

PHYLOGENETIC ANALYSES OF 18S rDNA SEQUENCES REVEAL A NEW COCCOID LINEAGE OF THE PRASINOPHYCEAE (CHLOROPHYTA)¹

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Phylogenetic analyses of 18S rDNA sequences from 25 prasinophytes, including 10 coccoid isolates, reveals that coccoid organisms are found in at least three prasinophyte lineages. The coccoid *Ostreococcus tauri* is included in the Mamiellales lineage and *Pycnococcus provasolii* is allied with the flagellate *Pseudoscourfieldia marina*. A previously undescribed prasinophyte lineage is comprised of the coccoid *Prasinococcus* cf. *capsulatus* (CCMP 1407) and other isolates tentatively identified as *Prasinococcus* sp. (CCMP 1202, CCMP 1614, and CCMP 1194), as well as three unnamed coccoids (CCMP 1193, CCMP 1413, and CCMP 1220). No flagellate organisms are known from this lineage. Organisms of this new lineage share some characteristics of both the Pycnococaceae and the Mamiellales, although relationships among these separate lineages were not supported by bootstrap analyses. An additional unnamed coccoid isolate (CCMP 1205) is separate from all major prasinophyte lineages. The analyses did not resolve the relationships among the major prasinophyte lineages, although they support previous conclusions that the Prasinophyceae are not monophyletic.

Key index words: coccoid algae; Prasinophyceae; rDNA; sequence analysis

Abbreviations: CCMP, Provasoli-Guillard National Center for Culture of Marine Phytoplankton; ML, maximum likelihood; MP, maximum parsimony; NJ, neighbor joining

The scaly flagellates that are included in the green algal class Prasinophyceae have long been considered primitive within the Chlorophyta and indeed the entire green plant lineage (Viridiplantae). These organisms have many characteristics that are considered ancestral in form, such as the structure of the flagellar apparatus, the cell covering of organic scales, and the flagellate vegetative cells (see Sym and Pienaar 1993 for a review). Because of these ancestral characters, this class has been difficult to delimit accurately and no defining synapomorphies have been identified for the Prasinophyceae (Mattox and Stewart 1984, Sym and Pienaar 1993).

Partly because of this lack of synapomorphic characters, the class Prasinophyceae has been under constant revision since its formal description by Moestrup and Throndsen (1988). The Prasinophyceae initially

comprised only the “scaly green flagellates,” but it was soon recognized that other types of organisms should also be included in the class. Similarities of cellular structure (Manton and Parke 1960), pigmentation (including the carotenoid prasinoxanthin), and pigment–protein complexes (Fawley et al. 1990) indicated that the naked flagellate *Micromonas pusilla* is closely allied to the prasinophyte order Mamiellales. Prasinoxanthin was assumed to be a distinguishing characteristic of the Mamiellales, because all organisms known to possess prasinoxanthin were found in this order (Foss et al. 1984). Subsequently, the scaled coccoid *Bathycoccus prasinos* was also included in the Mamiellales because of the shared scale morphology (Eikrem and Throndsen 1990) and the presence of prasinoxanthin (Egeland et al. 1995). Guillard et al. (1991) suggested that the scaleless coccoid *Pycnococcus provasolii* should be included in the Mamiellales as well, because it also possesses prasinoxanthin. The additional coccoid organisms *Prasinococcus capsulatus* (Miyashita et al. 1993), *Ostreococcus tauri* (Chrétiennot-Dinet et al. 1995), and *Prasinoderma coloniale* Hasegawa et Chihara (Hasegawa et al. 1996) have been identified as prasinophytes with presumed affinity with the Mamiellales, based once again on pigmentation.

