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PHYLOGENY OF THE GENUS *PYRAMIMONAS* (PRASINOPHYCEAE, CHLOROPHYTA) INFERRED FROM THE *rbc*L GENE[1]

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ABSTRACT

*A 1089-basepair fragment (approx. 75%) of the large subunit of the chloroplast-encoded gene, ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L), was sequenced from 16 species of the genus* Pyramimonas *Schmarda. Electron microscopic and biochemical studies of* Pyramimonas, *one of the most morphologically diverse genera within the potential sister groups to the chlorophyll* a– *and* b–*containing plants, suggest that this genus consists of at least four separate subgenera. Using the homologous sequence of* rbc*L from* Cymbomonas tetramitiformis *Schiller (Halosphaeraceae) as an outgroup and applying the maximum likelihood method, we show that the inferred topology is congruent with traditional delimitations of the taxa based on observations of periplast, internal ultrastructure, and biochemical features. A bootstrap analysis also supports division at the subgeneric level; however, the low bootstrap support associated with the deep nodes precludes resolution of these branches. A maximum likelihood relative rate test revealed that the* rbc*L gene in these single-celled green flagellates has a heterogeneous rate of substitution. The* rbc*L gene in species of the subgenus* Pyramimonas *has evolved at an accelerated rate relative to that of congenerics.*

Key index words: maximum likelihood; molecular phylogeny; PCR (polymerase chain reaction); Prasinophyceae; Pyramimonas; *relative rate test; Rubisco large subunit.*

Compared to multicellular plants, unicells generally have fewer morphological characters suitable for phylogenetic analysis. This is particularly true for closely related species and may partly explain the relative rarity of studies that have used morphological data to reveal protist relationships. However, recent developments in molecular biology introduce new possibilities for reconstructing the phylogeny of protists and other groups of organisms.

The large subunit of the chloroplast-encoded gene, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, *rbc*L), is often used in phylogenetic studies of plants. However, most of these studies have focused on relationships among the flowering plants (e.g. Doebley et al. 1990, Soltis et al. 1990, Donoghue et al. 1992, Gaut et al. 1992, Olmstead et al. 1992, Chase et al. 1993). To date, the *rbc*L gene has been sequenced in fewer than 20 taxa of cyanobacteria, prochlorophyceans, and single-celled, eukaryotic algae; in contrast, approximately 500 sequences of *rbc*L exist for the angiosperms (Clegg 1993). A list of species for which *rbc*L sequences exist is presented in Chase et al. (1993).

Phycologists generally consider the Prasinophyceae to be a potential sister group to other green algae and the land plants (see e.g. Stewart and Mattox 1978, Norris 1980, Melkonian 1990). Based on complete nuclear-encoded small-subunit ribosomal RNA, Steinkötter et al. (1994) found that the prasinophytes *Nephroselmis* Stein and *Pseudoscourfieldia* Manton branch deeply within the lineage of green plants. *Pyramimonas* represents the largest and one of the most morphologically diverse genera within the class Prasinophyceae. The genus is considered to be of monophyletic origin because of the inverse pyramidal to globular shape of the cell, the cupulate chloroplast with anterior lobes, the composition of the periplast with box and crown scales, and the presence of a scale reservoir. Since Schmarda's description of the genus in 1850, 32 species of *Pyramimonas* have been studied by electron microscopy (Hori et al., unpubl.). According to Moestrup and Hill (1991: table 2) another 13 marine or brackish water species are known from light microscopical observations. However, these species are inadequately described, and more information is needed before their taxonomic status can be reliably assessed.

Diversity within the genus is particularly reflected

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TABLE 1. *List of* Pyramimonas *species included in the phylogenetic analysis. Each species is appointed to its subgenus according to morphological and biochemical features (McFadden et al. 1986, 1987); the only exception is* Pyramimonas *sp. (Moestrup, unpubl.). Strain numbers refer to catalogue numbers of the Scandinavian Culture Centre of Algae and Protozoa. Genbank accession numbers are also given.*

Subgenus	Species	Strain no.	Accession no.
Vestigifera McFadden	*P. cyclotreta* Daugbjerg	K-0398	L34814
	P. mantoniae Moestrup & Hill	K-0254	L34810
	P. mitra Moestrup et Hill	K-0241	L34812
	P. moestrupii McFadden	K-0265	L34811
	P. orientalis McFadden	K-0003	L34813
	P. 'tychotreta' ined. Daugbjerg		L34778
	P. 'greenland' ined. Daugbjerg		L34818
Pyramimonas McFadden	*P. cyrtoptera* Daugbjerg		L34819
	P. octopus Moestrup et Aa. Kristiansen	K-0001	L34817
	P. propulsa Moestrup et Hill	K-0005	L34777
	P. tetrarhyncus Schmarda	K-0002	L34833
Trichocystis McFadden	*P. cirolanae* Pennick	K-0255	L34776
	P. grossii Parke	K-0253	L34779
	P. parkeae Norris et Pearson	K-0006	L34816
Punctatae McFadden	*P. olivacea* N. Carter emend. McFadden		L34815
Incertae sedis	*Pyramimonas* sp.		L34834
Outgroup	*Cymbomonas tetramitiformis*	K-0467	L34687

in the flagellar apparatus, the pyrenoid, and the arrangement and morphology of the nonmineralized organic scales covering both the cell body and flagella. These features are important in species delimitation. A number of publications have drawn attention to the existence of closely related groups (Melkonian and Robenek 1981, 1984, Pennick and Cann 1982, Pennick 1984, Inouye et al. 1985). McFadden et al. (1986, 1987) divided the genus into four subgenera. This division is based on scale morphology (up to seven different types), internal ultrastructure (e.g. flagella apparatus, pyrenoid, eyespots), and biochemical features (e.g. accessory pigments).

