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# PHYLOGENY OF THE GENUS *COLEOCHAETE* (COLEOCHAETALES, CHAROPHYTA) AND RELATED TAXA INFERRED BY ANALYSIS OF THE CHLOROPLAST GENE *rbcL*[1]

*Charles F. Delwiche,*[2] *Kenneth G. Karol, Matthew T. Cimino*

Department of Cell Biology and Molecular Genetics, University of Maryland–College Park, College Park, Maryland 20742-5815 USA

*and*

*Kenneth J. Sytsma*

Department of Botany, University of Wisconsin, Madison, Wisconsin 53706, USA

**The genus *Coleochaete* Bréb. is considered to be a key taxon in the evolution of green algae and embryophytes (land plants), but only a few of the approximately 15 species have been studied with molecular phylogenetic methods. We report here the sequences of the gene *rbcL* from six new cultures of *Coleochaete* and two of *Chaetosphaeridium* Klebahn. These sequences were combined with 32 additional sequences, and phylogenetic analyses were performed with maximum likelihood, distance optimality, and parsimony methods. Important subgroups within *Coleochaete* include two primary lineages, one marked by fully corticated zygotes and the other by naked or weakly corticated zygotes. In the first lineage there is a subclade with tightly joined filaments and distinctive ("T-shaped") cell division, an assemblage of strains that resembles the endophytic species *Coleochaete nitellarum* Jost, and a clade with loosely joined filaments and "Y-shaped" cell divisions. Consistent with recent multigene phylogenies, these analyses support the monophyly of the Coleochaetales, place the Charales as the sister taxon to land plants, and indicate that *Chaetosphaeridium* is far more closely related to *Coleochaete* than to *Mesostigma* Lauterborn.**

***Key index words:* *Chaetosphaeridium*; Charales; Charophyte; *Coleochaete*; Embryophyte; Land Plant; *Mesostigma*; Phylogeny; *rbcL*; Streptophyte**

The genus *Coleochaete* Bréb. (Chlorophyta; Charophyceae *sensu* Mattox and Stewart 1984) has been considered to be a possible sister group of embryophytes (land plants) at least since Bower (1908) explicitly compared its life history with that of embryophytes. The view of *Coleochaete* as a close relative of embryophytes fell out of favor in the middle part of the 20th century when the full diversity of green algal life histories became apparent but reemerged when ultrastructural studies revealed the presence of a plant-like phragmoplast during cell division in *Coleochaete*, as well as in *Chara*, *Nitella*, and some Zygnematales (Pickett-Heaps and Marchant 1972). This feature, combined with other ultrastructural and biochemical evidence, caused Mattox and Stewart (1984) to recognize an expanded class Charophyceae. Their Charophyceae included five orders, the Charales, Coleochaetales, Zygnematales, Klebsormidiales, and Chlorokybales, but excluded the embryophytes. The likely relationship between the charophycean algae and embryophytes was recognized (Pickett-Heaps 1975), but the embryophytes were excluded from the Charophyceae, presumably in large part because it was considered acceptable at the time to name paraphyletic groups. Subsequent cladistic analyses of morphological data identified a close relationship among the Coleochaetales, Charales, and embryophytes. Mishler and Churchill's (1985) analysis placed the genus *Coleochaete* as the sister taxon to embryophytes but was criticized because some of the characters coded for *Coleochaete* were not uniform within the genus (Mishler and Churchill 1987, Whittemore 1987). Revision of these analyses to include a greater diversity of *Coleochaete* placed the species *Coleochaete orbicularis* as the sister taxon to embryophytes, indicating a paraphyletic *Coleochaete*, but noted that this was based on a small number of characters of uncertain homology (Graham et al. 1991).

The Coleochaetales comprise three genera: *Coleochaete*, with approximately 13 well-characterized and an indeterminate number of poorly known or undescribed species (Pringsheim 1860, Szymanska 1989); *Chaetosphaeridium*, with three to five species that are well enough described to permit diagnosis (Thompson 1969); and the very rare genus *Awadhiella* (Nandan Prasad and Kumar Asthana 1979). Several other genera have been allied with the order, but their status remains unclear. The Coleochaetales show considerable morphological variation but are united by a suite of structural features, including a characteristic hair composed of a sheath that forms as an outgrowth of the cell wall and an inner bristle that is apparently continuously extruded from the base (Fig. 1) (Wesley 1928). The hair develops in close association with the chloroplast, with a portion of the chloroplast extending into the base of the hair (McBride 1974), and in many cases the chloroplast rotates around the base of the hair (Geitler 1961). The function of this hair re-

[1]Received 14 September 2001. Accepted 11 January 2002.

[2]Author for correspondence: e-mail delwiche@umd5.umd.edu.

mains unknown, with suggestions ranging from herbivore defense to nutrient assimilation, but the structure is extremely distinctive and unknown outside of the order (with the possible exception of the beaked mucilaginous cells of *Takakia*; McBride 1974).

The primary taxonomic treatment of *Coleochaete* remains Pringsheim's (1860) monograph, which recognized six species, *Coleochaete scutata*, *C. orbicularis*, *C. pulvinata*, *C. soluta*, *C. irregularis*, and C. *divergens*, although there has been some confusion as to the correct application of the name *C. orbicularis* (Szymanska and Spalik 1993, Cimino et al. unpublished data). Roughly seven clearly distinct species have been added in the subsequent literature, most notably *C. nitellarum*, which grows endophytically in the walls of *Nitella* and uncalcified *Chara* (Jost 1895); *C. conchata* (Möbius 1892); and the complex of species recently described by Szymanska (Szymanska 1988, 1989).