Several analyses of 18S rDNA and *rbdL* sequences have supported the inclusion of scaleless and coccoid organisms within the Prasinophyceae and have provided strong evidence that the Prasinophyceae are paraphyletic (Steinkötter et al. 1994, Daugbjerg et al. 1995, Melkonian and Surek 1995, Courties et al. 1998, Nakayama et al. 1998, Fawley et al. 1999). The affinities of all the prasinoxanthin-possessing organisms with the Mamiellales have not been supported by these studies. Sequence analyses based on 18S rRNA (Kantz et al. 1990, Nakayama et al. 1998) indicated that *Micromonas pusilla* should be included in the Mamiellales. An analysis of *rbdL* sequences provided strong support for including both *M. pusilla* and *Bathycoccus prasinos* in the Mamiellales (Daugbjerg et al. 1995). Likewise, 18S rDNA data place the coccoid *Ostreococcus tauri* with the Mamiellales (Courties et al. 1998). However, analyses of both the *rbdL* and 18S rDNA sequences indicated that the coccoid *Pycnococcus provasolii* and the flagellate *Pseudoscourfieldia marina* are not closely related to the Mamiellales, even though they possess prasinoxanthin (Daugbjerg et al. 1995, Fawley et al. 1999). Analysis of 18S rDNA sequences from *Prasinococcus* cf. *capsulatus* and one additional unnamed coccoid isolate also suggested that they are not closely related to the Mamiellales (Courties et al. 1998).

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In addition to the named coccoid prasinophytes, many other coccoid organisms presumed to be prasinophytes have been isolated. Several of these isolates have been assumed to be related to the Mamiellales based on the presence of the prasinoxanthin (Hooks et al. 1988, Fawley 1992). Partly because of the presence or absence of specific carotenoids, some of these organisms have been presumed to be similar to, or perhaps conspecific with, *Prasinococcus capsulatus* (Egeland et al. 1997, Courties et al. 1998).

Nakayama et al. (1998) discussed the need for including additional coccoid organisms in phylogenetic analyses of the Prasinophyceae. Based on differences in pigmentation, several distinct lineages of these organisms have been proposed (Fawley 1992, Egeland et al. 1997). In this study, we present evidence from 18S rDNA sequence data that coccoid organisms are found in at least three separate prasinophyte lineages. We also show that a previously undescribed prasinophyte lineage is made up solely of coccoid organisms.

MATERIALS AND METHODS

The coccoid isolates CCMP 1189, CCMP 1193, CCMP 1194, CCMP 1202, CCMP 1413, and CCMP 1614 were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Andersen et al. 1997). These were cultured as previously described (Fawley 1992). CCMP 1189 is listed in the collection as an unidentified coccoid/flagellate chlorophyte; CCMP1193, CCMP 1194, and CCMP 1202 are all identified as the coccoid *Prasinococcus capsulatus*; and CCMP 1413 is listed as an unidentified coccoid prasinophyte.

DNA isolation, PCR, and sequencing. Genomic DNA isolation, PCR, and sequencing were performed as previously described (Fawley et al. 1999), using the 18S rDNA specific primers NS1 (White et al. 1990) and 18L (Hamby and Zimmer 1991) to prime the PCR reactions. The sequences obtained from the coccoid organisms are available from GenBank, with accession numbers AF203398 through AF203403. Additional 18S rDNA sequences were obtained from GenBank (Table 1). Sequences were aligned using ClustalX (Thompson et al. 1997), with default settings, and corrected by eye using Xesee (Eric Cabot, University of Rochester). Alignments are available from GenBank under the accession numbers above.

Phylogenetic analyses. PAUP* V4.0 Beta2 (Swofford 1998) was used to perform phylogenetic analyses using maximum parsimony, neighbor joining (Saitou and Nei 1987), and maximum likelihood with quartet puzzling (Strimmer and von Haeseler 1996). Maximum parsimony analysis was performed with a heuristic search using the tree bisection-reconnection option and random sequence addition with 50 replications. For the MP analysis, gaps were treated as missing and all characters were unweighted and unordered. Neighbor joining was performed with distance matrix constructed by the Kimura (1980) two-parameter (K2P) model or by the LogDet/Paralinear model (Lake 1994, Lockhart et al. 1994). Maximum likelihood analysis employed the HKY85 (Hasegawa et al. 1985) model of nucleotide substitution, with transition/transversion ratio and nucleotide frequencies estimated from the data set and using the equal rates model. Bootstrapping with 1000 replicates was used to evaluate both the MP and NJ analyses. One thousand puzzling steps were used with quartet puzzling for ML analyses. All analyses were performed with the unalignable regions corresponding to positions 33–39, 94–101, 192–207, 677–686, 1358–1380, 1501–1508, and 1700–1745 removed. *Pavlova gyrans* (Haptophyta) and *Cyanophora paradoxa* (Glaucocystophyceae) were included in the analyses as outgroups, with *P. gyrans* used to root the trees as previously described (Fawley et al. 1999). TreeView (Roderic Page, University of Glasgow) was used to draw trees.