The objective of the present study is to reconstruct the phylogeny of the genus *Pyramimonas* based on analysis of a 1089-basepair fragment of the *rbc*L gene and to discuss the congruence between the phylogenetic information, the morphological and biochemical features currently used to divide the genus, and the present geographic distribution of polar taxa.

MATERIALS AND METHODS

Clonal cultures of *Pyramimonas* (not bacteria-free) were obtained from the Scandinavian Culture Centre of Algae and Protozoa (Department of Phycology, University of Copenhagen) or were isolated by ND or ØM. Two polar species of *Pyramimonas* (*P. 'tychotreta'* sp. ined. from the Weddell Sea, Antarctica, and *P. 'greenland'* sp. ined. from Disko Bay, West Greenland) and one species from Australia (*Pyramimonas* sp.) are undescribed. A 818-basepair fragment of the *rbc*L gene was sequenced from *Pyramimonas quadrifolia* Daugbjerg from Northern Foxe Basin (Arctic Canada) and was included in a separate phylogeny (not shown). Cultures were incubated at either 4° or 15° C and a 16:8 h LD cycle. A 10-mL sample of cells (approx. 1000 cells·mL^{-1}) in the exponential phase of growth was used for DNA extraction. Table 1 lists the taxa included as well as strain numbers.

TABLE 2. *Primers used for amplifications and sequencing.*

Primer code	Sequence (5′–3′)
RH-1S	ATG TCA CCA CAA ACA GAA ACT
Py31	GC TGG CTT CAA AGC TGG TGT
Ce127	ACT CCT CAA CCA GGT GTT CC
Hv362R	TGA ACC CAA ATA CGT TAC CCA
Ce622	TCA CAA CCA TTT ATG CGT TG
Ce800R	TGC ATA ATA ATA GGT ACA CC
Cym821R	AAA CCA CAA GTT AGG TAG TC
Ce1161R	CAT GTG CAA TAC GTG AAT ACC

DNA extraction, amplification, and sequencing. Prior to DNA extractions, each clonal culture was pelleted (1500–2000 rpm) and transferred to an Eppendorf tube and frozen at −80° C for approximately 24 h. The following day, 300 μL preheated 2X-CTAB isolation buffer was added to the thawed pellet. Extraction of DNA was done according to Doyle and Doyle (1987). Total cellular DNA was used for amplifications in 50-μL reaction volumes (67 mM Tris-HCl, pH 8.8, 2 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 200 μM dNTP, 0.5 μM of each primer, and 0.4 units of Boehringer-Mannheim Taq DNA polymerase). Double-stranded PCR products of 1161 basepairs were obtained by terminal primers RH-1S and Cen1161. Using this long fragment as a template, smaller fragments were obtained using internal primers. Primers used for amplifications and sequencing of the *rbc*L gene are listed in Table 2. R after a primer name indicates that the primer will sequence toward the 5′ end of *rbc*L. Numbers in the primer code refer to the position in the *rbc*L gene.

Single-stranded PCR products of about 500–600 basepairs were synthesized in asymmetric amplifications by diluting the limiting primer 100-fold. Conditions for balanced and unbalanced enzymatic amplifications were one initial cycle of denaturation at 94° C for 3 min, followed by 30 cycles of denaturation at 94° C for 1 min, annealing at 50° or 52° C for 1 min, and extension at 72° C for 2–3 min. Single-stranded products were concentrated using Millipore Ultrafree-MC filters (No. UFC3TTK00) and were resuspended in 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 the Sequenase 2.0 enzyme (United States Biochemical).

Alignment and phylogenetic analyses. No insertions/deletions were detected in the *rbc*L coding regions, and the sequences could be

