

## ORIGINAL PAPER

# *Prasinoderma singularis* sp. nov. (Prasinophyceae, Chlorophyta), a Solitary Coccoid Prasinophyte from the South-East Pacific Ocean

Fabien Jouenne<sup>a,b,1,2</sup>, Wenche Eikrem<sup>a,c</sup>, Florence Le Gall<sup>b</sup>,  
Dominique Marie<sup>b</sup>, Geir Johnsen<sup>d</sup>, and Daniel Vaultot<sup>b</sup>

<sup>a</sup>Norwegian Institute for Water Research, Gaustadalléen 21, 0349 OSLO, Norway

<sup>b</sup>UPMC (Paris 06) et CNRS, UMR 7144, Station Biologique de Roscoff, Place Georges Teissier, BP 74, 29682 ROSCOFF Cedex, France

<sup>c</sup>Department of Biology, University of Oslo, PO Box 1069, Blindern, 0316 OSLO, Norway

<sup>d</sup>Trondhjem biological station, Department of biology, Norwegian University of Science technology, Bynesveien 46, N-7491 TRONDHEIM, Norway

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During the BIOSOPE cruise in the South-East Pacific Ocean in 2004, several unidentified strains of prasinophytes were isolated into culture. Of these, nine strains composed a group for which the partial 18S rRNA gene sequence was related to *Prasinoderma coloniale*. The ultrastructure, morphology, division process, pigment composition, genome size and molecular genetic phylogeny of these nine strains were investigated, using *P. coloniale* as a reference. The 18S rRNA gene sequence of *P. singularis* sp. nov. shares only 96.9% of identity with that of *P. coloniale* and contains a conserved insertion of 567 bp length not recorded in *P. coloniale*. When compared to *P. coloniale*, *P. singularis* sp. nov. is morphologically characterized by the absence of colonies, smaller cells with a thinner cell wall, and a second cell type with a different cell covering.

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## Introduction

Prasinophytes (Chlorophyta) constitute a complex group of unicellular algae at the base of the green algal lineage. Molecular methods, such as environmental clone libraries of the 18S rRNA gene and fluorescent in situ hybridization (FISH), have demonstrated that they can be one of the

major components of picoeukaryotic communities in coastal waters (Not et al. 2004; Romari and Vaultot 2004). At present 9 prasinophyte clades are recognized, most corresponding to existing orders (Guillou et al. 2004; Viprey et al. 2008). The order Mamiellales (clade II following the nomenclature of Guillou et al. 2004) is the group that has received most attention, especially emblematic genera such as *Ostreococcus* or *Micromonas* (Courties et al. 1994; Throndsen and Kristiansen 1991). Other clades include clade V (Pycnococaceae, ex-Pseudoscourfieldiales, Fawley et al. 2000), clade VI (Prasinococcales, Fawley et al. 2000),

<sup>1</sup>Corresponding author; fax +33 2 98 292324

<sup>2</sup>Present address: Université de CAEN Basse-Normandie, Esplanade de la Paix, EP2 M, 14032 CAEN Cedex, France  
e-mail fabien.jouenne@unicaen.fr (F. Jouenne).

clade VII (Guillou et al. 2004), and clades VIII and IX that contain only environmental sequences (Viprey et al. 2008). Recently, two new classes, the Mamiellophyceae (Marin and Melkonian 2009) and Nephroselmidophyceae (Nakayama et al. 2007; initially named Nephrophyceae (Cavalier-Smith 1993)) were proposed to accommodate clade II (Mamiellales) and clade III (Nephroselmidales), respectively, leading the way for splitting of the paraphyletic class Prasinophyceae into new classes delineated along the current clade/order boundaries.

The class Prasinophyceae was originally associated with scaly flagellates having a flagellar pit (Sym and Pienaar 1993). Pigment and molecular genetic analyses, as well as observations of morphology and ultrastructure, have resulted in the progressive extension of the class to include organisms with additional morphologies. Several species within the Prasinophyceae are non-motile and lack scales such as the naked *Ostreococcus tauri* (Mamiellophyceae, Chretiennot-Dinet et al. 1995), the coccoid *Pycnococcus provasolii* (Clade V, Guillard et al. 1991), which is probably the vegetative phase of the scale-covered flagellate *Pseudoscourfieldia marina* (Fawley et al. 1999; Guillou et al. 2004; Zingone et al. 2002), and the coccoid species *Prasinococcus capsulatus* and *Prasinoderma coloniale* (Clade VI, Prasinococcales, Hasegawa et al. 1996; Miyashita et al. 1993). Prasinococcales have received little attention in comparison to other prasinophyte taxa. In addition to the original description of *P. coloniale* (Hasegawa et al. 1996), an investigation of the Golgi-decapore complex (Sieburth et al. 1999) and a study showing sensitivity to heavy metals (Satoh et al. 2005) for *P. capsulatus* are the only specific studies reported for this group. Information on pigments (Latasa et al. 2004) and the occurrence of environmental sequences (Viprey et al. 2008) of the Prasinococcales have appeared in general studies of prasinophytes.

During the BIOSOPE cruise in the South East Pacific (Claustre et al. 2008), many new strains of picoplankton were isolated and deposited in the Roscoff Culture Collection (Le Gall et al. 2008). Phylogenetic analyses based on partial 18S rRNA gene sequences have shown a relationship between nine strains isolated during the BIOSOPE cruise and *P. coloniale* (RCC 137). These strains, originating mainly from the vicinity of the upwelling off the Chilean coast, formed a separate clade with only 94.7% of identity to *P. coloniale* (Le Gall et al. 2008), suggesting that they could belong to a new species within the genus *Prasinoderma*. In the

present paper, the morphology, ultrastructure, pigment content, genome size, and 18S rRNA gene phylogeny of *P. coloniale* and of several of the nine new strains have been investigated and compared. Based on these results, a new species, *P. singularis* sp. nov., is characterized and described.

## Description

*Prasinoderma* Hasegawa et Chihara in Hasegawa et al. 1996, *Phycologia* 35: 171 emend. Jouenne

**Emended diagnosis:** Alga unicellularis; cellulae sphaericae (2.2-5.5 µm diametro), cum vaginis crassis et multis (28-171 nm); sine flagellis, sine squamis; chloroplastus unus, bilobatus, cupulatus; pyrenoides vagina amyli circumcincta, projectura ex cytoplasmate invasa; projectura bifurcata, cytoplasmate, mitochondria completa; reproductio asexualis per fissionem binariam et processum gemmationem; pigmenta chloroplasti ex chlorophyllis a et b, magnesio 2,4-divinylphaeoporphyrino, prasinoxanthino, micromonol et uriolide pro pigmento majore constantia.

Unicellular algae; cells spherical (2.2-5.5 µm) with thick and multilayered cell wall (28-171 nm thick); without flagella, without scales, chloroplast single, bilobed cupuliform, pyrenoid covered with a starch sheath, invaded by a projection from the cytoplasm; projection bifurcate, filled with cytoplasm and extension from the mitochondrion; asexual reproduction by unequal binary fission and "budding-like" process; chlorophylls a and b, Mg-2,4-divinylphaeoporphyrin a<sub>5</sub> monomethyl ester, prasinoxanthin, micromonol and uriolide are the major pigments.

*Prasinoderma singularis* Jouenne sp. nov.

**Diagnosis:** Cellulae solitariae, flavo-virentes, 2.2-4.0 µm diametro, cum vaginis crassis et multis (28-109 nm), planctonicae. Integumentum levis veritendunt secretionem. Species haec ab *P. coloniale* differt coloniis carentibus et secretionem.

Solitary, yellow-green, non-motile cells (2.2-4.0 µm) with multilayered cell wall (28-109 nm thick), planktonic. Cell covering smooth or sometimes with secretion. This species differs from *P. coloniale* by the absence of colonies and the secretion.

