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Molecular genetic delineation of *Phaeocystis* species (Prymnesiophyceae) using coding and non-coding regions of nuclear and plastid genomes

MARTIN LANGE[1], YUE-QIN CHEN[2] AND LINDA K. MEDLIN[1]

[1] *Alfred Wegener Institute for Polar and Marine Research, Postfach 120161, Columbusstrasse, 27515 Bremerhaven, Germany*
[2] *Key Laboratory of Gene Engineering of Education Ministry, Biotechnology Research Center, Zhongshan University, Guangzhou 510275, P. R. China*

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Sequence variation among 22 isolates representing a global distribution of the prymnesiophyte genus *Phaeocystis* has been compared using nuclear-encoded 18S rRNA genes and two non-coding regions: the ribosomal DNA internal transcribed spacer 1 (ITS1) separating the 18S rRNA and 5·8S rRNA genes and the plastid ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) spacer flanked by short stretches of the adjacent large and small subunits (*rbc*L and *rbc*S). 18S rRNA can only resolve major species complexes. The analysis suggests that an undescribed unicellular *Phaeocystis* sp. (isolate PLY 559) is a sister taxon to the Mediterranean unicellular *Phaeocystis jahnii*; this clade branched prior to the divergence of all other *Phaeocystis* species, including the colonial ones. Little divergence was seen among the multiple isolates sequenced from each colonial species complex. RUBISCO spacer regions are even more highly conserved among closely related colonial *Phaeocystis* species and are identical in *Phaeocystis antarctica*, *Phaeocystis pouchetii* and two warm-temperate strains of *Phaeocystis globosa*, with a single base substitution in two cold-temperate strains of *P. globosa*. The RUBISCO spacer sequences from two predominantly unicellular *Phaeocystis* isolates from the Mediterranean Sea and PLY 559 were clearly different from other *Phaeocystis* strains. In contrast, ITS1 exhibited substantial inter- and intraspecific sequence divergence and showed more resolution among the taxa. Distinctly different copies of the ITS1 region were found in *P. globosa*, even among cloned DNA from a single strain, suggesting that it is a species complex and making this region unsuitable for phylogenetic analysis in this species. However, among nine *P. antarctica* strains, four ITS1 haplotypes could be separated. Using the branching order in the ITS1 tree we have attempted to trace the biogeographic history of the dispersal of strains in Antarctic coastal waters.

Key words: biogeography, ITS1, *Phaeocystis*, phylogeny, Prymnesiophyceae, RUBISCO spacer, 18S rRNA

Introduction

Species separation in the ecologically important, cosmopolitan phytoplankton genus *Phaeocystis* is difficult using morphological features seen with light microscopy. The morphological features that can be used to separate the species can be found in Davidson & Marchant (1992) and in Zingone *et al.* (1999) and have been summarized in Table 1 here. The genus itself is easy to recognize if specimens are in the large gelatinous colony stage but *Phaeocystis* has a complex polymorphic life cycle with both colonial and single-cell stages (Kornmann, 1955; Rousseau *et al.*, 1994). Older colonial stages are more difficult to identify to species because the distinct colony morphology begins to break down. The single-cell stage is even more difficult to determine to either the genus or species level without electron microscopy. It still not clear how the many documented life stage forms in *Phaeocystis* interrelate with one another and the life cycle in Rousseau *et al.* (1994) is hypothetical. Only a few electron microscopic characters of the flagellate cell stage have been considered stable and are recommended for use in identifying *Phaeocystis* species unequivocally (Sournia, 1988). Recently, the species status of three colonial *Phaeocystis* taxa was corroborated by sequence variation in their nuclear-encoded 18S rRNA gene (Medlin *et al.*, 1994), by physiological differences (Baumann *et al.*, 1994) and, for two of the three colonial species, by differences in genome size (Vaulot *et al.*, 1994). Furthermore, two new unicellular *Phaeocystis* species from the Mediterranean Sea have been recognized by sequence variation in the 18S rRNA gene (Zingone *et al.*, 1999), by light and electron microscopic features (Zingone *et al.*, 1999) and, for at least one of them, by genome size (Vaulot *et al.*, 1994; Table 1).

Correspondence to: L. K. Medlin. e-mail: lmedlin@awi-bremerhaven.de

Table 1. Summary of salient morphological features and genome sizes of *Phaeocystis* species investigated in this study, extracted from Davidson & Marchant (1992), Vaulot *et al.* (1994 and personal communication) and Zingone *et al.* (1999) unless otherwise indicated

Species	*P. globosa*	*P. pouchetii*	*P. antarctica*	*P. cordata*	*P. jahnii*
Dominant morphology	Spherical colony	Cloud-like colony	Spherical colony	Unicellular	Unicellular, rarely colonial
Maximum size	8–9 mm	1·5–2 mm	9 mm	3·2 × 3·8 μm	3·5 × 5 μm
Mucilage in colony	Solid	Delicate	Solid	No colonies seen	Delicate, irregular, and loose aggregate
Distribution	Warm to cold-temperate	Arctic	Antarctic	Mediterranean	Mediterranean
DNA content	Group 1 = 32–35 pg $cell^{-1}$ Group 2 = 38 pg $cell^{-1}$ Group 3 = 42–44 pg $cell^{-1}$	31–36 crbc units[a] $cell^{-1}$	35–37 pg $cell^{-1}$	24 pg $cell^{-1}$	n.d.
Scale size: large	0·19–0·18 μm	0·25–0·24 μm	0·25–0·24 μm	0·27–0·21 μm	0·35–0·28 μm
Scale size: small	0·13–0·10 μm	0·19–0·15 μm[b]	n.d.	0·19–0·14 μm	0·18–0·14 μm
Flagellar length	10–15 μm	8 μm	10–12 μm	Unequal: 8·5–12; 5·5–6·50 μm	Unequal: 5·5–7·5; 4·5–60 μm

[a] Chicken red blood cells, taken from Jacobsen (2000).
[b] Taken from Jacobsen (2000).
n.d., not determined.