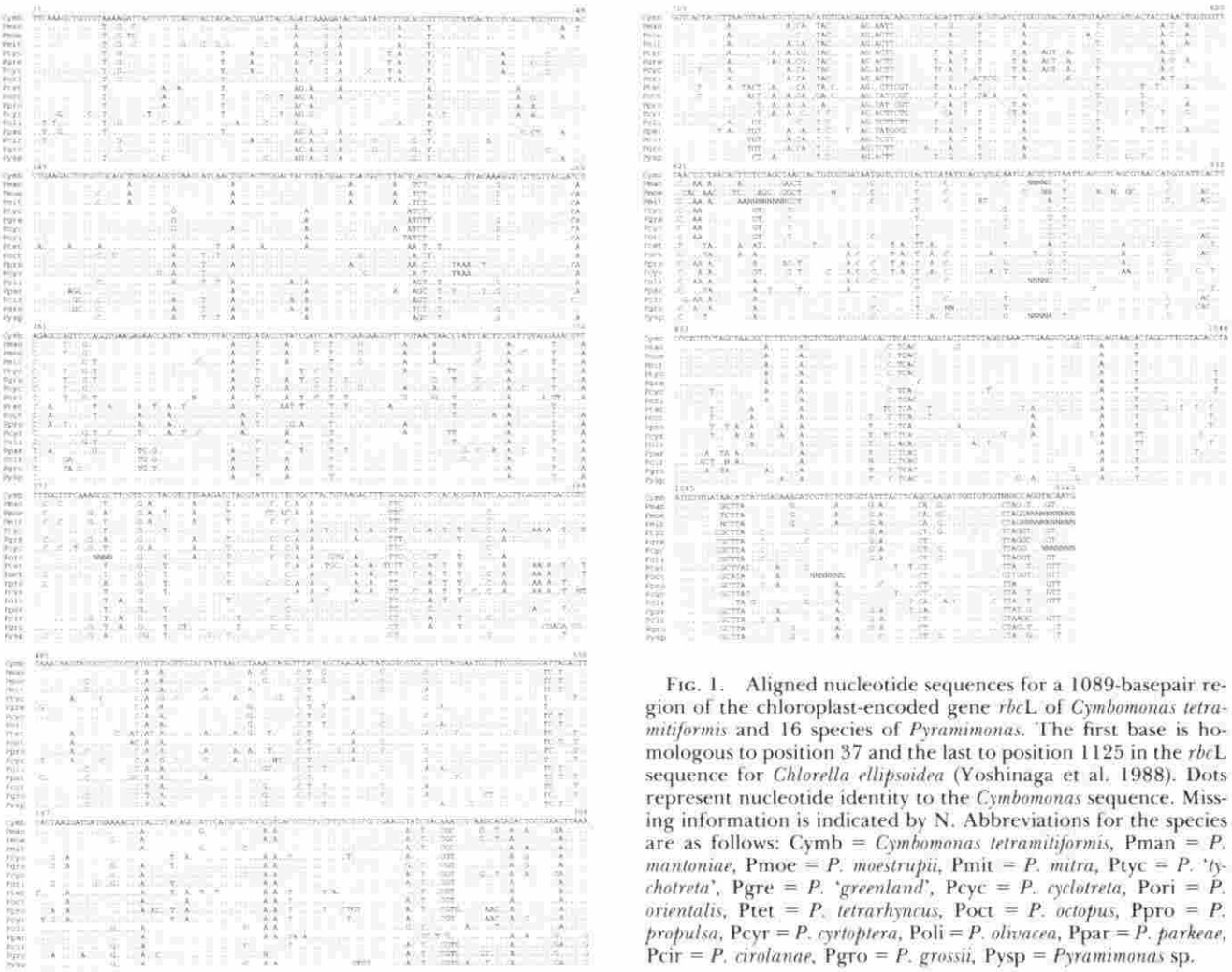

FIG. 1. Aligned nucleotide sequences for a 1089-basepair region of the chloroplast-encoded gene *rbc*L of *Cymbomonas tetramitiformis* and 16 species of *Pyramimonas*. The first base is homologous to position 37 and the last to position 1125 in the *rbc*L sequence for *Chlorella ellipsoidea* (Yoshinaga et al. 1988). Dots represent nucleotide identity to the *Cymbomonas* sequence. Missing information is indicated by N. Abbreviations for the species are as follows: Cymb = *Cymbomonas tetramitiformis*, Pman = *P. mantoniae*, Pmoe = *P. moestrupii*, Pmit = *P. mitra*, Ptyc = *P. 'tychotreta'*, Pgre = *P. 'greenland'*, Pcyc = *P. cyclotreta*, Pori = *P. orientalis*, Ptet = *P. tetrarhyncus*, Poct = *P. octopus*, Ppro = *P. propulsa*, Pcyr = *P. cyrtoptera*, Poli = *P. olivacea*, Ppar = *P. parkeae*, Pcir = *P. cirolanae*, Pgro = *P. grossii*, Pysp = *Pyramimonas* sp.

aligned unambiguously by eye using the computer programs ESEE (ver. 1.09d, Cabot) and CS3 (Siegismund, unpubl.). Computer simulations have shown that the maximum parsimony method may fail to reconstruct the correct (model) tree when the substitution rate of the gene varies significantly among the species studied (e.g. Saitou and Imanishi 1989, Kuhner and Felsenstein 1994, Tateno et al. 1994). A two-parameter maximum likelihood relative rate test (Muse and Weir 1992) was applied to test for unequal rates of substitutions within our *rbc*L sequence data. In each of the 120 pairwise comparisons, two sequences were tested for departures from rate equivalence; the sequence from a third taxon functioned as an outgroup. In all tests, *Cymbomonas tetramitiformis* was used as the outgroup. The likelihood ratio test is χ^2-distributed with 2 degrees of freedom ($P(\chi^2 > 5.99) < 0.05$). Due to the results of the relative rate tests, phylogenetic reconstructions were estimated by the maximum likelihood method, which has been shown to be robust against heterogeneity of substitution rates (Sourdis and Nei 1988, Saitou and Imanishi 1989, Hasegawa et al. 1991, Tateno et al. 1994). Log likelihood scores were obtained using fastDNAml (Gary Olsen et al., ver. 1.0.6, unpubl.). To find the optimal tree, searches were repeated by varying the transition-to-transversion ratio until the best log likelihood score was reached at least three times. One hundred bootstraps were conducted in order to obtain approximate confidence limits of the branching pattern.

The outgroup. Cymbomonas is considered a close relative of the genus *Pyramimonas* (Moestrup, unpubl.) and according to traditional taxonomic considerations, the two genera are referred to the family Halosphaeraceae. This family within the Prasinophyceae possesses unique features such as nine rows of limuloid-shaped flagellar scales, a cell body tightly covered by box-shaped scales, and a transitional helix ("coiled fiber") positioned in the proximal part of each flagellum.

