to possible placements of *Klebsormidium* tested in Table 2. Dotted lines around *Roya* and *Entransia* indicate anomalous placements discussed in text.

to such deletions. Deletion analyses involved the removal of the following groups from the data set: Desmidiaceae (Gerrath 1993); Mesotaeniaceae (Transeau 1951); Zygnemataceae (Transeau 1951); and Zygnemataceae plus Mesotaeniaceae. In each case, the tree(s) obtained in the search appeared to have been pruned of the removed taxa with little or no effect on the topology. The only changes observed involved the poor resolution of branches within the Desmidiaceae when the Mesotaeniaceae or Zygnemataceae were deleted, although monophyly of the four families within the Desmidiaceae was still evident. Conclusions regarding major clades of conjugating green algae (e.g. monophyly of placoderms, paraphyly of Zygnemataceae and saccoderm desmids) were unaltered by deletions of taxa.

Partitioning the data set by codon position showed no significant difference across partitions ( $P = .08$ ), nor for a partition of position 1 + position 2 versus position 3 ( $P = .4$ ). An MP analysis using only third codon positions yielded three equally parsimonious trees (2901 steps, consistency index [CI] = 0.311)

whose consensus was nearly identical to Fig. 1. Of 452 characters, 420 (93%) were parsimony informative. The one major change involved the placement of *Mougeotia* as a member of the Charales, which further reflects long-branch problems with this taxon. The only other branches that were altered from the topology in Fig. 1 were those with low bootstrap support. Topology of the well-supported clades found in Fig. 1 were retained: monophyly of Desmidiaceae, Peniaceae, Closteriaceae, Gonatozygaceae, and paraphyly of Zygnemataceae. *Entransia* was still found to be sister to *Chaetosphaeridium*. A strict consensus of the 69 equally parsimonious trees positions (677 steps, CI = 0.334), resulting from an MP search using positions one and two, was less resolved than the consensus tree derived from third codon positions. Of 902 characters, 133 (14.7%) were parsimony informative. Again, branches rearranged or collapsed from Fig. 1 were those with poor bootstrap support; and the consensus was not in conflict with well-supported clades of Fig. 1.

*Traditional family monophyly constraints.* Constraining the MP analysis so that the six traditional families were

each monophyletic (PAUP\* backbone constraints) produced 16 trees of 3833 steps, 220 steps longer and with a lower CI (0.2640) than that of the unconstrained tree (Fig. 1). The constrained ML scores for the fully resolved tree ( $-\ln = 18,137.92073$ ) and the tree with relationships within families unresolved ( $-\ln = 18,912.70647$ ) were significantly worse than the unconstrained tree in Fig. 2 ( $-\ln = 17,634.66154$ ). Within the families, topologies matched those obtained in the taxon deletion studies of the previous section. Relationships of genera were unperturbed except for the forced exclusion required by the imposed constraints that kept families monophyletic.

#### DISCUSSION

The *rbcL* data have implications for classification and phylogeny at various levels within the conjugating green algae.

*Desmidiaceae*. The *rbcL* data support the monophyly of the conjugating green algae as a whole, and the monophyly of the Desmidiaceae, or placoderm desmids. Within this order, two families, the Gonatozygaceae and Desmidiaceae, are strongly supported as monophyletic. The remaining two families, Closteriaceae (represented by one of two component genera [Gerrath 1993]) and monospecific Peniaceae, occupy positions within the phylogeny consistent with their independent status. Neither is nested within another family. The *rbcL* data strongly support the hypothesis that the Desmidiaceae is the most derived family, with the other three families derived earlier within the placoderm clade. Because all genera were each represented by a single species, these results do not explicitly test the monophyly of each genus.

These results corroborate the ultrastructural cell wall characters used by Mix (1972, 1973a, 1980) in her delineation of families of placoderm desmids. The *rbcL* data also support Mix's (1973a) distinction between putatively primitive placoderm families (Closteriaceae, Gonatozygaceae, and Peniaceae) and the morphologically complex Desmidiaceae, although the data refute her suggestion that a monophyletic suborder comprised the former three families. Genera with less elaborate wall ornamentation are primitive within the placoderm desmids, whereas the distinctly bipartite and elaborately sculptured walls of the Desmidiaceae are derived traits based on *rbcL* analysis.