Within the genus *Coleochaete* there are a number of growth forms (Fig. 2), including prostrate and pulvinate (i.e. heterotrichous) growth habit, several different patterns of cell division (notably "T-" and "Y-" shaped division), and organization of filaments into loose arrangements or the tight laterally joined pattern that has been compared with parenchyma (Pringsheim 1860, Wesley 1928, Graham 1982). Reproductive structures also show a range of forms. In some, but not all, species the zygotes are covered by neighboring filaments in postfertilization development of a cortex. In one species, *C. orbicularis*, the corticating filaments display wall ingrowths that resemble the placental transfer cell wall ingrowths of byrophytes, implying nutrient transfer between the haploid and diploid generations (Graham 1993).

Despite this structural and biological diversity, molecular phylogenetic studies have typically included only one or a few closely related species of *Coleochaete* (Bhattacharya and Medlin 1998, Sluiman and Guihal 1998), presumably in large part because of the inconvenience of isolating unialgal cultures from nature and the limited number of cultures available in public culture collections. A recent four-gene study of the origin of embyrophytes (Karol et al. 2001) included four species of *Coleochaete* and identified the Charales as the likely sister taxon to embryophytes, with Coleochaetales sister to that clade, but did not address phylogenetic relationships within the Coleochaetales. For an accurate understanding of the evolution of form, it is important that phylogenetic analyses include the spectrum of morphological diversity within a group. The small number of species available for study in *Coleochaete* made it difficult to use molecular phylogenetic methods to test the hypothesis that the genus is paraphyletic, which was posed by morphological cladistic analyses (with the species *C. orbicularis* sister to embryophytes), and virtually impossible to examine questions of intrageneric diversity and character evolution. Here we report a molecular phylogenetic analysis of the RUBISCO large subunit gene *rbcL* from newly isolated cultures of *Coleochaete* that more fully represent the diversity of the genus and consider the implications of this phylogeny for evolution within the Coleochaetales.

## MATERIALS AND METHODS

Cultures of *Coleochaete* and *Chaetosphaeridium* were isolated from nature by single-cell zoospore isolation from material collected from Lake Tomohawk, Oneida County, Wisconsin at Kemp Station (N 45° 50.419′ W 89° 40.683′). Structural and phenological information presented here comes from collections over a 15-year period beginning in 1986, and most of the new cultures were isolated during 1991–1993, with the exception of one culture (56a6), which was isolated in 1997. Individual thalli were removed from the substrate, placed in single drops (ca. 25 μL) of filtered Wood's Hole MBL freshwater culture medium (Nichols 1973), and monitored over a period of 2–3 days. Individual zoospores were captured with the use of a drawn Pasteur pipette and transferred into sterile Wood's Hole medium. Care was taken to isolate single zoospores, and cultures that showed signs of contamination were discarded. The parental thalli were documented photographically whenever possible. Additional cultures were obtained from the UTEX culture collection (Starr and Zeikus 1993). Taxonomic identification of all cultures was performed on the basis of morphology (Pringsheim 1860, Möbius 1892, Jost 1895, Szymanska 1988, 1989, 1990). Cultures were maintained in Wood's Hole medium supplemented with HEPES buffer, pH 7.5, at 15° C on a 16:8-h light:dark cycle or at room temperature in ambient light. A summary of the source and identification of cultures is shown in Table 1. All new cultures reported here have been submitted to the UTEX culture collection and also are available from the corresponding author by request. Vouchers have been deposited in the Norton-Brown Herbarium at the University of Maryland.

DNA was extracted from unialgal cultures via a modified CTAB extraction (Jones and Walker 1963, Murray and Thompson 1980, Doyle and Doyle 1987), by a similar extraction substituting a high-urea extraction buffer (8 M Urea, 0.15 M NaCl, 2% SDS w:v, 1 mM EDTA, 0.1 M Tris, pH 7.5) or with the Nucleon Phytopure resin-based extraction kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Quality of the extracted DNA was assessed via gel electrophoresis and ethidium bromide staining. Some amplifications from *Chaetosphaeridium* spp. were performed with nucleic acids extracted by boiling 10–20 cells for 1–2 min in water with polyvinypolypyrrolidone, followed by centrifugation and recovery of the supernatant.

Amplification of *rbcL* was performed with the primers RH1 (ATGTCACCACAAACAGAAACTAAAGC; Zurawski and Clegg 1987) and 1385R (AATTCAAATTTAATTTCTTTCC; Manhart 1994) or 1374R (TTTCTTTCCATACTTCACAA; Manhart, personal commmunication). All sequences were determined completely on both strands directly from PCR products. Sequences were initially obtained manually using a modified Sanger dideoxy protocol (Sanger et al. 1977, Conti et al. 1993) and subsequently checked and edited by automated sequencing (ABI 377, Perkin Elmer Biosystems, Foster City, CA, USA) performed by the University of Maryland Center for Agricultural Biotechnology.

Newly determined sequences were combined with sequences from the literature. Sequences were selected to represent all charophycean orders and embryophytes, with emphasis on the basal lineages of embryophytes. Outgroups were selected to represent the diversity of green algae, as well as *Cyanophora paradoxa* from the Glaucocystophyta. Red algal *rbcL* sequences were excluded from this study because of their probable history of horizontal gene transfer (Delwiche and Palmer 1996), as were sequences from secondary plastids (Delwiche 1999). To facilitate comparison with other studies, outgroup taxa were selected to match those used by Lemieux et al. (2000) and Karol et al. (2001) whenever possible. Two problematic genera were excluded from the study. Previous analyses suggested that the mode of sequence evolution was atypical in *Klebsormidium* and

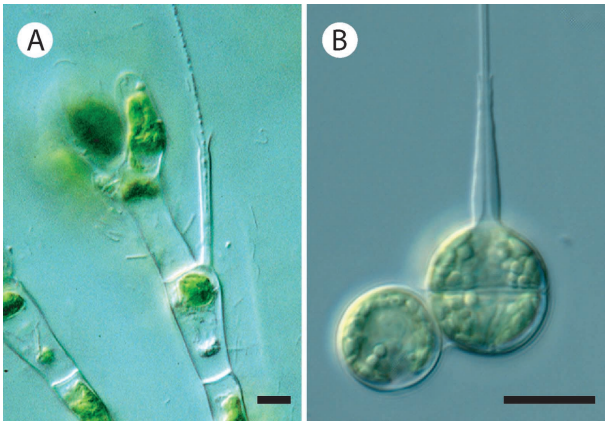


FIG. 1. The sheathed hair of the Coleochaetales. (A) *Coleochaete pulvinata.* (B) *Chaetosphaeridium ovalis* 5c5. Bars, 10 μm.