Sequence availability. The *rbc*L nucleotide sequences have been submitted to Genbank, and accession numbers are given in Table 1.

RESULTS

The alignment of the 1089-basepairs of the *rbc*L gene for the 16 species of *Pyramimonas* and of *Cymbomonas tetramitiformis* (Fig. 1) indicate that most of the sequence divergence (72%) is due to third position substitutions. First and second position substitutions account for 20.1 and 7.9%, respectively. The distribution of nucleotides expressed as percentages at first and second codon positions (Fig. 2A) shows that there is no bias in base composition of the 17 taxa since all four nucleotides occur with approximately the same frequency. The distribution of third codon positions (Fig. 2B) shows a marked bias toward thymine (ranging from 43.3 to 55.7%). The sum of T and A ranges from 67.0% in *P. moes-*

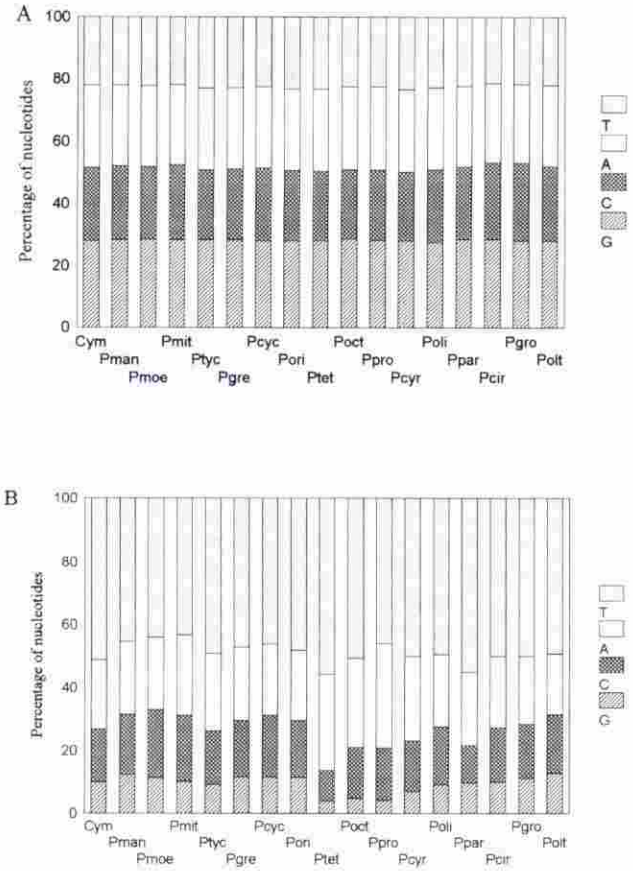

FIG. 2. Base composition in percentage of *rbc*L sequences at A) first and second codon positions and B) third codon positions. In the 17 species sequenced, the four nucleotides have almost the same frequency of occurrence in first and second codon positions, whereas there is a distinct bias toward thymine at third codon positions. See Figure 1 for abbreviations of species.

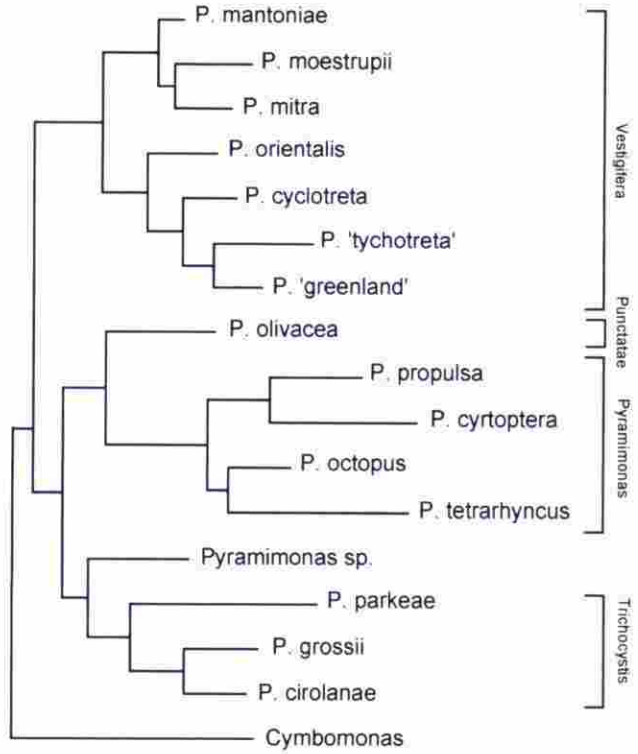

FIG. 3. Phylogenetic inference of 16 species of *Pyramimonas* inferred from maximum likelihood analysis of a 1089-basepair fragment of the *rbc*L gene. Subgenera as defined from morphological studies are indicated by brackets. The sequence of *Cymbomonas* was used to root the tree. The branch lengths are proportional to the number of character changes.

trupii McFadden to 86.5% in *P. tetrarhyncus* Schmarda. The ratio of transitions (Ts) to transversions (Tv) is very close to parity. The ratio of Ts to Tv in third codon positions is 1:1. Thus, the *rbc*L gene in these green algae does not exhibit a transition bias compared to the Ts/Tv ratio of 1.3:1 observed in higher plants (Chase et al. 1993). In second codon positions, 68.5% of the substitutions are transversions, whereas 57.8% of the substitutions in first codon positions are transitions. The total number of segregating sites is 416, of which more than half (66%) occur in third codon positions.