Phylogenetic relationships within the placoderm desmids are only partly resolved. The most strongly supported groups of genera in the Desmidiaceae include several clades of multicellular forms (filamentous or colonial). The strongly supported clade containing *Cosmocladium* (a colony) and *Sphaerosozma* (a filament) corroborates monophyly of this pair of genera based on small subunit (SSU) rDNA data (Bhattacharya et al. 1994). The filamentous *Groenbladia* is also a member of this clade in the *rbcL* tree. A second well-supported clade in the ML analysis includes four filamentous forms: *Desmidium*, *Hyalotheca*, *Onychonema*, and *Spondylosium*. Given the topology and support for

these multicellular clades, it is possible that a multicellular thallus evolved only once in the placoderm desmids. Interestingly, Krupp and Lang (1985a,b) found that a common mechanism of primary wall adhesion holds cells together in two filamentous placoderm genera (*Bambusina*, *Onychonema*), each with a very different type of component cell. It is surprising that the filamentous thallus type is a synapomorphy in placoderm desmids but not in the Zygnemataceae, a finding that opposes traditional classification of the latter family.

An interesting corollary to these findings is that multicellular forms whose component cells strongly resemble some unicellular genera (e.g. the "*Cosmarium*"-like cells of *Cosmocladium*) are not necessarily sister to these unicellular forms. This finding refutes the hypothesis of West (1904), Fritsch (1935), and Prescott et al. (1972, 1981) that such desmid genera are closely related. For example, Prescott et al. (1972, 1981) divided the filamentous forms into two sections, one related to *Cosmarium* "as progenitor" (*Onychonema*, *Sphaerosozma*, *Spondylosium*, and *Teilingia*), and one that "relates questionably" to *Staurastrum* (*Bambusina*, *Desmidium*, *Groenbladia*, *Haplozyga*, *Hyalotheca*, and *Phymatodocis*). Neither of these sections is supported by the *rbcL* data, and some clades with high bootstrap support directly contradict the sectional division (e.g. *Groenbladia* and *Sphaerosozma*, Fig. 2). However, some genera in well-supported multicellular clades share a common cell shape. For example, *Cosmocladium* and *Sphaerosozma* and *Onychonema* and *Spondylosium* have *Cosmarium*-like cells, but even these genera are not all members of one clade. The present analysis suggests that cell shape is quite malleable and that convergence and even reversal may be possible. A thorough test of the West/Fritsch/Prescott hypothesis awaits better resolution of relationships within the Desmidiaceae.

*Zygnematales*. In contrast with the relatively well-supported status of families within the Desmidiaceae, the phylogenetic relationships of genera in the Mesotaeniaceae and Zygnemataceae (*sensu* Bold and Wynne 1985) exhibit features at odds with traditional taxonomy. The two families are clearly paraphyletic. For example, *Spirotaenia* is part of a well-supported clade containing *Sirogonium* and *Spirogyra*; likewise, *Cylindrocystis* is sister to *Zygnemopsis*, and *Mesotaenium* is sister to *Mougeotia* (although support for the latter pair of genera is not as strong). As discussed by McCourt et al. (1995), members of these clades share similarities in chloroplast morphology (Randhawa 1959, Yamagishi 1963, Bold and Wynne 1985). Ribbon-shaped chloroplasts characterize the *Spirotaenia* clade, stellate chloroplasts are shared by *Cylindrocystis* and *Zygnemopsis*, and laminate chloroplasts are found in *Mesotaenium* and *Mougeotia*. Note that a similar chloroplast shape is not always a synapomorphy, and support for monophyly of genera sharing a chloroplast type is not supported by SSU rDNA data (Besendahl and Bhattacharya 1999). For example, the stellate chloroplast shape found in *Zygnema*

and *Zygogonium* is also found in a separate clade containing *Cylindrocystis*/*Zygnemopsis*.