**Holotype:** Fig. 1A. Inclusions of the authentic culture is deposited at the Roscoff Culture Collection at Station Biologique, Roscoff, France.

**Type locality:** Eutrophic, coastal surface water (0-40m): South-East Pacific Ocean (Chilean Coast), 72°25'12"W, 34°30'36"S

**Habitat:** Marine, planktonic

**Authentic culture:** Strain RCC 927. Deposited in the Roscoff Culture Collection (<http://www.sb-roscoff.fr/Phyto/RCC>) at the Station Biologique, Roscoff, France.

**Etymology:** *singularis* (solitary) refers to its non-colonial way of life.

## Results

### Cultures

Strains were isolated from different stations and depths during the BIOSOPE cruise (Table 1). The *Prasinoderma singularis* strains were mostly obtained from samples taken within the upwelling zone off the Chilean coast, from the surface down to 40 m.

### Morphology and Ultrastructure

In light microscopy, *P. singularis* appeared as single non-motile cells with a yellow-green chloroplast containing a conspicuous pyrenoid (Fig. 1A). This new species can be distinguished from *P. coloniale* (Fig. 1B) by the absence of colonies. *P. coloniale* formed loose sticky colonies (Fig. 1C), easily visible in culture flasks as a yellow-green layer accumulating at the bottom. None of the nine *P. singularis* strains have been observed to form such a layer in cultures. The size of *P. singularis* measured by light microscopy ranged from 2.2 to 4.0  $\mu\text{m}$ , whereas individual *P. coloniale* cells measured between 2.5 and 5.5  $\mu\text{m}$ .

In field emission scanning electron (FE-SEM) micrographs, *P. coloniale* appeared coccoid without scales or any other surface ornamentation (Fig. 2), some cells displaying a split (Fig. 2B). In *P. singularis*, two cell types have been observed in cultures (Fig. 3A). One type was indistinguishable from *P. coloniale* (smooth, coccoid cell with split, Fig. 3A and B), whereas the other was surrounded by an unknown secretion (Fig. 3A and C) and lacks a split. No flagellate stage has been observed in any of the cultures examined.

Asexual reproduction occurred by unequal binary fission resembling budding in yeast (Fig. 4). Initially, the cell displayed an equatorial line due to the formation of two daughter cells within the same cell wall (Fig. 4A), then a split appeared on the most recent layer of the old multilayered cell wall (Fig. 4B), and two new cell walls were synthesized inside the old cell wall (Fig. 4C); subsequently the smaller daughter cell began to extrude (Fig. 4D) and finally it was expelled from the old

cell wall (Fig. 4E-F). The largest daughter cell kept the old cell wall around its own new cell wall (Fig. 4G) and the two daughter cells separated (Fig. 4H).

All of the ultrastructural characters observed in the original description of *P. coloniale* (Hasegawa et al. 1996) were detected in the present study (Fig. 5A-F). These features such as the multilayered cell wall (Fig. 6A-D), the nucleus (Fig. 6A-C), the Golgi body (Fig. 6A), and the single cup-shaped chloroplast (Fig. 6A-B) containing a bifurcate intrusion with an extension of the single mitochondrion and surrounded by a starch sheath (Fig. 6A, 6E-F), were also present in *P. singularis*. The thickness of the cell wall (measured on thin section micrographs) ranged from 37 to 171 nm ( $73 \pm 31$  nm) in *P. coloniale*, while that of *P. singularis* was slightly thinner, varying between 28 and 109 nm ( $56 \pm 17$  nm).

### Photosynthetic Pigments

*Prasinoderma coloniale* strain RCC 137 and *P. singularis* strains RCC 927 and RCC 946 presented similar pigment suites as determined by HPLC. They contained chlorophylls *a*, *b* and MgDVP (Mg 3,8-divinylphaeoporphyrine *a5* monomethyl ester) (Table 2). Major light harvesting carotenoids were prasinoxanthin and a particularly high amount of urolide. Photoprotective pigments were also detected (zeaxanthin, lutein, violaxanthin, antheraxanthin). Micromonol (Egeland and Liaaen-Jensen 1995), present in both species, was recorded for the first time in the genus *Prasinoderma*. The amount of micromonol and photoprotective pigments like zeaxanthin and lutein, relative to chlorophyll *a*, were significantly higher in *P. coloniale* than *P. singularis*.

### Genome Size

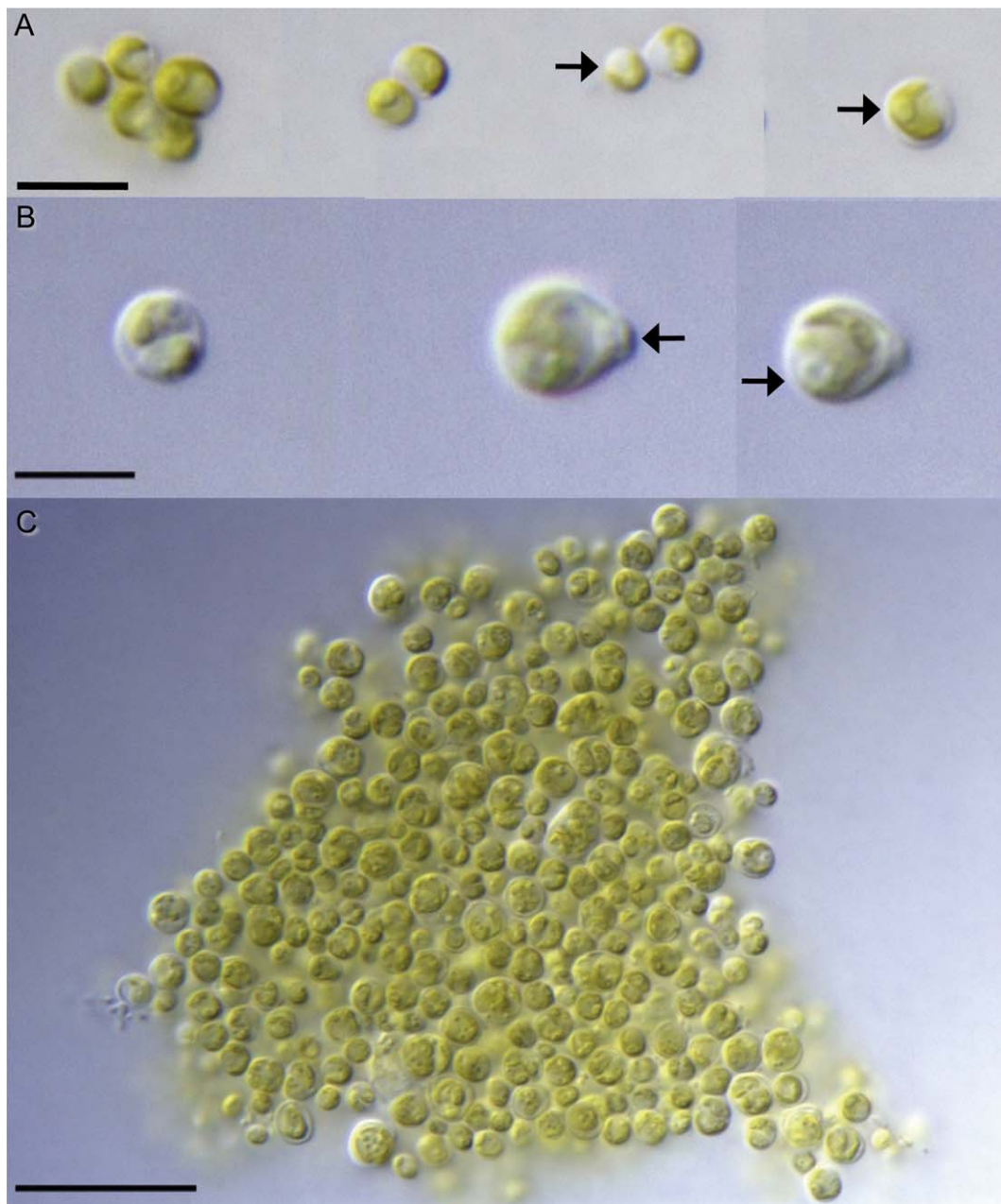
Genome sizes estimated by flow cytometry and compared to an internal standard (*Ostreococcus tauri* RCC 116, genome size 12.56 Mbp) were different for *Prasinoderma coloniale* and *P. singularis* (Fig. 7). Two peaks were observed for *P. coloniale* (ca. 21 and 36 Mbp), while only one was recorded for *P. singularis* (ca. 45 Mbp).