Maximum likelihood analysis. Using *Cymbomonas* as the outgroup, the best log likelihood score was −6667.15. The tree topology (Fig. 3) supports the monophyletic status of the subgenera of *Pyramimonas* (Table 1). Using the prasinophyte *Mesostigma viride* as the outgroup, *Cymbomonas* formed a sister group to the species of *Pyramimonas*, and the maximum likelihood inference did not alter the topology of the *Pyramimonas* ingroup (reconstruction not shown). *Pyramimonas* sp. and the species belonging to the subgenera *Pyramimonas*, *Punctatae*, and *Trichocystis* share a common ancestor separate from species of the subgenus *Vestigifera*. *Pyramimonas* sp. is the sister group of the *Trichocystis* species, and these are the sister groups to the *Punctatae/Pyramimonas* cluster.

Of the seven species of *Vestigifera* sequenced, the cold-water species *P. cyclotreta* Daugbjerg, *P. 'tychotreta'*, and *P. 'greenland'* form a sister group to *P. orientalis* McFadden, a cosmopolitan species. These two clades are the sister group to *P. mantoniae* Moestrup et Hill, *P. moestrupii*, and *P. mitra* Moestrup et Hill, species from temperate waters.

Including the 818-basepair fragment of the *rbc*L gene from *P. quadrifolia*, a maximum likelihood analysis showed this taxon to cluster with the other cold-water species of the subgenus *Vestigifera* cluster, though no bootstrap analysis for this relationship is provided. *Pyramimonas quadrifolia* formed a sister group with the *P. cyclotreta/P. 'tychotreta'/P. 'greenland'* cluster. Both *Pyramimonas cyclotreta* and *P. quadrifolia* were isolated from the same water samples collected in the Northern Foxe Basin, Arctic Canada (Daugbjerg and Moestrup 1992a, 1993).

The 50% majority-rule consensus tree of the 100 bootstraps (Fig. 4) shows that the branching order of the deep nodes is not well supported (bootstrap proportions below 50%). However, all species of *Pyramimonas* are still included in the subgenus in which they are traditionally placed based on morphological

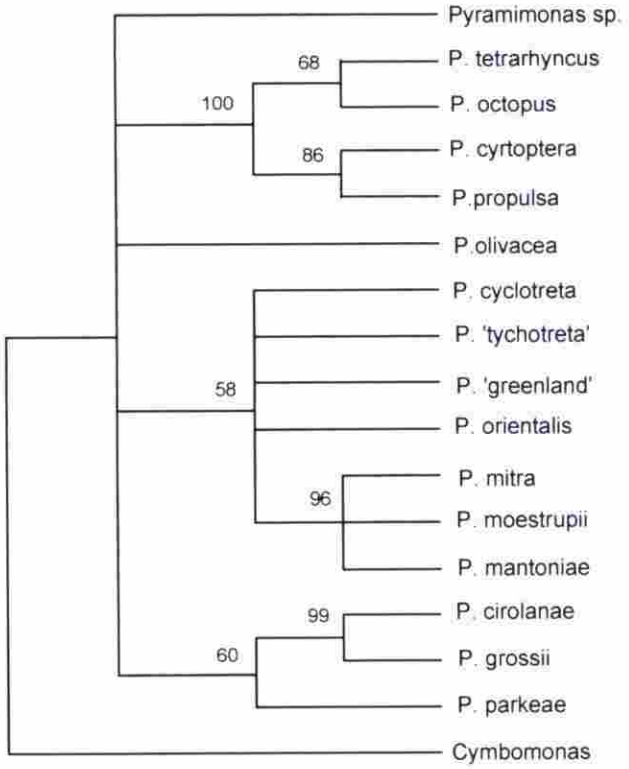

FIG. 4. Majority-rule (50%) consensus tree of a bootstrap analysis based on maximum likelihood. *Cymbomonas* was used to root the tree. Numbers associated with internal branches indicate the number of times a branch was recovered in 100 bootstrap replications.

and biochemical characteristics. In particular, the subgenus *Pyramimonas* is well supported, with a bootstrap proportion of 100%. The *Vestigifera* and *Trichocystis* clades occur 58 and 60 times, respectively, in the 100 bootstrap replicates. The *Pyramimonas* species from temperate waters have a distinct position in the subgenus *Vestigifera*, the clade having a bootstrap proportion of 96%. The two terminal clades in the subgenus *Pyramimonas* are also fairly well supported in terms of bootstrap proportions (the *P. cyrtoptera* Daugbjerg/*P. propulsa* Moestrup et Hill cluster by 86% and *P. tetrarhyncus*/*P. octopus* Moestrup et Aa. Kristiansen cluster by 68%).

Employing the neighbor-joining method (Kimura's two-parameter model) included in the PHYLIP package (Felsenstein 1993) resulted in a tree with the same topology as that given by maximum likelihood except for minor differences in the position of the *Vestigifera* species *P. cyclotreta* and *P. mitra* (not shown).