*Entransia* (McCourt et al. 2000), and these genera were excluded because they were not critical to the questions being addressed here.

Alignment was performed with CLUSTALX (Thompson et al. 1994) and checked by hand. Because indels were found only in two taxa, alignment was trivial. The alignment was composed entirely of protein-coding sequence. Phylogenetic analyses were performed primarily with PAUP* 4.0b8 (Swofford 2001) and MacClade 4.01 (Maddison and Maddison 2001), with supplementary analyses performed with fastDNAml (Olsen et al. 1994) and GCG (GCG 2000).

Parsimony analyses were performed with 100 random taxon addition sequence tree bisection and reconnection (TBR) searches retaining the shortest tree from each replicate. Minimum evolution analyses were performed using F84, maximum likelihood (ML) (GTR + i + Γ), and LogDet distances (Swofford et al. 1996). F84 and LogDet distance analyses were performed using the same fraction of invariable sites (i = 0.345903) estimated by ML (see below) using the GTR model, as well as with both all (i = 0.461197) and none (i = 0.0) of the invariant sites removed.

ML parameters were estimated using the "tree description" function in PAUP, and the likelihood ratio test was used to compare the performance of different nested models of sequence evolution (Swofford et al. 1996). Parameters were initially estimated using a minimum-evolution tree calculated with F84 distances. These model parameters were used in an ML analysis with 10 random addition-sequence nearest-neighbor interchange (NNI) searches, and parameters were reestimated on the resulting tree. This procedure was repeated for a total of three iterative rounds of searching and parameter estimation. A similar procedure was performed using the LogDet tree as the starting tree.

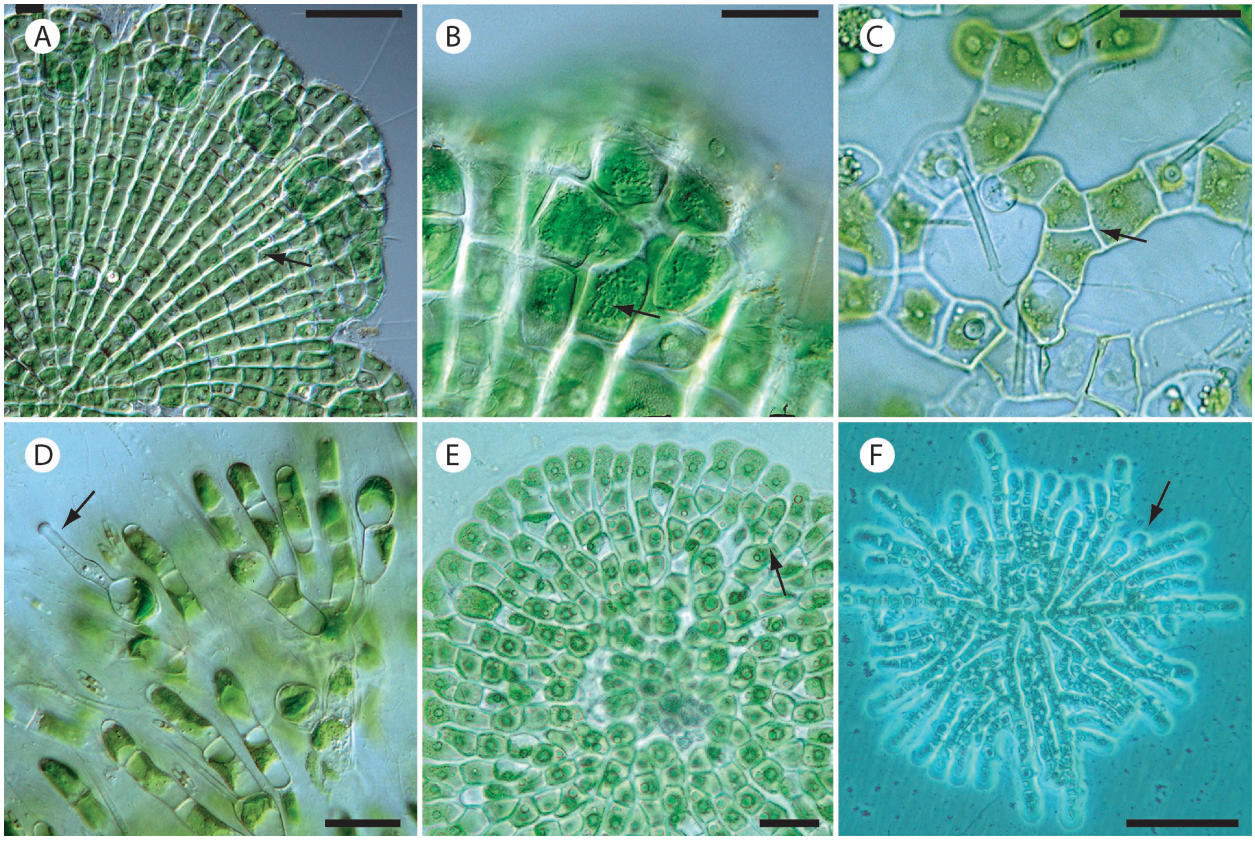


FIG. 2. Morphological diversity in *Coleochaete.* (A) *Coleochaete orbicularis* from nature with zygotes and antheridia. Arrow indicates a cell plate perpendicular to that from the previous division. Bar, 100 μm. (B) High magnification image of *C. orbicularis* zygote showing corticating cells. Arrow indicates putative placental transfer cell wall ingrowths. Bar, 32 μm. (C) *Coleochaete nitellarum* from nature, growing in the cell wall of *Nitella* sp. The visible hairs emerge from the *Nitella* cell wall but have been folded by pressure from the cover slip. Arrow indicates a cell plate perpendicular to that from the previous division. Bar, 32 μm. (D) *Coleochaete pulvinata* from nature, with an oogonium and trichogyne, as well as sheathed hairs. Arrow indicates the trichogyne on a receptive egg. Bar, 32 μm. (E) *Coleochaete sieminskiana* strain 10d1 from cultured material. Arrow indicates a cell plate formed at an angle with respect to the previous division. Bar, 32 μm. (F) *Coleochaete irregularis* 3d2 from cultured material. Arrow indicates a subapical branch. Bar, 32 μm.

