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Phylogeny of genera of Prasinophyceae and Pedinophyceae (Chlorophyta) deduced from molecular analysis of the *rbc*L gene

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SUMMARY

A molecular approach was used to study the phylogeny of 12 genera of Prasinophyceae and two genera of Pedinophyceae (Chlorophyta). The study was based on maximum likelihood and LogDet transformation analyses of a 1094-basepair fragment of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L), a chloroplast-encoded gene. With the inclusion of homologous sequences from two cyanobacteria (*Anabaena* PCC 7120 and *Anacystis nidulans* (Richter) Drout et Daily) and a prochlorophyte (*Prochlorothrix hollandica* Burger-Wiersma, Stal et Mur), the maximum likelihood reconstructions suggested that species referred to the family Mamiellaceae are secondarily reduced forms rather than the most ancestral eukaryotic green plants. The systematic position of *Micromonas pusilla* (Butcher) Manton et Parke based on morphology is ambiguous, but the *rbc*L-based inferences indicated that it is related to the Mamiellaceae. In spite of being morphologically very different, *Pycnococcus* and *Pseudoscourfieldia* appear to be closely related, and it is suggested that *Pseudoscourfieldia* be included in the family Pycnococcaceae. The phylogenetic framework when based on first and second codon positions identifies the Mamiellaceae, Pycnococcaceae, Halosphaeraceae, and Mesostigmataceae as monophyletic families, whereas the Chlorodendraceae appears to be of polyphyletic origin. However, this branching pattern was not confirmed by bootstrap analyses. The analysis based on a LogDet transformation matrix also supported the close relationship among species belonging to the Mamiellaceae (including *Micromonas pusilla*) and that the pedinophytes form a separate group. The branching pattern among most of the prasinophyte genera was not resolved giving a tree topology similar to those obtained in the bootstrap analyses. A relative rate test showed that the *rbc*L gene in the Pedinophyceae has evolved at a slower speed relative to that in the Prasinophyceae.

Key words: green algae, LogDet transformation, maximum likelihood, molecular phylogeny, PCR, Pedinophyceae, Prasinophyceae, *rbc*L, RUBISCO large subunit, relative rate test.

INTRODUCTION

Comparative ultrastructural studies have suggested that the Prasinophyceae (Micromonadophyceae) and Pedinophyceae (Chlorophyta) represent some of the earliest branching lineages among the extant eukaryotic plants containing chlorophyll *a* and *b* (e.g. Manton 1965; Moestrup and Throndsen 1988; Moestrup 1991). It is believed that the prasinophytes are related to the main line of evolution of the flowering plants (e.g. Stewart and Mattox 1975, 1978; Moestrup 1978; Norris 1980; Mattox and Stewart 1984; Melkonian 1984, 1990; O'Kelly and Floyd 1984).

The circumscription of the Prasinophyceae according to Moestrup and Throndsen (1988) may be summarized as follows: cells possess a flagellar pit, very long (600–900 nm) usually parallel basal bodies, tubular hair scales on the flagella, a parabasal position of the dictyosome(s), and flagella and cell body are typically covered by organic scales (often in layers and of several types). Some species deviate from at least one of these characteristics. For example, the four flagella in a newly observed prasinophyte (*Prasinopapilla* gen. ined.) from Japan do not emerge from a depression but project from an anterior protrusion (papilla), and the basal bodies are at an angle (Inouye, pers. comm. 1993).

The Pedinophyceae which at present contains three genera (*Resultor* Moestrup, *Pedinomonas* Korshikov and *Marsupiomonas* Jones, Leadbeater et Green) are thought to be evolutionarily isolated and without any clear relationship to other groups of green plants (Moestrup 1991). Cells of pedinophytes lack scales and have one emergent flagellum but two basal bodies slightly displaced rather than arranged end to end. During cell division, *Pedinomonas* has a closed mitosis (Pickett-Heaps and Ott 1974). The position of the eyespot opposite the flagella and in the cleavage plane of the

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cell during cytokinesis has been considered an ancient feature (Moestrup 1991).

Few studies have addressed the relationship between prasinophytes and the advanced green algae (Chlorophyceae, Charophyceae, Ulvophyceae and Pleurastrophyceae *sensu* Mattox and Stewart 1984) some of which gave rise to the land plants. Melkonian (1984) presented some ideas concerning the evolutionary relationships among different green algae based on features of the flagellar transition region and the flagellar apparatus. Mischler and Churchill (1985) compiled a data matrix based on morphological and biochemical characters for a cladistic analysis of major groups of green algae, bryophytes, gymno- and angiosperms. Kantz *et al.* (1990), Steinkötter *et al.* (1994) and Hori *et al.* (1990) have published molecular phylogenies based on sequences of 18S/28S and 5S ribosomal RNA. Only a few species of primitive green algae were included in the latter studies.

Before attempting to resolve relationships among major groups belonging to the Chlorophyta we present a phylogenetic study of the primitive green algae based on a 1094-basepair fragment of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L). This fragment was sequenced from 12 genera of prasinophytes (15 species) and two genera of pedinophytes. The aligned sequences were analyzed using the maximum likelihood (ML) (Felsenstein 1981) and the LogDet transformation methods (Steel 1994).

MATERIALS AND METHODS

Cultures

Most clonal cultures were obtained from the Scandinavian Culture Centre of Algae and Protozoa (Table 1). Non-axenic cultures were grown at temperatures of 4 or 15°C and a light : dark regime of 16 : 8 h.