*Teiling's hypotheses.* Teiling (1952) proposed that cell and chloroplast shape had evolved from short and circular cells in cross-section, through angular forms (pluriradiate desmids), to an "end-stage" of flattened, leaflike shapes with correspondingly lobed chloroplasts. This process represented, according to Teiling (1952), a progression toward greater capacity and efficiency for light capture and photosynthesis. His basic assumption was that more derived, flattened forms are better photosynthesizers, compared with the more primitive types with a lower surface to volume ratio. Cell shape evolution as inferred from the *rbcL* data conforms somewhat to Teiling's progression of shapes: filaments and unicells circular in cross-section are the primitive condition in the traditional Zygnemataceae and Mesotaeniaceae, and the angular and more flattened cell-shapes of the placoderm desmids are derived. However, the leaflike end-stage exemplified by *Cosmarium* and *Micrasterias* (Teiling 1952) is not found exclusively in the most derived Desmidiaceae. And whereas chloroplast shape generally follows the trend in cell shape, some of the omniradiate cells of saccoderms have fairly elaborate, ridged, spiral, or folded chloroplasts (e.g. *Roya*, *Cylindrocystis*, and *Spirotaenia*). The hypothesis that derived cell and chloroplast forms of placoderm desmids are better photosynthesizers and have achieved greater evolutionary success is difficult to reconcile with the ecological success of some of the so-called "primitive forms" such as *Spirogyra*, which is among the most widespread and species-rich of conjugating green algae (McCourt et al. 1986).

*Anomalous saccoderms and Entransia.* Two genera of saccoderm desmids are placed in unorthodox positions on the *rbcL* trees. The saccoderm desmid *Netrium* is sister to the placoderm desmids in the MP and ML trees, although this position is not well supported by bootstrap analyses. A second unusual placement is that of the saccoderm *Roya*, strongly supported as sister to a clade containing the two genera assigned by Mix (1973a, 1980) to the Gonatozygaceae. Genera of the Gonatozygaceae have been considered saccoderm desmids by some (Prescott et al. 1972), but the segmented and ornamented cell walls of these genera align them with the placoderms. *Roya* should be more closely examined for other characters that would corroborate or refute its inclusion within the Gonatozygaceae and placoderm desmids as a whole. The wall structure in *Roya* may represent a loss of ornamentation, or, if this absence is the retained plesiomorphic state, then the ornamentation in the Gonatozygaceae is nonhomologous with and evolved independently of that in the Closteriaceae, Peniaceae, and other desmids. The results of the present study confirm those of Park et al. (1996). In the present analysis, the Gonatozygaceae are clearly members of the placoderm clade. The *rbcL* data do not support the inclusion of the Gonatozygaceae as part of the Peniaceae as emended by Kouwets and Coesel (1984).

The placement of *Entransia* within the Coleochaetales and sister to *Chaetosphaeridium* (in MP and ML analyses) is unexpected, except so little is known of *Entransia* that one might not be surprised to find it anywhere on the tree in Fig. 2. *Entransia* is a monotypic genus collected once and described relatively recently by Hughes (1948), who declined to assign it to a group within the green algae. Transeau (1951) and Randhawa (1959) included it in their monographs of the Zygnemataceae, because of its unbranched filamentous thallus and distinctively shaped chloroplasts, but suggested that its inclusion in the family be considered provisional because it has never been observed conjugating. In fact, Kadlubowska (1972, 1984) excluded *Entransia* from her monographs of the Zygnemataceae. Although the phylogenetic connection of *Entransia* to conjugating green algae is tenuous, there is even less morphological evidence to support a sister relationship of *Entransia* with *Chaetosphaeridium*. *Chaetosphaeridium* has spherical cells that occur singly or in groups covered in mucilage and possesses sheathed bristles (Fritsch 1935). *Entransia* consists of unbranched filaments without bristles and has laminate chloroplasts bearing marginal, finger-like projections. The *rbcL* branch for *Entransia* is relatively long and its surprising placement in the charophycean outgroups may be an example of long-branch attraction (Felsenstein 1978). Deleting *Entransia* from the data set yielded no difference in tree topology (unlike the deletion of *Klebsormidium*). The *rbcL* data suggest that *Entransia* is at least highly divergent from the remainder of the conjugating green algae and warrants further study. *Entransia* and *Klebsormidium* may represent plesiomorphic filamentous forms within the streptophyte clade.

*Comparison with other molecular analyses.* Data from several molecular sources are generally congruent with respect to the phylogeny of the conjugating green algae. Surek et al. (1994), Bhattacharya et al. (1994), and Besendahl and Bhattacharya (1999) reported that zygnematalean genera (except *Spirogyra*) possess a group I intron in the SSU of nuclear rDNA. The conserved position, along with sequence analysis of the SSU and the intron, supported monophyly of the group. Besendahl and Bhattacharya (1999) also found that all sampled Desmidiaceae *sensu* Mix had apparently lost a conserved helix from the P2 domain of the SSU. As reported from studies of land plants and green algae (Nickrent and Soltis 1995, Chapman et al. 1998), *rbcL* sequences in the charophycean green algae appear to display a greater degree of sequence divergence, compared with that of the SSU rDNA gene. Nevertheless, bootstrap support in analyses using these two genes are in parallel and support nearly all the same clades.