### Phylogenetic Analyses

The full-length 18S rRNA gene sequences from *Prasinoderma* strains isolated from the South-East Pacific Ocean (RCC 927 and RCC 946, Table 1) share 96.9% identity with *P. coloniale*. A conserved insertion of 567 bp starting from position 1090 of RCC 927 18S rRNA gene occurs in *P. singularis* sequences but not in any of the available

**Table 1.** List of the Roscoff Culture Collection (RCC) strains used in this study. RCC 137 corresponds to the strain CCMP 1220 (Center for Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, Maine, USA, <https://ccmp.bigelow.org/>).

RCC	Name	Genus	Species	Sampling location	Longitude, Latitude		Depth (m)
137	CCMP1220	<i>Prasinoderma</i>	<i>Coloniale</i>	Gulf of Mexico	75°0'0"W	23°0'0"N	0
916	Biosope_34B2_FL2-5	<i>Prasinoderma</i>	<i>Coloniale</i>	West of Marquesas Island	141°16'12"W	8°19'12"S	10
941	Biosope_169_FL2-4	<i>Prasinoderma</i>	<i>singularis</i> sp. nov	South-East Pacific	78°7'12"W	33°21'0"S	40
910	Biosope_190_FL2-4	<i>Prasinoderma</i>	<i>singularis</i> sp. nov	South-East Pacific	75°50'24"W	33°34'48"S	5
936	Biosope_212_FLA5	<i>Prasinoderma</i>	<i>singularis</i> sp. nov	Coastal upwelling area (off the Chile)	73°20'24"W	33°51'36"S	30
934	Biosope_214_FLB3	<i>Prasinoderma</i>	<i>singularis</i> sp. nov	Coastal upwelling area (off the Chile)	73°20'24"W	33°51'36"S	30
933	Biosope_214_FLB6	<i>Prasinoderma</i>	<i>singularis</i> sp. nov	Coastal upwelling area (off the Chile)	73°20'24"W	33°51'36"S	30
929	Biosope_219_FL1-4	<i>Prasinoderma</i>	<i>singularis</i> sp. nov	Coastal upwelling area (off the Chile)	73°20'24"W	33°51'36"S	30
930	Biosope_212_FLA2	<i>Prasinoderma</i>	<i>singularis</i> sp. nov	Coastal upwelling area (off the Chile)	73°20'24"W	33°51'36"S	30
946	Biosope_235_FL1-4	<i>Prasinoderma</i>	<i>singularis</i> sp. nov	Coastal upwelling area (off the Chile)	72°25'12"W	34°30'36"S	0
927	Biosope_243_FL1-4	<i>Prasinoderma</i>	<i>singularis</i> sp. nov	Coastal upwelling area (off the Chile)	72°25'12"W	34°30'36"S	0

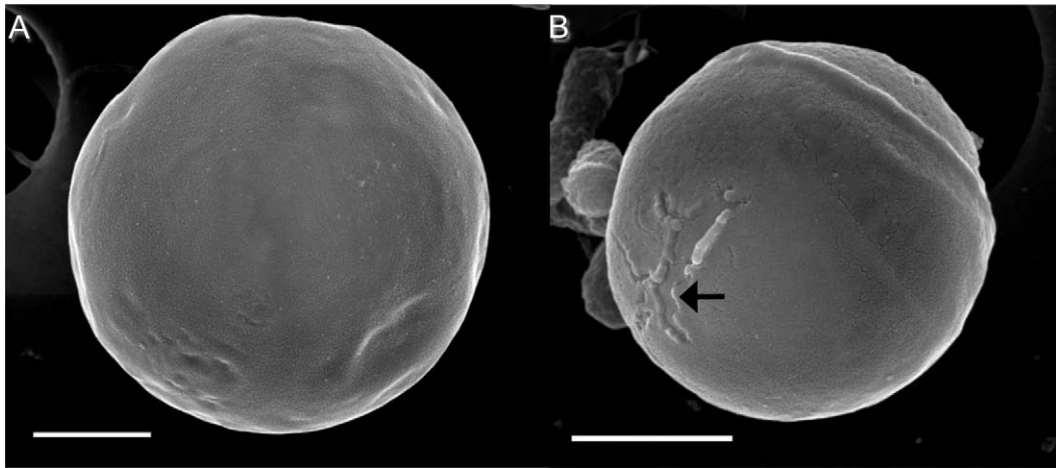


**Figure 1.** Light micrographs of *Prasinoderma coloniale* and *Prasinoderma singularis* sp. nov. **A.** Individual cells of *P. singularis* sp. nov. (RCC 927) with cup-shaped chloroplast and pyrenoid (right arrow). Potential expulsion of a daughter cell indicated by the left arrow. Scale bar = 5  $\mu$ m. **B.** Individual cells of *P. coloniale* (RCC 137) with cup-shaped chloroplast and pyrenoid (right arrow). Beginning of daughter cell expulsion indicated by left arrow. Scale bar = 5  $\mu$ m. **C.** Colony of *P. coloniale* (RCC 137). Scale bar = 20  $\mu$ m.

*P. coloniale* sequences. The phylogenetic analyses based on the 18S rRNA gene sequences confirms the affiliation of the two *P. singularis* strains to the order Prasinococcales and their placement on a branch separate from *P. coloniale* with high bootstrap values (Fig. 8).

## Discussion

According to 18S rRNA phylogeny, the new strains isolated from the South-East Pacific Ocean during the BIOSOPE cruise are affiliated to the Prasinococcales (Prasinophyceae Clade VI). At a



**Figure 2.** Scanning electron micrographs of *Prasinoderma coloniale* (RCC 137). Scale bars = 1  $\mu\text{m}$ . **A.** Spherical coccoid cell. **B.** Cell with split in cell wall (arrow).

lower taxonomic level, they belong to the genus *Prasinoderma* based on the following characters: (1) naked coccoid cells, (2) multilayered thick cell wall, (3) pyrenoid with bifurcate intrusion of cytoplasm and extension of the mitochondrion inside this pyrenoid, (4) pigment suite (mainly chl *a*, chl *b*, prasinoxanthin, Mg DVP, uriolide).