DNA extraction, amplification and sequencing

A 10 mL culture (in the exponential phase of growth, approx. 1000 cells/mL) was centrifuged at 1500–2000 rpm for 10–15 min in a Heraeus Labofuge 6000. The pellet was transferred to an Eppendorf tube and frozen at −80°C for *ca.* 24 h. The thawed pellet was then mixed with 300 μL preheated 2X CTAB (hexadecyltrimethyl-ammonium bromide) isolation buffer and further extraction of total genomic DNA followed the procedure described by Doyle and Doyle (1987). PCR reactions were performed as follows. Amplifications were in a 10 μL reaction volume (25 mmol/L Tris-HCl (pH 8.5), 1 mmol/L $MgCl_2$, 10 mmol/L KCl, 2.5 mg/mL BSA, 200 μmol/L dNTP, 0.5 mmol/L of each primer and 0.4 units of Boehringer-Mannheim Taq polymerase) in capillary tubes. Amplification conditions were achieved by 1 initial cycle of denaturation at 94°C for 3 min, followed by 30 cycles each comprising denaturation at 94°C for 1 s, annealing at 50 or 52°C for 1 s and extension at 72°C for 1 min. The double-stranded PCR product was visualized in a 2% NuSieve agarose gel containing 0.5 μg/mL ethidium bromide in a TE buffer (10 mmol/L Tris and 1 mmol/L EDTA). The resulting band was extracted as a plug from the gel with a disposable plastic pipette and transferred to 0.5 mL water. The gel plug containing the double-stranded PCR product was incubated at 70°C for 2 min and then vortexed gently. A 1 μL aliquot of resuspended PCR product was used as template in an asymmetrical amplification, 50 μL reaction volume (67 mmol/L Tris-HCl (pH 8.8), 2 mmol/L $MgCl_2$, 16.6 mmol/L $(NH_4)_2SO_4$, 10 mmol/L β-mercaptoethanol, 200 μm dNTP, 0.5 μmol/L and 0.05 μmol/L of the two primers and 0.4 units of Taq DNA polymerase). Conditions for single-stranded amplifications were 30 cycles of denaturation for 1 min at 94°C, annealing at 50 or 52°C for 1 min and extension for 3 min at 72°C. The single-stranded product was desalted and concentrated using Millipore Ultrafree-MC filters (no. UFC3TTK00) and resuspended in 9 or 16 μL distilled water. A 7 μL aliquot was used as a template in the dideoxy chain termination sequencing reaction (Sanger *et al.* 1977) with

Table 1. List of prasinophytes and pedinophytes included in the phylogenetic analysis

	Strain no.	Accession no.
Class Prasinophyceae		
Family Mamiellaceae		
Mantoniella squamata	K-0284	U30278
Bathycoccus prasinos‡	K-0417	U30275
Micromonas pusilla	K-0245	U30276
Mamiella sp.*		U30277
Family Pycnococcaceae		
Pseudoscourfieldia marina	K-0017	U30279
Pycnococcus provasolii	K-0249	U30280
Family Halosphaeraceae		
Cymbomonas tetramitiformis	K-0467	L34687
Pyramimonas orientalis	K-0266	L34813
Pyramimonas olivacea	K-0257	L34815
*Pterosperma cristatum**		U30281
Family Mesostigmataceae		
Mesostigma viride†	I-9239	
Family Chlorodendraceae		
Nephroselmis minuta	K-0022	U30286
Nephroselmis olivacea	K-0020	U30285
Tetraselmis marina	K-0377	U30284
Tetraselmis aff. maculata	K-0298	U30283
Class Pedinophyceae		
Family Pedinomonadaceae		
Resultor mikron	K-0279	U30288
Pedinomonas sp.	PCC441	U30287

Strain numbers refer to those used in the catalogue from the Scandinavian Culture Centre of Algae and Protozoa. * Extracted genomic DNA kindly provided by I. Inouye and T. Nakayama; † a clonal culture kindly provided by U. Schlösser; ‡ a clonal culture kindly provided by W. Eikrem and J. Throndsen. Genbank accession numbers are also given.

Sequenase 2.0 enzyme (United States Biochemical). Primers used for symmetrical and asymmetrical reactions have been reported elsewhere (Daugbjerg *et al.* 1994).

Alignment and phylogenetic analyses

The 1094-basepair fragment of the *rbc*L gene (approximately 75% of the total gene) were aligned unambiguously by eye and edited using the computer programs ESEE (version 1.0.9d, Cabot and Beckenbach 1989) and CS3 (Siegismund, unpubl. data 1992). Phylogenetic trees were inferred from ML using fastDNAmL (Olsen *et al.* 1994) and plotted using drawgram from the PHYLIP package, version 3.52c (Felsenstein 1993). Two approaches were conducted for phylogeny reconstruction using ML: (i) all 1094 nucleotides were included (Fig. 2a) and (ii) third codon positions were excluded (Fig. 3a). Third codon positions may add phylogenetic noise and thus disturb the phylogenetic signal (Miyamoto *et al.* 1994). No weighting of codon positions was performed. The input order of taxa was changed and the transition to transversion ratio was altered until the best log likelihood score was obtained. Trees were rooted using published sequences of two cyanobacteria (*Anabaena* PCC 7120, Curtis and Haselkorn 1983; and *Anacystis nidulans* (Richter) Drout et Daily, Shinozaki *et al.* 1983) and the prochlorophyte *Prochlorothrix hollandica* Burger-Wiersma, Stal et Mur (Morden and Golden 1991). Bootstrap analyses (100 replications) were used to assess the stability of relationships (Felsenstein 1985). The aligned *rbc*L sequence data matrix was also analyzed using the LogDet transformation method (Steel 1994) implemented in SplitsTree, version 1.0 written by D. M. Huson and R. Wetzel. All 1094-basepairs were considered in the LogDet transformation analysis.

The two-degrees-of-freedom method (2D) of Tajima (1993) was applied to test for a molecular evolutionary clock hypothesis. The 2D statistical test permits differences in transitions and transversions and does not assume a particular pattern in the substitution rate. It is also applicable when substitution rates vary among different sites. The test statistic in the 2D method approximately follows the Chi-squared distribution ($P(\chi^2>5.99)<0.05$) with two degrees of freedom. The *rbc*L sequence of *Anabaena* PCC 7120 (Curtis and Haselkorn 1983) was used as an outgroup in all of the 171 ((19*18)/2) pairwise comparisons including all nucleotide positions.

Sequence availability

Genbank accession numbers for the prasinophytes and pedinophytes studied are given in Table 1.