TABLE 1. GenBank accession numbers for published 18S rDNA sequences included in this study.

Organisms	GenBank accession no.
Haptophyta	
<i>Pavlova gyrans</i> Butcher	U40922
Glaucocystophyceae	
<i>Cyanophora paradoxa</i> Korsh.	X68483
Charophyceae	
<i>Chara foetida</i> A. Br.	X70704
<i>Coleochaete scutata</i> Bréb.	X68825
<i>Genicularia spirotaenia</i> (Ramb.) de Bary	X74753
<i>Klebsormidium flaccidum</i> (A. Br.) Silva, Mattox et Blackwell	M95613
<i>Nitella</i> sp.	M95615
<i>Staurastrum</i> sp.	X74752
Prasinophyceae	
<i>Cymbomonas tetramitiformis</i> Schiller	AB017126
<i>Halosphaera</i> sp.	AB017125
<i>Mamiella</i> sp.	AB017129
<i>Mantoniella antarctica</i> Marchant	AB017128
<i>Mantoniella squamata</i> (Manton et Parke) Desikachary	X73999
<i>Micromonas pusilla</i> (Butcher) Manton et Parke	AJ010408
<i>Nephroselmis olivacea</i> Stein	X74754
<i>Nephroselmis pyriformis</i> (Carter) Ett.	X75565
<i>Ostreococcus tauri</i> Courties et Chrétiennot-Dinet	Y15814
<i>Prasinococcus</i> cf. <i>capsulatus</i> Miyashita et Chihara (CCMP 1407)	U40919
<i>Pseudoscourfieldia marina</i> (Thronsdén) Manton	AF122888
<i>Pterosperma cristatum</i> Schiller	AB017127
<i>Pycnococcus provasolii</i> Guillard	AF122889
<i>Pyramimonas disomata</i> Butcher	AB017121
<i>Pyramimonas olivacea</i> N. Carter	AB017122
<i>Pyramimonas parkeae</i> Norris et Pearson	AB017124
<i>Scherffelia dubia</i> (Perty) Pascher	X68484
<i>Tetraselmis striata</i> Butcher	X70802
Unidentified coccoid (CCMP 1205)	U40921
Unidentified coccoid (CCMP 1220)	U40920
Chlorophyceae	
<i>Characium vacuolatum</i> Lee et Bold	M63001
<i>Chlamydomonas reinhardtii</i> Dangeard	M32703
<i>Hydrodictyon reticulatum</i> (L.) Lagerh.	M74497
<i>Neochloris aquatica</i> Starr	M62861
<i>Scenedesmus abundans</i> (Kirchn.) Chad.	X73995
Trebouxiophyceae	
<i>Chlorella vulgaris</i> Beij.	X13688
<i>Nanochlorum eucaryotum</i> Wilhelm, Eisenbeis, Wild et Zahn	X06425
<i>Pleurastrum terrestre</i> Fritsch et John	Z28973
<i>Trebouxia impressa</i> Ahmadjian	Z21551
Ulvophyceae	
<i>Acrosiphonia</i> sp.	U03757
<i>Gloeotilopsis planctonica</i> Iyengar et Philipose	Z28970
<i>Ulothrix zonata</i> (Weber et Mohr) Kütz.	Z47999
Embryophyta	
<i>Zamia pumila</i> L.	M20017
<i>Zea mays</i> L.	K02202

RESULTS

The alignment of 18S rDNA sequences totaled 1778 sites. Of these sites, 1660 were included in the phylogenetic analyses; 645 of the sites were variable, with 455 parsimony informative sites.

The results of the ML analysis, with bootstrap values for the MP and NJ analyses included, are shown in

Fig. 1. All three methods yielded strong support for major prasinophyte lineages and produced a topology for all green plant lineages similar to those reported in other studies (e.g. Nakayama et al. 1998). However, the relationships among the prasinophyte lineages were not consistent among the different analyses. Figure 2 shows one of the eight most parsimonious resolutions produced by MP analysis. The most parsimonious resolutions differed only in the arrangement of four terminal taxa in one lineage, as indicated. The MP analysis produced a different branching order for the prasinophyte lineages, but regions where the topologies varied did not have bootstrap values greater than 50%. The NJ analyses also produced a different branching order for the major prasinophyte lineages (not shown). In addition, one major prasinophyte lineage was a sister group to all other green plants in the MP analysis, although without bootstrap support (Fig. 2).