In the past decade sequencing of the nuclear-encoded ribosomal RNA (rRNA) genes and plastid-encoded ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) large and small subunit genes have been successfully used to identify micro-organisms and to infer phylogenetic relationships among taxa (Woese, 1987; Douglas & Durnford, 1989; Huss & Sogin, 1990; Fujiwara *et al.*, 1993; Leipe *et al.*, 1994; Birgitta *et al.*, 1998; Daugbjerg & Andersen, 1997; Sogin & Silbermann, 1998). However, these coding regions are normally used to distinguish between higher taxa and reach their limitation at the species level (Hillis & Moritz, 1990). Faster-evolving DNA regions, such as non-coding spacer regions, are therefore needed to resolve closely related or recently evolved species or to study species at the population level.

In land plants and green algae, only the large subunit gene of RUBISCO is plastid-encoded; the small subunit gene has been transferred to the nucleus (Reith & Cattolico, 1986). However, genes for both the large and small subunits of RUBISCO are plastid-encoded in all chlorophyll-*c*-containing algae (except dinoflagellates) and in the rhodophytes and are separated by a short spacer region (Reith & Cattolico, 1986). RUBISCO spacer sequences have been reported from cryptophytes (Douglas *et al.*, 1990), chloromonads (Boczar *et al.*, 1989), prymnesiophytes (Fujiwara *et al.*, 1993), phaeophyceans (Siemer *et al.*, 1998), diatoms (Chesnick *et al.*, 1996), pelagophytes (Bailey & Andersen, 1999) and rhodophytes (Muller *et al.*, 1998). These spacer regions are relatively small in size (38–204 bp) and have been found to be highly conserved at the population and species level in most algal groups. A relatively large RUBISCO spacer (545 bp) was found, however, in the cyanobacterium *Anabaena* 7120 (Curtis & Haselkorn, 1983).

The rDNA internal transcribed spacer regions (ITS1 and ITS2) separate three ribosomal genes in the rDNA cistron of eukaryotes. Although there is little agreement about the biological function of the internal transcribed spacers, growing evidence indicates that these highly variable regions between the ribosomal genes play an important role in ribosome processing (van der Sande *et al.*, 1992). ITS regions also evolve at a fast rate. For example, 0·8–2·0% base changes per Mya has been calculated for the chlorophyte *Cladophora albida* clade (Bakker *et al.*, 1995). With such high base substitution rates and well-documented length variation in ITS regions, it is possible to resolve differences among closely related species or different populations within species (e.g. Lee & Taylor, 1991; Pleyte *et al.*, 1992; Wesson *et al.*, 1993; Schlötterer *et al.*, 1994; Vogeler & DeSalle, 1994; Edvardsen & Medlin, 1998; Alice & Campbell, 1999; Booten *et al.*, 1999). The use of

ITS sequences for phylogenetic and biogeographic studies in marine algae is increasing, e.g. in marine and freshwater green algae (Bakker *et al.*, 1992, 1995; Kooistra *et al.*, 1992, 1993; van Oppen *et al.*, 1993; Coleman *et al.*, 1994; Pillmann *et al.*, 1997; Blomster *et al.*, 1998; Coat *et al.*, 1998; Gonzales *et al.*, 1998; Schagerl *et al.*, 1999), brown algae (van Oppen *et al.*, 1993; Peters *et al.*, 1997, Stache-Crain *et al.*, 1997; Siemer *et al.*, 1998), diatoms (Zechman *et al.*, 1994), dinoflagellates (Adachi *et al.*, 1997) and red algae (Steane *et al.*, 1991; Goff *et al.*, 1994; Vis & Sheath, 1997; Wattier *et al.*, 1997).

Differences in DNA content in 14 strains of *Phaeocystis globosa* as measured by flow cytometry (Vaulot *et al.*, 1994) suggested that at least one colonial form, *P. globosa*, could contain multiple cryptic species. Vaulot *et al.* (1994; D. Vaulot, personal communication) detected at least three groups within *P. globosa*, which all differed from *P. antarctica* on the basis of their DNA content (Table 1). Sequence data from 18S rRNA genes of *Phaeocystis* have been used to reconstruct the phylogeny and biogeographical history of its major, bloom-forming and most commonly recorded species (Medlin *et al.*, 1994; Lange, 1997; Zingone *et al.*, 1999). However, variation in the 18S rRNA gene between *P. globosa* and *P. pouchetii* and among strains of *P. antarctica* was minor and ranged from 0 to 5 nucleotides (Medlin *et al.*, 1994; Lange, 1997), making the resolution of closely related or recently evolved cryptic *Phaeocystis* species using these coding regions difficult.

Consequently, we sequenced 18S rRNA from additional strains of *P. globosa* and the RUBISCO spacer and the ITS1 spacer region of the ribosomal cistron from many *Phaeocystis* species to reconstruct the phylogenetic history of closely related *Phaeocystis* species/strains among the colonial species and to obtain an insight into the biogeographic history of *P. antarctica* and the separation of ancestral populations into two polar species, *P. antarctica* and *P. pouchetii*, using an analysis of both nuclear and plastid genomes.

Materials and methods

Cultures

Phaeocystis strains and other algae used in this study are listed in Table 2. Isolates were grown in K-medium (Keller *et al.*, 1987) or in an enriched seawater medium with major nutrients diluted by half or 50 times (von Stosch & Drebes, 1964) under a light/dark cycle suitable for growth at 0 °C, 10 °C and 15 °C (4:20, 12:12, 14:10, respectively). We included multiple representatives of three colonial species, *P. globosa*, *P. antarctic*a and *P. pouchetii*, and the only available cultures of three predominantly unicellular species, *P. cordata*, *P. jahnii* and *Phaeocystis* sp. (PLY 559). *Phaeocystis scrobiculata* Moestrup, known only from the flagellated stage in field material (Zingone *et al.*, 1999), is the only recognized species not included in our analysis.

Isolation of DNA

Cultures were harvested during logarithmic growth, frozen in liquid nitrogen until needed or used directly for DNA extraction. Total nucleic acids were obtained using a 3% CTAB (hexadecyltrimethylammonium bromide) procedure (Doyle & Doyle, 1990). Other extractions were performed by resuspending the cells in extraction buffer (100 mM Tris, pH 8·5, 100 mM NaCl, 50 mM EDTA) before total nucleic acids were extracted by vortexing the cells in the presence of 2% SDS and Tris-buffered phenol/chloroform/isoamyl alcohol (50:48:2 v/v/v). The supernatant was extracted twice with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol (48:2 v/v) prior to ethanol precipitation.

