Protist, Vol. 155, 193–214, June 2004
http://www.elsevier.de/protist
Published online 14 June 2004

Protist

ORIGINAL PAPER

# Diversity of Picoplanktonic Prasinophytes Assessed by Direct Nuclear SSU rDNA Sequencing of Environmental Samples and Novel Isolates Retrieved from Oceanic and Coastal Marine Ecosystems

**Laure Guillou[a,1,2], Wenche Eikrem[b], Marie-Josèphe Chrétiennot-Dinet[c], Florence Le Gall[d], Ramon Massana[a], Khadidja Romari[d], Carlos Pedrós-Alió[a], and Daniel Vaulot[d]**

[a] Institut de Ciències del Mar, Passeig Marítim de la Barceloneta 37-49, 08003 Barcelona, Spain
[b] Department of Biology, University of Oslo, P.O. Box 1069 Blindern, N-0316 Oslo, Norway
[c] Laboratoire d'Océanographie Biologique, UMR 7621 CNRS/INSU/UPMC, Laboratoire arago, BP 44, F-66651 Banyuls sur mer Cx, France
[d] Station Biologique, UMR 7127 CNRS/INSU/UPMC, BP 74, 29682 Roscoff Cx, France

Submitted July 25, 2003; Accepted January 13, 2004
Monitoring Editor: Michael Melkonian

**Picoplanktonic prasinophytes are well represented in culture collections and marine samples. In order to better characterize this ecologically important group, we compared the phylogenetic diversity of picoplanktonic prasinophyte strains available at the Roscoff Culture Collection (RCC) and that of nuclear SSU rDNA sequences from environmental clone libraries obtained from oceanic and coastal ecosystems. Among the 570 strains avalaible, 91 belonged to prasinophytes, 65 were partially sequenced, and we obtained the entire SSU rDNA sequence for a selection of 14 strains. Within the 18 available environmental clone libraries, the prasinophytes accounted for 12% of the total number of clones retrieved (142 partial sequences in total), and we selected 9 clones to obtain entire SSU rDNA sequence. Using this approach, we obtained a subsequent genetic database that revealed the presence of seven independent lineages among prasinophytes, including a novel clade (clade VII). This new clade groups the genus *Picocystis*, two unidentified coccoid strains, and 4 environmental sequences. For each of these seven lineages, at least one representative is available in culture. The three picoplanktonic genera *Ostreococcus*, *Micromonas,* and *Bathycoccus* (order Mamiellales), were the best represented prasinophytes both in cultures and genetic libraries. SSU rDNA phylogenetic analyses suggest that the genus *Bathycoccus* forms a very homogeneous group. In contrast, the genera *Micromonas* and *Ostreococcus* turned out to be quite complex, consisting of three and four independent lineages, respectively. This report of the overall diversity of picoeukaryotic prasinophytes reveals a group of ecologically important and diverse marine microorganims that are well represented by isolated cultures.**

[1] Corresponding author;
fax 33 2 98 29 23 24
e-mail lguillou@sb-roscoff.fr

[2] Present address: Station Biologique, UMR 7127 CNRS/INSU/UPMC, BP 74, 29682 Roscoff, France

1434-4610/04/155/02-193 $ 30.00/0

## Introduction

The separation of the Prasinophyceae from the rest of the green algae was first based on studies carried out with the light microscopy (reviewed by Chadefaud 1977, see also Christensen 1966). Additional important characters, such as the scaly coverage on flagella and cell body of many prasinophyceans, were added later by electron microscope investigations (see the pioneering study of Manton and Parke 1960). Since then, ultrastructural details like scales and flagellar hairs have been widely used to separate taxa (Marin et al. 1993; Melkonian 1990; Moestrup and Throndsen 1988; Sym and Pienaar 1993). Nevertheless, the taxonomical view of the class Prasinophyceae has profoundly changed in the last few years, with the description of several species presenting unusual morphological and pigment features. Some species lack flagella (e.g. *Bathycoccus prasinos*), others lack scales (e.g. *Micromonas pusilla*), and in some cases, both flagella and scales are missing (e.g. *Pycnococcus provasolii, Ostreococcus tauri*, *Prasinococcus capsulatus*, *Prasinoderma coloniale, Prasinococcus capsulatus,* and *Picocystis salinarum*). Combined with these morphological heterogeneities, extremely complex assemblages of accessory pigments have been described in this group (Egeland et al. 1997), with three different pigment types resulting from different proposed biosynthetic routes. Members of the order Mamiellales, for instance, belong to the Type 3 pigment group, characterized by the specific presence of carotenoids from the prasinoxanthin and uriolide series. Based upon these biochemical features, most coccoid species (i.e. without scales and flagella) were initially placed in this order. Genetic data, however, do not agree with this placement. Phylogenetic studies based upon the SSU rDNA gene have clearly demonstrated that the Prasinophyceae is a paraphyletic group (Steinkötter et al. 1994). At least six different clades have been defined, which emerge within the basal part of the Chlorophyta (Fawley et al. 2000; Nakayama et al. 1998). In fact, coccoid species lacking scales and containing Type 3 pigments appear in several distinct lineages. To date, there is no single morphological or biochemical character to unify these six clades. Thus, the term prasinophytes has been preferred to Prasinophyceae for scaly green flagellates by some authors (Marin and Melkonian 1999; Melkonian and Surek 1995; Nakayama et al. 1998; Steinkötter et al. 1994), although the class Prasinophyceae is still in use in recent papers that either introduce new taxa (Daugbjerg 2000; Moro et al. 2002; Sym and Pienaar 1999; Throndsen and Zingone 1997) or investigate phylogenetic relationships (Fawley et al. 2000; Nakayama et al. 2000; Zingone et al. 2002). In the present paper, we will use conservatively the term prasinophytes, awaiting a new definition of the Class Prasinophyceae.

Numerous prasinophytes are very small and belong to the picoplanktonic fraction, i.e. organisms with a diameter of less than 3 µm (Stockner and Antia 1986). The smallest free-living eukaryote described to date is the tiny prasinophyte *O. tauri*, with a cell diameter of less than 1 µm (Chrétiennot-Dinet et al. 1995; Courties et al. 1994). The ubiquity and abundance of prasinophytes within the picoplanktonic size class in marine waters have been demonstrated by electron microscopy, pigment analyses or, more recently, by direct gene sequencing of natural samples. The early work of Johnson and Sieburth (1982) proved their ubiquity in marine samples. Electron microscope sections revealed the presence of a coccoid prasinophyte recognizable by its scaly covering later identified as *Bathycoccus prasinos* (0.5 to 1.0 µm in diameter) and of *Micromonas pusilla* (1 to 1.5 µm in diameter) identified by its peculiar mucronate flagellum. In the following years, numerous uncharacterized picoplanktonic green algae, most of them possessing neither scales nor flagella, have been identified based on their plastid organization from different parts of the world, at times reaching high concentrations (Joint and Pipe 1984; Silver et al. 1986; Takahashi and Hori 1984). Further evidence of the importance of picoplanktonic green algae has been provided by pigment analyses. Large amounts of chlorophyll *b* (chl *b*) are generally measured by HPLC within the picoplanktonic fraction in many different oceanic and coastal ecosystems (Everitt et al. 1990; Peeken 1997; Rodríguez et al. 2003). However, since other accompanying carotenoid normally found in green algae (prasinoxanthin or lutein for example) occur only at low abundance in natural samples, the organisms that are responsible for such large amounts of chl *b* remain elusive.

Genetic tools based upon the amplification and sequencing of genes directly from natural samples provide a new and powerful way to analyze the diversity of the picoplankton. Recent work based on the nuclear SSU rDNA and plastidial *psb*A genes (Díez et al. 2001; Moon-van der Staay et al. 2001; Zeidner et al. 2003) revealed the presence of a large number of sequences from prasinophytes in oceanic waters; whereas, other chl *b*-containing lineages, such as the Chlorophyceae, Trebouxiophyceae, and Chlorarachniophyceae were, in general, absent. Sequences recovered from natural samples generally show little similarity to culture se-

quences deposited in GenBank. For instance, Moon-van der Staay et al. (2001) obtained two environmental sequences from the equatorial Pacific that formed a completely new clade within the prasinophytes. The most widespread SSU rDNA operational taxonomic unit (OTU) recovered by Díez et al. (2001) from the Mediterranean Sea and Antarctica, named ME1-2, had only 96.8% similarity to its closest relative, *Mantoniella squamata*. However, very few strains of picoplanktonic prasinophytes have been sequenced to date. For example, the SSU rDNA sequences (for which the most complete database is available) of key taxa such as the *Bathycoccus prasinos* were not available, and only one *Micromonas pusilla* strain had been sequenced. Isolating, sequencing, and describing representatives of the picoplanktonic prasinophytes was therefore necessary in order to increase our knowledge of this key algal class.

In this study, we have combined different approaches. First, we screened the Roscoff Culture Collection (RCC) that currently includes about 570 strains of marine cyanobacteria and microalgae with a strong emphasis on picoplankton (Vaulot et al. in press). Some strains representative of the picoplanktonic prasinophytes, such as *Pycnococcus provasolii*, *Pseudoscourfieldia marina*, *Bathycoccus prasinos*, *Micromonas* spp., and *Ostreococcus* spp. as well as some undescribed ones, were selected. Their SSU rDNA gene was completely sequenced. Second, we also characterized the morphology of some strains previously cited in the literature for which only the SSU rDNA sequence was available. For instance, we analyzed the morphology of strains CCMP 1407 and CCMP 1220 that were placed by their SSU rDNA gene sequences within the two distinct new coccoid lineages described by Fawley et al. (2000). Third, we analyzed a set of environmental SSU rDNA clones affiliated to the prasinophytes from both oceanic and coastal environments. Our work has led to the establishment of a more comprehensive overview of the phylogeny of the marine members of prasinophytes, which is found to encompass seven different clades, each one possessing cultured representatives.

## Results

Our first step was to obtain an extensive data set of prasinophyte SSU rDNA sequences both from cultures and from environmental clone libraries. Among the 570 strains maintained by the Roscoff Culture Collection (RCC), 91 belong to the prasinophytes. All strains were first screened by light microscopy and, when necessary, by transmission electron microscopy (TEM). In a second step, partial nuclear SSU rDNA sequences (about 500 bp) were obtained for 65 selected strains either directly from PCR products or, in most cases, after cloning the PCR amplified gene. In a third step, these partial sequences were used to select 14 strains, for which the complete SSU rDNA sequence was determined (Table 1). Among these 14 strains, four were initially cloned because their purity was doubtful (BLA77, RCC 434, RCC 356, and RCC 287). When the cloned DNA was restricted with *Hae*III, RCC 434 and RCC 287 gave a single RFLP pattern. Based upon partial SSU rDNA sequences, BLA77 was mixed with a diatom. This prasinophyte is now lost. Concerning RCC 356, four different RFLP patterns were retrieved. These clones differed by 1 to 4 nucleotides over 500 bp. We selected a clone representative of the most frequent RFLP pattern and sequenced its entire SSU rDNA. Additionally, three more strains (CCMP 1407, CCMP 1220, and CCMP 489) were characterized by electron microscopy since their SSU rDNA sequence was already available in GenBank, but no detailed morphological analysis had been performed until now.

Among nuclear SSU rDNA clone libraries from various oceanic and coastal environments (Table 2), the prasinophytes account for 12% of the total number of clones (142 partial sequences), constituting the most represented green alga group (Table 2). Other chl *b*-containing clades are only detected in some libraries from Blanes (Mediterranean Sea), 2 clones closely related to the Trebouxiophyceae species *Nannochlorum eucaryotum* (96.3% identity, as determined by BLAST search) and 9 belonging to the Chlorarachniophyceae (between 93 to 98% identity). Prasinophyte clones are detected from oceanic, coastal, and estuarine libraries in similar proportion (15%, 11%, and 19% of the total number of clones, respectively). In some specific cases, the percentage of prasinophytes is higher, either in coastal libraries such as in Roscoff (English Channel) during springtime or in oceanic libraries such as those from the Mediterranean Sea (25% of the total number of clones) and Antarctica (33% of the total number of clones). From this data set, we selected 9 partial clones and obtained complete SSU rDNA sequences for them.