Major prasinophyte lineages with strong bootstrap support included the lineages previously identified by Nakayama et al. (1998) as Clades I to IV (Fig. 3), as well as two new lineages, labeled Clades V and VI. Clade V comprised the flagellate *Pseudoscurfieldia marina* and the coccoid *Prasinococcus provasolii*. Only coccoid organisms comprised Clade VI, including those tentatively identified as *Prasinococcus* spp. Two major sister groups were detected in Clade VI, each of which had strong bootstrap support. An additional coccoid prasinophyte, CCMP 1205, was consistently separate from all other lineages.

The final coccoid organism included in the analyses, CCMP 1189, was resolved as in the Chlorophyceae. This result is consistent with pigment analysis, which indicated that CCMP 1189 does not possess prasinoxanthin (data not shown). This organism is also known to produce a quadriflagellate stage (Robert Andersen, personal communication).

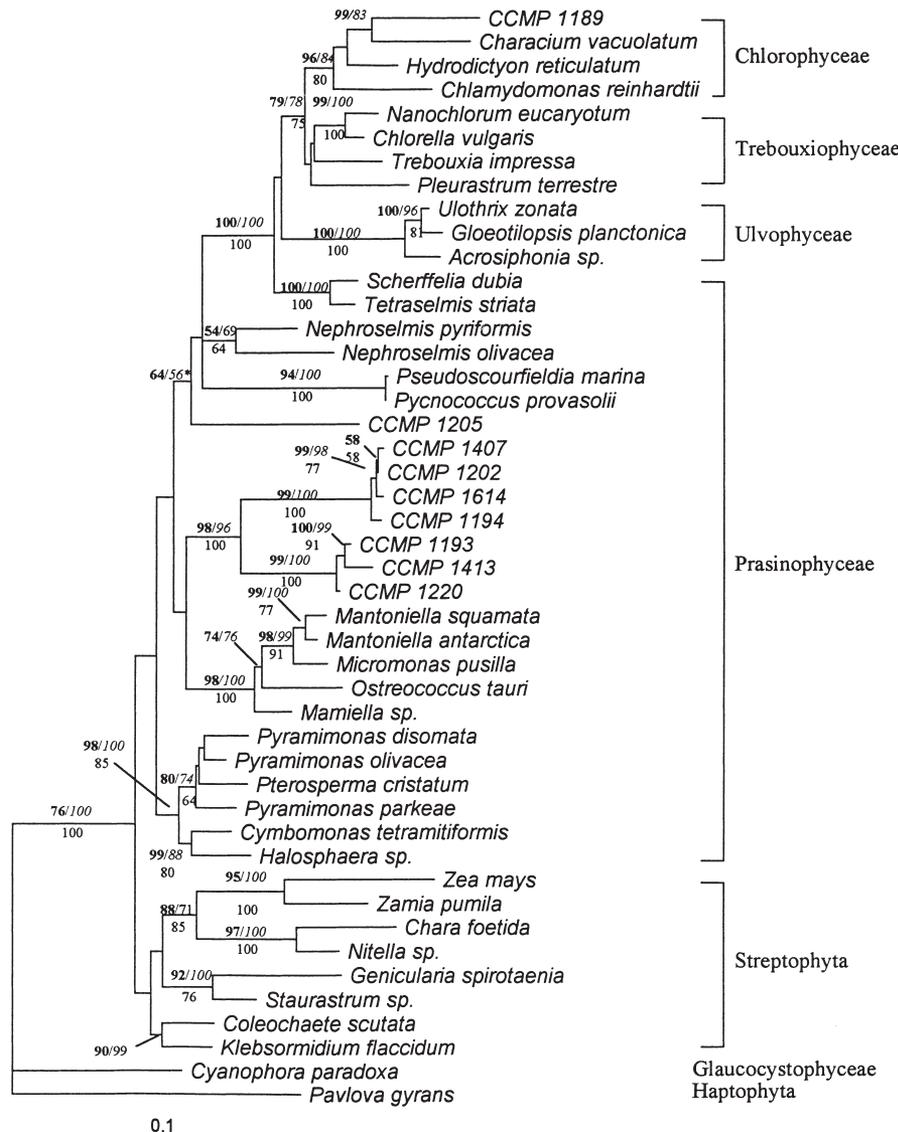


FIG. 1. Phylogenetic analyses of the 18S rDNA sequences of coccoid prasinophytes and additional green plants. The topology presented was produced using ML analysis with quartet puzzling. Nodes supported at greater than 