Amplification

Total nucleic acid preparations were used as templates for the amplification of the 18S rRNA gene and the ITS1 and RUBISCO spacers, except in a few cases where an entire *Phaeocystis* colony was used as template directly in the PCR reaction. The 18S rRNA genes were amplified following Chesnick *et al.* (1997). The ITS1 regions were amplified using a forward primer near the 3′ end of the 18S rRNA gene of *Phaeocystis* (5′ GCCGGACGCGACGCTCC 3′, position 1705–1719) and a reverse primer that matched a conserved region at the beginning of the 5·8S rRNA gene (5′ GCTACGTTCTTCATCGATGC 3′). RUBISCO spacer regions were amplified using primers complementary to conserved regions within the large and small RUBISCO subunits (Maggs *et al.*, 1992). One of the two primers used in each amplification was biotin-labelled prior to its synthesis. Each 100 μl PCR reaction contained 100 ng total nucleic acids and a PCR reaction mixture as described in Medlin *et al.* (1988). To amplify the ITS1 region of *Phaeocystis* it was essential to add 10 μl of 50% acetamide (Sigma) to the PCR reaction. The amplifications of the ITS1 region were performed in a Perkin-Elmer-Cetus thermocycler with an initial denaturation step of 95 °C for 6 min, after which the *Taq* polymerase was added as the heating block cooled to 60 °C, followed by 29 cycles of 72 °C for 4 min, 94 °C for 2 min, 45 °C for 2 min and a final extension step at 72 °C for 9 min.

For the Chinese isolate of *P. globosa*, purified gene products were ligated into the pUC18 vector following the manufacturer's instructions (Gene Company). A highly degenerate primer Phaeo LH_2 (5′ CGGAAGGATCATTACCGGTAT 3′) and D1a were used for clone selection. Several clones of each isolate were sequenced. The amplifications of RUBISCO spacer regions were performed with an initial denaturation step of 92 °C for 3 min, after which the *Taq* polymerase was added as the heating block cooled to 60 °C, followed by 29 cycles of 72 °C for 1 min, 94 °C for 45 s, 40 °C for 1 min and a final extension step of 72 °C for 5 min. Not all species could be amplified successfully for both spacer regions.

Sequence analysis

Single-stranded DNA was obtained by binding amplification products onto streptavidin-coated magnetic M-280

Table 2. Algal species used in the ITS and RUBISCO spacer analysis

Strains	Culture number, Culture facility[e–i], DNA-Prep. (Pxxx), or GenBank Acc. No. (GB-xxx)	Geographic origin (for numbers in parentheses see Fig. 4)
Phaeocystis globosa Scherffel[a,b]	SK 35[d]	North Sea, German Bight
Phaeocystis globosa[b]	CCMP1528[c]	Galapagos
Phaeocystis globosa[b]	SANTOU 97	Guangdong, China
clone 6	GB-AJ279505	Guangdong, China
clone 2	GB-AJ279504	Guangdong, China
clone 8	GB-AJ271218	Guangdong, China
clone 3	GB-AJ271217	Guangdong, China
Phaeocystis globosa[b]	CCMP 1524[c]	Thailand
clone 4	GB-AFJ279501	Thailand
clone 11	GB-AFJ279500	Thailand
Phaeocystis globosa[b]	CCMP 627[c]	Gulf of Mexico, 29°15′N, 85°54′W
clone 2	GB-AJ279502	Gulf of Mexico, 29°15′N, 85°54′W
clone 5	GB-AJ279503	Gulf of Mexico, 29°15′N, 85°54′W
Phaeocystis globosa[b]	CCMP 628[c]	Surinam, 6°45′N, 53°19′W
Phaeocystis globosa[a]	NIOZ1[d]	North Sea, 52°50′N, 4°45′E
Phaeocystis globosa[a]	P162[d]	South Africa
Phaeocystis globosa[a]	PLY 540[e]	East Atlantic, 47°37′N, 8°53′W
Phaeocystis globosa[b]	P277[h]	Palau
Phaeocystis pouchetii (Hariot) Lagerheim[a,b]	SK 34[d,h]	Greenland Sea, East Greenland Current
Phaeocystis pouchetii[b]	P361[f]	Svalbard (Norway)
clone 3	GB-AJ271046	
Phaeocystis pouchetii[a]	P360[f]	Raunefjorden (Norway)
Phaeocystis cordata Zingone[a,b]	Naples C2A1[g]	Mediterranean Sea, 40°49′N, 14°15′E
Phaeocystis jahnii Zingone[a]	B5[i]	Gulf of Naples
Phaeocystis sp.	PLY 559[e]	North Atlantic
Phaeocystis sp.	Med NS3[h]	Mediterranean Sea, 42°51′N, 3°47′E
Phaeocystis sp.	OLI 26 SA[g] OLI 26 SB[g] OLI 26 SF[g] OLI 26 SG[g]	Equatorial Pacific, 7°0′S, 150°0′W
Phaeocystis sp.[a]	P197[d,h]	Bergen (Norway)
Phaeocystis antarctica Karsten[a,b]	SK 22[d,h]	(6)Antarctica, 54°20′S, 3°20′W
Phaeocystis antarctica[b]	SK 23[d,h]	(8)Antarctica, 63°15′S, 58°20′W
Phaeocystis antarctica[a,b]	SK 20[d,h]	(7)Antarctica, 67°50′S, 20°51′W
Phaeocystis antarctica[a,b]	CCMP 1374[c,d]	(9)Antarctica, McMurdo Sound
Phaeocystis antarctica[b]	A1-3[d,h]	(1)Antarctica, 63°11·5′S, 85°45·3′E
Phaeocystis antarctica	D5[d,h]	Antarctica, 68°47·5′S, 73°30·2′E
Phaeocystis antarctica	DE3[d,h], DE10[d,h], DE11[d,h], DE12·2[d,h]	Antarctica, 68°33·3′S, 77°51·5′E
Phaeocystis antarctica	T4-3[d,h], T9-3[d,h]	Antarctica, 68°39·0′S, 72°21·2′E
Phaeocystis antarctica	MSIA1[d,h], MSIA2[d,h]	Antarctica, Prydz Bay
Phaeocystis antarctica	RG1·2[d,h], RG2·2[d,h], RG4·2[d,h]	Antarctica, 68°33·2′S, 77°53·0′E
Phaeocystis antarctica[b]	D4-5[d,h]	(2)Antarctica, 68°47·5′S, 73°30·2′E
Phaeocystis antarctica[b]	T4-2[d,h]	(4)Antarctica, 68°39·0′S, 72°21·2′E
Phaeocystis antarctica[b]	DE2[d,h]	(3)Antarctica, 68°33·3′S, 77°51·5′E
Phaeocystis antarctica[b]	T9-1[d,h]	(5)Antarctica, 68°39·0′S, 72°21·2′E
Emiliania huxleyi (Lohmann) Hay & Mohler	Texel B[e]	North Sea
Cylindrotheca closterium (Ehrenbeg) Reimann & Lewin[c]	XM-48[d]	Severn Estuary, UK
Phycodrys austrogeorgia Skottsberg	36/89[d]	King George Island
Desmarestia aculeata (Linnaeus) Lamouroux	3108[d]	Disko Island
Acrosiphonia arcta (Dillwyn) J.G. Agardh	1083[d]	Helgoland
Peridinium foliaceum symbiont (Stein) Biechler[a]	GB-U31876	
Odontella sinensis[a] (Greville) Grunow	GB-Z67753	
Ectocarpus siliculosus (*Dillwyn*) *Lyngbye*[a]	GB-U38834	
Gracilaria gracilis (Stackhouse) Steentoft, Irvine *et* Farnham[a]	GB-S58154	