Using the resulting data set of full length prasinophyte SSU rDNA sequences (62 sequences, Table 3), phylogenetic analyses were performed. The new sequences added in this study confirm that the prasinophytes are not monophyletic. Our phylogenetic analyses support the existence of seven clades within this algal class (Fig. 1). Clades I to VI

**Table 1.** Origin and size of the prasinophyte isolates used in this study. SD = serial dilution, NA = data not available, RCC = Roscoff Culture Collection, Station Biologique de Roscoff, Roscoff, France (http://www.sb-roscoff.fr/Phyto/collect.html).

| Taxon | Strain | Area | Dat | Latitude | Longitude | Depth | Length (µm) | Width (µm) | Strain isolated by | Method of isolation |
|---|---|---|---|---|---|---|---|---|---|---|
| *Bathycoccus prasinos* | ALMO2[a] | Alboran Sea | 05/91 | 36°11′N | 01°51′W | Surface | 1.4–1.5 | 1.4–1.5 | N. Simon | Filtration (< 1 µm) |
| *Bathycoccus prasinos* | BLA77[a] | Mediterranean Sea, Spanish coast | 28/02/01 | 41°40′N | 2°10′E | Surface | 1.4–2.0 | 2.1–3.1 | L. Guillou | Filtration (< 1 µm) |
| *Micromonas pusilla* | CCMP 489 | North Atlantic | 30/05/88 | 28°98′N | 64°36′W | 120 m | 1.2–2.4 | 1.2–1.6 | R. Selvin | NA |
| *Micromonas pusilla* | CCMP 490 | North Atlantic | 18/06/64 | 41°52′N | 70°67′W | – | 1.4–2.7 | 1.3–1.8 | R. Guillard | NA |
| *Micromonas* sp. | RCC 434 | Mediterranean Sea, Spanish coast | 20/03/01 | 41°40′N | 2°48′E | Surface | 2.1–2.7 | 1.5–2.2 | L. Guillou | Filtration (< 1 µm) |
| *Nephroselmis pyriformis* | RCC 499[a] | Mediterranean Sea, Spanish coast | 28/02/01 | 41°40′N | 2°10′E | Surface | 4–7 | 4–5 | L. Guillou | Filtration (< 3 µm) and SD |
| *Ostreococcus* sp. | RCC 143 | Tropical Atlantic | 01/10/91 | 21°02′N | 31°08′W | 120 m | 1.5–2.1 | 1.1–1.9 | F. Partensky | Filtration (< 1 µm) |
| *Ostreococcus* sp. | RCC 344 | North Atlantic, Moroccan upwelling | 12/09/99 | 30°08′N | 10°03′W | 5 m | 1.7–2.0 | 1.4–1.7 | F. Le Gall | Filtration (< 0.6 µm) |
| *Ostreococcus* sp. | RCC 356 | English Channel, Roscoff, France | 12/04/00 | 48°45′N | 03°57′W | Surface | 1.7–2.8 | 1.2–2.4 | F. Le Gall | Filtration (< 3 µm) and SD |
| *Ostreococcus* sp. | RCC 393 | Thyrrenian Sea | 28/09/99 | 41°54′N | 10°26′E | 90 m | 1.5–2.0 | 1.3–1.5 | F. Le Gall | Filtration (< 3 µm) and SD |
| *Ostreococcus* sp. | RCC 501 | Mediterranean Sea, Spanish coast | 28/02/01 | 41°40′N | 2°10′E | Surface | 1.3–2.1 | 1.2–2.0 | L. Guillou | Filtration (< 0.6 µm) and SD |
| *Prasinococcus* cf *capsulatus* | CCMP 1407 | North Atlantic | 25/08/84 | 29°97′N | 63°86′W | 84 m | 3.8–5.0 | 3.9–5.2 | M. Keller | NA |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Prasinoderma* cf *coloniale* | CCMP 1220 | North Atlantic | 25/02/81 | 23°00′N | 75°00′W | – | 2.3–3.2 | 2.2–3.5 | I. Pintner | NA |
| *Pseudoscourfieldia marina* | RCC 261 | Pacific Ocean, Takapoto atoll | 06/02/98 | 14°30′S | 145°20′W | 20 m | 3.3–5.3 | 2.2–4.0 | S. Le Gall | Filtration (< 3 µm) |
| *Pycnococcus provasolii* | RCC 244 | Mediterranean Sea | 26/05/96 | 39°12′N | 06°04′E | 75 m | 1.9–2.9 | 2.0–2.8 | L. Guillou | Filtration (< 3 µm) |
| *Tetraselmis* sp. | RCC 500 | Mediterranean Sea, Spanish coast | 25/02/01 | 41°40′N | 2°48′E | Surface | 5–8 | 4–7 | L. Guillou | Filtration (< 0.6 µm) and SD |
| Unknow coccoid | RCC 287 | Equatorial Pacific | 10/02/98 | 0° | 179°49′W | 120 m | 2.1–2.6 | 1.9–2.5 | S. Boulben | Filtration (< 3 µm) and SD |
| Unknown flagellate | RCC 391 | North Atlantic, Moroccan upwelling | 12/09/99 | 30°08′N | 10°03′W | 65 m | 3.3–4.4 | 2.9–3.8 | F. Le Gall | Filtration (< 3 µm) and SD |

[a] Strain lost

reported by Nakayama et al. (1998), Fawley et al. (2000), and Zingone et al. (2002), are well supported in our analyses. Clade VII is a new clade, grouping the recently described species *Picocystis salinarum* (Lewin et al. 2000), some environmental sequences, strain CCMP 1205, and the new strain RCC 287. Our analyses show however that the SSU rDNA gene failed to resolve relationships among these different clades (very low bootstrap values). For example, the monophyly of Chlorophyta (including the seven prasinophyte clades, Chlorophyceae, Trebouxiophyceae, and Ulvophyceae) is only well supported by bootstrap analyses with neighbor joining (NJ), but not with maximum parsimony (MP). Even if bootstrap values are generally low for the nodes separating the different clades, their phylogenetic position are similar in the three analyses performed in this study, with the exception of Clade III (Pseudoscourfieldiales, Nephroselmidaceae). This clade is allied with the Pycnococcaceae (Pseudoscourfieldiales) with maximum likelihood (ML) and MP, and placed between Clades VII and IV with NJ. This variable position is also reinforced by relatively low bootstrap values for this clade in all phylogenetic analyses. The Chlorodendrales is the closest lineage to the "advanced" Chlorophyta (Chlorophyceae, Trebouxiophyceae, and Ulvophyceae) with 100% bootstrap support for all phylogenetic analyses performed.

Clade I is a well-supported clade, mostly composed of nanoplanktonic species belonging to the order Pyramimonadales. One environmental sequence (BL010625.18), obtained from a Blanes library from June 2001, is closely related to *Pyramimonas*. This sequence shares, however, less than 97% similarity with its closest relatives *Pyramimonas propulsa*, *Pyramimonas australis*, *Pyramimonas olivacea*, and *Pyramimonas parkae*. Some species of *Pyramimonas* are very small, such as *Pyramimonas virginica* Pennick measuring 2.7–3.5 × 1.9–2.4 µm and *Pyramimonas obvata* Carter measuring 4 × 5 µm, but their SSU rDNA are not available. These cells can probably pass through a 3 µm filter. Furthermore, more species remain probably to be described (McFadden et al. 1986).

Clade III includes the genus *Nephroselmis* and strain RCC 499 (Fig. 2A and B) which has a SSU rDNA sequence identical to that of *Nephroselmis pyriformis* (strain MBIC 11099). RCC 499 possesses also typical morphological features of *Nephroselmis pyriformis*.

Clade IV is exclusively composed of members of the order Chlorodendrales, containing two genera: *Tetraselmis* and *Scherffelia*. The genus *Tetraselmis* is not monophyletic in our analyses, composed of

**Table 2.** Clone libraries analyzed in this study and relative contribution of prasinophytes. A: Molecular methods used for the screening of the clones: 1 = screening by RFLP using restriction enzyme *Hae*III and sequencing of a representative clone of each RFLP pattern; 2 = sequencing of all clones. B: Prefiltration used to collect the cells (in µm). C: Total number of clones analyzed. D: Number of clones belonging to the order Mamiellales. E: Number of clones belonging to other prasinophyte orders (except for the order Mamiellales). F: Relative contribution of the prasinophytes. G: Number of clones belonging to the Trebouxiophyceae or to the Chlorarachniophyceae.

| Name | Origin | Coordinates | Date | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|---|---|---|
| ME1[a] | Mediterranean Sea, Alboran | 36°14′N–04°15′W | 09/11/97 | 1 | < 5 | 64 | 16 | – | 25% | – |
| ANT37[a] | Antarctica, Weddel Sea | 60°32′S–44°12′W | 26/01/98 | 1 | < 1.6 | 58 | 19 | – | 33% | – |
| ANT12[a] | Antarctica, Scotia Sea | 58°16′S–44°27′W | 23/01/98 | 1 | < 1.6 | 67 | 8 | – | 12% | – |
| NA11[a] | North Atlantic | 59°30′N–21°08′W | 14/06/98 | 1 | < 2 | 17 | 1 | – | 6% | – |
| NA37[a] | North Atlantic | 59°34′N–21°03′W | 21/06/98 | 1 | < 2 | 20 | 2 | – | 10% | – |
| OLI11_75m[b] | Equatorial Pacific | 11°30′S–150°00′W | 07/11/94 | 2 | < 3 | 101 | – | 3 | 3% | – |
| | **Total oceanic sites** | | | | | **327** | **46** | **3** | **15%** | **–** |
| BL000921[c] | Mediterranean Sea, Blanes | 41°40′N–2°48′E | 21/09/00 | 1 | < 3 | 78 | 2 | – | 2% | 1 |
| BL001221[c] | Mediterranean Sea, Blanes | 41°40′N–2°48′E | 21/12/00 | 1 | < 3 | 111 | 8 | – | 7% | 4 |
| BL010320[c] | Mediterranean Sea, Blanes | 41°40′N–2°48′E | 20/03/01 | 1 | < 3 | 88 | 4 | – | 4% | 1 |
| BL010625[c] | Mediterranean Sea, Blanes | 41°40′N–2°48′E | 25/06/01 | 1 | < 3 | 106 | – | 4 | 4% | 5 |
| RA000412[d] | English Channel, Roscoff, Astan | 48°45′N–04°00′W | 12/04/00 | 2 | < 3 | 108 | 21 | – | 19% | – |
| RA000609[d] | English Channel, Roscoff, Astan | 48°45′N–04°00′W | 09/06/00 | 1 | < 3 | 47 | 4 | – | 8% | – |
| RA000907[d] | English Channel, Roscoff, Astan | 48°45′N–04°00′W | 07/09/00 | 1 | < 3 | 49 | 4 | 1 | 10% | – |
| RA001219[d] | English Channel, Roscoff, Astan | 48°45′N–04°00′W | 19/12/00 | 1 | < 3 | 46 | 4 | 2 | 13% | – |
| RA010412[d] | English Channel, Roscoff, Astan | 48°45′N–04°00′W | 12/04/01 | 1 | < 3 | 49 | 12 | – | 24% | – |
| RA010516[d] | English Channel, Roscoff, Astan | 48°45′N–04°00′W | 16/05/01 | 1 | < 3 | 48 | 13 | – | 27% | – |
| RA010613[d] | English Channel, Roscoff, Astan | 48°45′N–04°00′W | 13/06/01 | 1 | < 3 | 47 | 5 | 1 | 13% | – |
| | **Total coastal sites** | | | | | **777** | **77** | **8** | **11%** | **11** |
| RD010517[d] | English Channel, Roscoff, Dourduff | 48°38′N–03°51′W | 17/05/01 | 1 | < 3 | 43 | 8 | – | 19% | – |
| | **Total estuarine sites** | | | | | **43** | **8** | **–** | **19%** | **–** |
| | **TOTAL for all libraries** | | | | | **1148** | **131** | **11** | **12%** | **11** |

[a] Díez et al. (2001)
[b] Moon-van der Staay et al. (2001)
[c] Massana et al. (submitted)
[d] Romari and Vaulot (2004)

**Table 3.** GenBank accession numbers of complete SSU rDNA gene sequence used in this study (except for the RCC 356 clone 6, 7, and 8 that are partial). Partial sequences have been deposited under accession numbers: AY426829 to AY426945 for Blanes, and AY295353 to AY295760 for Roscoff.

| Species name with authority or clone identification | Strain name (when available) | Accession number |
|---|---|---|
| **Haptophyta** | | |
| *Phaeocystis globosa* Scherff. | SK35 | X77476 |
| *Pavlova gyrans* Butcher | CCMP 607 | U40922 |
| **Glaucocystophyceae** | | |
| *Cyanophora paradoxa* Korshikoff | Kies | X68483 |
| **Streptophyta** | | |
| *Chaetosphaeridium globosum* (Nordstedt) Klebahn | M 1311 | AJ250110 |
| *Chara foetida* Braun | | X70704 |
| *Chlorokybus atmophyticus* Geitler | UTEX LB 2591 | AF408244 |
| *Coleochaete scutata* Brébisson | SAG 110.80 | X68825 |
| *Entransia fimbriata* Hugues | UTEX LB 2352 | AF408243 |
| *Genicularia spirotaenia* (Ramb.) de Bary | 329 | X74753 |
| *Klebsormidium subtilissimum* (Rabenh.) Silva, Mattox et Blackwell | UTEX 462 | AF408241 |
| *Marchantia polymorpha* L. | | X75521 |
| *Mesostigma viride* Lauterborn | NIES 475 | AJ250109 |
| *Nitella capillaris* (Krocker) J. Groves & Bullock-Webster | | AJ250111 |
| *Staurastrum* sp. | M753 | X77452 |
| *Zamia pumila* L. | | M20017 |
| **Chlorophyceae** | | |
| *Chlamydomonas reinhardtii* Dangeard | | M32703 |
| *Neochloris aquatica* Starr | | M62861 |
| Unidentified coccoid/flagellate green alga | CCMP 1189 | AF203398 |
| **Trebouxiophyceae** | | |
| *Nanochlorum eucaryotum* Wilhelm et al. | Mainz 1 | X06425 |
| *Trebouxia impressa* Ahmadjian | UTEX 892 | Z21551 |
| *Chlorella minutissima* Fott et Nováková | C-1.1.9 | X56102 |
| **Prasinophytes** | | |
| *Bathycoccus prasinos* Eikrem et Throndsen | ALMO2 | AY425314 |
| *Bathycoccus prasinos* Eikrem et Throndsen | BLA77 | AY425315 |
| *Crustomastix* sp. | MBIC 10709 | MBIC[c] |
| *Cymbomonas tetramitiformis* Schiller | Shizugawa | AB017126 |
| *Dolichomastix tenuilepis* Throndsen et Zingone | | AF509625 |
| *Halosphaera* sp. | Shizugawa | AB017125 |
| *Mamiella* sp. | Shizugawa | AB017129 |
| *Mantoniella antarctica* Marchant | | AB017128 |
| *Mantoniella squamata* (Manton et Parke) Desikachary | CCAP 1965/1 | X73999 |
| *Micromonas pusilla* (Butcher) Manton et Parke | CCMP 489 | AJ010408 |
| *Micromonas* sp. | RCC 434 | AY425316 |
| *Micromonas* sp. | CCMP 490 | AY425320 |
| *Nephroselmis olivacea* Stein | SAG 40.89 | X74754 |
| *Nephroselmis pyriformis* Carter | RCC 499 | AY425306 |
| *Nephroselmis pyriformis* Carter | CCMP 717[a] | X75565 |
| *Nephroselmis pyriformis* Carter | MBIC 10641 | AB058378 |
| *Nephroselmis pyriformis* Carter | MBIC 11099 | AB058391 |
| *Ostreococcus* sp. | RCC 501 | AY425313 |
| *Ostreococcus* sp. | MBIC 10636 | AB058376 |
| *Ostreococcus* sp. | RCC 143 | AY425310 |
| *Ostreococcus* sp. | RCC 344 | AY425307 |
| *Ostreococcus* sp. | RCC 356 (clone 1) | AY425308 |
| | RCC 356 (clone 6) | AY465412 |
| | RCC 356 (clone 7) | AY465413 |
| | RCC 356 (clone 8) | AY465414 |
| *Ostreococcus* sp. | RCC 393 | AY425311 |
| *Ostreococcus tauri* Courties et Chrétiennot-Dinet | OTTH0595 | Derelle et al. (2002). Provided by H. Moreau |