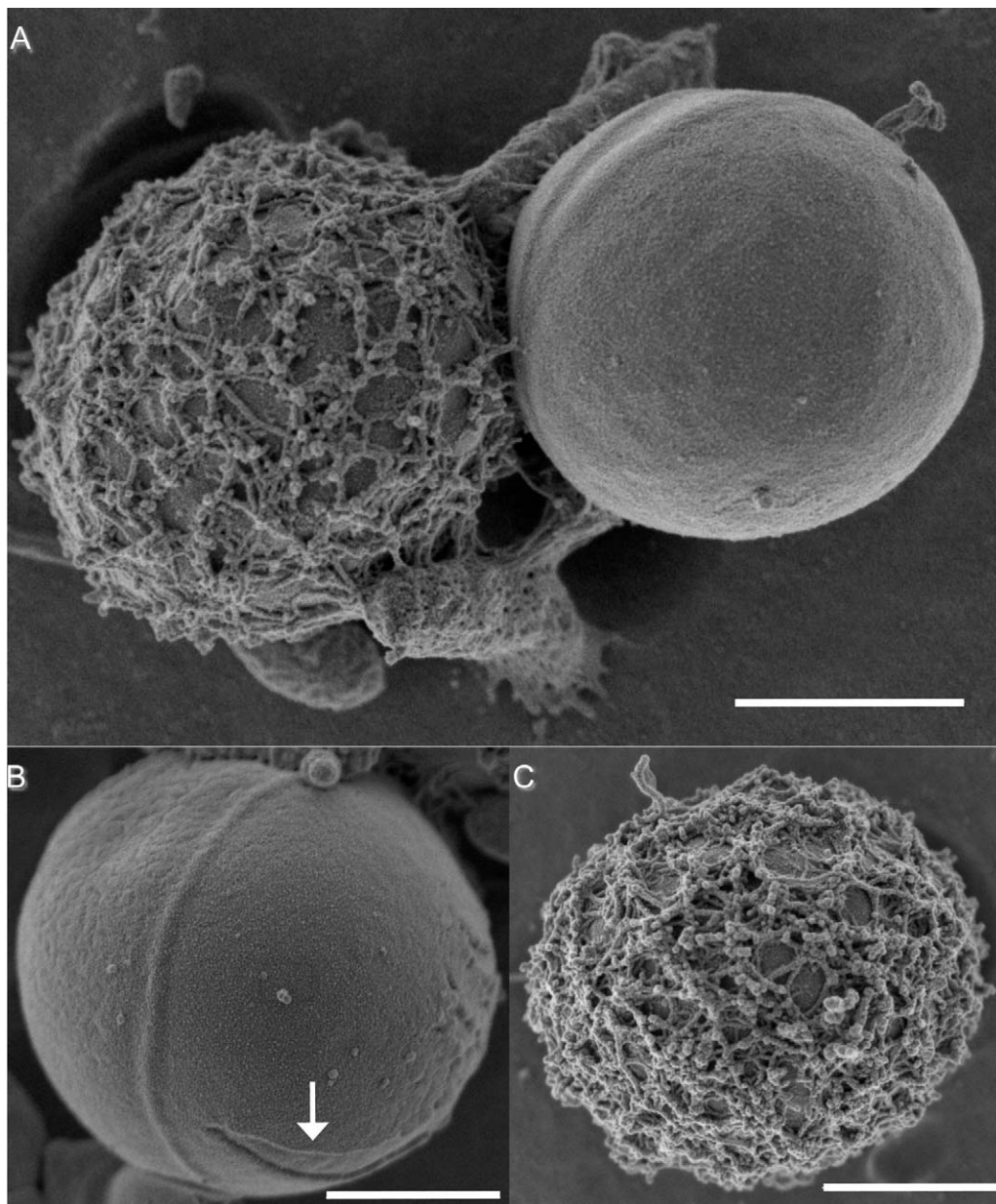
The position of these strains in a branch separate from *Prasinoderma coloniale* in the 18S rRNA gene phylogeny and the relatively low identity shared between their sequences and those of *P. coloniale* support the establishment of a new species within the genus *Prasinoderma*. Compared to *P. coloniale*, the new species *P. singularis* is morphologically characterized by the absence of colonies, smaller cells with a thinner cell wall, and a second cell type with a different cell covering.

In the original description of *P. coloniale*, the process of unequal cell division was described as “a new cell wall is formed around each of the daughter cells which are slightly unequal in size; the smaller daughter-cell is released through a small split in the parent cell wall; the larger cell remains within the parent cell wall and expands to fill the empty space; the larger cell is thus surrounded by two layers of cell wall” (Hasegawa et al. 1996). The exact nature of this process in the Prasinococcales is still under debate as some authors have observed daughter cell extrusion through a hole resulting in a scar in the thick parent wall (thin section micrographs, Hasegawa et al. 1996; Miyashita et al. 1993), while others have suggested the loss of the cell wall prior to separation of the two daughter cells (Sieburth et al. 1999). In the present study, division stages observed in scanning electron microscopy show

clear evidence for an unequal binary fission with a budding event.

With FE-SEM we detected a second cell type in *P. singularis* cultures that is entirely surrounded by a mucous-like substance. It is possible that the smaller daughter cell in *P. singularis* initially has only a thin veil of cell wall precursors, as described for *Prasinococcus capsulatus* (Sieburth et al. 1999) in which cell wall completion is accomplished after cell release (Miyashita et al. 1993). Patterns observed surrounding the cell could be an artefact due to sample chemical preparation on cell wall precursors. In Chlorellaceae, two types of daughter cell wall synthesis (early and late types) have been described (Yamamoto et al. 2005).

The pigment composition of *P. singularis* is very similar to that recorded in the original description of *P. coloniale*. The only difference is that Hasegawa et al. (1996) did not detect micromonol, which we found in both species. Micromonol is the corresponding allylic *prim* carotenol of micromonal and it has been previously recorded in three species of Prasinophyceae: *Micromonas pusilla* and *Mantoniella squamata* (Mamiellophyceae) as well as in *Pseudoscourfieldia marina* (Pycnococaceae) (Egeland et al. 1995). It is likely present in other prasinophytes but most of the time, micromonal is cited rather than micromonol (Latasa et al. 2004). Micromonol, along with uriolide, micromonal, and dihydrolutein, has been detected in the light-harvesting complex (LHC) of *M. squamata* and these minor carotenoids are thought to play an essential structural role in stabilisation of the LHC (Wilhelm et al. 1997). The amount of uriolide relative to chlorophyll *a* recorded in the present study

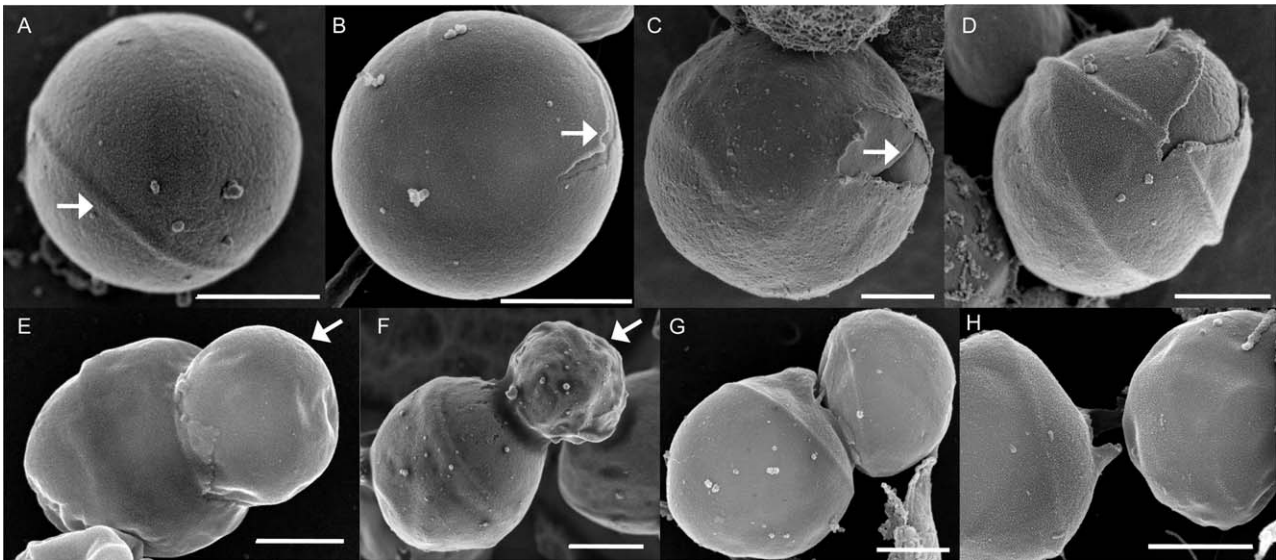


**Figure 3.** Scanning electron micrographs of *Prasinoderma singularis* sp. nov. Scale bars = 1  $\mu$ m. **A.** RCC 946. The two different cell types of *P. singularis* sp. nov. **B.** RCC 946. Spherical naked cell with split (arrow). **C.** RCC 927. Single cell surrounded by unknown secretion.

was three times higher than previous measurements in *P. coloniale* (Hasegawa et al. 1996) and two times higher than in its relative, *P. capsulatus* (Latasa et al. 2004). Uriolide has been considered as a rare carotenoid, specific of *P. capsulatus* and has not been recorded in the Pycnococcaceae (Fawley 1992; Latasa et al. 2004). Our data suggest that high amounts of uriolide could be used to discriminate the genus *Prasinoderma*. Violaxanthin was observed here in both *Prasinoderma* species,

as mentioned in the original description of *P. coloniale* (Hasegawa et al. 1996) but not in a more recent study including this species (Latasa et al. 2004). However, violaxanthin may go undetected due to less stable binding properties compared to other xanthophylls (Wilhelm et al. 1997).