Testing for equality of evolutionary rates. In the maximum likelihood relative rate tests including all nucleotides, 35 of 120 (29%) pairwise comparisons showed a heterogeneous rate of nucleotide substitution (Fig. 5, above diagonal). The heterogeneity of substitution rates is observed between the lineage containing the subgenus *Pyramimonas* and the other major lineages of *Pyramimonas*. Hence, in species belonging to the subgenus *Pyramimonas*, the *rbc*L locus has evolved at a different rate relative to the

	Pcyc	Pman	Pmit	Pmoe	Pori	Ptyc	Pgre	Pcyr	Poct	Ppro	Ptet	Pcir	Pgro	Ppar	Poli	Pysp
Pcyc	-	3.37	0.92	1.95	1.58	0.53	0.82	7.21	0.99	4.29	10.15	0.96	0.01	0.28	2.66	3.43
Pman	2.78	-	0.95	3.92	0.96	3.51	2.2	15.21	4.37	11.23	18.4	1.87	1.57	2.03	0.49	2.58
Pmit	0.93	4.23	-	1.41	0.55	1.6	0.71	11.63	3.79	8.32	14.83	1.09	0.78	1.59	0.59	2.09
Pmoe	0.021	4.56	1.03	-	0.42	2.45	3.31	9.42	3.38	6.04	11.72	2.42	1.13	1.62	3.49	5.64
Pori	0.55	2.86	0.22	0.62	-	4.21	3.17	12.08	4.71	7.45	15.95	1.62	0.19	1.38	1.91	3.96
Ptyc	0.87	4.34	1.08	1.29	0.93	-	1.71	9.35	0.39	3.75	10.57	1.38	0.66	0.11	3.83	3.8
Pgre	0.81	1.03	0.037	0.92	0.47	2.35	-	9.96	1.91	6.78	13.44	0.1	0.49	0.23	1.97	1.57
Pcyr	8.82	16.34	10.38	6.2	9.93	11.53	13.29	-	5.62	2.86	0.37	11.12	8.43	7.19	18.93	17.42
Poct	1.99	4.78	3.12	0.73	1.87	0.32	2.73	8.13	-	2.58	11.45	3.23	1.38	0.73	7.26	6.96
Ppro	5.96	13.78	8.54	4.34	6.59	4.38	8.8	1.07	3.31	-	3.51	7.61	4.74	4.2	14.35	15.68
Ptet	10.63	17.56	12.51	7.54	11.38	9.19	12.41	1.1	9.34	1.49	-	15.08	11.5	10.23	25.11	21.6
Pcir	2.97	2.76	1.02	1.36	0.47	1.43	0.62	11.08	3.55	7.76	14.08	-	2.55	0.52	2.23	1.53
Pgro	2.66	5.22	1.77	1.17	0.34	0.42	1.33	7.96	1	4.72	9.4	5.21	-	1.02	2.03	3.81
Ppar	1.97	3.39	1.94	0.65	0.27	0.2	0.92	7.45	0.82	4.48	10.33	0.69	0.33	-	2.83	3.13
Poli	2.74	0.45	2.19	4.4	3.97	5.71	2.29	22.79	10.93	19.17	24.34	4.9	5.21	5.1	-	2.04
Pysp	5.83	4.27	2.19	4.29	2.75	3.74	2.61	17.18	7.04	14.8	20.72	7.5	3.37	3	7.5	-

FIG. 5. Relative rate tests. Results of the two-parameter maximum likelihood test are given above the diagonal, and the results of Tajima's two-dimensional model are given below the diagonal. The test statistics are χ^2-distributed with 2 degrees of freedom. Shaded cells show significant values.

other lineages, although *P. octopus* deviates slightly from this pattern. The branch lengths from the maximum likelihood estimate show that species of subgenus *Pyramimonas* have evolved at an accelerated rate relative to congenerics.

Applying the two-dimensional statistical model of Tajima (1993) in testing for a molecular evolutionary clock gave almost identical results, with 37 of 120 (31%) significant hits at the 5% level (Fig. 5, below diagonal). Only two of 35 significant comparisons differed between the two rate models. The two additional significant results found by the statistical model of Tajima are the comparisons of *Pyramimonas* sp. with *P. olivacea* N. Carter emend McFadden and *Pyramimonas* sp. with *P. cirolanae* Pennick. In both models, the chi-square estimates are of similar values (cf. Fig. 5).

DISCUSSION

The *rbc*L-based phylogenetic reconstruction (Fig. 3) and the taxonomic groupings based on morphological and biochemical characteristics of the genus *Pyramimonas* (Table 1) are congruent. Although the relatively high bootstrap proportions support the taxonomic groups of *Pyramimonas*, the bootstrap analysis provides no conclusive evidence of the branching order among the subgenera; the deep nodes form a bush in the 50% majority-rule consensus tree (Fig. 4).

Hillis and Bull (1993) conducted computer simulations under a wide range of conditions (e.g. differences in mutation rate, the symmetry of the tree topology) as well as with data for species with known phylogenetic relationships. They suggested that bootstrap proportions represent conservative estimates of true clades when the bootstrap proportions are above 50%. The bootstrap proportions mapped on the branches in Figure 4 may therefore underestimate the potential phylogenetic signal in this *rbc*L data set.