**Table 3.** (Continued).

| Species name with authority or clone identification | Strain name (when available) | Accession number |
|---|---|---|
| *Picocystis salinarum* Lewin | IM214 | AF153314 |
| *Picocystis salinarum* Lewin | L7 | AF153313 |
| *Picocystis salinarum* Lewin | SSFB | AF125167 |
| *Prasinococcus cf. capsulatus* Miyashita et Chihara | CCMP 1407[b] | U40919 |
| *Prasinococcus* sp. | CCMP 1202 | AF203401 |
| *Prasinococcus* sp. | CCMP 1194 | AF203400 |
| *Prasinococcus* sp. | CCMP 1614 | AF203403 |
| *Prasinoderma cf coloniale* Hasegawa et Chihara | CCMP 1220[b] | U40920 |
| *Prasinoderma coloniale* | MBIC 10720 | AB058379 |
| Unknown prasinophyte | MBIC 10879 "*Prasinopapilla vacuolata*" | MBIC[c] |
| Prasinophyte symbiont of radiolarian | Host: cf. Spongodrymus 333 | AF166381 |
| Prasinophyte symbiont of radiolarian | Host: cf. Spongodrymus 331 | AF166380 |
| Prasinophyte symbiont of radiolarian | Host: cf. Spongodrymus 257 | AF166379 |
| *Pseudoscourfieldia marina* (Throndsen) Manton | K-0017 | AJ132619 |
| *Pseudoscourfieldia marina* (Throndsen) Manton | K-0017 | AF122888 |
| *Pseudoscourfieldia marina* (Throndsen) Manton | RCC 261 | AY425304 |
| *Pterosperma cristatum* Schiller | Yokohama | AB017127 |
| *Pterosperma cristatum* Schiller | NIES 221 | AJ010407 |
| *Pycnococcus provasolii* Guillard | CCMP 1198 | AJ010406 |
| *Pycnococcus provasolii* Guillard | CCMP 1199 | AF122889 |
| *Pycnococcus provasolii* Guillard | CCMP 1203 | X91264 |
| *Pycnococcus provasolii* Guillard | RCC 244 | AY425305 |
| *Pyramimonas australis* Andreoli & Moro | | AJ404886 |
| *Pyramimonas disomata* Butcher | Singapore | AB017121 |
| *Pyramimonas olivacea* Carter | Shizugawa | AB017122 |
| *Pyramimonas parkeae* Norris et Pearson | Hachijo | AB017124 |
| *Pyramimonas propulsa* Moestrup et Hill | NIES 251 | AB017123 |
| *Scherffelia dubia* (Perty) Pascher | | X68484 |
| *Tetraselmis convolutae* (Parke & Manton) Norris et al. | 208 | U05039 |
| *Tetraselmis* sp. | RCC 500 | AY425299 |
| *Tetraselmis* sp. | MBIC 11125 | AB058392 |
| *Tetraselmis* sp. | RG-07 | U41900 |
| *Tetraselmis striata* Butcher | PLY 443 | X70802 |
| Unidentified coccoid green alga | CCMP 1205 | U40921 |
| Unidentified coccoid green alga | MBIC 10622 | AB058375 |
| Unidentified coccoid green alga | RCC 287 | AY425302 |
| Unidentified coccoid prasinophyte | CCMP 1193 | AF203399 |
| Unidentified coccoid prasinophyte | CCMP 1413 | AF203402 |
| Unidentified flagellated prasinophyte | RCC 391 | AY425321 |
| **Ulvophyceae** | | |
| *Acrosiphonia duriuscula* (Ruprecht) Yendo | | AB049418 |
| *Ulothrix zonata* (Weber et Mohr) Kützing | SAG 38.86 | Z47999 |
| **Environmental sequences** | | |
| OLI11059 | | AJ402345 |
| OLI11305 | | AJ402358 |
| OLI11345 | | AJ402359 |
| RA000412.97 | | AY425319 |
| RA000412.150 | | AY425312 |
| RA001219.46 | | AY425303 |
| RA000412.37 | | AY425317 |
| RA010412.39 | | AY425309 |
| BL000921.10 | | AY425318 |
| BL010625.18 | | AY425322 |
| BL010625.1 | | AY425300 |
| BL010625.2 | | AY425301 |

[a]Listed in GenBank as *Pseudoscourfieldia marina*.
[b]Strains morphologically characterized in this study.
[c]Sequences from MBIC (not deposited in GenBank), available at http://seasquirt.mbio.co.jp/mbic/

two clades separated by a sequence of *Scherffelia dubia*. Our sequences are allied with two free-living *Tetraselmis* (MBIC 11125 and RCC 500) and strains similar in size and shape to descriptions of *Tetraselmis cordiformis* that have been described as symbionts of radiolarians (Gast et al. 2000). The environmental sequence BL010625.1 differs only by one nucleotide from strain RCC 500 (Fig. 2C and D). Both of them originate from the same coastal sample (Blanes, 25 June 2001). RCC 500 has the typical morphological features of the genus *Tetraselmis*. The sequence closest to BL010625.2 is *Tetraselmis* sp. MBIC 11125 (98.8% similarity). This strain appears somewhat distant from the other *Tetraselmis* species (*Tetraselmis convolutae* and *Tetraselmis striata*) in the tree. The size of *Tetraselmis* is generally between 5 to 20 µm (Butcher 1959), and no picoplanktonic species has been described until now. Our strain varied between 4 and 8 µm (Table 1).

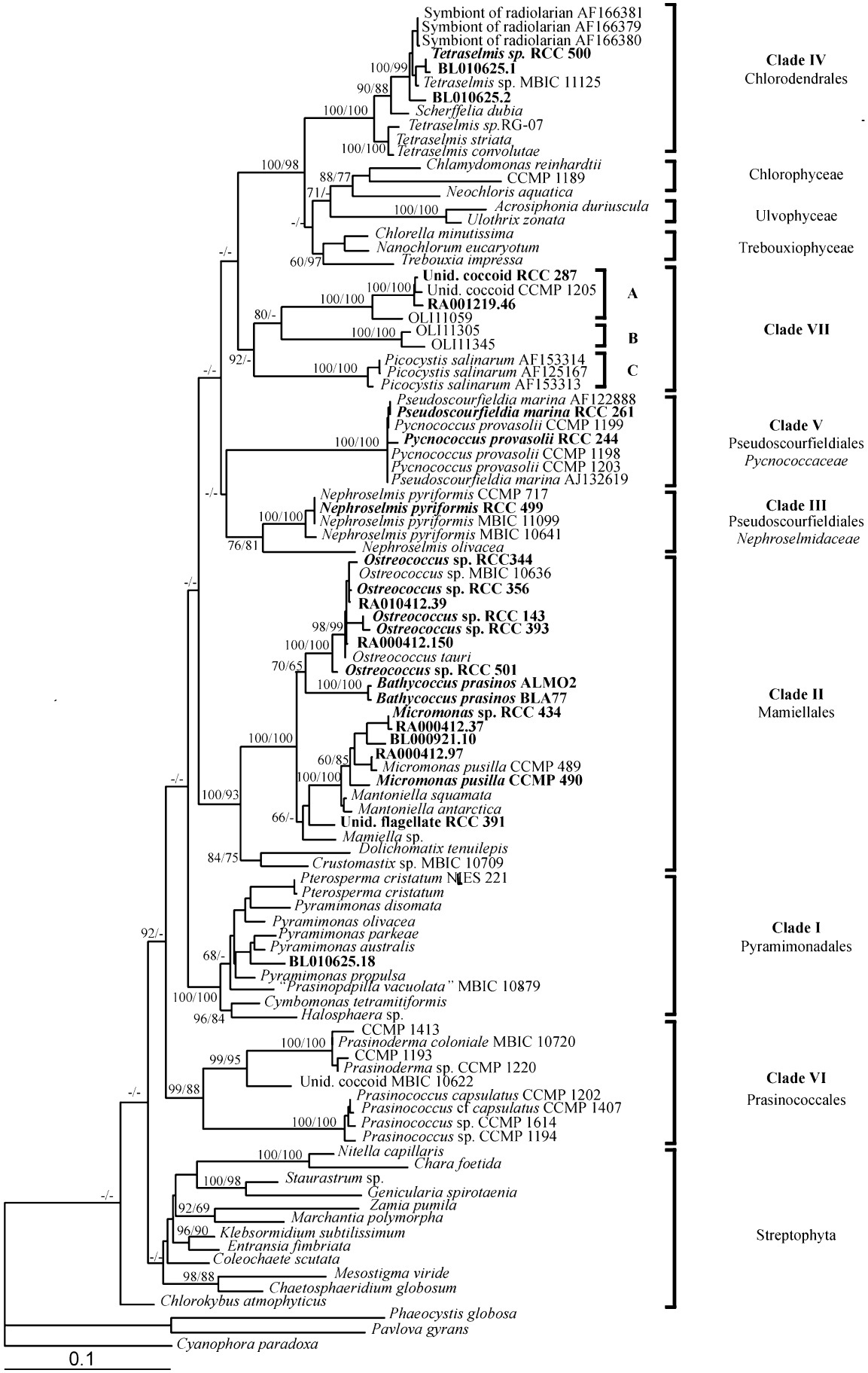
Clade V is composed of members of the family Pycnococcaceae (Guillard) Fawley belonging to the order Pseudoscourfieldiales. It contains sequences of *Pycnococcus provasolii* Guillard and *Pseudoscourfieldia marina* that are nearly identical (99.1 to 99.8% similarity). These two species may represent different growth forms or alternate life-cycle stages of the same organism (Fawley et al. 1999). In fact, sequence differences may be attributed to sequencing errors or to the presence of different copies of the SSU rRNA gene within the same strain. As an example, the two sequences AF122888 and AJ132619, that differ by 3 bases, correspond in fact to the same strain (K-0017).

RCC 244 (*Pycnococcus provasolii*) is very small (1–3 µm) and coccoid, without scales or flagellum (not shown). *Pseudoscourfieldia marina* (RCC 261) has two flagella, is covered by different types of scales, and is larger (3–5.3 µm, Fig. 2E). Both flagella have hairscales (Fig. 2F). An inclusion of the mitochondrion is visible inside the plastid (Fig. 2G). No environmental sequences have been obtained for this clade, although *Pseudoscourfieldia* is common in dilution cultures from coastal areas.

Clade VI was described by Fawley et al. (2000). Strain CCMP 1202 was identified by Sieburth et al. (1999) as *Prasinococcus capsulatus*. Two additional strains belonging to this clade are characterized in the present study. CCMP 1407 presents the general characteristics of *Prasinococcus capsulatus* described by Miyashita et al. (1993). Cells are spherical and covered by a thick gelatinous matrix (Fig. 3A). The mitochondrion presents thin invaginations inside the pyrenoid (Fig. 3B). A decapore is present, located at the opposite side of the pyrenoid (Fig. 3C). *Prasinococcus* is somewhat larger than picoplankton, measuring 3.5 to 5.2 µm. CCMP 1220 was tentatively characterized as *Prasinococcus capsulatus* by Sieburth et al. (1999) based on pigment type and surface antigens. However, we found that this strain resembles more closely the genus *Prasinoderma* (Hasegawa et al. 1996). The cells are spherical, without scales, and surrounded by one or more layers of cell walls (Fig. 3D). A single mitochondrion, a nucleus, and a Golgi apparatus are located in the center of the cell, enclosed within the two lobes of the chloroplast. A pyrenoid is situated close to the mitochondrion. Asexual reproduction is achieved by unequal binary fission in which one of the daughter cells retains the parent wall, while the other is released with a newly produced cell wall (Fig. 3D). No environmental sequences have been obtained for this clade.

Clade VII is a new group, composed of three different lineages labeled A, B, and C. Monophyly of Clade VII is well supported by ML and bootstrap analysis with NJ (Fig. 1). These three lineages are also grouped together by MP, but with a low bootstrap value (52%). It is possible that this clade will split into separate lineages as more sequences become available in the future. Lineage A contains two environmental sequences from oceanic (OLI11059 from the equatorial Pacific) and coastal (RA001219-46 from Roscoff) ecosystems and two cultured strains (RCC 287 and CCMP 1205). Both strains are coccoid, without scales (see Fig. 4A and 4B for RCC 287). Pigments of RCC 287 are typical for green algae, with lutein, zeaxanthin, violaxanthin, and neoxanthin as major carotenoids (Latasa et al. in prep). Lineage B is composed by two environmental sequences from the equatorial Pacific (OLI11305 and OLI11345). Lineage C is composed by the recently described species *Picocystis salinarum*, isolated from a saline lake of California (Lewin et al. 2000).