[a] Used for RUBISCO spacer sequencing. [b] Used for ITS sequencing. [c] Provasoli-Guillard Culture Center for Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine, USA. [d] Culture no longer available. [e] Plymouth Culture Collection, Plymouth Marine Laboratory, Plymouth, UK. [f] University of Bergen, Norway. [g] Université Pierre et Marie Curie, Station Biologique, Roscoff, France. [h] CSIRO Division of Fisheries, Hobart, Tasmania, Australia. [i] Stazione Zoologica Anton Dohrn, Naples, Italy.

Dyna-beads, following a slightly modified protocol of DYNAL (Oslo, Norway). Thirty microlitres of beads were washed with 200 μl of binding buffer (10 mM Tris HCl, pH 7·5, 1 mM EDTA, 2·0 M NaCl); all liquid was removed by magnetic separation. Beads were resuspended in 40 μl binding buffer plus 90 μl of the PCR

reaction solution containing the amplification products, followed by a gentle shaking of the solution for 30 min at room temperature. The beads were washed twice with 200 μl of binding buffer, then all liquid removed and the beads resuspended in 20 μl freshly prepared 0·1 N NaOH for 10 min at room temperature. Liquid was removed and stored on ice and an additional denaturation step with 100 μl 0·1 N NaOH was performed followed by washing the beads three times with 200 l sterile water. Beads were finally resuspended in 11 μl sterile water and used directly as template for sequencing. In some cases, DNA from the first denaturation step was precipitated with 500 μl ice-cold 100% ethanol, kept for at least 1 h at –70 °C and spun in a microcentrifuge for 30 min at 13000 *g* at 4 °C. DNA was resuspended in 10 μl sterile water and used directly for sequencing.

The 18S rRNA coding regions and ITS1 and RUBISCO spacer regions were sequenced using the dideoxynucleotide chain-termination sequencing method (Sanger *et al.*, 1977) with the T7-Sequencing Kit (Pharmacia) and the same primers as in the amplification reactions plus internal primers for the 18S rRNA gene (Elwood *et al.*, 1985). Primer annealing was performed by heating samples at 60 °C for 10 min and subsequently allowing them to cool at room temperature for 10 min. Sequencing reactions were run as described in Medlin *et al.* (1994).

All sequences were aligned manually using the Olsen sequence editor (Larsen *et al.*, 1993). Both the RUBISCO and the ITS1 data sets were submitted to CLUSTAL-W for alignment and then corrected by eye. The 18S rRNA sequences were manually aligned to an algal database originating from the Ribosomal Database Project, which contains over 500 published and unpublished chlorophyll $a+c$ algae, using maximum primary and secondary structural similarity. A final data set of 37 species/strains and 1764 nucleotides was used for phylogenetic analyses with the Class Pavlovophyceae as outgroup for the 18S rRNA gene. A recent analysis of all available prymnesiophyte 18S rRNA sequences has shown that the Class Pavlovophyceae is the sister group to the Class Prymnesiophyceae and is thus an appropriate outgroup for the Class Prymnesiophyceae (Edvardsen *et al.*, 2000). The Phaeocystales are members of the Class Prymnesiophyceae (Edvardsen *et al.*, 2000). RUBISCO spacer sequences from 12 *Phaeocystis* species/strains and one diatom contaminant from *Phaeocystis* sp. P197 were aligned with RUBISCO spacer sequences from other prymnesiophytes (Fujiwara *et al.*, 1993; Barker, 1996), diatoms (Hwang & Tabita, 1991; Chesnick *et al.*, 1996), phaeophyceans (Valentin & Zetsche, 1990) and rhodophytes (Destombe & Douglas, 1991), which were used as the outgroup for the RUBISCO spacer analysis. ITS1 spacer regions were aligned only within the genus *Phaeocystis*, using relationships inferred from the 18S rRNA data set to determine the appropriate outgroup. For the ITS analysis, two different data sets were used. The first data set analysed all available sequences and the bases marked as weighting mask 1 in Fig. 5. The second data set used only the polar species and the base positions marked as weighting mask 2 in Fig. 5. Within the polar data set more positions could be used for the analysis because more bases were considered to be unambiguously aligned.