Flow cytometry revealed a difference in genome size between the two *Prasinoderma* species. Moreover, in contrast to *P. singularis*, in *P. coloniale* two distinct DNA peaks were observed (Fig. 7a),



**Figure 4.** Scanning electron micrographs of the binary fission within the genus *Prasinoderma*. Scale bars = 1  $\mu\text{m}$ . **A.** Coccoid cell showing the equatorial line (arrow) caused by the formation of two daughter cells within the same cell wall (*P. singularis* sp. nov. RCC 936). **B.** Early stage of split formation (arrow) (*P. singularis* sp. nov. RCC 946). **C.** Opening of the split and apparition of the limits (arrow) of the two new cell walls synthesized inside the old cell wall (*P. singularis* sp. nov. RCC 946). **D.** Early stage of the expulsion of the smaller daughter cell (*P. singularis* sp. nov. RCC 946). **E,F.** Expulsion of daughter cell (arrow) from the old cell wall (*P. coloniale* RCC 137 and *P. singularis* sp. nov. RCC 927). **G.** The larger daughter cell keeps the old cell wall around its own new cell wall (*P. coloniale* RCC 916). **H.** Separation of the two daughter cells (*P. coloniale* RCC 916).

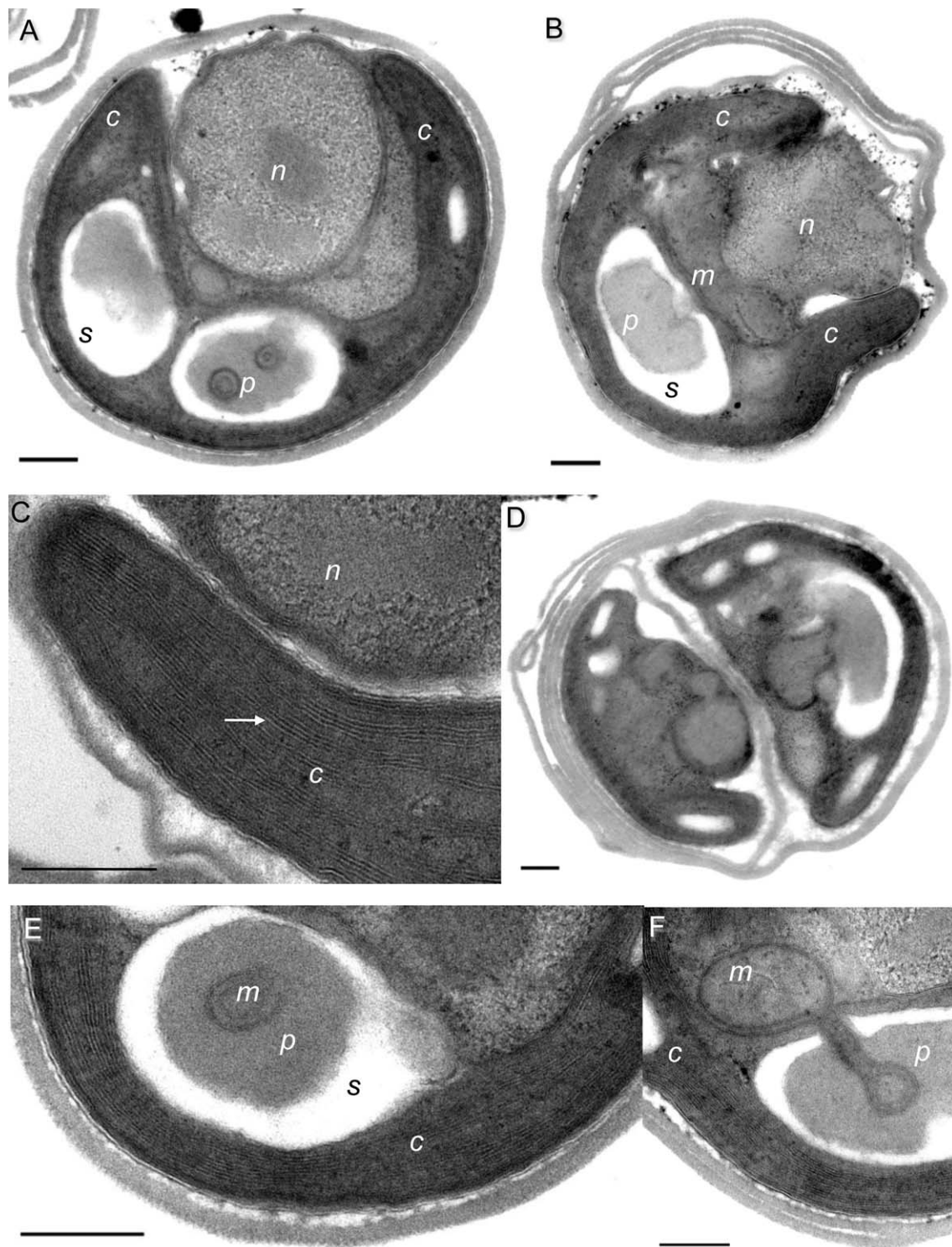
suggesting the occurrence of a haplo-diplontic life cycle. This hypothesis will require further study since the peak with the lower DNA content in *P. coloniale* could also result from the incomplete isolation of a fraction of the cell nuclei due to the multilayered structure of the cell wall.

**Table 2.** Pigment-to-Chl *a* ratios of *Prasinoderma* species. Significant differences are shown by \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ) and \*\*\* ( $p < 0.001$ ),  $n = 3$ .