TABLE 1. Sources of algal tissue and DNA sequences.

| Classification | Source | Strain | Genbank no. |
|---|---|---|---|
| Coleochaetales | | | |
| *Coleochaete scutata* | New | 34c1 | AY051138 |
| *C. orbicularis* | UTEX | LB 2651 | L13477 |
| *C. sieminskiana* | New | 10d1 | AF408249 |
| *C. irregularis* | New | 3d2 | AF408248 |
| *C. nitellarum* | UTEX | LB 1261 | AY051140 |
| *C.* sp. | New | 18b3 | AY051141 |
| *C.* sp. | New | 18a1 | AY051139 |
| *C. pulvinata* | New | 56a6 | AY051142 |
| *Chaetosphaeridium ovalis* | New | 5c5 | AF408251 |
| *Ch. globosum* | New | 1c11 | AY072816 |
| Charales | | | |
| *Tolypella prolifera* | | | AF097175 |
| *Nitella translucens* | | | L13482 |
| *Chara connivens* | | | AF097161 |
| *Nitellopsis obtusa* | | | U27530 |
| Embryophyta | | | |
| *Marchantia polymorpha* | | | NC_001319 |
| *Sphaerocarpos texanus* | | | U87090 |
| *Mnium horrida* | | | U87082 |
| *Megaceros vincentianus* | | | U87080 |
| *Anthoceros punctatus* | | | U87063 |
| *Angiopteris lygodiifolia* | | | X58429 |
| *Isoetes melanopoda* | | | L11054 |
| *Oryza sativa* | | | NC_001320 |
| *Ginkgo biloba* | | | D10733 |
| *Gnetum gnemon* | | | L12680 |
| Zygnematales | | | |
| *Spirogyra maxima* | | | L11057 |
| *Zygnema peliosporum* | | | U38701 |
| *Mesotaenium caldariorum* | | | U38696 |
| *Gontatozygon monotaenium* | | | U71438 |
| *Roya angelica* | | | U38694 |
| Chlorokybales | | | |
| *Chlorokybus atmosphyticus* | | | AF408255 |
| Mesostigmatales | | | |
| *Mesostigma viride* | | 50.1 | AF408256 |
| *Mesostigma viride* | | 296 | NC_002186 |
| Outgroups | | | |
| *Mantoniella squamata* | | | U30278 |
| *Nephroselmis olivacea* | | | NC_000927 |
| *Chlorella vulgaris* | | | NC_001865 |
| *Ulva curvata* | | | AF189071 |
| *Bryopsis maxima* | | | X55877 |
| *Cyanophora paradoxa* | | | X53045 |

Parsimony bootstrap analysis was performed with 1000 replicates each with 10 random addition sequence NNI searches. Minimum evolution bootstrapping was performed with 1000 replicates and TBR branch swapping starting with a neighbor-joining tree. ML bootstrapping was performed with 100 replicates, each using NNI branch swapping starting from a single random taxon addition tree.

## RESULTS

Morphological diversity representing much of that known from the literature was found in cultures isolated from Lake Tomohawk. Twenty-five new cultures of *Coleochaete* and *Chaetosphaeridium* were isolated, representing at least eight species. This study presents data from nine representative cultures of *Coleochaete*, six of which are new to this study, emphasizing those that could be assigned with reasonable confidence to a named species on the basis of morphology and excluding redundant isolates. Among the morphological species that were not isolated from Lake Tomohawk were *Coleochaete conchata* Möbius and *Coleochaete divergens* Prings., neither of which was found in repeated sampling of the lake over a 15-year period. The new accessions show substantial sequence diversity among morphological species but little sequence variation within morphological species. Within *Coleochaete*, nucleotide sequence identity ranged from 90.1% between *C. scutata* and *C. irregularis* to 100% between two accessions of *C. orbicularis* that are distinguishable only by cell size. In comparisons of *Coleochaete* and *Chaetosphaeridium*, the smallest observed sequence identity was 85.7% between *Coleochaete scutata* and *Chaetosphaeridium globosum*.

The alignment included 1354 characters, with a base composition of 29% A, 17% C, 23% G, and 31% T. There were 599 parsimony informative, 130 parsimony uninformative, and 624 constant characters. A single parsimony tree (not shown) was found, with length 3805 and had a consistency index of 0.313. On this parsimony tree there were 640 first-, 252 second-, and 2913 third-codon position changes.

The best ML model of sequence evolution as determined with the likelihood ratio test was the general time-reversible model (Rodríguez et al. 1990) with invariant sites (i) and gamma-distributed ($\Gamma$) corrections for variation in the rate of sequence evolution among sites (Swofford et al. 1996) (Table 2). During iterative parameter estimation, parameters converged on identical values after the first ML search regardless of the starting tree.

Using the GTR + i + $\Gamma$ model of sequence evolution, the pairwise distance between *Coleochaete scutata* and *C. irregularis* was 0.148 (inferred substitutions per site), and that between *Coleochaete scutata* and *Chaetosphaeridium globosum* was 0.255. These values compare with distances of 0.190 between *Ginkgo biloba* and *Oryza sativa* and 0.107 between *Chara* and *Nitella*.