Surek et al. (1994) sampled SSU sequences in four genera of conjugating green algae and found that *Mesotaenium* and *Mougeotia* were grouped together as a sister clade to two placoderms, *Genicularia* and *Staurostrum*, a topology not in conflict with the *rbcL* trees. Bhattacharya et al. (1994) sampled additional genera (eight total), and their neighbor-joining tree grouped

*Mesotaenium* (saccoderm) and *Zygnemopsis* (Zygnemataceae) as a sister clade to *Mougeotia* (Zygnemataceae). The MP or ML trees using *rbcL* sequences place these three genera in the same clade (along with *Cylindrocystis*) but with *Mesotaenium* sister to *Mougeotia* (Figs. 1 and 2). Bhattacharya et al. (1994) sampled five placoderm desmids, which formed a monophyletic group with *Genicularia* sister to the remaining four genera; this result is congruent with the *rbcL* tree. In their study, as in the present one, *Cosmoladium* and *Sphaerosoma* were members of a strongly supported clade, to the exclusion of the unicellular *Cosmarium*. MP analysis of the *rbcL* data, using only the genera in Bhattacharya et al. (1994), yielded a tree nearly identical with the SSU tree (data not shown).

Besendahl and Bhattacharya (1999) included several additional genera from all three traditional families of conjugating green algae; the resulting trees shared features of topology with those presented here but did differ in several important ways. Their study found strong support for some of the sister relationships shown in Figs. 1 and 2: (a) *Cosmoladium* plus *Sphaerosoma*; (b) *Cylindrocystis* plus *Zygnemopsis*; and (c) *Genicularia* plus *Gonatozygon*. The sister relationship of *Mesotaenium* and *Mougeotia* was not supported by their analysis, which they stated argued against the hypothesis that chloroplast shape is a synapomorphy. Although the latter hypothesis cannot be universally true if the SSU rDNA topology is correct, chloroplast shape is nonetheless a shared trait of several clades based on one or both sets of data (i.e. a *Cylindrocystis/Zygnemopsis* and a *Sirogonium/Spirogyra/Spirotaenia* clade).

The relatively ancient divergence time of the Zygnemataceae from other streptophytes (>500 million years) and number of changes in the third position might lead one to expect that this position would be saturated and therefore contain little signal relative to first and second positions. However, our analysis found the opposite to be true: the tree derived from third positions only was well resolved and nearly identical to the tree obtained using all three positions. This result is less surprising when compared with a study of liverwort phylogeny using *rbcL* sequences (Lewis et al. 1997) and seed plant phylogeny using photosystem genes (Sanderson et al. 2000). In these studies, although the third position was highly variable, it nevertheless yielded well-resolved trees that were generally congruent with trees based on the entire data set. We concur with Lewis et al. (1997) that third-codon position contains significant phylogenetic signal across several hundred million years of green plant evolution.

**Classification and plesiomorphic characters.** A cardinal characteristic of phylogenetic systematic classification is that taxa should be defined on the basis of derived characters (synapomorphies) rather than shared primitive ones (symplesiomorphies) (Theriot 1992). Thus, in retrospect, it should not be surprising that the *rbcL* data support the monophyly of groups (placoderms and included families) that share a derived cell wall structure (i.e. either moderately or elaborately sculptured and

segmented). In contrast, the unsegmented, homogeneous, and unsculptured cell walls in the Zygnemataceae and Mesotaeniaceae represent a primitive (plesiomorphic) character state (i.e. outgroups lack the segmentation and ornamentation of placoderms), and it is not surprising that these traditional families are revealed to be nonmonophyletic by cladistic analysis.