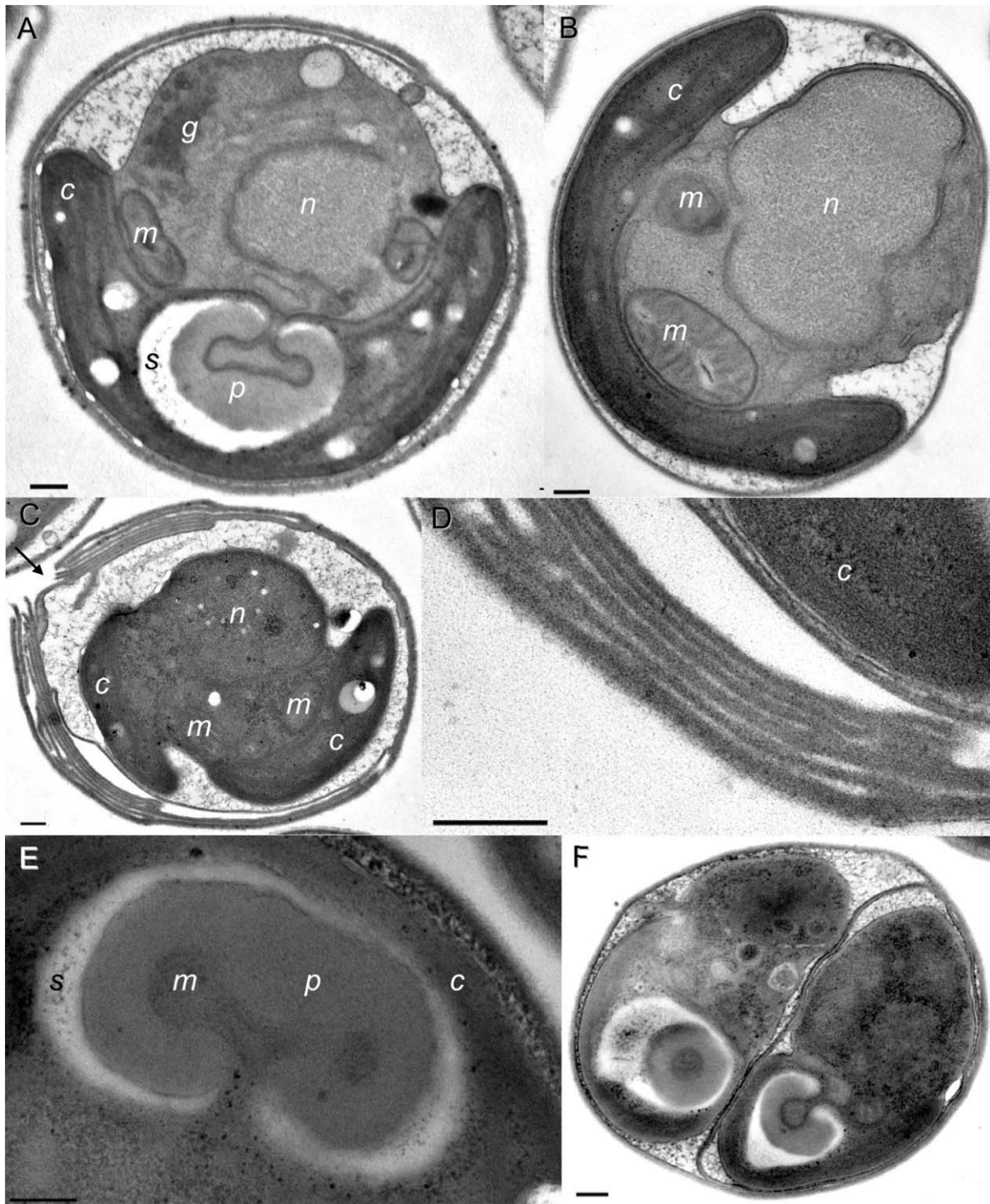
Pigments	Pigment / Chl <i>a</i> ratios	
	<i>P. coloniale</i> RCC 137	<i>P. singularis</i> sp. nov. RCC 927/RCC 946
MgDVP	0.69	0.54
Chlorophyll b	0.66	0.61
Prasinoxanthin	0.56	0.43
Neoxanthin	0.20	0.23
Uriolide	0.17	0.19
Micromonol	0.12	0.08*
Zeaxanthin	0.04	0.02***
Lutein	0.04	0.01***
Violaxanthin	0.03	0.09**
Antheraxanthin	0.03	0.02

The phylogeny based on full-length 18S rRNA gene sequences is consistent with previous analyses of prasinophytes (Guillou et al. 2004). *Prasinoderma singularis* is clearly affiliated to clade VI of the prasinophytes (order Prasinococcales) and forms a separate branch. The intron observed in the 18S rRNA gene sequence of *P. singularis* sp. nov. seems to belong to group I introns. The intron is located at position 1139 relative to *Ostreococcus tauri* 18S rRNA sequence (coming from the whole genome sequence; Derelle et al. 2006). This position corresponds to position 1190 on *E. coli* 16S rRNA gene sequence, commonly used to classify introns (Jackson et al. 2002). Thus, according to the intron nomenclature (Johansen and Haugen 2001), the *P. singularis* intron can be named Psi.S1189. Among prasinophytes, notably from freshwater, introns have already been reported for a few species (*Nephroselmis olivacea*, *Scherffelia dubia*, *Monomastix* sp., *Pterosperma cristatum* and an unclassified prasinophyte according to Comparative RNA Web Site and Project, <http://www.rna.icmb.utexas.edu/>). However, the position of these other chlorophyte introns is different from that of *P. singularis*. This feature can be used to design a specific probe for

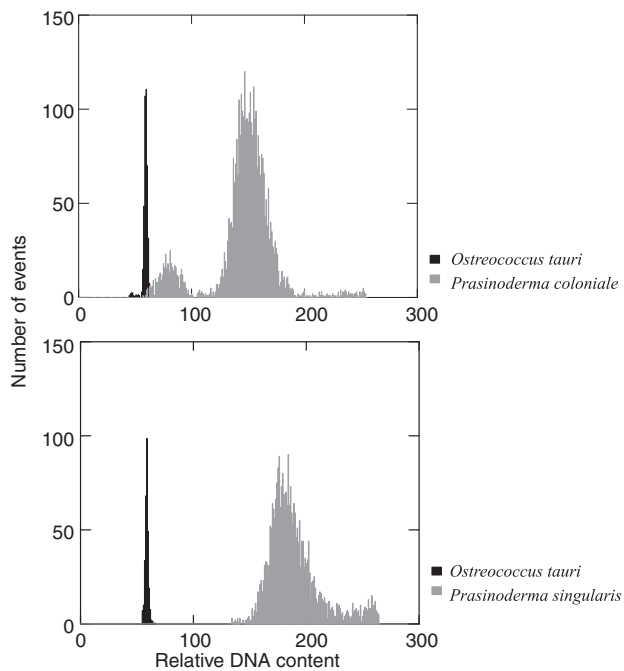




**Figure 5.** *Prasinoderma coloniale* (RCC 137), transmission electron micrographs of thin sections. Abbreviations: c, chloroplast; m, mitochondrion; n, nucleus; p, pyrenoid; s, starch sheath; g, Golgi apparatus. Scale bars = 0.2  $\mu\text{m}$ . **A.** Cell containing cup shaped chloroplast with pyrenoid and starch sheath enclosed by a thick cell wall. **B.** Section through cell showing the location of the mitochondrion in close proximity of the chloroplast. Note multilayered cell wall. **C.** Thylakoids (arrows) are arranged in lamella of three in the chloroplast. **D.** Dividing cell with two daughter cells within the parental cell wall. **E.** Details of the cell wall showing the external thick layer. **F.** Detail of chloroplast showing the mitochondrion protruding into the pyrenoid.



**Figure 6.** *Prasinoderma singularis* sp. nov. (RCC 927), transmission electron micrographs of thin sections. Abbreviations as in Figure 5. Scale bars = 0.2  $\mu$ m. **A.** Cell containing nucleus, chloroplast with pyrenoid surrounded by starch sheath, mitochondrion protruding into the pyrenoid and a Golgi apparatus. **B.** Section through cell showing the location of the mitochondrion in close proximity to the chloroplast and thin cell wall. **C.** Multilayered cell wall with split (arrow). **D.** Detail of the multilayered cell wall with at least six distinct layers. **E.** Bifurcate mitochondrion protrusion into the pyrenoid. **F.** Dividing cell with two daughter cells enclosed by the old cell wall.



**Figure 7.** Flow cytometry analysis of DNA content of cell nuclei for *Prasinoderma* species, compared to *Ostreococcus tauri* RCC 116 which has a genome size of 12.56 Mbp (Derelle et al. 2006).

detection with hybridization or quantitative PCR to determine the distribution of *P. singularis* in marine waters.