The preceding analyses show that species assigned to the subgenus *Pyramimonas* are closely related. Species assigned to this subgenus also share a characteristic swimming behavior and habitat type (benthic or semibenthic) rarely encountered elsewhere in the genus. Light microscopical observations show that this group of *Pyramimonas* settle quickly to a surface (Belcher 1969, Gardiner 1980, Moestrup et al. 1987, Moestrup and Hill 1991, Daugbjerg and Moestrup 1992b), whereas other species swim for longer periods before settling, or they settle rarely. Five species have been referred to the subgenus *Pyramimonas* (only *P. amylifera* Conrad was not included in the present study). *Pyramimonas tetrarhyncus* (freshwater) possesses 4 flagella, unlike the four marine species that have either 8 or 16, a remarkable number of flagella for autotrophic nanoflagellates. The larger number of flagella may be an adaptation for living in a benthic or semibenthic environment, the typical habitat for species of this subgenus. More flagella increase the surface area, which may provide better adherence to sand grains (*P. octopus*, *P. tetrarhyncus*, and *P. amylifera*) or to an ice-layer (*P. cyrtoptera*). In this context it should be noted that species of the subgenus *Punctatae* (*P. olivacea* and *P. mucifera* Sym et Pienaar) are also primarily benthic. However, both of these monads are quadriflagellate. The benthic or semibenthic species of *Pyramimonas* share a similar type of swimming behavior, and it may be that habitat type is determined by swimming behavior. The type of habitat correlates well with the topology shown in Figure 3 and provides support for a relationship between the subgenus *Punctatae* and the subgenus *Pyramimonas*. The phylogenetic reconstruction indicates that the primitive type of habitat is presumably planktonic.

In their revision of the genus *Pyramimonas*, McFadden et al. (1986, 1987) examined the photosynthetic pigments in a number of species. They showed that both *P. propulsa* (as *P. amylifera* in McFadden et al. 1986) and *P. olivacea* possess siphonein rather than lutein as their accessory xanthophyll. The sharing of siphonein suggests a phylogenetic relationship between the two taxa as indicated by the maximum likelihood tree (Fig. 3). Though the accessory xanthophyll has not been determined for *P. cyrtoptera*, *P. tetrarhyncus*, *P. octopus*, and *P. punctatae*, it appears likely that the common ancestor to the subgenera *Pyramimonas* and *Punctatae* possessed siphonein, thus indicating a monophyletic origin of this photosynthetic pigment. Other species of *Pyramimonas* included in the analysis possess lutein, except for *P. mantoniae*, *P. mitra*, *P. cyclotreta*, *P.* '*tychotreta*', *P.* '*greenland*', and *Pyramimonas* sp., in which the xanthophyll has not been determined.

In a comparative study of the eyespot in species of *Pyramimonas*, Hori et al. (unpubl.) have shown that species referred to either *Trichocystis* or *Punctatae* possess the same type of eyespot/nucleus arrangement in the cell. According to these authors, the only distinguishable morphological feature separating these two groups is the presence of trichocysts in the *Trichocystis* group. However, the bilayered eyespot is intervened by a thylakoid in *P. olivacea* (*Punctatae*), whereas such an arrangement is not seen in *Trichocystis* species. The tree topology (Fig. 3) does not support a close relationship between the *Trichocystis* and *Punctatae* subgenera. Additional studies are needed to determine whether the two subgenera should be kept separate or united. In the case of combining the two, subgenus *Trichocystis* has priority.

The flagellar apparatus is important for the systematics and phylogeny of flagellates, and it has formed the basis for comparative studies of green algae (Mattox and Stewart 1984, Melkonian 1984, O'Kelly and Floyd 1984, Inouye et al. 1990, Sym and Pienaar 1991). Ultrastructural studies of the absolute configuration of the flagellar apparatus in

Pyramimonas, *Halosphaera* Schmitz, and *Pterosperma* Pouchet (Halosphaeraceae) led Sym and Pienaar (1991) to suggest that the subgenera *Pyramimonas*, *Punctatae*, and *Trichocystis* should be considered primitive and that the subgenus *Vestigifera* should be considered derived. The maximum likelihood reconstruction proposes a relationship among *Pyramimonas*, *Punctatae*, and *Trichocystis*, which share the 3-1 type of configuration, basal bodies interconnected by fibrillar bands, and a duct fiber associated with basal body number 1 and the 1d microtubular root. With respect to the flagellar configuration, the topology merely shows two separate lineages (the *Punctatae/Pyramimonas/Trichocystis* cluster and the *Vestigifera* cluster with a diamond configuration of the basal bodies), which separated early in the evolution of the genus *Pyramimonas*. Thus, the *rbc*L-based reconstruction provides no conclusive phylogenetic evidence for the primitive or derived condition of the type of flagellar configuration.

The bootstrap analysis (Fig. 4) shows the position of the undescribed species *Pyramimonas* sp. to be unresolved. This taxon has a number of features unique in the genus. Probably the two most distinctive traits are the rhizoplast/microbody complex and the morphology of the pyrenoid. The rhizoplast/microbody complex continues from the proximal part of the basal bodies to the posterior part of the cell in close association with the pyrenoid. The pyrenoid is not encircled by starch grains as is typical for species of *Pyramimonas* (see e.g. Inouye et al. 1985), and the pyrenoid matrix appears very diffuse. The minute underlayer body scales may represent a new type, but further study is required. To accommodate the morphological and molecular observations presented here, a new subgenus may have to be established for this taxon, which is known only from Australian waters (Moestrup, unpubl.).