50% bootstrap or puzzling values for at least two different analyses are labeled with puzzling values for the ML analysis (percent, based on 1000 puzzling steps) shown in bold face above the internodes, bootstrap percentages (1000 replicates) for NJ with K2P distances shown in italics above the internodes and bootstrap percentages (1000 replicates) for unweighted MP shown below the internodes. The topology that resulted from the NJ analysis with LogDet distances was identical to NJ with K2P distances. The node marked with an asterisk was supported with LogDet distances but not K2P distances. The analyses were rooted using the haptophyte *Pavlova gyrans* as the outgroup. Class-level affinities are shown on the right.

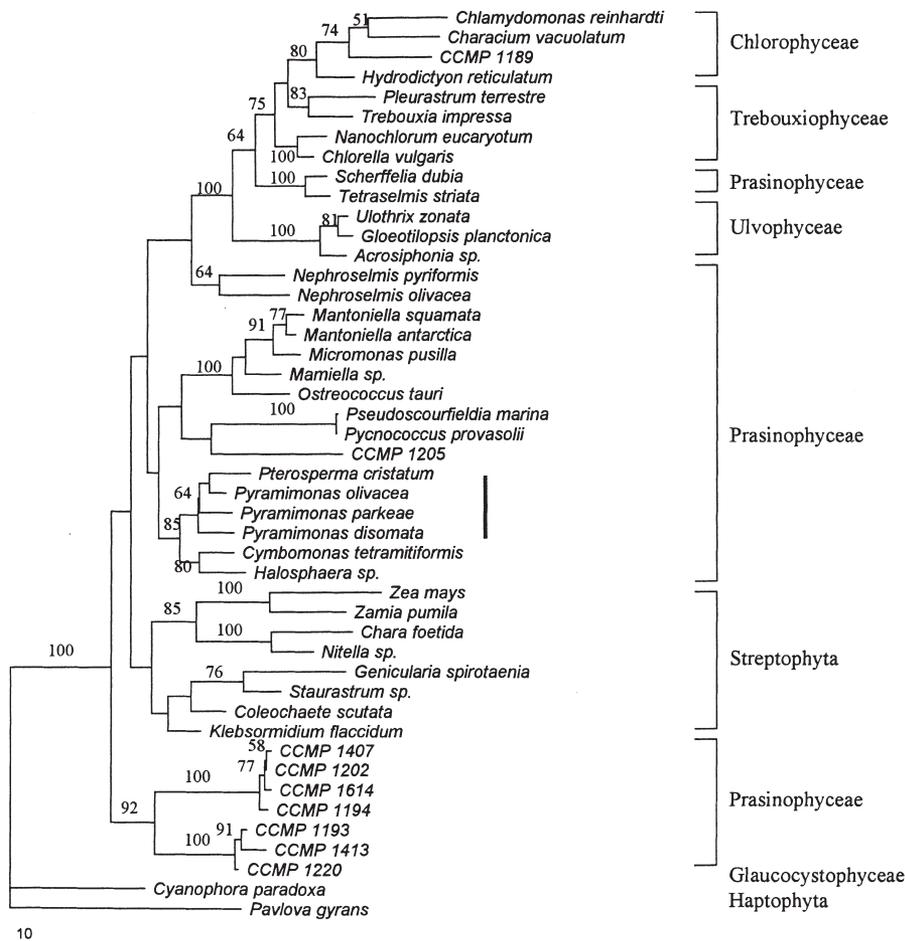


FIG. 2. One of eight most parsimonious resolutions (length = 2198, consistency index = 0.448, retention index = 0.626 and rescaled consistency index = 0.280) produced by unweighted MP analysis of the 18S rDNA sequence data. Bootstrap values greater than 50% are shown above the internodes. The eight resolutions produced equivalent topographies except for the region indicated by the solid bar. Class-level affinities are shown on the right.