The nine *P. singularis* strains investigated in the present study were isolated during the BIOSOPE cruise in 2004 near the surface (depth  $\leq 40$  m) from mesotrophic or eutrophic areas (Table 1), either in the Chilean upwelling itself or on the eastern border of the South Pacific gyre (Le Gall et al. 2008), suggesting that this new species requires relatively high levels of nutrients and light. During the same cruise, *P. coloniale* was isolated near the Marquesas islands, in warmer and more oligotrophic waters (Le Gall et al. 2008). The original descriptions of the genera *Prasinococcus* and *Prasinoderma* were based on strains isolated during a cruise in the western North Pacific, from the south of Japan ( $31^{\circ}\text{N}$ ) to the north-east of Australia ( $12^{\circ}\text{S}$ ) (Hasegawa et al. 1996; Miyashita et al. 1993). Four strains of *P. capsulatus* had also been isolated in the early 1980's from the western North Atlantic (Cayman Trench, Gulf Stream and Sargasso Sea), in samples collected between 20 and 84 m depth and one strain of *P. coloniale* from the Gulf of Mexico at 81 m depth (Sieburth et al. 1999). Among the strains deposited in the Roscoff Culture Collection, some Prasinococcales originate

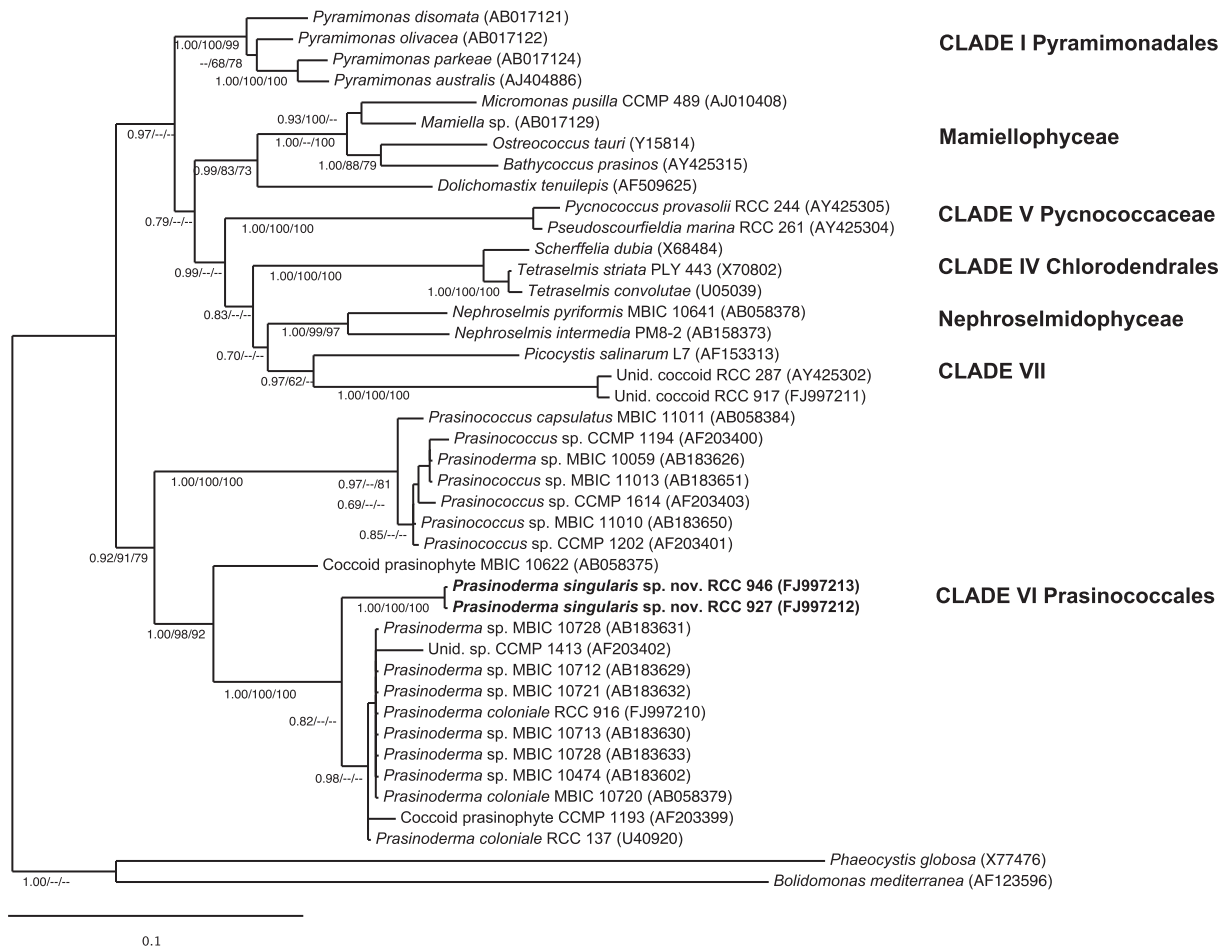
from the Indian Ocean, some from the Mediterranean Sea, and some from the Pacific Ocean. These observations suggest that with the exception of *P. singularis*, this order is probably characteristic of moderately oligotrophic waters. Surprisingly, no Prasinococcales 18S rRNA gene sequences have been recovered from marine samples using general primers (Vaultot et al. 2008). It was only the use of Chloroplastida-biased primers that allowed recovery of Prasinococcales sequences from different areas of the Mediterranean Sea (Viprey et al. 2008). In the Arabian Sea, oligonucleotide probes for Prasinococcales-targeting plastid 16S rRNA detected by dot blot hybridization yielded signals mostly close to the detection limits (Fuller et al. 2006). However, during the BIOSOPE cruise, the same probe detected very high levels (64% of the signal) of Prasinococcales in the vicinity of the Marquesas islands where *P. coloniale* strains have been isolated (Le Gall et al. 2008) and in surface waters of the centre of the gyre, but nowhere else (Lepère et al. 2009). Prasinococcales have also been detected sporadically by 18S rRNA probes revealed with FISH in the English Channel but always at low abundance (Not et al. 2004). These sparse data on Prasinococcales suggest that this order is likely to be a minor component of the phytoplankton, probably more characteristic of oligotrophic/mesotrophic areas and that some of its member species can bloom under specific conditions, such as those encountered off the Marquesas islands. Global surveys using molecular probes may shed light on its actual distribution.

## Methods

**Culture strains:** Samples were collected during the BIOSOPE cruise from Tahiti to Chile (Claustre et al. 2008). Enrichment of filtered seawater and single cell sorting by flow cytometry were used to obtain cultures (Le Gall et al. 2008). These strains were first characterized by optical microscopy and subsequently deposited in the Roscoff Culture Collection (<http://www.sb-roscoff.fr/Phyto/RCC>). All cultures were grown under the same conditions, in polystyrene tubes (12 mL) or polystyrene tissue culture flasks (50 mL) in K medium (Keller et al. 1987) at  $20^{\circ}\text{C}$  with a 12:12 light:dark cycle, under  $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  (Sylvania Daylight neons).

**Light microscopy:** Live cells were observed with an Olympus BX 51 microscope, an x100 objective using differential interference contrast (DIC), and a SPOT RT-slider digital camera (Diagnostics Instruments, Sterling Heights, MI).