Maximum likelihood analyses for the 18S rRNA gene and the RUBISCO spacer were performed using the fastDNAml program (v. 1.0) (Larsen *et al.*, 1993, using the Felsenstein model). The ITS data set was analysed using the Modeltest program to determine the optimal evolution model for this data set from 40 available models (Posada & Crandall, 1998). This test found that the Felsenstein 1981 model of evolution best fitted the ITS data set in which all *Phaeocystis* sequences were included, whereas the Tamura and Nei model of evolution best fitted the second data set with only the polar strains. Both models allowed for a gamma distribution of the data. Options, empirically determined from the Modeltest program, were entered into the distance and maximum likelihood analyses settings in PAUP* v.4.0 (Swofford, 1999) where appropriate and used to run the bootstrap analyses. Maximum parsimony analyses were implemented with the PAUP* v.4.0 computer program (Swofford, 1999) using a re-scaled consistency index to weight the data. Introduced gaps were treated as missing data; informative characters were treated as multistate unordered. All trees were obtained using the tree-bisection-reconnection (TBR) branch swapping option in a heuristic search with random taxon addition. Distance analyses for the 18S rRNA gene and the RUBISCO spacer were performed using PHYLIP (Felsenstein, 1993), whereas the distance options in PAUP were used for the ITS analysis because we incorporated settings from the Modeltest program where appropriate. Dissimilarity values (Fitch & Margoliash, 1967), based on pair-wise comparisons of sequences, were transformed into distances using the Kimura two-parameter-models (Kimura, 1980). Distance matrices were converted into trees using the neighbor-joining method (Felsenstein, 1993). Stability of monophyletic groups in maximum parsimony and distance trees was estimated with a bootstrap analysis (500 replicates) (Felsenstein, 1985) and in a maximum likelihood analysis with 100 replicates.

Results

18S rRNA sequences

Fig. 1 shows the phylogenetic relationships among all available strains of *Phaeocystis*. The colonial species/strains fall into one clade (clade 2) that is sister taxon to the new unicellular species *Phaeocystis cordata*, which does not form colonies (Zingone *et al.*, 1999). Together, these taxa are sister to another clade (clade 1) consisting of two other *Phaeocystis* strains, one of which is unicellular and undescribed (PLY 559) and the other of which, *P. jahnii*, can occasionally form loosely aggregated colonies (Zingone *et al.*, 1999). The true colonial forms belong to three species: *P. globosa*, *P. antarctica* and *P. pouchetii*. No haptophytes in the other sister clades are colony formers: the formation of colonies is unique to *Phaeocystis* and is a derived condition. A thicker line on the tree (Fig. 1) indicates the warm temperature preference of some *Phaeocystis* spp.

Variation in the 18S rRNA gene occurs within and between each cluster of strains of a single

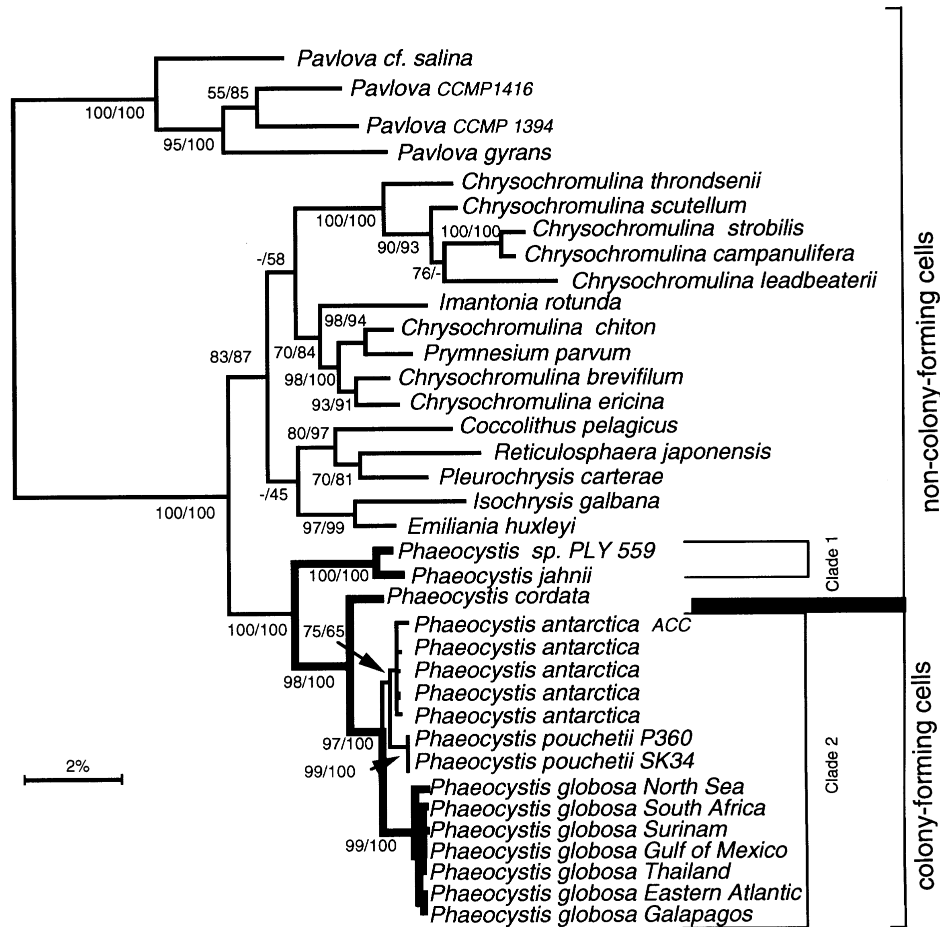

Fig. 1. Maximum likelihood phylogeny (fastDNAml) of 17 *Phaeocystis* species/strains and other prymnesiophytes inferred from 18S rRNA. The Class Pavlovophyceae was used as outgroup. Numbers placed to the left of the nodes are bootstrap values based on a neighbor-joining analysis (PHYLIP) and maximum parsimony analysis (PAUP, tree length = 167, CI = 0·952, RI = 0·956). Bootstrap values are placed on the nodes that are identical and recovered in all three analyses. Scale bar corresponds to 2 base changes per 100 nucleotides. Lines leading to warm-water taxa in the genus *Phaeocystis* are in enlarged font.

species. A bootstrap value of greater than 70 indicates that there is a probability of 95 % that the clade is real (Hillus & Bull, 1993). The base positions separating one species cluster from another are consistent and unique within and between each species cluster and thus, each species cluster is supported by moderate to strong bootstrap support. There is little variation among the strains of each true colonial species (0–5 bases).