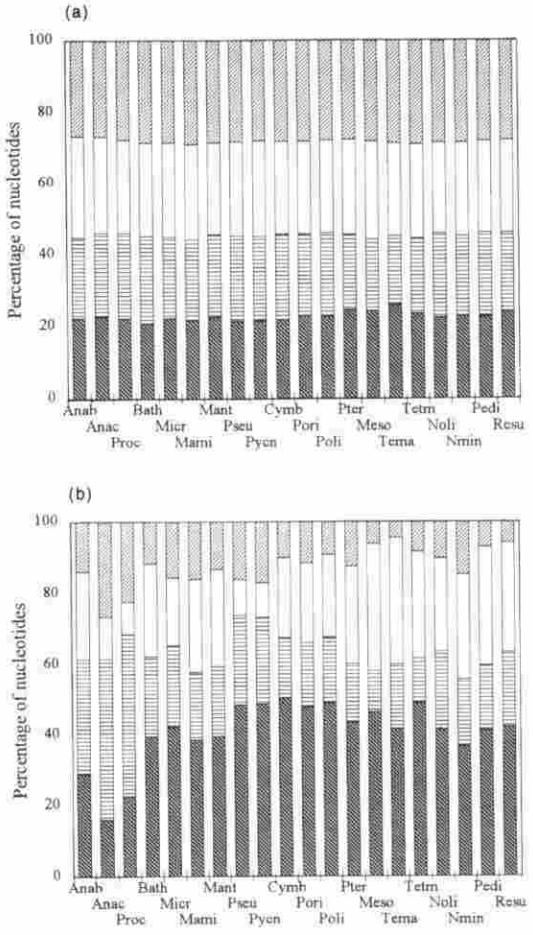

Fig. 1. Base composition of guanine (▩), adenine (□), cytosine (▤) and thymine (▤) in percentage of *rbc*L sequences at first/second (a) and third (b) codon positions for the 20 taxa included in the phylogenetic analyses. No compositional bias is present in first/second codon positions as the four nucleotides have an equal frequency of occurrence in the pro- and eukaryotes studied. The three photosynthetic prokaryotes show a bias towards adenine whereas the ancestral green algae exhibit a marked bias towards thymine in third codon positions. The percentage of pyrimidines (C+T) in (b) is similar between pro- and eukaryotes (*ca* 63%). Abbreviations for the species are as follows: Anab, *Anabaena* PCC 7120; Anac, *Anacystis nidulans*; Proc, *Prochlorothrix hollandica*; Bath, *Bathycoccus prasinos*; Micr, *Micromonas pusilla*; Mami, *Mamiella* sp.; Mant, *Mantoniella squamata*; Pseu, *Pseudoscourfieldia marina*; Pycn, *Pycnococcus provasolii*; Cymb, *Cymbomonas tetramitiformis*; Pori, *Pyramimonas orientalis*; Poli, *Pyramimonas olivacea*; Pter, *Pterosperma cristatum*; Meso, *Mesostigma viride*; Tema, *Tetraselmis* aff. *maculata*; Tetm, *Tetraselmis marina*; Noli, *Nephroselmis olivacea*; Nmin, *Nephroselmis minuta*; Pedi, *Pedinomonas* sp.; Resu, *Resultor mikron*.

RESULTS

Distribution of the 1094 nucleotides (expressed as percentages) at first and second codon positions, and in third codon positions, are illustrated in Figs 1a,b, respectively. There is no compositional bias at first and second codon position: similar frequency of each of the four nucleotides among the species studied (cf. Fig. 1a). The primitive green algae exhibited a marked bias towards thymine in third codon positions, ranging from 36.9% in *Nephroselmis minuta* (N. Carter) Butcher to 50.7% in *Cymbomonas tetramitiformis* Schiller (Fig. 1b). Cytosine was the most frequent nucleotide

in the cyanobacteria *Anabaena* PCC 7120 and *Anacystis nidulans* and in the prochlorophyte *Prochlorothrix hollandica* (ranging from 32.7% to 46.1%). Despite the different nucleotide bias in third codon positions between the photosynthetic prokaryotes and the eukaryotes, these organisms had almost the same frequency of pyrimidines (approximately 63%, Fig. 1b). Of the 1094 nucleotides sequenced in the *rbc*L gene, 611 sites segregated and approximately two thirds (67%) of the total number of substitutions occurred in third codon positions. The empirical ratio of transitions (Ts) to transversions (Tv) for all codon positions was 0.8 : 1 whereas that for first and second codon positions only was 0.7 : 1. Of the 364 amino acids, 165 sites (45%) were non-synonymous.

Maximum likelihood inference

When all 1094-basepairs were included in a ML analysis the best log likelihood score was −11 780.64, obtained with a Ts : Tv ratio of 0.8 : 1, a ratio identical to that observed empirically. The tree topology from this analysis is shown in Fig. 2a and a bootstrap analysis based on 100 replications is shown in Fig. 2b. The ML inference (Fig. 2a) shows that the Prasinophyceae and Pedinophyceae form two separate groups whereas the bootstrap analysis shows a trifurcation, as the *Mesostigma*/*Tetraselmis* spp. clade was separated from the main cluster of prasinophytes (Fig. 2b). However, this cluster was supported by a bootstrap proportion of only 52%. The three species sequenced from the family Mamiellaceae (*Bathycoccus prasinos* Eikrem et Throndsen, *Mantoniella squamata* (Manton et Parke) Desikachary and *Mamiella* sp.) appeared to be monophyletic, and this clustering was well supported by a bootstrap proportion of 90%. The picoplanktonic flagellate *Micromonas pusilla* (Butcher) Manton et Parke also appears to be related to species of this family (Figs 2a,b). *Pseudoscourfieldia* and *Pycnococcus* form a monophyletic sister group to the Mamiellaceae (Moestrup 1984).

A close relationship between *Pycnococcus* and *Pseudoscourfieldia*, two morphologically very different species, was strongly suggested by a bootstrap proportion of 100% (Fig. 2b). Both the ML analysis and the bootstrap analysis indicate that *Bathycoccus prasinos* (Eikrem and Throndsen 1990) occupies the most basal position within the Mamiellaceae. From Fig. 2a it is indicated that *Mamiella* sp. forms a sister group to the *Micromonas pusilla*/*Mantoniella squamata* cluster, but following the bootstrap analysis this relationship was not sustained (Fig. 2b).