Clade II corresponds to the order Mamiellales. A very large fraction of environmental sequences and cultured strains belong to this clade (78% of the total number of "green" clones, 131 clones in total for all libraries). Representatives from the Mamiellales form the dominant clade in all environmental libraries with the exception of the equatorial Pacific and one library from Blanes (June 2001). In the latter two cases, no Mamiellales sequences were detected, and other prasinophyte groups are present (Pyramimonadales for Blanes and Clade VII for the equatorial Pacific). The monophyly of the Mamiellales is well supported by all phylogenetic analyses (Fig. 1). Clade II is composed of three different subclades including (1) *Crustomastix* and *Dolichomastix* in the basal part, (2) the two non-flagellated genera


Symbiont of radiolarian AF166381
Symbiont of radiolarian AF166379
Symbiont of radiolarian AF166380
Tetraselmis sp. RCC 500
BL010625.1
Tetraselmis sp. MBIC 11125
BL010625.2
Scherffelia dubia
Tetraselmis sp.RG-07
Tetraselmis striata
Tetraselmis convolutae
Chlamydomonas reinhardtii
CCMP 1189
Neochloris aquatica
Acrosiphonia duriuscula
Ulothrix zonata
Chlorella minutissima
Nanochlorum eucaryotum
Trebouxia impressa
Unid. coccoid RCC 287
Unid. coccoid CCMP 1205
RA001219.46
OLI11059
OLI11305
OLI11345
Picocystis salinarum AF153314
Picocystis salinarum AF125167
Picocystis salinarum AF153313
Pseudoscourfieldia marina AF122888
Pseudoscourfieldia marina RCC 261
Pycnococcus provasolii CCMP 1199
Pycnococcus provasolii RCC 244
Pycnococcus provasolii CCMP 1198
Pycnococcus provasolii CCMP 1203
Pseudoscourfieldia marina AJ132619
Nephroselmis pyriformis CCMP 717
Nephroselmis pyriformis RCC 499
Nephroselmis pyriformis MBIC 11099
Nephroselmis pyriformis MBIC 10641
Nephroselmis olivacea
Ostreococcus sp. RCC344
Ostreococcus sp. MBIC 10636
Ostreococcus sp. RCC 356
RA010412.39
Ostreococcus sp. RCC 143
Ostreococcus sp. RCC 393
RA000412.150
Ostreococcus tauri
Ostreococcus sp. RCC 501
Bathycoccus prasinos ALMO2
Bathycoccus prasinos BLA77
Micromonas sp. RCC 434
RA000412.37
BL000921.10
RA000412.97
Micromonas pusilla CCMP 489
Micromonas pusilla CCMP 490
Mantoniella squamata
Mantoniella antarctica
Unid. flagellate RCC 391
Mamiella sp.
Dolichomatix tenuilepis
Crustomastix sp. MBIC 10709
Pterosperma cristatum NIES 221
Pterosperma cristatum
Pyramimonas disomata
Pyramimonas olivacea
Pyramimonas parkeae
Pyramimonas australis
BL010625.18
Pyramimonas propulsa
"Prasinopapilla vacuolata" MBIC 10879
Cymbomonas tetramitiformis
Halosphaera sp.
CCMP 1413
Prasinoderma coloniale MBIC 10720
CCMP 1193
Prasinoderma sp. CCMP 1220
Unid. coccoid MBIC 10622
Prasinococcus capsulatus CCMP 1202
Prasinococcus cf capsulatus CCMP 1407
Prasinococcus sp. CCMP 1614
Prasinococcus sp. CCMP 1194
Nitella capillaris
Chara foetida
Staurastrum sp.
Genicularia spirotaenia
Zamia pumila
Marchantia polymorpha
Klebsormidium subtilissimum
Entransia fimbriata
Coleochaete scutata
Mesostigma viride
Chaetosphaeridium globosum
Chlorokybus atmophyticus
Phaeocystis globosa
Pavlova gyrans
Cyanophora paradoxa
0.1
Clade IV
Chlorodendrales
Chlorophyceae
Ulvophyceae
Trebouxiophyceae
Clade VII
A
B
C
Clade V
Pseudoscourfieldiales
Pycnococcaceae
Clade III
Pseudoscourfieldiales
Nephroselmidaceae
Clade II
Mamiellales
Clade I
Pyramimonadales
Clade VI
Prasinococcales
Streptophyta

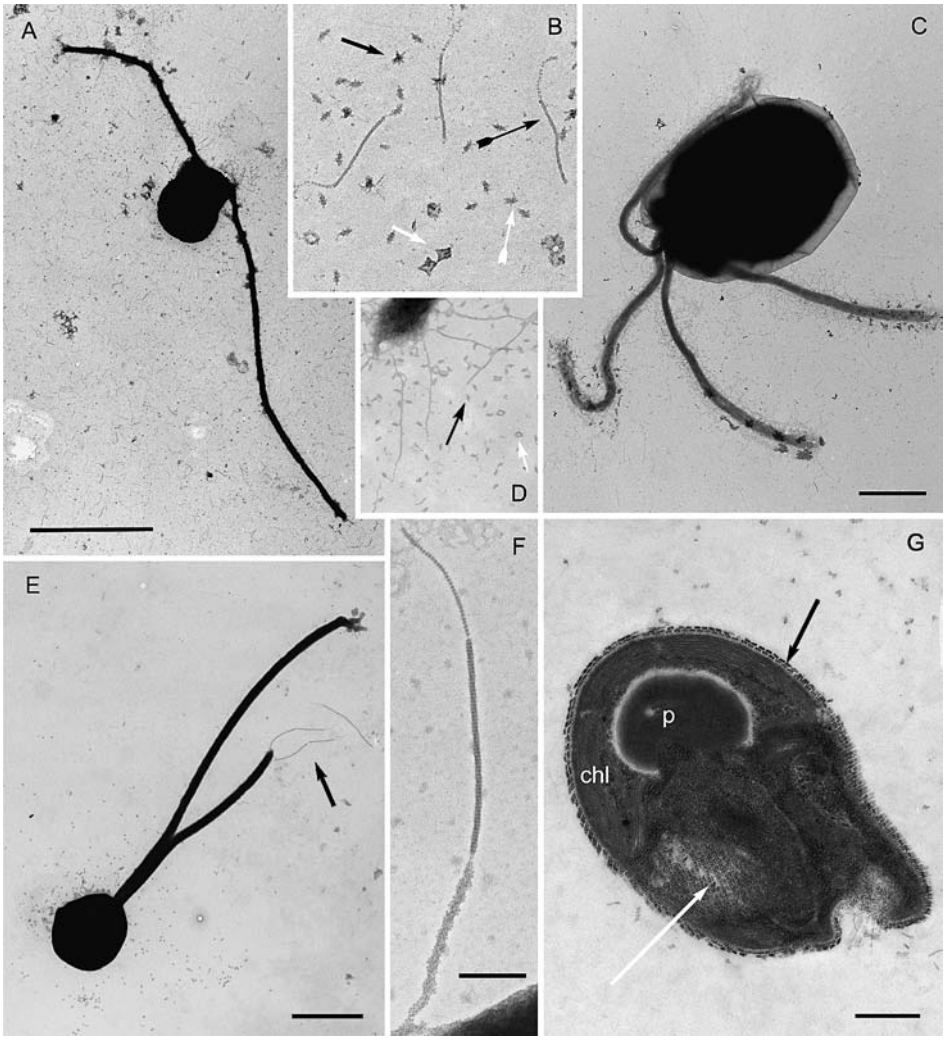



**Figure 2.** Transmission electron micrographs of *Nephroselmis pyrifomis* (RCC 499), *Tetraselmis* sp. (RCC 500), and *Pseudoscourfeldia marina* (RCC 261) representatives of Clades V, III, and IV respectively. Scale terminology according to Melkonian (1990) and Marin and Melkonian (1994) **A.** Shadow-cast whole-mount of *Nephroselmis pyriformis* (RCC 499). Bar = 5 µm. **B.** Stained whole-mount of *Nephroselmis pyriformis* (RCC 499) showing scales covering the cell, under layer scales (white plain arrow), stellate scales (black plain arrow), rod shaped double scales (white tailed arrow), and T-hair scales (black tailed arrow). Bar = 0.2 µm. **C.** Stained whole-mount of *Tetraselmis* sp. (RCC 500). Bar = 2 µm. **D.** Close up of the flagellum of *Tetraselmis* sp. (RCC 500) showing pentagonal under-layer scales (white arrow), rod shaped double scales (black arrow), and T-hair scales covering the flagella. Bar = 0.5 µm. **E.** Stained whole-mount of *Pseudoscourfieldia marina* (RCC 261) with flagella and flagellar hairs (arrow). Bar = 2 µm. **F.** Close up of $P_1$-flagellar hair scale from *Pseudoscourfieldia marina* (RCC 261), note tripartition. Bar = 0.2 µm **G.** Section through *Pseudoscourfieldia marina* (RCC 261) showing chloroplast (chl) with pyrenoid (p), and scaly covering with pentagonal under layer scales (white arrow) and rod shaped double scales (black arrow). Bar = 0.5 µm.

---

◄ **Figure 1.** Phylogeny of prasinophytes based on 89 full-length SSU rDNA sequences and 1668 total characters (913 constant, 201 parsimony-uninformative, 554 parsimony-informative). The phylogenetic tree shown was inferred by the maximum likelihood (ML) method based on a TrN (Tamura and Nei 1993) model of DNA substitutions with the following parameters: proportion of invariable sites (I) = 0.3836, gamma distribution shape parameter = 0.5482, and substitution models of R(b) [A–G] = 2.4483, R(e) [C–T] = 4.5947, and 1.0 for all other substitution rates (–lnL = 19030.8468). Total number of rearrangements tried = 77025. New sequences obtained from this study are in bold. Bootstrap values for major clades are indicated above internodes and correspond to neighbor joining (NJ) and maximum parsimony (MP), respectively. Bootstrap value < 60% are indicated by hyphens. Clade numbering follows that of Fawley et al. (2000). CCMP 1220 and CCMP 1407, which fall inside the two dichotomic branches of Clade VI, were characterized by electron microscopy as *Prasinoderma* cf. *coloniale* and *Prasinococcus* cf. *capsulatus*, respectively. The scale bar indicates 0.1% sequence divergence.

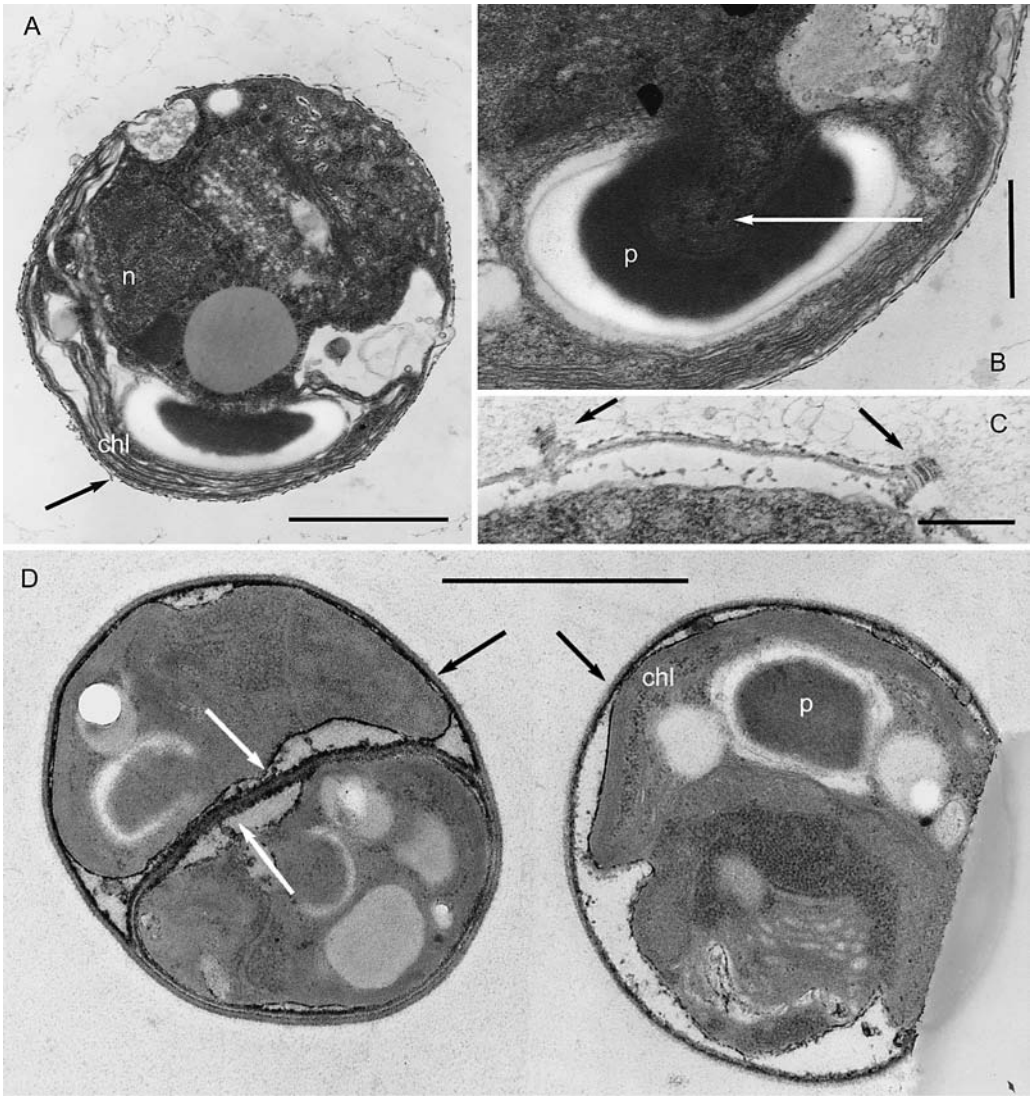



**Figure 3.** Transmission electron micrographs of representative strains from the order Prasinococcales, Clade VI. **A.** Section through *Prasinococcus* cf *capsulatus* (CCMP 1407) with chloroplast (chl) and nucleus (n). Bar = 1 µm. **B.** Detail of the chloroplast of *Prasinococcus* cf *capsulatus* (CCMP 1407) with pyrenoid (p) surrounded by starch. Note that part of the cytoplasm penetrates into the pyrenoid (white arrow). Bar = 0.5 µm. **C.** The decapore (arrows) of *Prasinoderma* (CCMP 1407). Bar = 0.2 µm. **D.** Section through *Prasinoderma* sp. (CCMP 1220). Cell to the right is enclosed by a cell wall and contains a chloroplast (chl) with a pyrenoid (p). Cell to the left is dividing within the cell wall (white arrows). Bar = 1 µm.