DISCUSSION

The results of the 18S rDNA sequence analysis clearly show that organisms with a coccoid vegetative phase are found in at least three lineages of the Prasinophyceae (Fig. 3). The coccoid *Ostreococcus tauri* is clearly allied with the Mamiellales (Clade II), *Pycnococcus provasolii* is related to *Pseudosourfieldia marina* (Clade V), and several coccoid isolates comprise a separate lineage (Clade VI). These results for *O. tauri* and *Py. provasolii* are consistent with the results of previous investigations examining smaller groups of taxa, as outlined below.

The tiny (as small as 0.8 μm by 0.5 μm) coccoid *O. tauri* was originally described by Chrétiennot-Dinet et al. (1995) as a prasinophyte based on the presence of the pigment Mg-3,8-divinyl phaeoporphyrin a_5 monomethyl ester, which is known only from the Prasinophyceae among green algae. Phylogenetic analyses of 18S rDNA sequences of a limited set of prasinophyte taxa placed *O. tauri* with the Mamiellales (Courties et al. 1998). *Ostreococcus tauri* reportedly has a pigment suite that includes siphonaxanthin-like pigments (Chrétiennot-Dinet et al. 1995) and is quite different from the suite of prasinoxanthin, uriolide, and additional unusual minor carotenoids that is characteristic of the Mamiellales (Fawley 1992, Egeland et al. 1997). Our more extensive analyses clearly support the placement of *O. tauri* within the

Mamiellales, despite the reported pigment differences. However, we feel that the pigments of *O. tauri* should be reexamined. The original pigment identifications (Chrétiennot-Dinet et al. 1995) were made using spectra obtained in HPLC solvent mixture, which would not produce definitive results. Also, in the HPLC separation of pigments from *O. tauri* a pigment coeluted with violaxanthin (Chrétiennot-Dinet et al. 1995), which is the expected position of prasinoxanthin (Fawley 1992). Courties et al. (1998) further placed *O. tauri* in the Mamiellaceae, a family characterized by the presence of scales (Melkonian 1990). However, our analyses, as well as previous analyses by Nakayama et al. (1998) indicate that the Mamiellaceae are paraphyletic (Fig. 3). On the other hand, the scaleless *O. tauri* could be placed in the Micromonadaceae, a family characterized by a lack of scales (Melkonian 1990). This placement is not supported by our analyses (Fig. 3). Clearly, a more exhaustive phylogenetic analysis of the Mamiellales is needed, which should include more isolates of each genus, as well as the scaly coccoid *Bathycoccus prasinos*.

As previously reported (Fawley et al. 1999), the coccoid *Py. provasolii* is closely related to the flagellate *Ps. marina* in the family Pycnococcaceae. Earlier analyses of 18S rDNA sequences (Steinkötter et al. 1994, Nakayama et al. 1998), which did not indicate this relationship, had