The family Halosphaeraceae (with three genera represented in this study: *Pterosperma* Pouchet, *Cymbomonas* Schiller, and *Pyramimonas* Schmarda) appeared to be polyphyletic. The two species of *Pyramimonas* (*P. orientalis* McFadden, Hill et Wetherbee and *P. olivacea* N. Carter) and *Cymbomonas tetramitiformis* formed a cluster, whereas *Pterosperma cristatum* Schiller was related to the two species of *Nephroselmis* (Fig. 2a), a genus currently referred to the family Chlorodendraceae. The Chlorodendraceae, with two

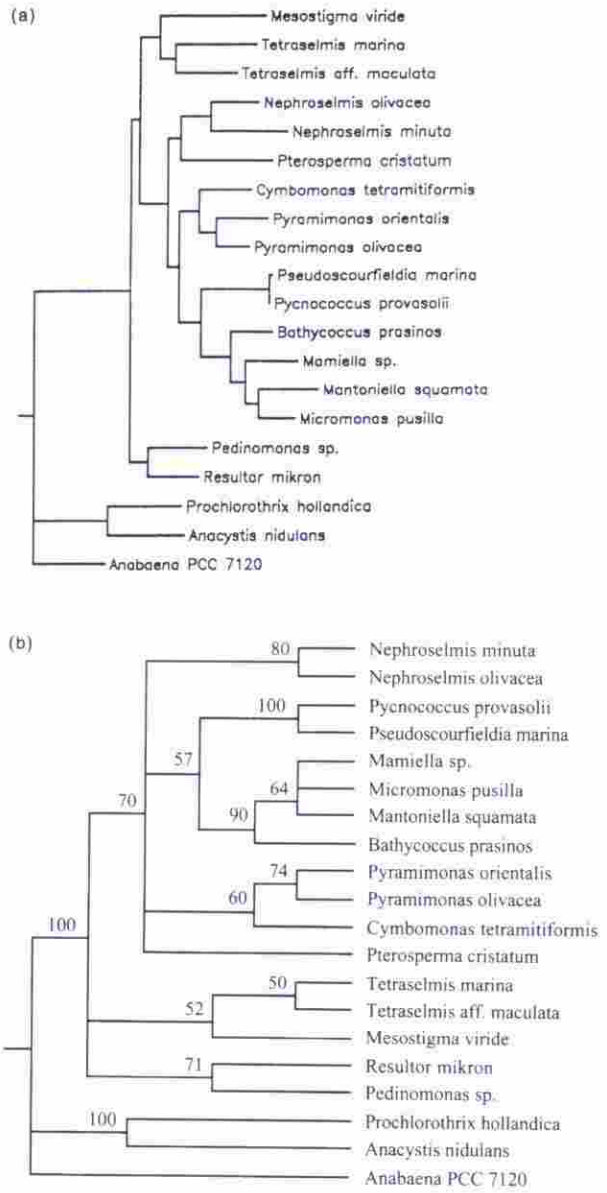

Fig. 2. Phylogenetic reconstruction of genera of Prasinophyceae and Pedinophyceae as inferred from maximum likelihood analysis. (a) A total of 1094 nucleotides of the *rbc*L-gene were considered and homologous sequences from *Anabaena* PCC 7120, *Anacystis nidulans* and *Prochlorothrix hollandica* were used to root the tree. The branch lengths are proportional to the number of character changes. (b) Majority-rule (50%) consensus tree of a bootstrap analysis based on the maximum likelihood method. Numbers associated with internal branches indicate the number of times a branch was recovered in 100 bootstrap replications.

genera sequenced (*Nephroselmis* and *Tetraselmis*), also appeared to be polyphyletic, as the two thecate species of *Tetraselmis* formed a clade with *Mesostigma viride* of the family Mesostigmataceae.

A maximum likelihood reconstruction based on first and second positions only is shown in Fig. 3a. The best log likelihood score was −4817.50 obtained with a Ts : Tv ratio of

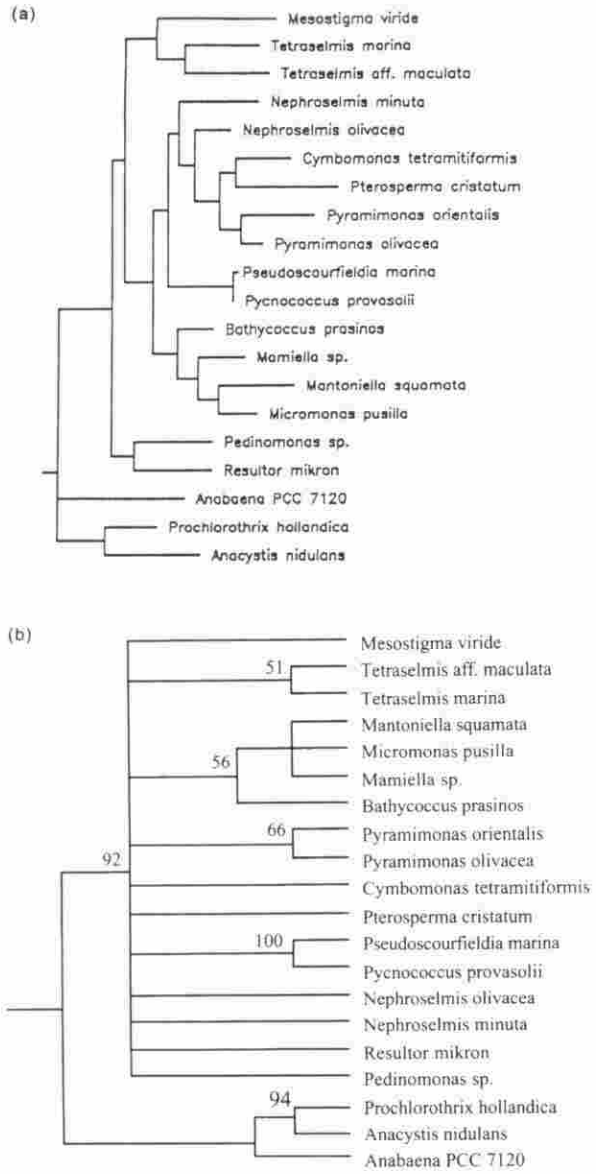

Fig. 3. Reconstruction based on the maximum likelihood method. (a) Phylogenetic analysis based on first and second codon positions only (equal to 730 basepairs of the *rbc*L-gene). Homologous sequences from the tree photosynthetic prokaryotes were used to root the tree. The branch lengths are proportional to the number of character changes. (b) Majority-rule (50%) consensus tree of a bootstrap analysis based on the maximum likelihood method. Numbers associated with internal branches indicate the number of times a branch was recovered in the 100 bootstrap replications.