NNI searches were effective at finding the ML tree, finding the most likely tree in 43 of 60 (71%) independent NNI ML searches performed during iterative parameter estimation. ML analyses of the *rbcL* data identified a tree of Ln likelihood −18063.15169 (Fig. 3) that shows the embryophytes, Charales, and Coleochaetales to be strongly supported monophyletic groups. Consistent with Karol et al. (2001), *Coleochaete* and *Chaetosphaeridium* form a monophyletic Coleochaetales and appear as a monophyletic group with strong (92%) bootstrap support. There is no special association between *Coleochaete orbicularis* and embryophytes, as had been proposed on the basis of previous morphological cladistic analyses (Mishler and Churchill 1985). In fact, *Coleochaete orbicularis* is nested deeply within the Coleochaetales, with *C. scutata* as its sister taxon. Also consistent with Karol et al. (2001), the Charales are the sister group to embryophytes but with weak (41%) bootstrap support, and the Coleochaetales are the sister to that group. The clade composed of Coleochaetales, Charales, and embryophytes has moderate (56%) bootstrap support.

TABLE 2. Maximum likelihood parameter estimation.

| | JC | K2P | HKY85 | GTR |
|---|---|---|---|---|
| **Equal rates** | 19796.2035 | 19499.4624 | 19421.4420 | 18836.9612 |
| Δ Substitution | — | 593.4821[a] | 156.0408[a] | 1168.9615[a] |
| +Γ | 18017.9341 | 17697.9787 | 17399.6257 | 17314.2328 |
| Δ equal→Γ | 3556.5387[a] | 3602.9674[a] | 4043.6324[a] | 2494.3157[a] |
| +i | 18280.0830 | 17970.5886 | 17786.2251 | 17589.8033 |
| Δ equal→i | 3032.2408[a] | 3057.7476[a] | 3270.4336[a] | 3045.4567[a] |
| +i+Γ | 18001.3564 | 17677.6636 | 17356.8588 | *17281.7461*[b] |
| Δ i → i+Γ | 557.4533[a] | 585.8499[a] | 858.7327[a] | 616.1144[a] |
| Δ Γ→ i+Γ | 33.1554[a] | 40.6301[a] | 85.5339[a] | 64.9734[a] |
| **ss** | 18142.1957 | 17832.3749 | 17566.4931 | 17391.1656 |
| Δ equal → ss | 3308.0155[a] | 3334.1750[a] | 3709.8978[a] | 2891.5912[a] |

[a]Significant at $P < 1\%$.
[b]Using preferred model.

*Coleochaete* is divided into two distinct clades: a "*C. scutata*" clade, consisting of species with fully corticated zygotes, and a "*C. irregularis*" clade, composed of species with naked or slightly corticated zygotes (Fig. 3). This feature is consistent among the species in these two subclades. Closely related to *C. scutata* and *C. orbicularis* are three filamentous species, including the strain identified as *C. nitellarum* in the UTEX culture collection (18a1, 18b3, and UTEX LB1261). These three strains are paraphyletic with respect to *C. scutata* and *C. orbicularis* in the ML analyses with moderate (64%) bootstrap support but form a monophyletic group in LogDet analyses. *Coleochaete soluta* and *C. pulvinata* form a strongly supported (95%) group in all analyses.

*Chaetosphaeridium* shows no tendency to cluster with *Mesostigma*, a grouping proposed on the basis of analyses of small subunit (SSU) rRNA (Marin and Melkonian 1999) but with negligible bootstrap support in our analyses. The Zygnematales are monophyletic but with lower bootstrap support (56%) than for the other classically recognized orders. At the base of the charophytes fall the monotypic *Chlorokybus* and a strongly supported monophyletic group composed of two strains of the scaly flagellate *Mesostigma*. The branching order among the major clades (orders) finds only moderate bootstrap support, but the support for the monophyly of the Charophyceae as a whole (including both embryophytes and *Mesostigma*) is strong (93%).

## DISCUSSION

*Culture isolation.* The three species of *Coleochaete* previously available in unialgal culture, *C. scutata*, *C. orbicularis*, and *C. nitellarum*, constitute a monophyletic group in our analyses (Fig. 3). They also represent only a part of the structural diversity in *Coleochaete* but have been the subjects of nearly all molecular studies. The availability of cultures more broadly representing the genus should facilitate comparative studies of the group.

The new cultures reported in this study were isolated from a single site (Lake Tomohawk). This simplified the process of isolating new cultures and will facilitate future comparisons of algal biodiversity among sites. Lake Tomohawk has a rich morphological diversity of *Coleochaete*, and research facilities are available nearby, making it a practical long-term study site. Although some species seem not to be present in Lake Tomohawk (e.g. *C. conchata* and *C. divergens*), the species isolated cover the range of morphological diversity described in the literature. The factors controlling the distribution of species of *Coleochaete* remain poorly understood, but it seems that most species can occur sympatrically, and the cultures obtained in this study make it possible to test morphological hypotheses. Additional work currently in progress is addressing the global biogeography of *Coleochaete* and suggests that the data presented here accurately reflect the diversity of the genus (data not shown).

The *rbcL* data indicate that the Coleochaetales are a monophyletic group, but both morphological and molecular phylogenetic data indicate that these new isolates are markedly distinct entities. For example, the pairwise sequence divergence between *Coleochaete scutata* and *C. irregularis* (0.148) is nearly as great as that between *Ginkgo*, a gymnosperm, and *Oryza*, an angiosperm (0.190). Despite the fact that *rbcL* has been used for phylogenetic analyses at the family level or above in angiosperms and vascular plants, the measure of sequence variation among species of *Coleochaete* makes *rbcL* an appropriate molecule for phylogenetic analyses in this group.