*Ostreococcus* and *Bathycoccus*, and (3) four flagellated genera: *Mamiella*, *Mantoniella*, *Micromonas*, and the new genus represented by isolate RCC 391 (Fig. 1). All these genera have scales, except for *Ostreococcus* and *Micromonas*. The basal position of *Crustomastix* and *Dolichomastix* within the Mamiellales confirms the work of Zingone et al. (2002). Partial sequences available from environmental libraries and strains in culture provide a broad overview of the diversity of picoplanktonic species within this clade (Fig. 5). With the exception of one sequence that is closely related to *Mantoniella* (ANT37-3), all other sequences group with three genera: *Bathycoccus* (33 clones), *Ostreococcus* (24 clones), and *Micromonas* (66 clones). *Bathycoccus* and *Ostreococcus* are sister taxa in all the phylogenetic analyses (Fig. 5). *Bathycoccus* is a very homogeneous clade, characterized by little divergent sequences. For example, sequences from the Mediterranean Sea and the English Channel are almost identical

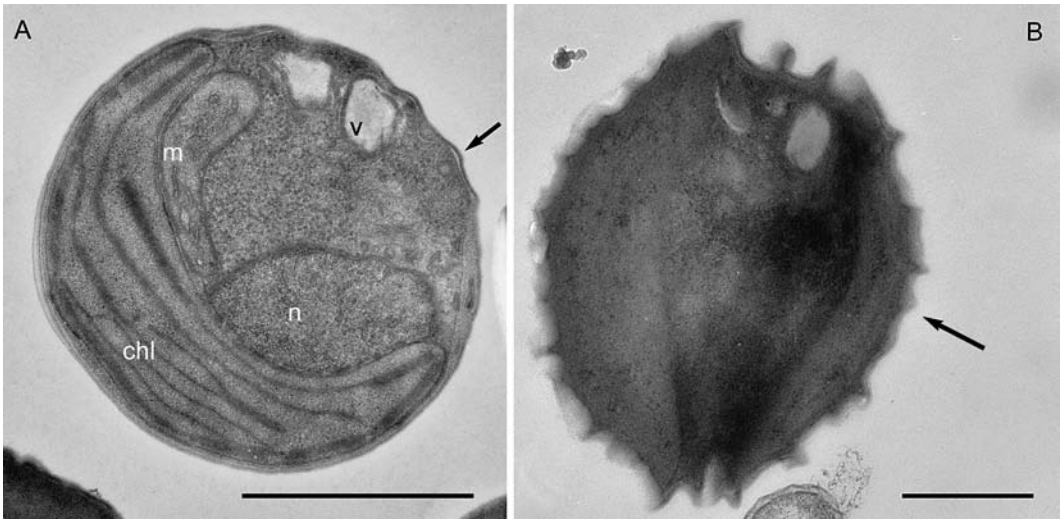
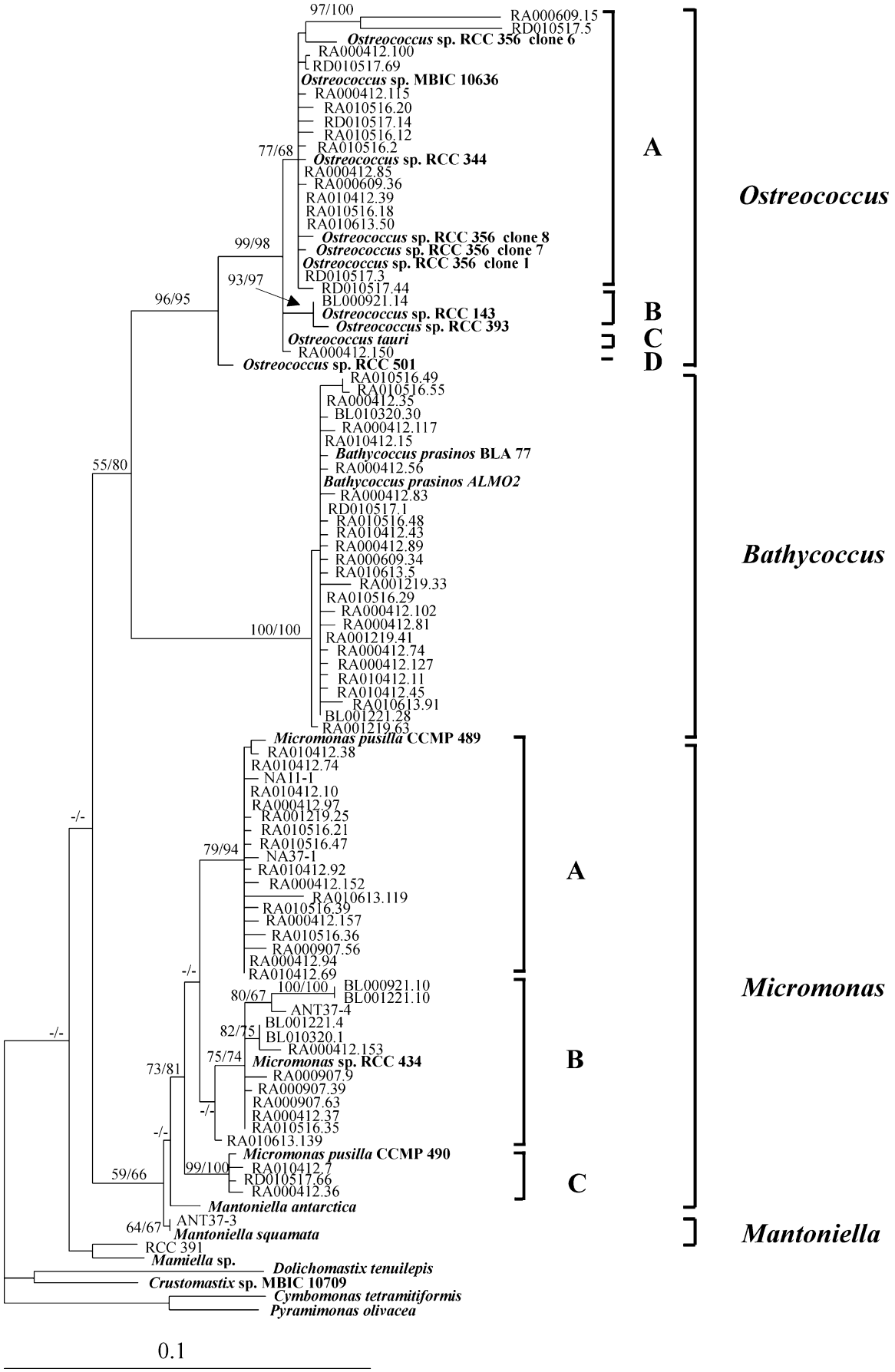



**Figure 4.** Transmission electron micrographs of RCC 287 sp. nov. belonging to Clade VII. **A.** Section showing cell of RCC 287 with a single chloroplast (chl), a mitochondrion (m), a nucleus (n), vesicles (v), and cell wall (arrow). Bar = 1 µm. **B.** Section through resting cell of RCC 287. Note wavy wall (arrow). Bar = 0.5 µm.

(generally only 1 to 2 bases difference). The two strains isolated in culture (ALMO2 and BLA77), and since lost, were very similar and corresponded to the general description of *Bathycoccus prasinos*. Both are non-motile organisms, covered by one type of circular scales with radiating and concentric ribs, forming a spider web-like structure (Fig. 6A). The cells contain one nucleus, one mitochondrion, and a Golgi body. The chloroplast is single and a starch grain can be present (results not shown).

The genus *Ostreococcus* is more diverse and composed of four different clades. For each of these groups, cultured isolates are available. Clade A is composed of environmental sequences originating from the English Channel and strains obtained in culture from the same site (RCC 356), Moroccan upwelling (RCC 344), and Pacific Ocean (MBIC 10636). Clade B is composed of two strains, RCC 393 and RCC 143, and one environmental sequence from Blanes. Clade C is composed of the type species *Ostreococcus tauri*, originating from the Thau Lagoon, Mediterranean coast, France, and one environmental sequence from Roscoff (RA000412.150). Finally, Clade D is composed of a single strain, RCC 501, isolated from the Mediterranean Sea. *Ostreococcus* clade A possesses the largest number of environmental sequences. One strain isolated on 12 April 2000 from Roscoff (RCC 356 clone 1) has a sequence identical to several environmental clones retrieved from the same sample on the same date. The different SSU rDNA clones retrieved from the RCC 356 isolate are placed in the same Clade A. Nucleotide differences vary from 1 to 2 (clones 7 and 8) up to 5 (clone 6). Most of these differences can be attributed to probable PCR and sequencing errors, since they are located in very conserved regions of the SSU rDNA gene. All *Ostreococcus* isolates (strains RCC 356, RCC 393, RCC 143, and RCC 501) are coccoid, small (less than 2 µm), non-motile, naked (no cell wall or scaly covering), and contain one chloroplast, one nucleus, one mitochondrion, and one Golgi body (Fig. 6B). A starch grain may be present in the chloroplast.

The genus *Micromonas* turns out to be quite complex, consisting of three independant lineages (Fig. 5). Clade A is relatively homogeneous genetically and composed of environmental sequences originating from Roscoff and the Atlantic Ocean. It is represented by strain CCMP 489 collected from the Sargasso Sea and identified by H. A. Thomsen as *Micromonas pusilla*. (http://ccmp.bigelow.org and Fig. 6C). Clade B is more heterogeneous genetically and composed of environmental sequences originating from Blanes, Roscoff, the Antarctic, and the Mediterranean Sea. One representative culture, RCC 434, was isolated from Blanes. This strain alternates between flagellate and non-motile morphologies. The flagellate form cannot be distinguished from *Micromonas pusilla* (Fig. 6D). Clade C is composed of environmental sequences from Roscoff and from strain CCMP 490, which was isolated from the North Atlantic, and displays the typical morphological features of *Micromonas pusilla* (Fig. 6E). Its swimming behavior was precisely described by Manton and Parke (1960). The cells swim rapidly, then frequently move in circles for a while,


97/100
RA000609.15
RD010517.5
Ostreococcus sp. RCC 356 clone 6
RA000412.100
RD010517.69
Ostreococcus sp. MBIC 10636
RA000412.115
RA010516.20
RD010517.14
RA010516.12
77/68
RA010516.2
Ostreococcus sp. RCC 344
RA000412.85
RA000609.36
RA010412.39
RA010516.18
RA010613.50
Ostreococcus sp. RCC 356 clone 8
99/98
Ostreococcus sp. RCC 356 clone 7
Ostreococcus sp. RCC 356 clone 1
RD010517.3
93/97
RD010517.44
96/95
BL000921.14
Ostreococcus sp. RCC 143
Ostreococcus sp. RCC 393
Ostreococcus tauri
RA000412.150
Ostreococcus sp. RCC 501
A
B
C
D
Ostreococcus
RA010516.49
RA010516.55
RA000412.35
BL010320.30
RA000412.117
RA010412.15
Bathycoccus prasinos BLA 77
55/80
RA000412.56
Bathycoccus prasinos ALMO2
RA000412.83
RD010517.1
RA010516.48
RA010412.43
RA000412.89
RA000609.34
RA010613.5
RA001219.33
RA010516.29
RA000412.102
100/100
RA000412.81
RA001219.41
RA000412.74
RA000412.127
RA010412.11
RA010412.45
RA010613.91
BL001221.28
RA001219.63
Bathycoccus
Micromonas pusilla CCMP 489
RA010412.38
RA010412.74
NA11-1
RA010412.10
RA000412.97
-/-
RA001219.25
RA010516.21
79/94
RA010516.47
NA37-1
RA010412.92
RA000412.152
RA010613.119
RA010516.39
RA000412.157
RA010516.36
RA000907.56
RA000412.94
-/-
RA010412.69
A
100/100
BL000921.10
80/67
BL001221.10
ANT37-4
-/-
BL001221.4
82/75
BL010320.1
RA000412.153
75/74
Micromonas sp. RCC 434
73/81
RA000907.9
RA000907.39
RA000907.63
-/-
RA000412.37
RA010516.35
-/-
RA010613.139
B
Micromonas
Micromonas pusilla CCMP 490
99/100
RA010412.7
59/66
RD010517.66
RA000412.36
C
Mantoniella antarctica
64/67
ANT37-3
Mantoniella squamata
Mantoniella
RCC 391
Mamiella sp.
Dolichomastix tenuilepis
Crustomastix sp. MBIC 10709
Cymbomonas tetramitiformis
Pyramimonas olivacea
0.1