0.7 : 1, identical to the ratio observed empirically. A bootstrap analysis is shown in Fig. 3b. With the exclusion of third codon positions *Pycnococcus* and *Pseudoscourfieldia* form a sister group with the two species of *Nephroselmis* and the Halosphaeraceae rather than being the sister group to the Mamiellaceae (cf. Fig. 2a). *Pterosperma* forms a clade with *Cymbomonas* and thus the family Halosphaeraceae appears to be of monophyletic origin when the reconstruction is based on first and second codon positions. The family Chlorodendraceae still seems to be polyphyletic. The separate branching of the clade with *Mesostigma* and the two species of *Tetraselmis* is observed both in Fig. 2a and Fig. 3a. The reconstruction illustrated in Fig. 3a also shows the split between pedinophytes and prasinophytes. However, the branching order as described above is not sustained by the bootstrap analysis as the 50% majority rule consensus tree reveals a more or less collapsed topology. Only the close relationship between *Pseudoscourfieldia* and *Pycnococcus* is validated (Fig. 3b).

Inference from LogDet transformation

The split-graph obtained when using the LogDet transformation method to compute the distance matrix is displayed as Fig. 4. It is based on all 1094 nucleotide positions and has a fit of 68.2%. The fit measurement indicates how well the split-graph represents the distance matrix from which it was estimated (a 100% indicates a perfect fit). Using the photosynthetic prokaryotes as the outgroup the tree topology shows a star-like branching pattern. The branching is essentially identical to the bootstrap analysis based on first and second codon positions only (Fig. 3b) in that it does not resolve relationships among the genera of primitive green algae. However, it does support that the two genera of pedinophytes are more closely related to each other than to any of the prasinophyte genera included. The split-graph also identifies that species belonging to the Mamiellaceae are closely related but it does not reveal the relationship within the family. The close relationship between *Pseudoscourfieldia marina* and *Pycnococcus provasolii* suggested by the ML analyses is also corroborated by the LogDet transformation method.

Testing for equality of evolutionary rates

When all nucleotide positions were included in the pairwise comparisons using the two-degrees-of-freedom method (Tajima 1993) 31 of 171 (18.1%) had a heterogeneous rate of nucleotide substitution (data not shown). When the pairwise comparisons were restricted to involve only the green algae, the number of significant hits decreased to 16 (11.8%). Ten of these involve either *Resultor* or *Pedinomonas*, implying that the *rbc*L gene in the pedinophytes evolved at a different speed relative to that in prasinophytes.

DISCUSSION

Use of chloroplast-encoded genes for phylogenetic reconstructions

The use of chloroplast-encoded genes for phylogenetic inference has some important implications. According to the endosymbiotic theory, chloroplasts in the eukaryotic cell originated when a phagotrophic protozoan host engulfed a photosynthetic prokaryote some 700–900 million years ago (Cavalier-Smith 1993). For the past 2 decades it has often been debated whether chloroplasts have a mono- or polyphyletic origin. If they had a polyphyletic origin, chloroplast-

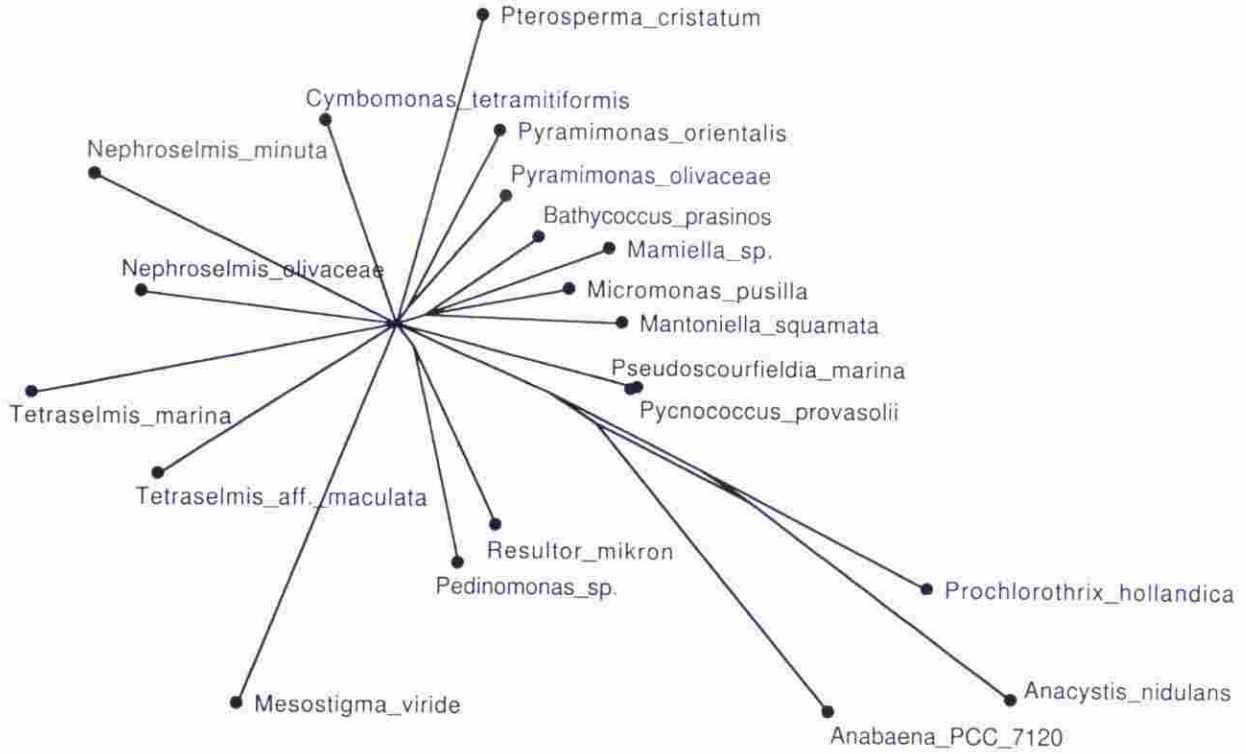

Fig. 4. Phylogenetic reconstruction based on the LogDet transformation method of all 1094 nucleotides of the *rbc*L-gene. The three cyanobacteria were used to root the tree.

encoded genes cannot form the basis of phylogenetic studies that include major groups of plants, since they would not share a common ancestry. For an overview of the various hypotheses regarding mono- versus polyphyly of chloroplasts as revealed by nucleotide sequence analyses the reader is referred to Morden and Golden 1989, 1991; Assali *et al.* 1990, 1991; Douglas 1992; Martin *et al.* 1992; Morden *et al.* 1992; Palenik and Haselkorn 1992; Urbach *et al.* 1992; Cavalier-Smith 1993; Kowallik 1993; Valentin *et al.* 1993; Ueda and Shibuya 1993 (plus references cited therein) and thus it will only be commented on briefly below.