*Chaetosphaeridium* shares a number of striking morphological characteristics with *Coleochaete* (most notably the distinctive sheathed hair), but some authors have questioned its relationship to *Coleochaete* (Graham et al. 1991, Marin and Melkonian 1999). *Chaetosphaeridium* has proven to be somewhat difficult to work with, and much of its biology remains poorly understood. Unfortunately, neither of the two new strains of *Chaetosphaeridium* presented here is markedly different in terms of morphology or in sequence divergence from those previously available in culture. Nonetheless, a number of clearly distinct morphological species of *Chaetosphaeridium* have been described from nature (Thompson 1969) and, given the substantial *rbcL* sequence divergence between *Coleochaete* and *Chaeto-*

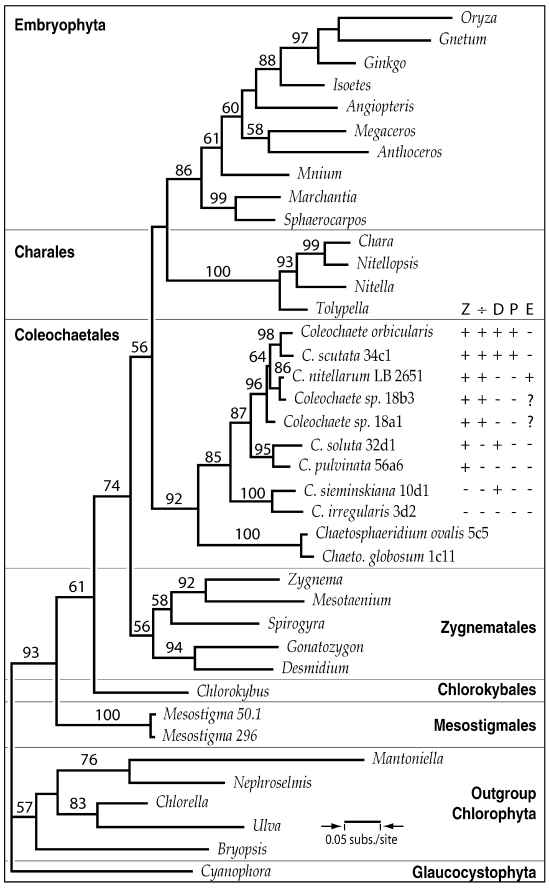


FIG. 3. ML tree, showing branch lengths (see scale bar) and with bootstrap values above 50% shown above the corresponding branch. The parsimony tree differed from the ML tree only in the relative placement of *Gnetum* and *Ginkgo* and in the placement of *Bryopsis*. The LogDet tree differs from the ML tree: 1) in the presence of a monophyletic group with moderate (66%) support composed of the three morphologically similar accessions, *Coleochaete nitellarum* LB1261, *C.* sp. 18b3, and *C.* sp. 18a1; 2) in the placement of *Chlorokybus*, which forms a strongly supported (90%) monophyletic group with *Mesostigma* in LogDet analyses; and 3) in poorly supported details of embryophyte phylogeny. Distance analyses using F84 and GTR distances found a similar tree topology to that found by LogDet but with moderate (<75%) to weak (<50%) support for all features that differed from the ML tree. Characters coded for the genus *Coleochaete* are as follows: Z, corticated zygotes; ÷, perpendicular cell division; D, discoidal habit; P, parenchyma *sensu* Graham (1982); E, endophytic habit.

*sphaeridium*, it would be valuable to isolate representatives of these species.

*Phylogenetic analyses.* Parameter estimation from starting trees determined by parsimony and LogDet analyses converged on similar model parameters. The data set is too large to permit exhaustive searching, so it could not be guaranteed that the topology and parameters presented here represent a global optimum. However, because most of the searches performed during parameter estimation were performed after the model parameters had converged on their final values, these searches can also be used to evaluate the effectiveness of NNI searches for these data. The fact that 71% of these searches converged on the same tree indicates that NNI branch swapping with random taxon addition sequences is an effective search strategy for these data. This also suggests that this tree topology (Fig. 3) and set of model parameters (Table 2) correspond to a global optimum.

Parsimony, minimum evolution using several different distance measures, and ML analyses produced generally congruent trees. The ML tree is presented (Fig. 3) because likelihood is thought to perform relatively well under difficult analytical conditions (Swofford et al. 1996) and because the use of an explicit model of sequence evolution means that both the branching order and branch lengths are meaningful and easy to interpret. The agreement between analytical methods suggests that the tree topology shown is a good representation of the data within the shared assumptions of the analytical methods.

In minimum evolution and parsimony analyses, which are less computationally intensive, bootstrap analyses were performed with 1000 replicates, TBR branch swapping, and 10 random addition sequences per replicate. However, because ML is more computationally intensive, bootstrapping was performed with a relatively small number of replicates (100), NNI searches, and a single random addition sequence. This method has the advantage of being relatively fast, although is still expected to yield accurate and unbiased bootstrap values. The use of a low number of replicates will yield bootstrap values that are accurate but less precise than those determined with a larger number of replicates (Hedges 1992). Because bootstrap values are not normally interpreted at high precision (i.e. a 1% difference in bootstrap values is rarely considered to be important), a relatively small number of replicates (100) can give useful bootstrap values (Brown 1994). Similarly, NNI branch swapping is fast but does not search the tree space as thoroughly as TBR branch swapping. However, in an analysis that does not have a large number of local optima ("islands"), NNI branch swapping may be adequate (Swofford et al. 1996).

The use of a relatively weak search strategy in bootstrapping is conservative, as the failure to detect the globally optimal tree in any given bootstrap replicate will tend to lead to an underestimate of bootstrap values as long as the searches are not biased by the stepwise addition process. This effect is expected to be strongest for low bootstrap values, and high bootstrap values should be relatively unaffected. Because the most informative bootstrap values are those that are relatively high and the interpretation of precise values is context dependent (Hillis and Bull 1993), the approach used here yields useful values with a relatively low computational burden.