RUBISCO spacer

The spacer region separating the RUBISCO large and small subunits is 54 nucleotides in length in the three colonial species and is in the range reported in the literature from other algae (Destombe & Douglas, 1991; Fujiwara *et al.*, 1993; Goff *et al.*, 1994). A possible ribosome binding sequence (5′ AGGAG 3′) (Shine & Dalgarno, 1974) upstream of the initiation codon of the small subunit gene has been identified (Fig. 2). Similar regions have been found in all RUBISCO spacers investigated (see Bailey & Andersen, 1999).

The RUBISCO spacer in the colonial strains of *P. globosa*, *P. antarctica* and *P. pouchetii* was identical (Fig. 2). Only one base substitution was found in two cold-temperate strains of *P. globosa*. In contrast, the RUBISCO spacer of *P. cordata*, *P. jahnii* and the undescribed *Phaeocystis* species strain PLY 559 ranged from 59 to 79 nucleotides in length and differed from the other colonial *Phaeocystis* species by containing two insertions near the 5′ end and multiple base substitutions (Fig. 2).

A clearly different RUBISCO sequence was obtained from one strain of *Phaeocystis* (P197) (Fig. 2), in repeated amplifications of this region from a single, well-washed *Phaeocystis* colony. Comparisons with other RUBISCO spacer sequences (Fig. 3) indicate the likelihood that we have amplified a diatom contaminant, which we believe resides inside the *Phaeocystis* colony. The RUBISCO spacer sequence from this isolate (Fig. 2) is basal to the known diatoms in our maximum likelihood tree (the diatom endosymbiont from *Peridinium foli-*

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                                     3' end of the rbcL gene                    50                              100
position                                                                         ⊥                               ⊥
weighting mask                       ......................................111111............................1111111111111111111....111111111111111111111111111...111...111111
1  Ph. cordata                       cagcaactgcaaaccgttaa--------------------TAA------------------------------CGTAAC-GGGTTCAAAAT------CGC--GTTAAAATTATTAAA-TTTCA---TTT---AAGGAG
2  Ph. jahnii                        cagcaactgcaaacagataa--------------------TCACTATCTAACTTAGAATCTT-----------AATTACAAGATTCTAAGA------CGA--GTTCAAACTATCAAA-ATTCA---TTT---AAGGAG
3  Ph. sp. PLY 559                   cagcaactgcaNaNNgataa--------------------TCNNTATCTAACTTAGANTCTT-----------AATTACAAGATTCTAAGA------CGA--GTTCAAACTATCAAA-ATTCA---TTT---AAGGAG
4  Ph. antarctica SK 20              cagcaactgcaaaccgttaa--------------------TAA------------------------------------CGGACTTAATTT------CGC--GTTTAAACTATTAAA-TTTCA---TTT---AAGGAG
5  Ph. antarctica CCMP 1374          cagcaactgcaaaccgttaa--------------------TAA------------------------------------CGGACTTAATTT------CGC--GTTTAAACTATTAAA-TTTCA---TTT---AAGGAG
6  Ph. antarctica SK 22              cagcaactgcaaaccgttaa--------------------TAA------------------------------------CGGACTTAATTT------CGC--GTTTAAACTATTAAA-TTTCA---TTT---AAGGAG
7  Ph. pouchetii SK 34               cagcaactgcaaaccgttaa--------------------TAA------------------------------------CGGACTTAATTT------CGC--GTTTAAACTATTAAA-TTTCA---TTT---AAGGAG
8  Ph. pouchetii P360                cagcaactgcaaaccgttaa--------------------TAA------------------------------------CGGACTTAATTT------CGC--GTTTAAACTATTAAA-TTTCA---TTT---AAGGAG
9  Ph. globosa P162                  cagcaactgcaaaccgttaa--------------------TAA------------------------------------CGGACTTAATTT------CGC--GTTTAAACTATTAAA-TTTCA---TTT---AAGGAG
10 Ph. globosa PCC540                cagcaactgcaaaccgttaa--------------------TAA------------------------------------CGGACTTAATTT------CGC--GTTTAAACTATTAAA-TTTCA---TTT---AAGGAG
11 Ph. globosa NIOZ1                 cagcaactgcaaaccgttaa--------------------TAA------------------------------------CGGACCTAATTT------CGC--GTTTAAACTATTAAA-TTTCA---TTT---AAGGAG
12 Ph. globosa SK 35                 cagcaactgcaaaccgttaa--------------------TAA------------------------------------CGGACCTAATTT------CGC--GTTTAAACTATTAAA-TTTCA---TTT---AAGGAG
13 Emiliania huxleyi                                  taa--------------------TAAACTTACGACATTGTCTTAAGTTAAATTATTTTC------CGGGCAT--TTA-------GCCCGAATAT-CTTATAAATTTTCA---TTT---ACGGAG
14 Pleurochrysis carterae            caccaacagctaaccgataa------------------TTATTAA-TTAT--TATTGGTATAGACTTTAA-------------GGGCCTAATATTAATTAGGC--TCTTAT--TTATAAATTTTCA---TTT--AATGGAG
15 Ectocarpus siliculosus            ttgcaacaggaagtagataa +(154 NUCLEOTIDES)+ -----------------------------------------------------------TATA--TTTA-----------TCTCT-AAGTAA
16 Diatom from Phaeocystis sp. P197  caccaacagcaaacgtataa---------------------TTAA-----------------------------------------------------------GTT---ACTTTTAAT----CAA-ATTT--AAAGGAG
17 Peridinium foliaceum symbiont     caccaacagcaaacgtataa--------------------TAA------------------------------------------------------------GTT---ACTTTTAAA-----AA-AATT--AAAGGAG
18 Odontella sinensis                cacctacagcaaacgtataa--------------------TAA------------------------------------------------------------GTT---ACTTTTAAT-----AA-AAATA-AAAGGAG
19 Cylindrotheca sp.                 catcaacagcaaacgtataa--------------------TAAAAAAA-------------------------------------------------------GTT---ACTTTTAAA-----AA-AATT--AAAGGAG
20 Gracilaria gracilis               ctccaacagctaatgtatagtgt----------------ATATCTATTATTTATCTTTTGATTAGCTTTATTTTCATAATATTTAATTTGAATGAAAATAAAATGTTAATTTTTTTAACAGACACAAAAATATTATAAGGAG