Immunological evidence was recently presented for a close relationship of the polypeptides of the antenna complex associated with photosystem I in the chlorophyll *a*/*b*-containing Chlorophyta, the chlorophyll *a*/*c*-containing Chromophyta and the red-algae which contain chlorophyll *a* and phycobilisomes (Wolfe *et al.* 1994). This observation suggests that the light-harvesting proteins in these photosynthetic eukaryotes had a common ancestor. It does not imply, however, that the algae themselves are monophyletic because secondary endosymbiotic events have probably occurred numerous times in the evolution of algae (e.g. cryptophytes, chlorarachniophytes, dinoflagellates). Sequence analyses of *psb*A, *tuf*A, *atp*B and 16S rRNA (all chloroplast-encoded genes) support a single cyanobacterium-like ancestor of the chloroplasts (Morden *et al.* 1992). Analyses of the large and small subunit of RUBISCO (*rbc*LS) including purple bacteria, cyanobacteria, prochlorophytes, and major groups of algae and higher plants display a conflicting picture as the phylogenetic trees indicate a polyphyletic origin of the chloroplasts (e.g. Morden *et al.* 1992; Ueda and Shibuya 1993). This opposed observation can be explained by a lateral gene transfer of the *rbc*LS operon from an α- or β-purple bacterium to the chloroplast lineage(s) leading to rhodophytes, chromophytes and cryptophytes as hypothesized by Morden *et al.* (1992, fig. 9). All molecular studies confirm that the chloroplast in chlorophyll *a*/*b* containing plants originated from a cyanobacterium-like ancestor and it is very likely that the green plant lineage arose from a single endosymbiotic event. Accordingly *rbc*L gene sequences from cyanobacteria are suited as the outgroup when elucidating phylogenetic hypotheses among green plants whereas they are probably not a useful outgroup in analyses of chromophytes, cryptophytes and rhodophytes. We decided not to include *Cyanophora* Korshikov (a glaucophyte) among the outgroup taxa because it forms a sister group relationship with chlorophytes and land plants when based on *rbc*L data while it is the sister group to rhodophytes, cryptophytes, chromophytes and euglenophytes when based on chloroplast 16S rRNA (Douglas and Turner 1991; Morden *et al.* 1992).

Relative rate test and methods for phylogenetic reconstructions

Among the primitive green algae most of the significant differences in substitution rates were noted when comparing pedinophytes with prasinophytes, suggesting that the *rbc*L

gene evolved at different rates in the two classes of green algae. The relative branch lengths in Fig. 2a indicate that the gene coding for the large subunit of RUBISCO in the pedinophytes evolved at a slower rate compared to that in the prasinophytes. Significant variation in substitution rates of the *rbc*L gene has also been observed among certain groups of land plants (e.g. Bousquet *et al.* 1992a,b; Gaut *et al.* 1992; Martin *et al.* 1993). For example, the *rbc*L gene although considered conservative, may have an overall substitution rate five times greater in grasses than in palms (Gaut *et al.* 1992). In addition, difference in evolutionary rates of the *rbc*L gene may also occur at the subgeneric level as witnessed by work done by Daugbjerg *et al.* (1994) on members of the genus *Pyramimonas* (Prasinophyceae). It is well documented that heterogeneous substitution rates have important implications for phylogenetic reconstructions (e.g. Felsenstein 1981; Saitou and Imanishi 1989; Jin and Nei 1990; Fukami-Kobayashi and Tateno 1991; Hasegawa *et al.* 1991; Tateno *et al.* 1994; Kuhner and Felsenstein 1994). The results of computer simulations indicate that ML performs better than maximum parsimony (MP) under conditions of unequal rates of nucleotide substitution among lineages (branches), whether based on nucleotide sequences (Saitou and Imanishi 1989; Tateno *et al.* 1994) or amino acids (Hasegawa and Fujiwara 1993).

A recent simulation study by Huelsenbeck (1995) included 26 of the most commonly used tree-building algorithms and it was concluded that ML out-performed the other methods. Even when its assumptions were violated (e.g. rate heterogeneity among sites) it was robust in a large proportion of the parameter space; see Huelsenbeck (1995) for details and use of terminology. The newly developed LogDet transformation method seems superior to standard algorithms under some conditions (MP, ML and distance based methods) in being consistent for sequences with a nucleotide compositional bias (e.g. the low frequency of adenine in third codon positions of *Pseudoscourfieldia* and *Pycnococcus* compared to that of other primitive green algae, cf. Fig. 1b). It does not group sequences which irrespective of the evolutionary history of the organisms have obtained the same nucleotide composition (Lockhart *et al.* 1994). The results of the relative rate test and the ability of the LogDet transformation to effectively reconstruct phylogenies independent of a nucleotide bias were taken into account when selecting methods for phylogenetic analyses of the *rbc*L gene.

Implications from phylogenetic reconstructions of the primitive green algae

Third codon positions evolve more rapidly than first and second codon positions (Miyamoto *et al.* 1994) and they may cause a random phylogenetic signal (evolutionary noise) which can effect the tree topology. To study the effect of third codon positions phylogenetic analyses were performed both with and without this site. The ML inference of the primitive green algae presented in Fig. 3a is largely congruent with the classification shown in Table 1, while that based on all 1094 nucleotides deviates slightly (Fig. 2a). The classification scheme outlined in Table 1 is based mainly on Moestrup and Throndsen (1988) and Moestrup (1991). At present we are reluctant to suggest an updated classification of prasinophyte orders until their relationships have been further addressed in terms of analyses based on additional molecular markers and/or in combination with morphological data.