Like the data presented here, the analysis of Karol et al. (2001) covers a broad range of charophycean algae, but it incorporated data from four genes (*rbcL*, *atpB*, *nad5*, and SSU rRNA) and consequently had roughly four times the number of characters, albeit

from fewer taxa. The higher level phylogenetic relationships observed in that study were similar to those reported here (Fig. 3), with the Charales sister to embryophytes and the Colechaetales sister to the group comprised of Charales and embryophytes (the Streptophyta *sensu* Jeffrey [1967]), but bootstrap values for these groups were higher than those observed here. This is consistent with expectation, as bootstrap analysis provides a measure of the amount of information supporting a particular taxon bipartition and the additional length of sequence available to Karol et al. (2001) would be expected to yield a more robust tree.

*Coleochaete diversity.* There is reason to believe that the Coleochaetales are an ancient group. Although neither *Coleochaete* nor *Chaetosphaeridium* has an extensive fossil record, the subgroups of the Charales represented by *Chara* and *Nitella* can be reliably traced back over 200 million years B.P., whereas the Charales as a whole have an unequivocal fossil record over 420 million years old (Feist and Feist 1997). The branching pattern of the *rbcL* tree (Fig. 3) and levels of sequence divergence (a GTR distance of 0.255 between *Coleochaete scutata and Chaetosphaeridium globosum*, compared with 0.107 between *Chara connivens* and *Nitella translucens*) suggest that the Coleochaetales are a group of at least comparable age.

The major division within the genus *Coleochaete* seen in the *rbcL* phylogeny occurs between the *C. irregularis* clade and the remainder of the genus. This division seems to correspond closely with a morphological feature of *Coleochaete* thought to be of evolutionary significance, namely the cortication of the zygote (Fig. 2B). The cortication of the zygote is one of the structural features that led early botanists to compare *Coleochaete* with embryophytes. In most species of *Coleochaete*, the egg develops when an apical (or marginal) cell enlarges and develops a trichogyne (Fig. 2D). Sperms of *C. soluta* are attracted to the trichogyne, apparently by some form of pheromone, and elongate from nearly spherical to the shape of a long-necked gourd before fertilization (personal observation). After fertilization of the egg, nearby filaments will branch and grow toward the zygote and eventually fully invest it with cortical filaments (Pringsheim 1860, Graham 1993). Similar postfertilization cortication of the zygote occurs in all of the species in the clade that includes both *C. scutata* and *C. soluta*, as indicated in Figure 3.

In contrast, the zygotes of species in the other major clade of *Coleochaete*, which includes both *C. irregularis* and *C. sieminskiana*, do not become fully corticated. They typically are retained on the parental thallus by overgrowth of adjoining filaments or, in the case of *C. irregularis*, by sparse overgrowth by a few nearby filaments. Despite this variation in zygote development, the zygote in all species does undergo considerable enlargement after fertilization, and more than four (typically 16 or 32) meiospores are produced upon germination (Pringsheim 1860, Hopkins and McBride 1976). Szymanska (1988, 1989) investigated several species of *Coleochaete* that seem to be closely related to *C. sieminskiana*. These species, together with *C. irregularis*, represent a major component of *Coleochaete* diversity but have received very little investigation. Our observations suggest that there may be a substantial suite of characters that distinguish these two groups (typified by *C. scutata* and *C. irregularis*), but most of these putative characters will require significant effort to investigate and verify.

*Coleochaete orbicularis* and *C. scutata* have such closely spaced filaments that their organization has been compared with parenchyma (Graham 1982). In these species, cell division occurs in the distal cell in each filament (or cell file), is closely coordinated, and creates a marginal meristem. The presence of this parenchyma-like organization is another distinctive feature of *Coleochaete* and is one of the characters that specifically linked *C. orbicularis* to embryophytes in morphological analyses (Graham et al. 1991). Other morphological characters linking *C. orbicularis* with embyrophytes include multicellular antheridia (which are also found in *C. scutata*) and putative placental transfer cell wall ingrowths (Graham et al. 1991).

The *rbcL* phylogeny indicates that taxa with a flattened discoidal habit are not monophyletic (Fig. 3). A discoidal growth form occurs in *Coleochaete scutata* and *C. orbicularis*, as well as in *C. soluta* and *C. sieminskiana*, both of which are species with more loosely joined filaments. However, the thalli of *C. scutata* and *C. orbicularis* are noteworthy for their particularly orderly and box-like cells (Fig. 2A). As these species grow radially, the circumference of the disk increases, and the width of each filament has to increase to remain in contact with its neighbors. To accommodate this increase in circumference, periodic circumferential divisions permit the filaments to maintain a roughly constant size (Pringsheim 1860, Wesley 1928). The radial and circumferential divisions occur at precise right angles, and this pattern of cell division has been viewed as developmentally significant (Graham 1982, Brown et al. 1994).

This pattern of cell division is not unique to the discoidal species *Coleochaete scutata* and *C. orbicularis* but may mark a monophyletic group that includes *C. nitellarum* (Fig. 3). *Coleochaete nitellarum* is composed of relatively loosely arranged filaments that develop internally in the walls of uncalcified members of the Charales. Although *C. nitellarum* does not form discoidal thalli, it does undergo cell division with cell plates forming at a precise right angle to the previous plate (Fig. 2C) in a manner reminiscent of *C. scutata* and *C. orbicularis*. In the *rbcL* analyses, *C. nitellarum* and two new isolates that may represent undescribed species fall in a strongly supported monophyletic group with *C. orbicularis* and *C. scutata*. In contrast, the species *C. soluta* and *C. sieminskiana* form discoidal thalli superficially similar to those of *C. scutata* and *C. orbicularis* but have filaments that are less tightly conjoined than are those of *C. scutata* and *C. orbicularis*. These species undergo a qualitatively different pattern of cell division with cell plates segregating a lobed outgrowth of

the cell wall and rarely form cell plates at precise right angles as is seen in *C. scutata, C. orbicularis*, and *C. nitellarum*.