                                      150   5' end of the rbcS gene            200                          ↓                        ↓          250        ↓
position                               ⊥                                        ⊥
weighting mask                       11111111.....11111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
1  Ph. cordata                       TAAYATA------atgaaactaactcaaggagctttctctttccttcctgacttaactgatgagcaaatcacaaagcaaattcaatacgctttaaacaacaactgggctgtttctgta
2  Ph. jahnii                        TAACACA------atgNNactaactcaaggagcttttctttttcttcctgatctaactgatgaacaaatcacaaagcaaattcaatatgctttaaacaagaactgggctgtttctatt
3  Ph. sp. PLY 559                   TAACACA------atgaaactaactcaaggagcttttctttttcttcctgatctaactgaNNaacaaatcacaaagcaaattcaatatgctttaaacaagaactgggctgtttctatt
4  Ph. antarctica SK 20              TAATATA------atgaaactaactcaaggagcttttctttccttcctgacttaactgacgagcaaatcacaaagcagatccaatacgctttaaataagagttgggctgtatctata
5  Ph. antarctica CCMP 1374          TAATATA------atgaaactaactcaaggagcttttctttccttcctgacttaactgacgagcaaatcacaaagcagatccaatacgctttaaataagagttgsgctgtatctata
6  Ph. antarctica SK 22              TAATATA------atgaaactaactcaaggagcttttctttccttcctgacttaactgacgagcaaatcacaaagcagatccaatacgctttaaataaaagttgggctgtatcttta
7  Ph. pouchetii SK 34               TAATATA------atgaaactaactcaaggagcttttctttccttcctgacttaactgacgagcaaatcacaaagcagatccaatacgctttaaataaaagttgggctgtatcttta
8  Ph. pouchetii P360                TAATATA------atgaaactaactcaaggagcttttctttccttcctgacttaactgacgagcaaatcacaaagcagatccaatacgctttaaataaaagttgggctgtatcttta
9  Ph. globosa P162                  TAATATA------atgaaactaactcaaggagcttttctttccttcctgacttaactgacgagcaaatcacaaagcaaatccaatacgctttaaataagagttgggctgtatcttta
10 Ph. globosa PCC540                TAATATA------atgaaactaactcaaggagcttttctttccttcctgacttaactgacgagcaaatcacaaagcaaatccaatacgctttaaataagagttgggctgtatcttta
11 Ph. globosa NIOZ1                 TAATATA------atgaaactaactcaaggagcttttctttccttcctgacttaactgacgagcaaatcacaaagcaaatccaatacgctttaaataagagttgggctgtatcttta
12 Ph. globosa SK 35                 TAATATA------atgaaactaactcaaggagcttttctttccttcctgacttaactgacgagcaaatcayaaagcaaatccaatacgctttaaataagagttgggctgtatcttta
13 Emiliania huxleyi                 TAACAG-------atgaaactaacacaaggatgcttttctttccttcctgacctaactgacgagcaaattacgaagcagattcaatatgctttaaacaagtcttgggctctatctatc
14 Pleurochrysis carterae            TAAC-TA------atgaaactaacacaaggagctttctcatttcttcctgacttaactgatgcgcaagtaactaagcaaatccagtacgctttaaataagagttgggctatttcgatt
15 Ectocarpus siliculosus            TATTTGAATAGTGatgagagttacacaaggatgtttttcgtttttaccagatttaagtgatgatcaaattaaacaacaagtttcttacgctatgagcaaaggttgggcggttagtgta
16 Diatom from Phaeocystis sp. P197  TTTTTGAATA---gtgtgacttacacaaggttgcttctctttcttaccagatttaactgaccaacaaattgaaaaacaagttgcttatgccatttcaaaaggctgggcaatgaacgtt
17 Peridinium foliaceum symbiont     TATTTGAATA---gtgagacttagagaaggttgtttctcgttcttaccagatttaactgatcaacaaattgaaaaacaaatcacgtacgcaatcaacaaaggttgggcgatgaacgtt
18 Odontella sinensis                TATTTGAATA---gtgagacttacacaaggttgtttctcttttta
19 Cylindrotheca sp.                 TATTTGAATA---gtgagacttacacaaggttgtttctcgttctta
20 Gracilaria gracilis               TTTTT-AATA---gtgagattaacacaagg
Odontella sinensis: sequence from K.V. Kowallik, unpublished
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Fig. 2. Alignment of the RUBISCO spacer from the prymnesiophytes *Phaeocystis globosa* (strains SK 35, P 162, PCC 540, NIOZ 1), *P. antarctica* (strains SK 20, SK 22, CCMP 1374), *P. pouchetii* (strains SK 34, P 360), *P. cordata*, *P. jahnii*, *Phaeocystis* sp. PLY 559, *Pleurochrysis carterae* and *Emiliania huxleyi*, the phaeophycean *Ectocarpus siliculosus*, the diatoms *Cylindrotheca* sp., *Odontella sinensis*, the *Peridinium foliaceum* symbiont, a suspected diatom contaminant in the *Phaeocystis* P197 and the rhodophyte *Gracilaria gracilis* as outgroup. Base substitutions and insertions among *Phaeocystis* species are in bold. Shine–Dalgarno sequence near the 3′ end of the RUBISCO spacer is underlined. Arrows indicate synapomorphies for cold-water *Phaeocystis* clade (position 221, position 242, position 268). All positions were used for these phylogenetic analyses. RUBISCO spacer is shown in capital letters, whereas flanking coding regions are in lower case.