With respect to relationships and monophyly/non-monophyly in the two ML reconstructions, the main differences relate to the position of *Pterosperma cristatum* and thus the mono- or polyphyletic status of the family Halosphaeraceae, and the sister group relationship of the Mamiellaceae. Following the bootstrap analyses (Figs 2b and 3b) and the LogDet method (Fig. 4), decisive implications concerning relationships within families of the Prasinophyceae are not possible. However, the ML inferences prompt a number of new and interesting observations.

Mamiellaceae and relationship of *Micromonas pusilla*

Due to the simple scaly covering that characterizes the Mamiellaceae, this family is often considered the most primitive of the prasinophytes (Melkonian 1984, 1990; Moestrup 1990). However, the ML reconstructions suggest that these species are secondarily reduced forms. This implies that the underlayer of diamond-shaped scales on the flagella and cell body were secondarily lost and that *Bathycoccus* has lost both the pyrenoid and flagella. Although *Bathycoccus prasinos*, a non-motile picoplanktonic unicell, is the most ancestral taxon in the family, it probably does not qualify as the most primitive of all green plants as suggested by Moestrup (1990), who cited the classification of the green algae proposed by van den Hoek *et al.* (1988). The branching order of Mamiellales species is identical in the two phylogenetic approaches based on ML whereas it was not resolved in the LogDet analysis. Moestrup (1991) suggested that *Micromonas pusilla* represents a reduced form that is related to the Mamiellaceae, since most of the single emerging flagellum consists of only the central pair of microtubules, and *Micromonas* lacks body and flagellar scales entirely. In its pigmentation, *Micromonas* resembles *Mantoniella squamata*, *Mamiella gilva* (Parke et Rayns) Moestrup, *Pycnococcus provasolii* Guillard and *Pseudoscourfieldia marina* (Throndsen) Manton in having chl_a, chl_b, Mg DV and prasinoxanthin as the major xanthophyll (Guillard *et al.* 1991; Fawley 1992). However, uriolide, 'unknown A' and 'unidentified M1' are distinctive pigments of *Micromonas pusilla*, *Mantoniella squamata* and *Mamiella gilva* (Fawley 1992) separating these taxa from *Pseudoscourfieldia marina* and *Pycnococcus provasolii*. Hence, *rbc*L nucleotide sequence data and the observations on pigmentation mentioned above indicate that *Micromonas pusilla* is related to species of the Mamiellaceae, and it may perhaps be in-

cluded in this family (as shown in Table 1). When comparing the topology in Fig. 2a and Fig. 3a two different scenarios are suggested for the evolution of pigments in the Mamiellaceae and *Pycnococcus*/*Pseudoscourfieldia*. Using Fig. 2a it is suggested that uriolide, 'unknown A' and 'unidentified M1' pigments evolved in the ancestor to the Mamiellaceae whereas the topology in Fig. 3a leads one to propose that the three pigments were lost in the ancestor giving rise to the *Pycnococcus*/*Pseudoscourfieldia* clade, *Nephroselmis* spp. and the Halosphaeraceae.

Relationship between *Pycnococcus* and *Pseudoscourfieldia*

A cytoplasmic channel into the pyrenoid incorporating a lobe of the mitochondrion was thought to be a unique feature of *Pycnococcus provasolii* (Guillard *et al.* 1991). A very similar structure in *Pseudoscourfieldia marina* was described in detail by Moestrup and Throndsen (1988) and has been commented on by Fawley (1992) and Sym and Pienaar (1993). In *Pseudoscourfieldia* the pyrenoid matrix may be invaginated by up to three short branches. The existence of this shared feature, together with the similarities in pigmentation and the *rbc*L-based inferences (Figs 2a,b; 3a,b and 4), argue for a close relationship between *Pycnococcus* and *Pseudoscourfieldia* despite their very different morphology. *Pycnococcus provasolii* is a coccoid, picoplanktonic organism covered by a thin wall whereas *Pseudoscourfieldia marina* is a scaly biflagellate with two posterior, unequal flagella. Phylogenetic analysis of the D3 (divergent) domain and neighboring core region of the large subunit 23S-like ribosomal RNA (nuclear coded rDNA) also points to a common ancestry between *Pseudoscourfieldia* and *Pycnococcus* (Daugbjerg, unpubl. data). A HPLC analysis of pigment contents suggests the idea that *Pseudoscourfieldia marina* and *Pycnococcus provasolii* are closely related; the absorbance profiles are identical (Fawley 1992; fig. 1A and 1B). We therefore propose to include *Pseudoscourfieldia* with *Pycnococcus* in the family Pycnococcaceae Guillard (Guillard *et al.* 1991). There is a single observation of the monad stage of *Pycnococcus provasolii* (Guillard *et al.* 1991), and further studies are required to determine whether the single flagellum reported by these authors has the distinctive scaly covering of *Pseudoscourfieldia* in which rod-shaped 'double scales' cover the square or pentagonal underlayer scales.

Evolution of rod flagellar scales

The rod-shaped scales of *Pseudoscourfieldia* are also present on the flagella of species referred to *Nephroselmis* Stein, *Tetraselmis* Stein and *Scherffelia* Pascher (Chlorodendraceae *sensu* Moestrup and Throndsen 1988). If Fig. 2a is a true phylogenetic representation of the prasinophytes, the rod-shaped scales either evolved three times or were lost four times. If Fig. 3a is a closer approximation of phylogeny, the rod-shaped scales evolved only twice or they were lost three times. Figure 3a therefore presents the most parsimonious solution with respect to the flagellar rod scales.

Sequence divergence of species of *Tetralmis*, *Nephroselmis* and *Pyramimonas*

Norris *et al.* (1980) and Mattox and Stewart (1984) considered the genus *Prasinocladus* Kuckuck a synonym of *Tetraselmis*. Cells of both genera are covered by a periplast of fused scales and the reconstructions in Fig. 2a and Fig. 3a confirm that these flagellates are closely related and diverged early among the primitive green algae. The LogDet based analysis shown in Fig. 4 does not reveal the branching order among these taxa. The sequence divergence of 17% between the *rbc*L gene of *Tetraselmis aff. maculata* and *T. marina* (Cienk.) Norris, Hori et Chihara (formerly *Prasinocladus marinus*) is similar to that between the two species of *Nephroselmis* (16%, *N. olivacea* Stein and *N. minuta*), and only slightly higher than between the two species of *Pyramimonas* examined (11%, *P. orientalis* and *P. olivacea*).