Other character states of the conjugating green algae are less readily identified as primitive because of the lack of corresponding characters in outgroups that allow character polarization (Maddison et al. 1984). For example, the number of germlings varies from one (all Zygnemataceae), to two (most placoderms examined) to four (most saccoderms examined). Zygospores and germlings clearly homologous with those of the Zygnematales are not known outside the conjugating green algae and the primitive condition of this trait must be inferred from phylogenetic patterns based upon other characters. The molecular analyses suggest that the number of germlings exhibits several instances of homoplasy. The distribution of thallus character states on the *rbcL* tree offers the hypothesis that the filamentous genera of the Zygnemataceae *sensu* Smith (1933) are somehow constrained to produce a single germling from zygotic meiosis, whereas the saccoderm and placoderm desmids (at least the unicellular genera studied) generally produce more than one surviving germling after meiosis.

**Classification based on rbcL tree.** In the last 100 years, several elaborate phylogenetic schemes have been proposed for the conjugating green algae (de Bary 1858, Palla 1894, West and West 1898, West 1916, West and Fritsch 1927, Fritsch 1935, Randhawa 1959, Yamagishi 1963, Prescott et al. 1972). The schemes conflict on many points, in ways that can be traced to the authors' views on the thallus type of the ancestral "conjugate" and whether taxa should be grouped on the basis of thallus type or chloroplast morphology.

West (1899, 1904) was convinced that the ancestral conjugate was filamentous. He proposed that this ancestral filament preceded degenerate unicellular forms. The reasoning underlying his scheme was not always made explicit, but it appears that he inferred a progression from filaments to simple unicells (saccoderm desmids, which included *Genicularia* and *Gonatozygon*) to more elaborate unicells (placoderm desmids). Apparently the intermediate degree of ornamentation of cell walls of *Genicularia* and *Gonatozygon* was not considered important in this progression; rather the transient filamentous habit of these genera was seen as transitional between the primitive filamentous and the advanced unicellular thallus. In general features, West's phylogeny is reminiscent of the *rbcL* tree; he might have approved of it. In the *rbcL* analyses, placoderms are derived from unicellular ancestors without wall segmentation or ornamentation (i.e. *Netrium*), and the less elaborately ornamented Peniaceae, Gonatozygaceae, and Closteriaceae are primitive. Filamentous placoderms evolved the multicellular thallus secondarily from a unicellular placoderm ancestor.

Yamagishi (1963) detailed an evolutionary scheme that had its roots in a hypothesis of Palla (1894), in which three evolutionary lineages were characterized by one of three basic chloroplast shapes (ribbonlike, stellate, and laminate). Yamagishi (1963) further suggested that each line of filamentous forms evolved from a unicellular ancestor (i.e. saccoderm) with the same chloroplast type. *Zygnema* and other forms with stellate chloroplasts evolved from *Cylindrocystis*, *Mougeotia*, and others with laminate plastids from *Mesotaenium*, and *Spirogyra* and others with ribbonlike plastids from *Spirotaenia*. Placoderm desmids were considered an offshoot of the *Cylindrocystis* line via *Netrium*. Yamagishi's scheme is consistent with the sister-group relationships implied by the *rbdL* tree: *Spirogyra* and *Spirotaenia*, *Cylindrocystis* and *Zygnema*, *Mesotaenium* and *Mougeotia*, and *Netrium* and placoderm desmids. (However, Besendahl and Bhattacharya [1999] failed to find these pairs.) Because Yamagishi's classification included explicitly polyphyletic families, it is not consistent with a phylogenetic classification.

The West and Yamagishi schemes conflict in their views of the ancestral conjugate—filamentous for West, unicellular for Yamagishi. In neither case is the reasoning based on a clear outgroup polarity assessment, a concept without explicit formulation in their eras. The topology of *rbdL* trees is not congruent with either West's or Yamagishi's phylogeny, although certain trends are consistent with both (as noted above). The basal portions of the charophycean or zygnematalean clade are not clearly resolved by the *rbdL* analysis such that the ancestral character state of the conjugate thallus can be inferred. This issue awaits further analysis of morphological and molecular data, although even with such data the answer may remain elusive unless a clearly primitive clade or taxon with one of the two thallus types can be identified.

Our proposed phylogenetic classification of orders and families of conjugating green algae is shown in Fig. 3, which includes only the zygnemophycean genera that were sampled in this study. Boldface type indicates those taxa strongly supported as monophyletic by the ML analysis. The *rbdL* analysis also supports several other unranked groups of genera as clades, indicated by brackets in Fig. 3.