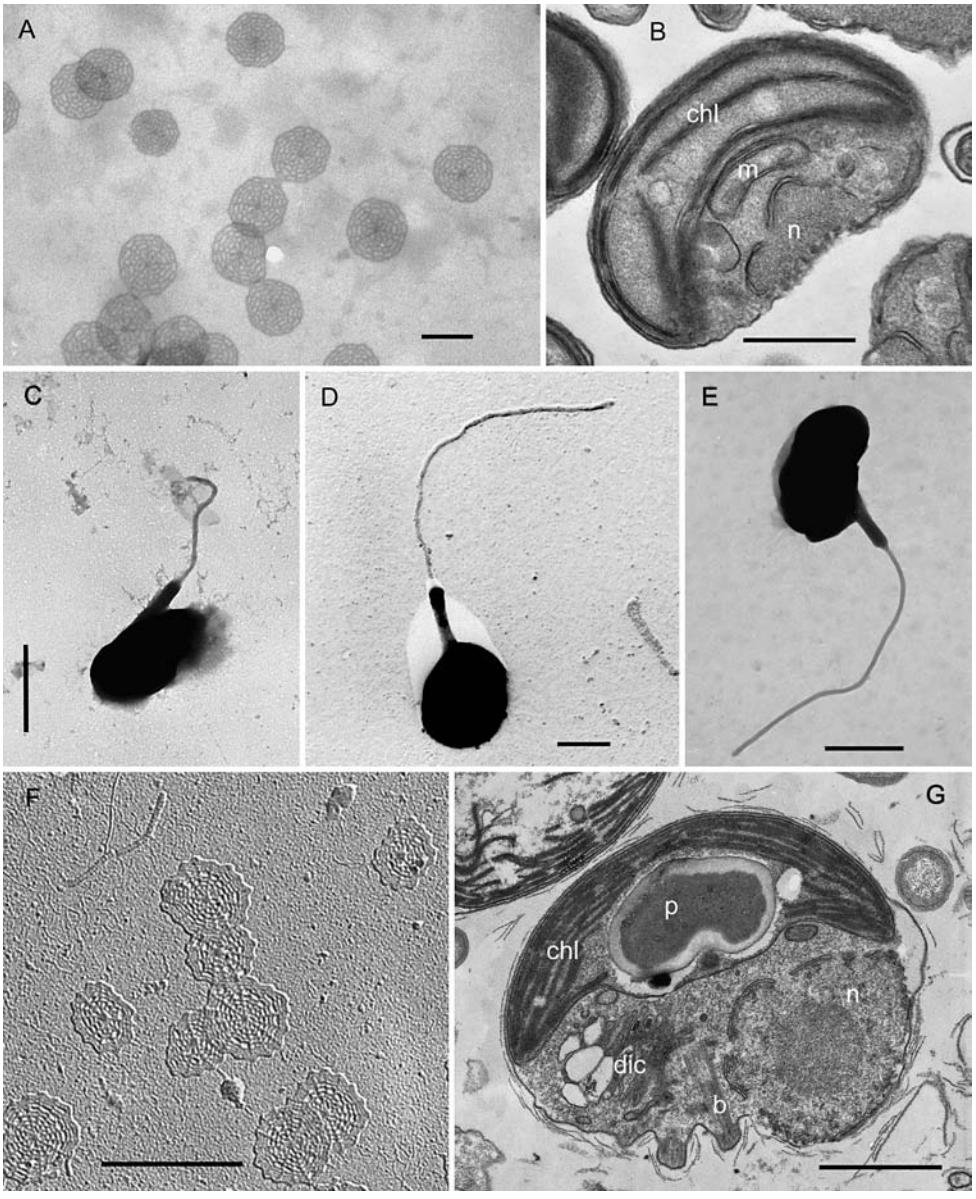



**Figure 6.** Transmission electron micrographs of strains representative from the order Mamiellales, Clade II. **A.** Stained whole-mount of scales covering the cell body of *Bathycoccus prasinos* (ALMO2). Bar = 0.1 µm. **B.** Thin section through *Ostreococcus* sp. (RCC 143) with chloroplast (chl), mitochondrion (m) and nucleus (n). Bar = 0.5 µm. **C.** . Stained whole-mount of *Micromonas* sp. (CCMP 489). Bar = 1 µm. **D.** Shadow-cast whole-mount of *Micromonas* sp. (RCC 434). Bar = 1 µm. **E.** Stained whole-mount of *Micromonas* sp. (CCMP 490). Bar = 1 µm. **F.** Shadow-cast whole-mount of scales covering the cell body of RCC 391. Bar = 0.5 µm. **G.** Thin section through RCC 391 showing the nucleus (n), chloroplast (chl) with pyrenoid (p), dictyosome (dic) and flagella bases (b). Bar = 1 µm.

◄ **Figure 5.** Phylogeny of Mamiellales based on partial 93 SSU rDNA sequences and 531 total characters. New sequences obtained from this study are in bold. The phylogenetic tree shown was inferred by the maximum likelihood (ML) method based on a TrNef (TrN equal base frequencies) model of DNA substitutions with a gamma distribution shape parameter of 0.4030and substitution rates of R(b) [A–G] = 3.1526, R(e) [C–T] = 5.3505, and 1.0 for all other substitution rates (–lnL = 2982.5193). Total number of rearrangements tried = 106 569.Bootstrap values for major clades are indicated above internodes and correspond to NJ and MP, respectively. Bootstrap values <60% are indicated by hyphens. The scale bar indicates 0.1% sequence divergence.

and then change direction. Flagellate cells belonging to the three clades A, B, and C, present the same swimming behavior.

Finally, a strain corresponding to a new genus has also been isolated (RCC 391). The cells have two flagella and swim like *Mamiella*. The cell body and flagella are covered by scales (Fig. 6F). The cells contain one nucleus, one mitochondrion, and one Golgi body. The single chloroplast has a pyrenoid (Fig. 6G).

Sequences from the ME1 genetic library (Díez et al. 2001) are not included in the phylogenetic analysis shown in Fig. 5 because the sequences available covered a different region of the SSU rDNA gene. However, comparison to full length sequences indicates that ME1-1 is closely related to *Ostreococcus* (the exact *Ostreococcus* clade is uncertain), ME1-2 to RCC 434 (clade B, *Micromonas*), and ME1-3 to *Bathycoccus*.

## Discussion

In many cases, the image of the microbial diversity provided by isolated cultures vs. direct gene sequencing from natural samples is quite divergent, as exemplified by the pioneering work of Giovannoni et al. (1990). However, this is not the case for the prasinophytes. In fact, for each clade containing environmental sequences (with the exception of Clade VIIB, which is only composed of two environmental sequences from the equatorial Pacific), one isolate is available in culture. This contrasts to what has been recently established for other eukaryotic groups, such as the novel alveolates and novel stramenopiles, for which many clades are only known from their environmental sequences (Díez et al. 2001; López-García et al. 2001; Moon-van der Staay et al. 2001). Culture conditions used for phytoplankton appear to be particularly efficient in order to isolate members of the prasinophytes and which turned out to be even more diverse in culture than in genetic libraries. As an example, *Pycnococcus provasolii*, which was initially isolated from the western North Atlantic Ocean and the Gulf of Mexico (Guillard et al. 1991), was also isolated during this work from the Mediterranean Sea, and more recently, from Roscoff. However, we could not find any environmental sequence belonging to Clade V from Roscoff, even after the analysis of seven genetic libraries generated from samples taken at different times of the year. In addition, we also isolated completely new taxa, such as RCC 391 or RCC 287 which is closely related to CCMP 1205 in Clade VIIA, a new genus that will be formally described in a separate paper. Therefore, isolation efforts must continue because they are still providing novel information about eukaryotic species richness.

Among picoplanktonic prasinophytes, we found that *Micromonas*, *Bathycoccus* and *Ostreococcus*, belonging to the Mamiellales, were the most common genera both in genetic libraries and among isolates, which is in agreement with previously published work. The ubiquity of *Micromonas pusilla* was demonstrated some years ago by serial dilution cultures of natural samples (Throndsen 1976). This very small green alga was detected at concentrations between 1 and 10 cells $ml^{-1}$ in oceanic ecosystems, such as the Caribbean Sea, the Sargasso Sea, the equatorial Pacific Ocean, and the Western North Pacific (Furuya and Marumo 1983; Throndsen 1976), between 10 and $10^3$ cells $ml^{-1}$ in Arctic waters (Throndsen 1970), and between $10^3$ and $10^4$ cells $ml^{-1}$ in several coastal areas, such as the Norwegian coast, the Gulf of Naples, the Barents Sea, and the Fraser River plume in the Strait of Georgia (Harrison et al. 1991; Throndsen 1976; Throndsen and Kristiansen 1991; Zingone et al. 1999). This tiny species can be recognized from the presence of a characteristic flagellum with a short, wide base and a long, thin distal end, and its particular swimming behavior (Manton and Parke 1960). Our phylogenetic analyses demonstrate the heterogeneity within this genus, composed of at least of three groups that cannot be distinguished by their morphology or swimming behavior. Environmental sequences suggest that different groups may co-occur within the same sample, such as in Roscoff in April 2000, when clones belonging to the three different groups were retrieved. Group B, represented by strain RCC 434, was the most abundant OTU (named ME1-2) in the five oceanic libraries analyzed by Díez et al. (2001). Genetic heterogeneity within *Micromonas pusilla*-like strains may explain genetic heterogeneity among viruses specific to this species (Cottrell and Suttle 1991) or differences in cross-reactivity of immunofluorescence assays using specific antibodies (Shapiro et al. 1989). Another member of the Mamiellales that was well represented in our genetic libraries is *Bathycoccus prasinos*, also observed by TEM by Johnson and Sieburth (1982) in field samples. In addition, it has been observed in samples from the coast of Norway and the Barents Sea. The genus *Ostreococcus* also provided very interesting results. The species *O. tauri* was initially isolated and described from the Thau Lagoon, on the French Mediterranean coast (Courties et al. 1994). We found that this genus is not only widely distributed, but also more diverse genetically than realized before. It encompasses at least four different groups,

which are impossible to distinguish based on ultrastructural features alone. From an ecological point of view, it is well known that *O. tauri* can be a successful competitor in certain environments. For example, it represents, on average, 30% of the total photosynthetic biomass of the Thau Lagoon throughout the year (Vaquer et al. 1996).

*Ostreococcus*, *Micromonas*, and *Bathycoccus* are three successful genera with several features in common. First, they belong to the same order and clade (Mamiellales, Clade II). Second, they contain the same complex assemblage of accessory pigments, namely MgDVP (Mg 2,4 divinyl-phaeoporphyrin $a_5$ monomethyl ester), prasinoxanthin, uriolide, micromonal, dihydrolutein, lutein, zeaxanthin, violaxanthin, and neoxanthin (Latasa et al. in prep). These pigments are in fact shared by all Mamiellales, with the exception of the unusual genus *Crustomastix* (Zingone et al. 2002). The main light-harvesting carotenoid in Mamiellales appears to be prasinoxanthin. The other xanthophylls could play important roles in photoprotective and light-harvesting processes, regulating photosynthesis under fluctuating light conditions (Böhme et al. 2002). Third, *Micromonas* (1.0–3.0 $\times$ 0.75–2 µm), *Bathycoccus* (1.5–2.5 $\times$ 1.0–2.0 µm), and *Ostreococcus* (0.6–2.8 $\times$ 0.6–2.4 µm) are all very small, which may confer an ecological advantage to acquire nutrients since their surface to volume ratio is larger (Raven 1986).

Large amounts of chl *b* are generally measured both in oceanic and coastal waters (Higgins and Mackey 2000; Peeken 1997; Rodríguez et al. 2002, 2003; Suzuki et al. 2002). This chl *b* could be attributed to members of prasinophytes, Chlorophyceae, Trebouxiophyceae, Chlorarachniophyceae, Euglenophyceae, and to the coccoid cyanobacterium *Prochlorococcus*. By using mathematical algorithms such as CHEMTAX (Mackey et al. 1996), the relative contribution of each phytoplankton group to chl *a* can be estimated. However, these groups do not correspond exactly to taxonomic classes since they are defined based on specific marker pigments. Some pigments are really specific for a single taxonomic entity, such as the DV chl *a* and *b* for *Prochlorococcus* spp., prasinoxanthin and uriolide for the prasinophytes (pigment type III), and lutein for all other green algae (Mackey et al. 1998). However, some are found across several unrelated classes (e.g. zeaxanthin). Moreover, pigment ratios used in the CHEMTAX algorithms still must be calibrated since they are based on the analyses of a limited number of strains isolated in culture. For instance, a large variability of the prasinoxanthin to chl *a* ratio has been observed within the genus *Micromonas*, e.g. 0.13 for the strain RCC 434 (Latasa et al. in prep) and 0.61 for another strain (Zingone et al. 2002). Moreover these pigment ratios vary widely with environmental conditions, and values in the field may fall outside the range observed in culture.