Mono- or polyphyly of Halosphaeraceae

The polyphyletic origin of *Pterosperma*, *Cymbomonas* and *Pyramimonas* (family Halosphaeraceae) suggested by analysis of all 1094 nucleotides (Fig. 2a) is unexpected, since species referred to this family share a number of distinct morphological features thought to reflect a common origin. These include a transitional helix in the flagellar transition region, limulus-shaped scales arranged in nine longitudinal rows on the flagella, and a duct fibre associated with basal body 1 and flagellar root 1d (*sensu* Moestrup and Hori 1989). The monophyletic status of the Halosphaeraceae in Fig. 3a is more in keeping with our understanding from ultrastructural data but it was not revealed by the LogDet analysis (Fig. 4).

Inouye and Hori (1991) used high-speed video to study flagellar beat and swimming patterns in four genera of the Halosphaeraceae. They proposed that *Pyramimonas* (forward swimming and with biphasic cycle of the ciliary beat) is an advanced genus while *Pterosperma* (backward swimming with a unidirectional flagellar beat) is the most primitive. *Cymbomonas* and *Prasinopapilla* gen. ined. are intermediate (forward or backward swimming with flagellar beat). The phylogenetic implications posed by observations of swimming patterns could not be justified by analyses of the *rbc*L-gene.

Other phylogenetic studies of primitive green algae

Few morphological studies have focused on the phylogeny of the primitive green algae. Melkonian (1984) conducted a comparative study of the flagellar apparatus based on the transition region, the disposition of the microtubular roots and the direction of the effective stroke of the flagella. Melkonian's illustrations (1984, figs 33 and 34) display a network with no discrimination between primitive and derived states for the characters examined. This study has elements of both congruency and incongruency with the findings presented here. The main difference between Melkonian's fig.

34 and Fig. 2a, 3a of the present paper relates to the position of *Mesostigma* and *Pyramimonas*. Figure 2a and 3a shows *Mesostigma* to be more closely related to *Tetraselmis* than to *Pyramimonas*. In addition *Pyramimonas* does not appear to be related to *Nephroselmis* and *Tetraselmis* via *Mamiella* as suggested by Melkonian (1984).

In a comprehensive review of the Prasinophyceae, Sym and Pienaar (1993) considered the morphology of two possible ancestral green flagellates (AGF). Their scenarios, by admission, are highly speculative and the ancestral green flagellate is thought to be (i) a backward swimming, scaly, asymmetrical biflagellated cell with two roots on basal body 1 and a single multilayered structure; or (ii) a backward swimming scaly quadriflagellated cell with cruciate roots and a large 1d root with a multilayered structure. The tree topology varies accordingly and either doubling or halving in the number of flagella took place in the evolutionary schemes suggested by Sym and Pienaar (1993, figs 26, 28). The *rbc*L-based phylogeny presented in the present paper and the tree based on 18S rRNA (figs 1 and 2 in Steinkötter *et al.* 1994) both suggest that the AGT was biflagellated. The molecular phylogenies thus do not support the idea that the AGF had four flagella as proposed by O'Kelly (1992) and discussed by Sym and Pienaar (1993).

In 1990 Kantz *et al.* published a study based on nuclear-encoded ribosomal RNA sequences. Some information relevant to the tree topology and discussion presented by these authors has subsequently become apparent. In particular, the culture of *Pedinomonas minutissima* Skuja (CCMP VA3) sequenced by Kantz *et al.* has been studied ultrastructurally and was found to be a member of the Chlorarachniophyceae (Moestrup, unpubl. data 1993): the 18S rRNA sequence of *Pyramimonas parkeae* Norris et Pearson (UTEX 2287) used by Kantz *et al.* differs markedly from that of another culture of *P. parkeae* sequenced by Inouye and co-workers (Inouye, pers. comm. 1993). These findings influence the tree topology and thus the phylogenetic conclusions presented by Kantz *et al.* (1990). Steinkötter *et al.* (1994) compared homologous sequences of complete 18S rRNA from four genera of prasinophytes (*Pseudoscourfieldia, Nephroselmis, Tetraselmis* and *Scherffelia*) with those from other green algae (including Chlorophyceae, Ulvophyceae, Pleurastrophyceae and Charophyceae) and some land plants. Steinkötter *et al.* concluded that the Prasinophyceae (*sensu* Moestrup and Throndsen 1988) is polyphyletic and that *Pseudoscourfieldia marina* and *Nephroselmis olivaceae* represent the earliest diverging clade of species included in their investigation. The deep branching of *P. marina* and *N. olivaceae* seen in the 18S rRNA tree is not supported by the branching pattern based on the rbcL data matrix. In the latter, *Tetraselmis* branches off before *P. marina* and *N. olivaceae*. Future studies will have to address this topological inconsistency as well as others and it serves to illustrate that gene trees do not necessarily depict the phylogeny of the organisms. Rather we may expect differences when comparing genealogies because they reflect different genes subjected to different selective constraints.

CONCLUSIONS

Surely the fossil record has proved of little direct use because of the size and fragile nature of these single-celled flagellates. The origin of the green plants remains enigmatic, since an extant heterotrophic flagellate resembling a prasinophyte has yet to be discovered. This study presents a molecular phylogeny of most genera of prasinophytes and pedinophytes and the *rbc*L nucleotide data matrix may be used in future studies including advanced taxa of green algae (Chlorophyceae, Ulvophyceae, Pleurastophyceae, Trentepholiophyceae and Charophyceae) and land plants. Such an investigation will also have to address in more detail whether the pedinophytes represent a blind evolutionary lineage, their ancestors not leading to more advanced green plants as suggested by Moestrup (1991). Eventually, studies on genes coding for *rbc*L, 18S rRNA, etc. should be combined with the evolutionary trees obtained from ultrastructural studies, to present a phylogeny which may be more reliable than any tree based on results from any single set of data only.

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