Though the species of *Pyramimonas* included in this study account for most of the diversity within the genus, it would be interesting to include a few additional species in any future phylogenetic study. To date, the phylogenetic position of *Pyramimonas longicauda* van der Meel and *P. virginica* Pennick is not well understood. Based on the configuration of the flagellar apparatus, the missing fibrillar bands interconnecting the basal bodies, and the eyespot arrangement, *P. longicauda* is probably related to species of *Pyramimonas* referred to the subgenus *Vestigifera* with the rhombic type and the group III nucleus and eyespot arrangement (Hori et al., unpubl.). *Pyramimonas longicauda* is distinct from the species of *Vestigifera*, as it does not have footprint scales, the cell body is fully covered by underlayer scales, and it has a separate type of pyrenoid (Inouye et al. 1984). Hori et al. (unpubl.) have found these morphological differences to *Vestigifera* species significant to warrant a separate subgenus for *P. longicauda* (*Longicauda*). *Pyramimonas virginica*, the only known ultraplanktonic species in the genus, possesses trichocysts that are indistinguishable from the trichocysts observed in the subgenus *Trichocystis*. The arrangement of the basal bodies, the structure of the flagellar roots, the position of the eyespot, and the structure of the pyrenoid sets *P. virginica* apart from other known species of *Pyramimonas*, and a separate subgenus (Virginica) will be suggested for this species (Hori et al., unpubl.). *Pyramimonas mucifera* is unusual in having a planktonic and a benthic stage. The morphology of the planktonic stage resembles that of other species of *Pyramimonas*, but the benthic form more closely resembles the nonprasinophycean genera *Hafniomonas* Ettl et Moestrup and *Oltmannsiellopsis* Chihara et Inouye in being loosely packed in mucilage (Sym and Pienaar 1991). Though these authors suggest that *P. mucifera* is probably a member of the subgenus *Punctatae*, there are a number of differences between *P. olivacea* and *P. mucifera* that may indicate a divergent relationship (e.g. two types of underlayer body scales, the number of starch grains surrounding the pyrenoid, the anterior and posterior invasions of thylakoids into the pyrenoid, intervening of thylakoids between the bilayered eyespots, the muciferous vesicles).

The two methods employed in testing for equality of evolutionary rates gave almost identical results, thus arguing for the existence of different rates of nucleotide substitution of the *rbc*L locus in the genus *Pyramimonas*. Heterogeneous rates have also been noted in grasses, a major monocot lineage (Gaut et al. 1992), and it appears likely that different rates of change of the *rbc*L gene is not a rare molecular event. The relative speed-up in grasses (five times that of palms) led Gaut et al. (1992) to suggest that maximum parsimony-based reconstructions should be used with caution on *rbc*L data sets.

The maximum likelihood analysis (Fig. 3) indicates a close relationship between the cryophilic species in the subgenus *Vestigifera* as opposed to those from temperate waters (*P. mantoniae*, *P. moestrupii*, and *P. mitra*). The close relationship between the Antarctic species (*P. 'tychotreta'*) and Arctic species (*P. 'greenland'*) from Disko Bay (West Greenland) is unexpected. A hypothesis in which the Arctic species of *Pyramimonas* formed a cluster would have been easier to explain; autecological studies show that *P. cyclotreta* (Daugbjerg and Moestrup 1992a) and *P. 'tychotreta'* (Daugbjerg, unpubl. data) are unable to survive at water temperatures above 7–8° C, making it unlikely that the ancestral form spread across the equator in surface waters. However, if one accepts the present hypothesis of the relationship between *P. 'tychotreta'* and *P. 'greenland'*, this would argue for transportation of unicellular organisms between the polar marine ecosystems. *Pyramimonas gelidicola* (McFadden et al. 1982), from Antarctic waters, is known to have alternating stages in its life cycle, a flagellate stage, and an encystment stage (van den Hoff et al. 1989). Dispersal of species over long distances may therefore be possible in the

form of cysts. However, cyst stages have not been observed for other polar species of *Pyramimonas*.

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IDENTIFICATION OF GROUP- AND STRAIN-SPECIFIC GENETIC MARKERS FOR GLOBALLY DISTRIBUTED *ALEXANDRIUM* (DINOPHYCEAE). II. SEQUENCE ANALYSIS OF A FRAGMENT OF THE LSU rRNA GENE[1]

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ABSTRACT

A fragment of the large-subunit (LSU) ribosomal RNA gene (rDNA) from the marine dinoflagellates Alexandrium tamarense *(Lebour) Balech,* A. catenella *(Whedon et Kofoid) Balech,* A. fundyense *Balech,* A. affine *(Fukuyo et Inoue) Balech,* A. minutum *Halim,* A. lusitanicum *Balech, and* A. andersoni *Balech was cloned and sequenced to assess inter- and intraspecific relationships. Cultures examined were from North America, western Europe, Thailand, Japan, Australia, and the ballast water of several cargo vessels and included both toxic and nontoxic isolates. Parsimony analyses revealed eight major classes of sequences, or "ribotypes," indicative of both species- and strain-specific genetic markers. Five ribotypes subdivided members of the* A. tamarense/catenella/fundyense *species cluster (the "tamarensis complex") but did not correlate with morphospecies designations. The three remaining ribotypes were associated with cultures that clearly differ morphologically from the tamarensis complex. These distinct sequences were typified by 1)* A. affine, *2)* A. minutum *and* A. lusitanicum, *and 3)* A. andersoni. *LSU rDNA from* A. minutum *and* A. lusitanicum *was indistinguishable. An isolate's ability to produce toxin, or lack thereof, was consistent within phylogenetic terminal taxa. Results of this study are in complete agreement with conclusions from previous work using restriction fragment-length polymorphism analysis of small-subunit rRNA genes, but the LSU rDNA sequences provided finer-scale species and population resolution.*

The five divergent lineages of the tamarensis complex appeared indicative of regional populations; representa-

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