The *rbdL* data support the classification scheme of Mix (1973a) for the families of placoderms. In addition, the recognition of the Desmidiaceae as a monophyletic group (Gerrath 1993) is corroborated by the *rbdL* data. Based on the *rbdL* and SSU studies to date, the Mesotaeniaceae should be disbanded as a family in favor of the Zygnemataceae, which has priority (Kützing 1843). Nevertheless, the informal name "saccoderm desmids" remains a useful term in reference to the unicellular grade. The saccoderm genus *Roya* should be included in the Gonatozygaceae. The placement of *Netrium* is unclear, but it may be sister to the placoderms. By convention, *Netrium* is listed in Fig. 3 in the Zygnemataceae with the other saccoderm desmids (except *Roya*), but its position is noted as *insertae sedis*. Similarly noted is the

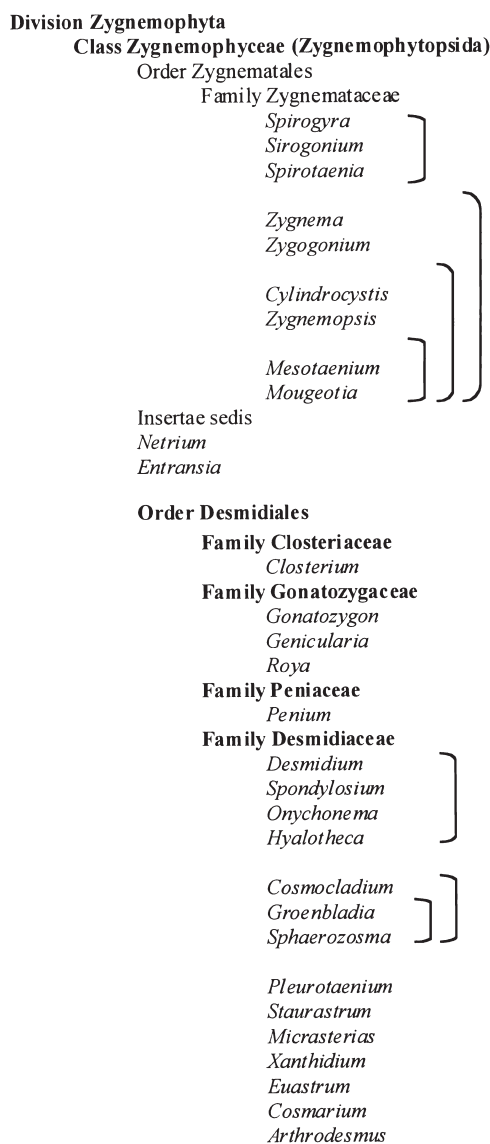


FIG. 3. Phylogenetic classification of conjugating green algae and genera sampled in the present analysis of *rbdL* sequences. Classification above the level of order follows Kenrick and Crane (1997). Taxa in boldface are strongly supported as monophyletic (>75% bootstrap) in the ML analysis. Brackets indicate unranked groups of genera supported as monophyletic (>75% bootstrap support in FIG. 2).

uncertain position of *Entransia*, which may be a plesiomorphic or nozygnemophycean taxon. Support for clades within the Zygnemataceae as redefined here is not robust, however, and further studies may support its division into the two clades (Fig. 1) that contain either ribbonlike chloroplasts (e.g. *Spirogyra*) or stellate/laminate chloroplasts (e.g. *Mougeotia* and *Zygnema*).

We thank Drs. James Manhart and Kenneth Sytsma for advice and technical support. Joseph Gerrath, Richard Moe, and Paul Silva provided advice on nomenclature. Members of the Green Plant Phylogeny Research Coordination Group (USDA grant 94-37105-0713) provided helpful advice regarding taxon sam-

pling and analysis in the broader context of green plant phylogeny. Dr. Charles F. Delwiche provided unpublished sequences for Coleochaetales. We thank Drs. Michael Melkonian, Barbara Surek, and Debashish Bhattacharya for preprints of articles. This research was supported by an NSF grant (BSR-9020117) to R. M. M. and R. W. H.; REU supplements supported undergraduate researchers. The Competitive Grants Program of University Research Council and the College of Liberal Arts and Sciences of DePaul University provided support to R. M. M. We dedicate this article to Robert W. Hoshaw, who began the research in collaboration with M. F. W. and R. M. M., but died before seeing it come to fruition. Hoshaw would have been especially happy with undergraduate (J. B. and A. G.) and graduate student (K. G. K.) participation in the research.

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