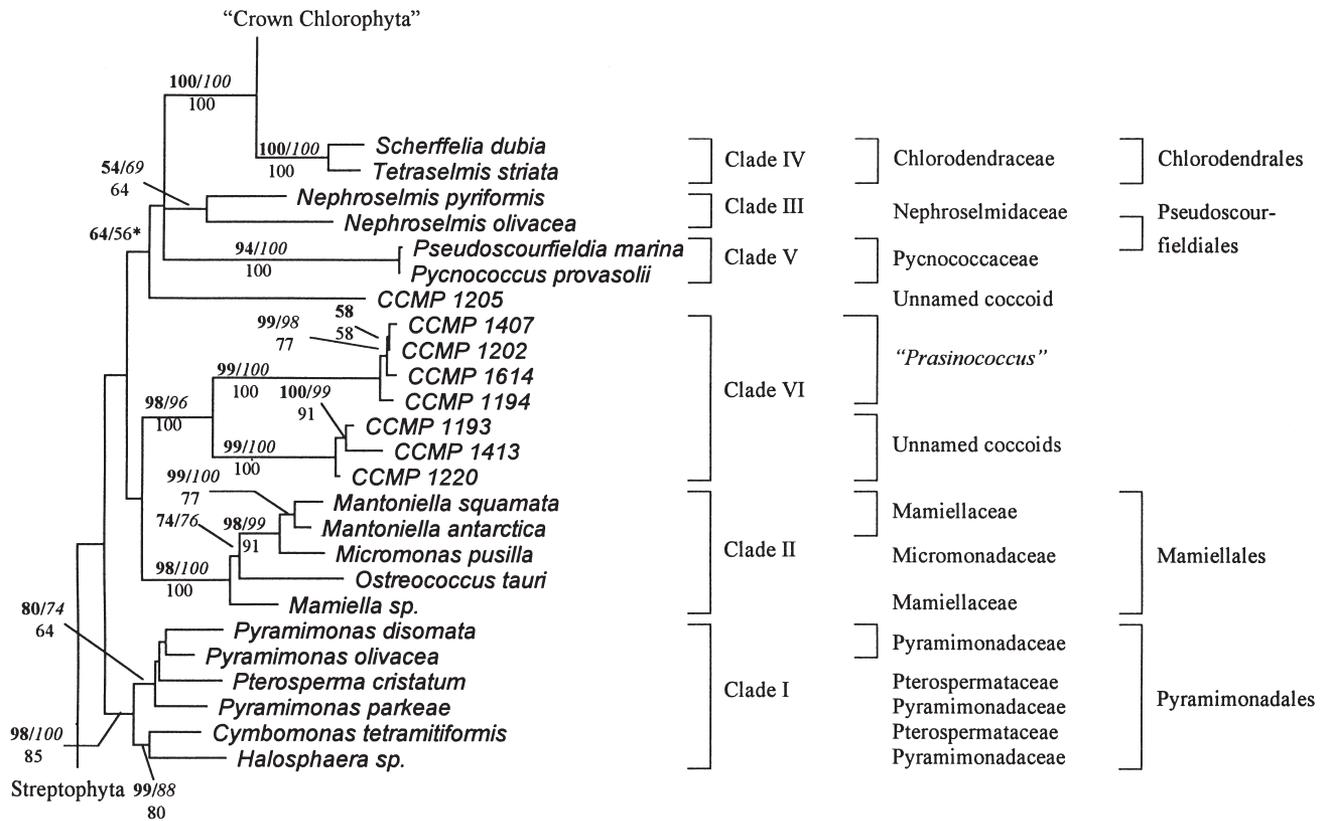


FIG. 3. Phylogenetic analyses of the 18S rDNA sequences as in Fig. 1, except that only the prasinophyte taxa are shown. Chlorophyceae, Ulvophyceae, and Trebouxiophyceae are combined as "Crown Chlorophyta" and streptophyte taxa are reduced to "Streptophyta" after the phylogenetic analysis. Major lineages of prasinophyte taxa are shown on the right as Clades I to VI. Current family and ordinal taxonomy of the Prasinophyceae (Melkonian 1990, with subsequent modifications) is indicated on the far right.

used a flagellate (CCMP 717, *Nephroselmis pyriformis*) that had been incorrectly identified as *Ps. marina* (Fawley et al. 1999). Guillard et al. (1991) originally placed *Py. provasolii* in the Mamiellales because of the presence of prasinoxanthin. However, no close relationship of *Pycnococcus* and the Mamiellales is indicated by our analyses or other analyses of 18S rDNA (Nakayama et al. 1998) and *rbcL* (Daugbjerg et al. 1995) data. On the other hand, *Ps. marina* has traditionally been placed with *Nephroselmis* in either the Pseudoscourfieldiales (Melkonian 1990) or the Chlorodendrales (Moestrup and Thronsen 1988) because of similarity of scale structure. Our analyses, as well as the results of *rbcL* sequence analyses (Daugbjerg et al. 1995), do not support this relationship, either. At the present time, the Pycnococcaceae are seen as a separate lineage among prasinophytes (Clade V, Fig. 3). Certain features, such as the presence of prasinoxanthin (Fawley 1992) and characteristics of the photosynthetic light-harvesting complexes (Fawley 1993), are shared between the Pycnococcaceae and the Mamiellales, but there is no strong support for this relationship from the sequence data.