Environmental analyses by HPLC have provided contrasting results for the pigments related to the prasinophytes. In some oceanic and coastal waters, prasinoxanthin (and consequently, the number of species representing the Mamiellales, Clade V, and Clade VI) is low (Andersen et al. 1996; Higgins and Mackey 2000; Letelier et al. 1993), whereas in other studies, prasinoxanthin is much more abundant (Rodríguez et al. 2003; Suzuki et al. 2002). Dominance of Mamiellales species in all marine environments must be taken with precaution. Species of clades lacking prasinoxanthin, such as the Pyramimonadales (Clade I) or the Clade VII may be also abundant in some cases (Bird and Karl 1991; Rodríguez et al. 2002). Absence of Mamiellales species was observed twice in the genetic libraries analyzed in this paper (in the equatorial Pacific where clones belonging to Clade VII dominated and in Blanes, where *Pyramimonas* was present). HPLC pigment data from the equatorial Pacific sample indicate that prasinoxanthin was below the limit of detection, despite relatively high concentration of chl *b* (H. Claustre, unpublished data). Interestingly, high concentration of chl *b* and virtual absence of prasinoxanthin have also been reported several times from the central and western equatorial Pacific (Bidigare and Ondrusek 1996; Everitt et al. 1990; Higgins and Mackey 2000). In contrast, in congruence with clone libraries, the Mamiellales was the major group quantified by fluorescent *in situ* hybridization from Roscoff between July 2000 and September 2001, and prasinoxanthin was found to be high in the smallest size fraction (0.2 to 3 µm) throughout the year at this station (Not et al. submitted).

Prasinophytes are important components of the picophytoplankton. The large number of clades among major genera, such as *Micromonas* or *Ostreococcus*, may also result from the existence of different ecotypes. These ecotypes, as it was demonstrated for the oceanic cyanobacterium *Prochlorococcus* (Moore et al. 1998), may exhibit specific adaptations to environmental conditions (such as light, temperature, or salinity), which could help to understand their geographical distribution. Prasinophytes offer also the special advantage of being easy to culture, and constitute interesting models that could be used to understand better the evolution of photosynthetic lineages, including land plants. The best example is probably provided by *Ostreococcus tauri*, which possesses the smallest

genome described among free-living eukaryotes with a very compact organization (Derelle et al. 2002). The availability of its full sequence in the near future should prompt many studies linking genotype and ecotype as is the case for *Prochlorococcus* (Dufresne et al. 2003; Rocap et al. 2003).

## Methods

**Origin of the strains and culture conditions:** Strains listed in Table 1 were used for several purposes (phylogenetic analyses, morphological analyses, or both). Most of these strains were selected from the Roscoff Culture Collection (RCC, Roscoff, France, http://www.sb-roscoff.fr/Phyto/ collect.html). Others were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP 489, CCMP 490, CCMP 1407, and CCMP 1220). All these strains are maintained at 19–20 °C under a 12:12 h LD (Light:Dark) regime (Table 1), with the exception of RCC 356 that grows at 15 °C. Light is provided by Sylvania Daylight fluorescent bulbs. Light intensity is different according to the depth from where the strains were isolated (4 µE $m^{-2}$ $s^{-1}$ for RCC 143, RCC 393, and RCC 244; 40 µE $m^{-2}$ $s^{-1}$ for RCC 261, and 100 µE $m^{-2}$ $s^{-1}$ for all other strains). CCMP 1407, RCC 244, BLA77, RCC 499, RCC 500, and RCC 501 grow in f/2 medium (Guillard and Ryther 1962), whereas all the other strains grow in K medium (Keller et al. 1987). All RCC strains (except for BLA77 and ALMO 02 that have been lost subsequently) are freely available.

**Selection of prasinophyte strains within the RCC:** Strains were selected as follows: (1) We prescreened strains by light microscopy and whole mount observations done by transmission electronic microscopy (TEM, see protocol below); (2) Strains of special interest or strains that could not be recognized based upon morphological characters were partially sequenced. For that, DNA was extracted by a classical CTAB protocol (Doyle and Doyle 1987). The entire SSU rDNA gene was amplified using the eukaryotic primers Euk 328f (5′-ACC TGG TTG ATC CTG CCA G-3′) and Euk 329r (5′-TGA TCC TTC YGC AGG TTC AC-3′) as described in Moon-van der Staay et al. (2001). In most cases, we then cloned the PCR products using the TOPO TA cloning kit (Invitrogen) following the manufacturer's recommendations. Genetic polymorphism was then assessed by analyzing several clones (usually 10) by RFLP (Díez et al. 2001). For that, the entire SSU rDNA amplified by PCR was digested with 1 U $µl^{-1}$ of the restriction enzyme *Hae*III (Gibco BRL) for 6 to 12 h at 37 °C. The digested products were separated by electrophoresis at 80 V for 2 to 3 h on a 2.5% low-melting-point agarose gel. Partial sequences of PCR products or of clones representative of each RFLP pattern obtained were determined by Qiagen Genomics Sequencing Services, using the internal primer Euk 528f (5′-GCG GTA ATT CCA GCT CCA A-3′; Elwood et al. 1985); (3) All these partial sequences were then compared with sequences available in GenBank as well as with environmental sequences (see below) by a quick phylogenetic analysis (see below). At least, one strain by phylogenetic clade was selected and the entire nuclear SSU rDNA was sequenced (Qiagen Genomics Sequencing Services).

**Environmental clone libraries:** Sequences analyzed in this study represent a selection of clones that were extracted from 18 clones libraries either already published or submitted in separate papers (see Table 2). Information on sampling procedures, clone library construction, and analyses is available in the corresponding papers. Information regarding location, date, prefiltration, screening of the clones, and a brief analysis of the clone composition in each library appears in Table 2. For coastal genetic libraries (Roscoff and Blanes), only partial sequences were initially available. Based on a quick phylogenetic analysis (see below), a subset of clones were selected for full length sequencing (Qiagen Genomics Sequencing Services), in order to obtain at least one environmental sequence per clade.

**TEM:** Whole-mounts were prepared by placing a drop of the culture on a carbon- and formvar-coated grid. The drop was allowed to dry on the grid and was subsequently rinsed in distilled water. The whole-mounts were shadowed with gold-palladium using an Edward's speedivac 12 E6 coating unit, angle ca. 30 or contrasted for 20 min in uranyle acetate. Thin sections were prepared as described in Guillou et al. (1999), except for Figures 4A, 4B, 6B, and 6G, for which the following protocol was used: fixation in glutaraldehyde for 2 h, rinsing 3 × 30 min in medium and 2 × 10 min in 0.1 M Na cacodylate buffer (pH 8), post-fixing in 1% osmium tetroxide and 1.5 % ferricyanide in 0.1M Na cacodylate buffer for 3 h, rinsing 3 × 15 min in Na cacodylate buffer and 2 × 10 min in distilled water. Dehydration was accomplished in an ethanol series starting at 30% and gradually rising to 96%. The dehydration was concluded with 4 × 10 min in 100% ethanol and 2 × 10 min in propylene oxide. The pellets were left over night in a 1:1 mixture of propylene oxide and Epon embedding resin. Finally, the cells were transferred 3 × 1 h in Epon before polymerization at 50 °C for 12 h. The thin-sections were contrasted in lead citrate. Thin sections and whole-mounts were

viewed in a Jeol 1200ex at the laboratories for Biosciences, Department of Biology, University of Oslo.

**Phylogenetic analyses:** Sequences of different green algae were compared using three different phylogenetic analyses: maximum parsimony (MP), neighbor joining (NJ), and maximum likelihood (ML). *Cyanophora paradoxa* (Glaucocystophyceae), *Pavlova gyrans* (Prymnesiophyceae), and *Phaeocystis globosa* (Prymnesiophyceae) were used to root the trees. Sequences were aligned automatically using CLUSTALW, and the alignment was refined by hand using the BioEdit sequence editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Hypervariable regions (Helix 6, 9, E10-1, E21-1, E21-3, E21-3, 41 and 43) were realigned using Mfold (http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html). The alignment is available at http://www.sb-roscoff.fr/Phyto/Databases/index.php3. Poorly aligned positions and divergent regions were eliminated using Gblocks (Castresana 2000) using the following parameters: minimum length of a block = 5, allowed gap positions = half. Gaps were treated as missing characters. Different nested models of DNA substitution and associated parameters were estimated using Modeltest 3.0 (Posada and Crandall 1998). These parameters were used to process the NJ and ML. A heuristic search procedure using the tree bisection/reconnection branch swapping algorithm (settings as in MP) was performed to find the optimal ML tree topology. NJ, MP, and ML were done using the PAUP*4.0b10 version (Swofford 2002). Bootstrap values for NJ and MP were estimated from 1,000 replicates. For MP, the number of rearrangements was limited to 5,000 for each bootstrap replicate. The starting trees was obtained by randomized stepwise addition (number of replicates = 20).

## Acknowledgements

This work was supported by a Marie Curie fellowship (EVKE-CT-1999-50004) to LG, the EU project PICODIV (EVK3-CT-1999-00021), and the following French programs: PicManche funded by the Région Bretagne, the CNRS Aventis initiative, the "Souchothèque de Bretagne" funded in the frame of the Plan Etat-Région by the Région Bretagne and the Département du Finistère, the Centre de Ressources Biologiques, and the CNRS PROOF programs PROSOPE and BIOSOPE. We thank H. Moreau for providing the SSU rDNA sequence from *Ostreococcus tauri* and F. Rodríguez for critically reading this manuscript.

## References

**Andersen RA, Bidigare RR, Keller MD, Latasa M** (1996) A comparison of HPLC pigment analysis and electron microscopic observations for oligotrophic waters of the North Atlantic and Pacific Oceans. Deep-Sea Res Part II **43**: 517–537

**Bidigare RR, Ondrusek ME** (1996) Spatial and temporal variability of phytoplankton pigment distributions in the central equatorial Pacific Ocean. Deep-Sea Res Part II **43**: 809–833

**Bird DF, Karl DM** (1991) Massive prasinophyte bloom in the northern Gerlache Strait. Antarct J US **26**: 152–154

**Böhme K, Wilhelm C, Goss R** (2002) Light regulation of carotenoid biosynthesis in the prasinophycean alga *Mantoniella squamata*. Photochem Photobiol Sci **1**: 619–628

**Butcher RW** (1959) An Introductory Account of the Smaller Algae of Bristish coastal Waters. Part I. Introduction and Chlorophyceae. Fish Invest ser IV, HMSO, London

**Castresana J** (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol **17**: 540–552

**Chadefaud M** (1977) Les Prasinophycées. Remarques historiques, critiques et phylogénétiques. Bull Soc Phycol France **22**: 1–18

**Chrétiennot-Dinet M-J, Courties C, Vaquer A, Neveux J, Claustre H, Lautier J, Machado MC** (1995) A new marine picoeucaryote: *Ostreococcus tauri* gen. et sp. nov. (Chlorophyta, Prasinopjhyceae). Phycologia **34**: 285–292

**Christensen T** (1966) Alger. In: Böscher TW, Lange M, Sørensen T (eds) Systematisk Botanik, Vol 2, no 2. Munksgaard, Copenhagen

**Cottrell MT, Suttle CA** (1991) Wide-spread occurrence and clonal variation in viruses which cause lysis of a cosmopolitan, eukaryotic marine phytoplankter, *Micromonas pusilla*. Mar Ecol Prog Ser **78**: 1–9

**Courties C, Vaquer A, Troussellier M, Lautier J, Chrétiennot-Dinet MJ, Neveux J, Machado C, Claustre H** (1994) Smallest eukaryotic organism. Nature **370**: 255

**Daugbjerg N** (2000) *Pyramimonas tychotreta*, sp. nov. (Prasinophyceae), a new marine species from Antarctica: light and electron microscopy of the motile stage and notes on growth rates. J Phycol **36**: 160–171

**Derelle E, Ferraz C, Lagoda P, Eychenié S, Cooke R, Regad F, Sabau X, Courties C, Delseny M, Demaille J, Picard A, Moreau H** (2002) DNA libraries for sequencing the genome of *Ostreococcus tauri* (Chlorophyta, Prasinophyceae): the smallest free-living eukaryotic cell. J Phycol **38**: 1150–1156

**Díez B, Pedrós-Alió C, Massana R** (2001) Study of genetic diversity of eukayoric picoplankton in different oceanic regions by small-subunit rRNA gene cloning and sequencing. Appl Environ Microbiol **67**: 2932–2941

**Doyle JJ, Doyle JL** (1987) A rapid DNA isolation for small quantities of fresh leaf tissue. Phytochem Bull **19**: 11–15

**Dufresne A, Salanoubat M, Partensky F, Artiguenave F, Axman IM, Barbe V, Duprat S, Galperin MY, Koonin EV, Le Gall F, Makarova KS, Ostrowski M, Oztas S, Robert C, Rogozin IB, Scanlan DJ, Tandeau de Marsac N, Weissenbach J, Wincker P, Wolf YI, Hess WR** (2003) Genome sequence of the cyanobacterium *Prochlorococcus marinus* SS120, a nearly minimal oxyphototrophic genome. Proc Natl Acad Sci USA **100**: 10020–10025

**Egeland ES, Guillard RRL, Liaaen-Jensen S** (1997) Additional carotenoid prototype representatives and a general chemosystematic evaluation of carotenoids in Prasinophyceae (Chlorophyta). Phytochemistry **44**: 1087–1097

**Elwood HJ, Olsen GJ, Sogin ML** (1985) The small-subunit ribosomal RNA gene sequences from the hypotrichous ciliate *Oxytricha nova* and *Stylonychia pustulata*. Mol Biol Evol **2**: 399–410

**Everitt DA, Wright SW, Volkman JK, Thomas DP, Lindstrom EJ** (1990) Phytoplankton community compositions in the western equatorial Pacific determined from chlorophyll and carotenoid pigment distributions. Deep-Sea Res Part A **37**: 975–997

**Fawley MW, Qin M, Yun Y** (1999) The relationship between *Pseudoscourfieldia marina* and *Pycnococcus provasolii* (Prasinophyceae, Chlorophyta): evidence from 18S rDNA sequence data. J Phycol **35**: 838–843