The species *Coleochaete nitellarum* is also distinctive because of its endophytic growth habit. Zoospores and meiospores of *C. nitellarum* will settle on uncalcified members of the Charales (e.g. *Nitella*). In the initial development of the thallus, they extend a tube that is capable of penetrating beneath the outer layer of the *Nitella* cell wall (Jost 1895). In subsequent growth, *C. nitellarum* spreads through the *Nitella* cell wall, living endophytically but apparently not parasitically (*C. nitellarum* grows vigorously in axenic culture and in nature is only found in the outer layer of the *Nitella* cell wall). Curiously, *C. nitellarum* does produce sheathed hairs that penetrate the overlying *Nitella* cell wall and extend into the surrounding medium. In this study, several cultures of *Coleochaete* (*C.* sp. 18b3, *C.* sp. 18a1, and *C. nitellarum* UTEX 1261) were identified that were morphologically distinct but displayed early development reminiscent of that of *C. nitellarum*. Although closely related in *rbcL* analyses, these strains display substantial sequence divergence and may represent a previously unrecognized endophytic species complex.

The sister taxon to *Coleochaete soluta* is *C. pulvinata*, a pulvinate species. In this growth form the thallus is organized in three dimensions with both prostrate and upright filaments but the pattern of cell division is qualitatively similar to that in *C. soluta*. The pulvinate growth form is represented in this study only by the species *C. pulvinata* (Fig. 2D). The sister taxon of *C. sieminskiana* (Fig. 2E) is *C. irregularis*, a flattened species that can form somewhat discoidal thalli (Fig. 2F) but unlike most species of *Coleochaete* undergoes subapical branching.

*The genus* Chaetosphaeridium. *Chaetosphaeridium* is placed in these analyses as the sister taxon to *Coleochaete* (Fig. 3). This is consistent with conventional classification and with multigene analyses based on a moderate number of taxa (Karol et al. 2001). Morphologically, the two genera are supported as a monophyletic group by branched filaments that undergo tip growth after division of the apical, subapical, or presubapical cell (Thompson 1969, Graham et al. 2000); by sheathed hairs associated with rotating plastids (Geitler 1961, McBride 1974); and by the structure of their zoospores (Moestrup 1974, Pickett-Heaps 1975). *Chaetosphaeridium* has been reported to be oogamous with eggs released from the parental thallus (Thompson 1969), but this report has not been verified.

Despite these similarities, cladistic analyses have not always placed *Coleochaete* and *Chaetosphaeridium* together (Mishler and Churchill 1985, Graham et al. 1991). In fact, some analyses of SSU rRNA have placed *Chaetosphaeridium* in a monophyletic group with the unicellular flagellate *Mesostigma*. Primarily on this basis, Marin and Melkonian (1999) proposed a new class, the Mesostigmatophyceae, separating *Chaetosphaeridium* from the Coleochaetales. One morphological feature that seemed to unite the Mesostigmatophyceae was the presence of "maple leaf" shaped scales on the flagella of both taxa. However, that study apparently did not consider the flagellar scales of *Coleochaete* or *Chara* (Pickett-Heaps 1975, Graham and McBride 1979, Graham and Taylor 1986), both of which more closely resemble those of *Chaetosphaeridium* than do those of *Mesostigma*. Additional work is required to reconcile the rRNA analyses with the data presented here, but analyses of other protein-coding genes also place *Chaetosphaeridium* in a monophyletic group with *Coleochaete* (Karol et al. 2001).

*Reconciling morphological and molecular analyses.* On the surface, morphological and molecular analyses of the Coleochaetales would appear to have produced quite different results. For example, formal analyses based on morphological characters have sometimes found the genus *Coleochaete* to be paraphyletic, placing the species *Coleochaete orbicularis* as the sister taxon to embryophytes (Mishler and Churchill 1985, 1987, Whittemore 1987, Graham et al. 1991). However, these previous analyses were based on a small number of characters, and analyses of robustness of tree topology showed relatively poor resolution and were consistent with either a monophyletic or paraphyletic *Coleochaete* (Graham et al. 1991). Thus, the conflict may be more apparent than real, with the topologies found in morphological analyses reflecting chance resolution of a small number of characters rather than genuinely incompatible data.

A similar situation holds for *Chaetosphaeridium*, which, although long considered by taxonomists to be closely allied to *Coleochaete*, does not form a monophyletic group with *Coleochaete* in many morphological analyses. This is probably because it is not known to form a phragmoplast during cell division and because fertilization has been reported to follow release of the egg from the parental thallus, with the zygote developing entirely independently (Thompson 1969, Graham et al. 1991). There are, however, striking similarities between *Chaetosphaeridium* and *Coleochaete*, including the sheathed hair, chloroplast ultrastructure, and zoospore ultrastructure and scale morphology. Other potentially informative traits, such as sperm ultrastructure, zygote structure, and germination, and genome organization remain largely unstudied in *Chaetosphaeridium*. This leads to many uncoded characters in morphological analyses and, consequently, places undue weight on those few characters that are available. Interestingly, older more intuitive treatments (e.g., Smith 1950) placed *Chaetosphaeridium* and *Coleochaete* close together, presumably because they implicitly used a larger number of less formally defined characters.

Thus, the apparent conflict between molecular and morphological data is largely attributable to poor resolution in morphological studies resulting from data sets with a small number of characters and substantial missing data. Although informative, the *rbcL* analyses presented here do not provide full resolution of phylogeny within the group. One approach to improve this resolution would be to perform combined analy-

sis of the molecular and morphological data. This, however, has the disadvantage of introducing circularity into the study; because one major objective is to understand the evolution of form within the Charophyceae, it is more desirable to determine phylogeny on the basis of nonmorphological data (Givnish and Sytsma 1997a,b,c, Givnish et al. 1997). Molecular phylogenetic analyses based on other genes can provide additional information that can be used to test the *rbcL* phylogeny and resolve branches that are weakly supported here (Fig. 3). Such a multigene analysis of the Charophyceae, although based on a smaller number of taxa than presented here, has been presented by Karol et al. (2001) and suggests that a fully resolved taxon-rich analysis of charophyte diversity is practical and will be attainable in the near future.

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