**Transmission electron microscopy:** For thin sections, cells were fixed in 1-2% glutaraldehyde (final concentration) in growth medium K (Keller et al. 1987) and immediately centrifuged (6000 rpm) for 30 min at room temperature. Pellets were rinsed three times in K medium (5 min each) and then three times in 0.1 M sodium cacodylate (5 min each). After



**Figure 8.** Phylogenetic analysis of *Prasinoderma singularis* strains based on full-length 18S rRNA sequences (1645 bp for RCC927 and 1665 bp for RCC946, without the intron for both). Maximum likelihood, neighbor-joining and maximum parsimony trees were computed and respective bootstrap values are displayed next to the branches (see Methods for more details). Only values higher than 60% are shown. Genbank accession numbers are shown in brackets. Strain MBIC 10059 (AB183626) had been initially identified as *Prasinoderma* sp., but our phylogenetic analysis now shows a stronger affiliation to the *Prasinococcus* branch.

post-fixation with a final concentration of 1% osmium tetroxide and 1% potassium ferricyanide in 0.1 M sodium cacodylate for 2 hours at 4 °C, the pellets were rinsed three times (5, 10, and 5 min) in 0.1 M cacodylic acid buffer and twice in MilliQ water (5 min each). Samples were dehydrated by serial transfers through progressive aqueous-ethanol series (30%, 50%, 70%, 90%, 96%, once and finally 100%, three times) and rinsed twice with propylene oxide (5 min each). Samples were left overnight in a 1:1 mixture of propylene oxide and Epon's resin (EMBed-812 based on EPON-812, Luft 1961) and they were finally embedded in EPON and polymerized at 60 °C overnight. Ultrathin sections were made using a Leica Ultracut UCT microtome (Wetzlar, Germany), using a diamond knife. Sections were mounted on copper grids coated with Formvar film and stained with uranyl acetate (saturated solution in 50% ethanol) and lead citrate (saturated solution in 0.1 M NaOH). Sections were viewed with a JEOL 1400 (Tokyo, Japan) transmission electron microscope (TEM) at the Station Biologique de Roscoff and a FEI/PHILIPS CM-100 TEM (Hillsboro, Oregon, USA) at the Electron Microscopy

Unit of the Department of Molecular Biosciences, University of Oslo.

**Scanning electron microscopy:** Cultured cells were sampled during late exponential growth phase. Cells were fixed in 1-2% glutaraldehyde and 5-10 mL were gravity-filtered onto Nuclepore filters (13 mm diameter, 2 µm pore size, volumes used depended on cell density and filter clogging). The filter was rinsed with growth medium and subsequently in 0.1 M cacodylate (10 min each). Samples were post-fixed for 30 min in 0.5 mL of 1% osmium tetroxide in 0.1 M cacodylate. Three rinses in 0.1 M cacodylate were conducted (5 min each) and then dehydration was performed by serial transfers through progressive aqueous-ethanol series (70%, 90%, 96%, once and finally 100%, three times; 10 min each). The filter-holders were dried in a Critical Point Dryer (Baltec CPD 030, Balzers, Liechtenstein) and the filters were subsequently mounted on stubs using carbon tabs. Finally the cells were sputtered with gold-palladium or platinum and observed in a HITACHI S-4800 (Pleasanton, California, USA) field-emission scanning electron microscope at the Electron Microscopy Unit

of the Department of Molecular Biosciences, University of Oslo.

**Pigments:** Cultured cells (strains RCC 137, RCC 916, RCC 927 and RCC 946) were grown under standard conditions (see above) and sampled during late exponential growth phase. Three 100 mL samples for each strain were filtered and stored at  $-80^{\circ}\text{C}$  prior to analysis. Assessment of the pigment composition was performed using a Hewlett-Packard HPLC 1100 Series system, equipped with a quaternary pump system and diode array detector. Pigments were separated on a Waters Symmetry  $\text{C}_8$  column (150 x 4.6 mm, 3.5  $\mu\text{m}$  particle size) using a previously described HPLC method (Zapata et al. 2000). Frozen filters from algal cultures were extracted in a Teflon-lined screw-capped tube with 5–7 mL 95% methanol using a spatula for filter grinding. The tube was then placed in a beaker with ice and water and placed in an ultrasonic bath for 5 min. All sample preparations were conducted under subdued light. Extracts were then filtered through Millipore 0.45  $\mu\text{m}$  pore size filters to remove cell and filter debris and 200  $\mu\text{L}$  of the final extract were injected into the HPLC system. Chlorophylls and carotenoids were detected by absorbance at 440 nm and identified by a diode array detector ( $\lambda=350\text{--}750$  nm, 1.2 nm spectral resolution). HPLC calibration was performed using standards prepared in the laboratory (see Rodriguez et al. 2006) or obtained from SIGMA-ALDRICH (UK).

**Flow cytometry:** The DNA content of both species, *Prasinoderma coloniale* (RCC 137 and RCC 916) and *P. singularis* (RCC927, RCC 946, RCC 910, RCC 929, RCC 930 and RCC 936) was estimated by flow cytometry. Nuclei were released by injection of 50  $\mu\text{L}$  of *Prasinoderma* cultures into 450  $\mu\text{L}$  of Nuclei Isolation Buffer (NIB, previously described in Marie et al. 2000) twice diluted with distilled water. 5  $\mu\text{L}$  of an *Ostreococcus tauri* (RCC 116) culture were added as an internal standard (genome size = 12.56 Mbp). The nucleic acid specific stain SYBR Green I (Molecular Probes) was added at a final dilution of 1: 50000 of the commercial solution. Samples were incubated for 15 minutes before analysis by flow cytometry. Samples were run at a rate of 50  $\mu\text{L}/\text{min}$  on a FACS Canto II flow cytometer (Becton Dickinson) equipped with a 488 nm excitation and the standard filter setup. For *P. coloniale* two DNA peaks were observed (Fig. 7a) and its genome size was estimated from the dominant one which had the highest DNA content.

**Phylogenetic analyses:** All strains of *P. singularis* (Table 1) have very similar partial 18S rRNA gene sequences (Le Gall et al. 2008). Therefore two strains (RCC 927 and 946) were chosen for complete sequence determination. Cultures were grown in 50 mL flasks for 1–2 weeks and recovered by centrifugation at 11000 x g for 10 min. DNA was extracted using 3% Cetyl Trimethyl Ammonium Bromide (CTAB, Doyle and Doyle 1990). DNA was then stored in a freezer ( $-80^{\circ}\text{C}$ ).

The 18S rDNA gene was amplified by polymerase chain reaction (PCR) using the primer set Euk328f and Euk329r (Romari and Vaulot 2004) and the HotStarTaq Master Mix (Qiagen, Courtaboeuf, France). For PCR, a 15 min initial activation step of the polymerase at  $95^{\circ}\text{C}$  was followed by 40 cycles including 1 min of denaturation at  $94^{\circ}\text{C}$ , 45 s of annealing at  $57^{\circ}\text{C}$  and 75 s extension at  $72^{\circ}\text{C}$ . The PCR program was finished by a final extension of 10 min at  $72^{\circ}\text{C}$  followed by cooling to  $4^{\circ}\text{C}$ . PCR products were purified with the Qiaquick PCR purification kit (Qiagen) and controlled by electrophoresis on a 1% agarose gel. Full-length 18S rRNA gene sequences were determined from purified PCR products using Big Dye Terminator V3.1 (Applied Biosystems, Foster city, California, USA) and the primers Euk 528f (Elwood et al. 1985), Euk328f

and Euk329r run on an ABI prism 3100 sequencer (Applied Biosystems, Courtaboeuf, France).

Consensus sequences were determined and then compared with sequences available in the NCBI (National Center for Biotechnology Information) database using BLAST (Altschul et al. 1990). The identity (%) was calculated as the ratio of identical bp between sequences of *Prasinoderma coloniale* 18S rRNA gene (MBIC 10720 AB058379) and of *P. singularis* (RCC 927 FJ997212 and RCC 946 FJ997213). Consensus sequences were edited in BioEdit 7.0.5.3 and aligned using CLUSTALW2 (Hall 1999; Larkin et al. 2007). The TrN+I+G (Tamura-Nei) model was selected with ModelTest version 3.7 and used as a model of nucleotide substitution for the phylogenetic inference of each sequence by the maximum-likelihood (ML) method and Bayesian inference (Posada and Crandall 1998). Bayesian inference was conducted using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). Neighbour Joining (NJ) and Maximum of Parsimony (MP) trees were inferred using PAUP 4.0b10 using the PaupUp graphical interface (Calendini and Martin 2005; Swofford 2000). Sequences have been submitted to GenBank under accession number FJ997210–FJ997213.

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