**Fawley MW, Yun Y, Qin M** (2000) Phylogenetic analyses of 18S rDNA sequences reveal a new coccoid lineage of the Prasinophyceae (Chlorophyta). J Phycol **36**: 387–393

**Furuya K, Marumo R** (1983) The structure of the phytoplankton community in the subsurface chlorophyll maxima in the western North Pacific Ocean. J Plankton Res **5**: 393–406

**Gast RJ, McDonnell TA, Caron DA** (2000) srDNA-based taxonomic affinities of algal symbionts from a planktonic foraminifer and a solitary radiolarian. J Phycol **36**: 172–177

**Giovannoni SJ, Britschgi TB, Moyer CL, Field KG** (1990) Genetic diversity in Sargasso Sea bacterioplankton. Nature **345**: 60–63

**Guillard RRL, Ryther JH** (1962) Studies on of marine planktonic diatoms. 1 *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gram. Can J Microbiol **8**: 229–239

**Guillard RRL, Keller MD, O'Kelly CJ, Floyd GL** (1991) *Pycnococcus provasolii* gen. et sp. nov., a coccoid prasinoxanthin-containing phytoplankter from the western North Atlantic and Gulf of Mexico. J Phycol **27**: 39–47

**Guillou L, Chrétiennot-Dinet MJ, Medlin LK, Claustre H, Loiseaux-de-Goër S, Vaulot D** (1999) *Bolidomonas*: a new genus with two species belonging to a new algal class, the Bolidophyceae (Heterokonta). J Phycol **35**: 368–381

**Harrison PJ, Clifford PJ, Cochlan WP, Yin K, John MAS, Thompson PA, Sibbald MJ, Albright LJ** (1991) Nutrient and plankton dynamics in the Fraser river plume, Strait of Georgia, British Columbia. Mar Ecol Prog Ser **70**: 291–304

**Hasegawa T, Miyashita H, Kawachi M, Ikemoto H, Kurano N, Miyachi S, Chihara M** (1996) *Prasinoderma coloniale* gen. et sp. nov., a new pelagic coccoid prasinophyte from the western Pacific Ocean. Phycologia **35**: 170–176

**Higgins HW, Mackey DJ** (2000) Algal class abundance, estimated from chlorophyll and carotenoid pigments, in the western Equatorial Pacific under El Niño and non-El Niño conditions. Deep-Sea Res Part I **47**: 1461–1483

**Johnson PW, Sieburth JMN** (1982) *In situ* morphology and occurrence of eucaryotic phototrophs of bacterial size in the picoplankton of estuarine and oceanic waters. J Phycol **18**: 318–327

**Joint IR, Pipe RK** (1984) An electron microscope study of a natural population of picoplankton from the Celtic Sea. Mar Ecol Prog Ser **20**: 113–118

**Keller MD, Selvin RC, Claus W, Guillard RRL** (1987) Media for the culture of oceanic ultraphytoplankton. J Phycol **23**: 633–638

**Latasa M, Scharek R, Le Gall F, Guillou L** (submitted) Pigment suites and taxonomic groups in Prasinophyceae

**Letelier RM, Bidigare RR, Hebel DV, Ondrusek M, Winn CD, Karl DM** (1993) Temporal variability of phytoplankton community structure based on pigment analysis. Limnol Oceanogr **38**: 1420–1437

**Lewin RA, Krienitz L, Goericke R, Takeda H, Hepperle D** (2000) *Picocystis salinarum* gen. et sp. nov. (Chlorophyta) – a new picoplanktonic green alga. Phycologia **39**: 560–565

**López-García P, Rodríguez-Valera F, Pedrós-Alío C, Moreira D** (2001) Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. Nature **409**: 603–607

**Mackey MD, Higgins HW, Wright SW** (1996) CHEMTAX-a program for estimating class abundances from chemical markers: application to HPLC measurements of phytoplankton. Mar Ecol Prog Ser **144**: 265–283

**Mackey DJ, Higgins HW, Mackey MD, Holdsworth D** (1998) Algal class abundances in the western equatorial Pacific: estimation from HPLC measurements of chloroplast pigments using CHEMTAX. Deep-Sea Res Part I **45**: 1441–1468

**Manton I, Parke M** (1960) Further observations on small green flagellates with special reference to possible relatives of *Chromulina pusilla* Butcher. J Mar Biol Assoc UK **39**: 275–298

**Marin B, Melkonian M** (1994) Flagellar hairs in prasinophytes (Chlorophyta): ultrastructure and the distribution on the flagellar surface. J Phycol **30**: 659–678

**Marin B, Melkonian M** (1999) Mesostigmatophyceae, a new class of streptophyte green algae revealed by SSU rRNA sequence comparisons. Protist **150**: 399–417

**Marin B, Matzke C, Melkonian M** (1993) Flagellar hairs of *Tetraselmis* (Prasinophyceae): ultrastructural types and intrageneric variation. Phycologia **32**: 213–222

**Massana R, Balagué V, Guillou L, Pedrós-Alió C** (Submitted) Picoeukaryotic diversity in a Mediterranean coastal site studied by molecular and culturing approaches.

**McFadden GI, Hill DRA, Wetherbee R** (1986) A study of the genus *Pyramimonas* (Prasinophyceae) from south-eastern Australia. Nord J Bot **6**: 209–234

**Melkonian M** (1990) Phylum Chlorophyta Class Prasinophyceae. In Margulis L, Corliss JO, Melkonian M, Chapman DJ (eds) Handbook of Protoctista. Jones and Bartlett Publ, Boston, pp 600–607

**Melkonian M, Surek B** (1995) Phylogeny of the Chlorophyta: congruence between ultrastructural and molecular evidence. Bull Soc zool Fr **120**: 191–208

**Miyashita H, Ikemoto H, Kurano N, Miyachi S, Chihara M** (1993) *Prasinococcus capsulatus* gen. et sp. nov., a new marine coccoid prasinophyte. J Gen Appl Microbiol **39**: 571–582

**Moestrup Ø, Throndsen J** (1988) Light and electron microscopical studies on *Pseudoscourfieldia marina*, a primitive scaly green flagellate (Prasinophyceae) with posterior flagella. Can J Bot **66**: 1415–1434

**Moon-van der Staay SY, Watcher RD, Vaulot D** (2001) Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. Nature **409**: 607–610

**Moore LR, Rocap G, Chisholm SW** (1998) Physiology and molecular phylogeny of coexisting *Prochlorococcus* ecotypes. Nature **393**: 464–467

**Moro I, La Rocca N, Dalla Valle L, Moschin E, Negrisolo E, Andreoli C** (2002) *Pyramimonas australis* sp. nov. (Prasinophyceae, Chlorophyta) from Antarctica: fine structure and molecular phylogeny. Eur J Phycol **37**: 103–114

**Nakayama T, Marin B, Kranz HD, Surek B, Huss VAR, Inouye I, Melkonian M** (1998) The basal position of scaly green flagellates among the green algae (Chlorophyta) is revealed by analyses of nuclear-encoded SSU rRNA sequences. Protist **149**: 367–380

**Nakayama T, Kawachi M, Inouye I** (2000) Taxonomy and the phylogenetic position of a new prasinophycean alga, *Crustomastix didyma* gen. & sp. nov. (Chlorophyta). Phycologia **39**: 337–348

**Not F, Latasa M, Marie D, Cariou T, Vaulot D, Simon N** (submitted) Picoplanktonic Prasinophyceae abundance in the western English Channel determined by fluorescent *in situ* hybridization (FISH)

**Peeken I** (1997) Photosynthetic pigment fingerprints as indicators of phytoplankton biomass and development in different water masses of the Southern Ocean during austral spring. Deep-Sea Res Part II **44**: 261–282

**Posada D, Crandall KA** (1998) Modeltest: testing the model of DNA substitution. Bioinformatics **14**: 817–818

**Raven JA** (1986) Physiological Consequences of Extremely Small Size for Autotrophic Organisms in the Sea. In Platt T, Li WKW (eds) Photosynthetic Picoplankton. Canadian Bulletin of Fisheries Aquatic Sciences, pp 1–70

**Rocap G, Larimer FW, Lamerdin J, Malfatti S, Chain P, Ahlgren NA, Arellano A, Coleman M, Hauser L, Hess WR, Johnson ZI, Land M, Lindell D, Post AF, Regala W, Shah M, Shaw SL, Steglich C, Sullivan MB, Ting CS, Tolonen A, Webb EA, Zinser ER, Chisholm SW** (2003) Genome divergence in two *Prochlorococcus* ecotypes reflects oceanic niche differentiation. Nature **424**: 1042–1047

**Rodríguez F, Varela M, Zapata M** (2002) Phytoplankton assemblages in the Gerlache and Bransfield Straits (Antarctic Peninsula) determined by light microscopy and CHEMTAX analysis of HPLC pigment data. Deep-Sea Res Part II **49**: 723–747

**Rodríguez F, Pazos Y, Moroño A, Maneiro J, Zapata M** (2003) Temporal variation in phytoplankton assemblages and pigment composition in a fixed station in the ría of Pontevedra (NW Spain). Estuar Coast Shelf Sci **58**: 715–730

**Romari K, Vaulot D** (2004) Composition and temporal variability of picoeukaryotes communities at the coastal site of the English Channel from 18S rDNA sequences. Limnol Oceanogr **49**: 784–798

**Shapiro LP, Campbell L, Haugen EM** (1989) Immunochemical recognition of phytoplankton species. Mar Ecol Prog Ser **57**: 219–224

**Sieburth JM, Keller MD, Johnson PW, Myklestad SM** (1999) Widespread occurrence of the oceanic ultraplankter, *Prasinococcus capsulatus* (Prasinophyceae), the diagnostic "golgi-decapore complex" and the newly described polysaccharide "capsulan". J Phycol **35**: 1032–1043

**Silver MW, Gowing MM, Davoll PJ** (1986) The Association of Photosynthetic Picoplankton and Ultraplankton with Pelagic Detritus through the Water Column (0–2000 m). In Platt T, Li WKW (eds) Photosynthetic Picoplankton. Canadian Bulletin of Fisheries and Aquatic Sciences, pp 311–341

**Steinkötter J, Bhattacharya D, Semmelroth I, Bibeau C, Melkonian M** (1994) Prasinophytes form independent lineages within the Chlorophyta: evidence from ribosomal RNA sequence comparisons. J Phycol **30**: 340–345

**Stockner JG, Antia NJ** (1986) Algal picoplankton from marine and freshwater ecosystems: a multidisciplinary perspective. Can J Fish Aquat Sci **43**: 2472–2503

**Suzuki K, Minami C, Liu H, Saino T** (2002) Temporal and spatial patterns of chemotaxonomic algal pigments in the subarctic Pacific and the Bering Sea during the early summer of 1999. Deep-Sea Res Part II **49**: 5685–5704

**Swofford DL** (2002) PAUP*. Phylogenetic analysis using parsimony (*and others methods). version 4, Sinauer associates, Sunderland, MA

**Sym SD, Pienaar RN** (1993) The Class Prasinophyceae. In Round FE, Chapman DJ (eds) Progress in Phycological Research, Volume 9, Biopress Ltd, Bristol, pp 281–376

**Sym SD, Pienaar RN** (1999) An additional punctuate species of *Pyramimonas*, *P. formosa*, sp. nov., and its impact of the subgenera punctatae and *Pyramimonas* (Prasinophyceae, Chlorophyta). J Phycol **35**: 1313–1321

**Takahashi M, Hori T** (1984) Abundance of picophytoplankton in the subsurface chlorophyll maximum layer in subtropical and tropical waters. Mar Biol **79**: 177–186

**Tamura K, Nei M** (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol **10**: 512–526

**Throndsen J** (1970) Flagellates from Arctic waters. Nytt Mag Bot **17**: 49–57

**Throndsen J** (1976) Occurrence and productivity of small marine flagellates. Norw J Bot **23**: 269–293

**Throndsen J, Kristiansen S** (1991) *Micromonas pusilla* (Prasinophyceae) as part of picoplankton and nanoplankton communities of the Barents Sea. Proceedings of the Pro Mare Symposium on Polar Marine Ecology **10**: 201–207

**Throndsen J, Zingone A** (1997) *Dolichomastix tenuilepis* sp. nov., a first insight into the microanatomy of the genus *Dolichomastix* (Mamiellales, Prasinophyceae, Chlorophyta). Phycologia **36**: 244–254

**Vaquer A, Troussellier M, Courties C, Bibent B** (1996) Standing stock and dynamics of picophytoplankton in the Thau lagoon (northwest Mediterranean coast). Limnol Oceanogr **41**: 1821–1828

**Vaulot D, Le Gall F, Marie D, Guillou L, Partensky F** (in press) The Roscoff Culture Collection (RCC): a collection dedicated to marine picoplankton. Nova Hedwigia

**Zeidner G, Preston CM, Delong EF, Massana R, Post AF, Scanlan DJ, Béjà O** (2003) Molecular diversity among marine picophytoplankton as revealed by *psbA* analyses. Environ Microbiol **5**: 212–216

**Zingone A, Sarno D, Forlani G** (1999) Seasonal dynamics in the abundance of *Micromonas pusilla* (Prasinophyceae) and its viruses in the gulf of Naples (Mediterranean Sea). J Plankton Res **21**: 2143–2159

**Zingone A, Borra M, Brunet C, Forlani G, Kooistra WHCF, Procaccini G** (2002) Phylogenetic position of *Crustomastix stigmatica* sp. nov. and *Dolichomastix tenuilepis* in relation to the Mamiellales (Prasinophyceae, Chlorophyta). J Phycol **38**: 1024–1039