The coccoid *Prasinococcus capsulatus* has also been placed in the Mamiellales based on the presence of prasinoxanthin and the additional carotenoid, uri-

olide (Miyashita et al. 1993). The 18S rDNA analyses of Potter et al. (1997) and Courties et al. (1998) indicated that a coccoid tentatively identified as *Pr. capsulatus* (CCMP 1407) is in a lineage with an unidentified coccoid, CCMP 1220, and not closely related to the Mamiellales. These results are supported by our more extensive analyses. Several coccoids, most of which have been tentatively identified by the Provasolii-Guillard Center for Culture of Marine Phytoplankton as *Pr. capsulatus* or *Prasinococcus* sp. (CCMP 1202, CCMP 1407, CCMP 1614, and CCMP 1194), form a well-supported lineage that is a sister group to another lineage made up of the unidentified coccoids CCMP 1193, CCMP 1413, and CCMP 1220 (CCMP 1193 was erroneously identified as *Pr. capsulatus* by the CCMP). The lineage that includes all of these coccoids (Clade VI, Fig. 3) is not closely allied with any other prasinophyte lineage in our analyses. Organisms of Clade VI and the Mamiellales have similar, but not identical, pigmentation (Hooks et al. 1988, Fawley 1992 and unpublished data, Miyashita et al. 1993, Egeland et al. 1997). Preliminary results also suggest that Clade VI organisms share the unusual light-harvesting complex with the Mamiellales (unpublished data). On the other hand, *Pr. capsulatus* shares with the Pycnococcaceae and the

cocoid *Prasinoderma coloniale* unusual cytological features including an intrusion of mitochondrial membranes into the pyrenoid (Fawley 1992, Miyashita et al. 1993, Hasegawa et al. 1996) and an unusual method of cell division in which one product of division is retained within the old cell wall and the other is extruded from the cell (Miyashita et al. 1993, Hasegawa et al. 1996). Our analyses do not support a close relationship among *Pr. capsulatus*, the Mamiellales, and the Pycnococaceae. However, the type specimen of *Pr. capsulatus* should be included in the analyses before conclusions are reached.

A final prasinophyte cocoid, CCMP 1205, is not allied with any other prasinophyte taxon. Although the pigments of this cocoid are not yet known, based on HPLC retention times the pigment suite of this organism is different from other cocoid prasinophytes (unpublished data).

Additional lineages (Clades I, III and IV), which are composed of only flagellate taxa, have been thoroughly discussed by Nakayama et al. (1998). Our analyses add no additional taxa to these clades, nor do they change the topologies within the clades. Our analyses also show that most of the families in the traditional prasinophyte classification are not monophyletic (Fig. 3), in agreement with the results of previous 18S rDNA (Nakayama et al. 1998) and *rbcl* (Daugbjerg et al. 1995) analyses.

The phylogenetic analyses of 18S rDNA sequences of Nakayama et al. (1998) revealed considerable support for the branching pattern of the major prasinophyte clades. However, in our analyses relationships among the various prasinophyte lineages lacked bootstrap support. In fact, the different types of analyses produced different topologies (e.g. Figs. 1 and 2). Based on our results, we feel that it is premature to speculate on the relationships of the various prasinophyte lineages. Additional taxa need to be added to both the *rbcl* and 18S rDNA data sets, and perhaps the sequences of additional should be determined. In particular, more species representing the diverse pigment suites (Ricketts 1970) of the genus *Nephroselmis* need to be included, as well as members of the Pedinophyceae (Moestrup 1991) and type specimens of the cocoids *Prasinococcus capsulatus* and *Prasinoderma coloniale*. Additional new cocoid prasinophytes have been isolated (Laure Guillou, personal communication) and additional flagellate prasinophytes have been observed (e.g. Thomsen and Buck 1998). These organisms should be added to the analyses as isolates and sequences become available.

In conclusion, the addition of several cocoid prasinophytes to analyses of prasinophyte phylogeny reveals that the cocoid form has evolved several times in this class of green algae. At least three separate lineages of prasinophytes have cocoid members. In addition, these analyses indicate that a previously undescribed prasinophyte lineage is comprised entirely of cocoid organisms, including isolates tentatively identified as *Pr. capsulatus*, as well as other highly divergent unnamed organisms. This new cocoid lineage shares characteristics with both the Mamiellales and

the Pycnococaceae, but our analyses do not support a close relationship among these lineages.

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