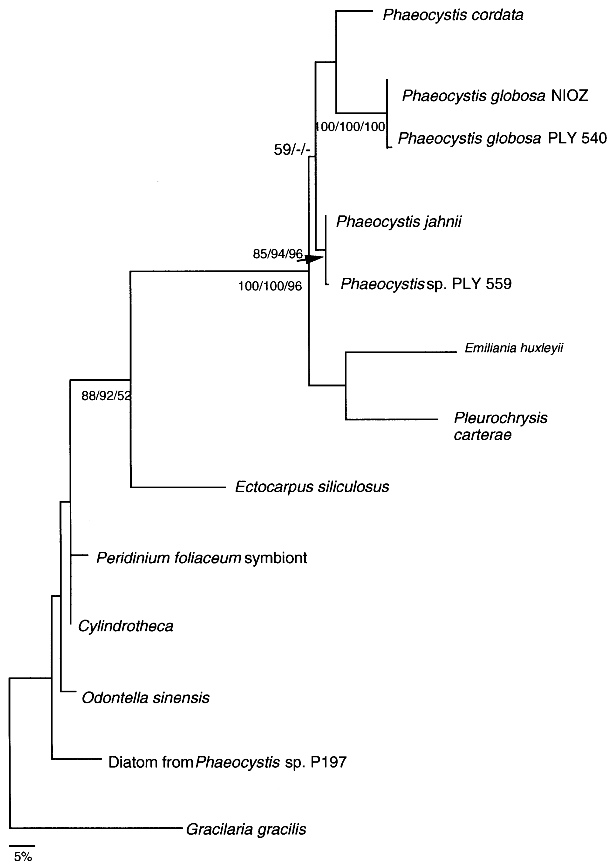

Fig. 3. Maximum likelihood tree inferred from RUBISCO spacer sequences and a small part of the small subunit RUBISCO gene from *Phaeocystis globosa* (strains PCC 540, NIOZ 1), *Phaeocystis cordata*, *Phaeocystis jahnii*, *Phaeocystis* sp. PLY 559, *Pleurochrysis carterae*, *Emiliania huxleyi*, *Ectocarpus siliculosus*, *Cylindrotheca* sp., *Odontella sinensis*, the *Peridinium foliaceum* symbiont, a suspected diatom contaminant from *Phaeocystis* P197 and the rhodophyte *Gracilaria gracilis* as outgroup. The RUBISCO spacer sequences from *P. globosa* (strains SK 35, P 162, NIOZ 1), *P. antarctica* (strains SK 20, SK 22, CCMP 1374) and *P. pouchetii* (strains SK 34, P 360) are identical to that of *P. globosa* strain PCC 540 and were not included in the analyses. Bootstrap values are placed at the nodes from a maximum likelihood analysis (100 replicates), a neighbor-joining analysis (500 replicates) and a maximum parsimony analysis (500 replicates). Scale bar corresponds to 5 changes per 100 nucleotide positions.

aceum (Chesnick *et al*., 1996), *Odontella sinensis* and *Cylindrotheca* sp.) and is most closely related to that of the centric diatom *Odontella* (Figs 2, 3). In the maximum parsimony and neighbor-joining analyses all the diatoms fall into one clade and the contaminant is at the base of the clade (data not shown).

Quite unexpectedly, 105 nucleotides of the 5′ end of the *rbc*S gene showed more variation among our isolates. As expected, many base substitutions were found in the unicellular *Phaeocystis* strains compared with the colonial *Phaeocystis* species/strains, which correlated well with morphological features (Zingone *et al*., 1999), 18S rDNA sequence data and flow cytometric measurements of genome size (Vaulot *et al*., 1994; Table 1). Also three informative sites in the 5′ end of the *rbc*S gene were found that could be used to separate the colonial *Phaeocystis* species (Fig. 2). One site (position 221) separates the *Phaeocystis* warm-water species complex from *Phaeocystis* cold-water species complex, whereas position 242 separates *P. antarctica* strain SK 22 and *P. pouchetii* strains SK 34 and P360 from the remaining Antarctic *Phaeocystis* strains (SK 20 and CCMP 1374). Position 258 separates *P. antarctica* SK 20 and CCMP 1374 from the remaining cold-water species. *P. antarctica* strain SK 22 was isolated on the western side of Bouvet Island within the Antarctic Circumpolar Current (ACC) south of the Polar Front (Fig. 4, location 6).

The relationships among our taxa using the RUBISCO spacer are shown in Fig. 3. The relationships recovered are similar to those found in the 18S rRNA tree (Fig. 1), except that all colonial forms are identical (represented in the tree by *P. globosa* strain NIOZ) except for the single base substitution in two cold-temperate forms of *P. globosa* (represented in the tree by *P. globosa* strain PLY 540). The unicellular forms of *Phaeocystis* diverge before the colonial forms, as in the 18S rRNA tree.

ITS1

ITS1 regions from nine *P. antarctica* strains collected from different locations around Antarctica (Fig. 4, Table 2), one *P. pouchetii* strain from the Greenland Sea, five temperate/tropical strains of *P. globosa* and one of the unicellular species *P. cordata* were sequenced. In addition, we obtained, from GenBank, multiple ITS1 sequences from three *P. globosa* strains from China, Thailand and the Gulf of Mexico and an additional sequence of ITS1 from our Greenland strain of *P. pouchetii* (Table 2). These sequences were obtained from clone libraries. Boundaries of coding and non-coding regions were determined by comparison with published 18S rRNA genes of *Phaeocystis* (Medlin *et al*., 1994) and the 5·8S rRNA gene of the chlorophyte *Cladophora albida* (Bakker *et al*., 1992). ITS1 ranged in size from 259 to 273 nucleotides for *P. antarctica*, from 279 to 301 nucleotides for *P. globosa*, and was 256 bp in *P. cordata* (Table 3, Fig. 5). The 3′ end of ITS1 from the directly sequenced PCR products of *P. pouchetii* was highly variable and could not be reliably aligned. Thus, the total size of the ITS1 region from this species was not determined. Nevertheless, many base substitutions and several deletion/insertion events were found within ITS1, clearly separating *P. antarctica*, *P. pouchetii* and *P. globosa* from one another and from *P. cordata* (Fig. 5, Table 3).

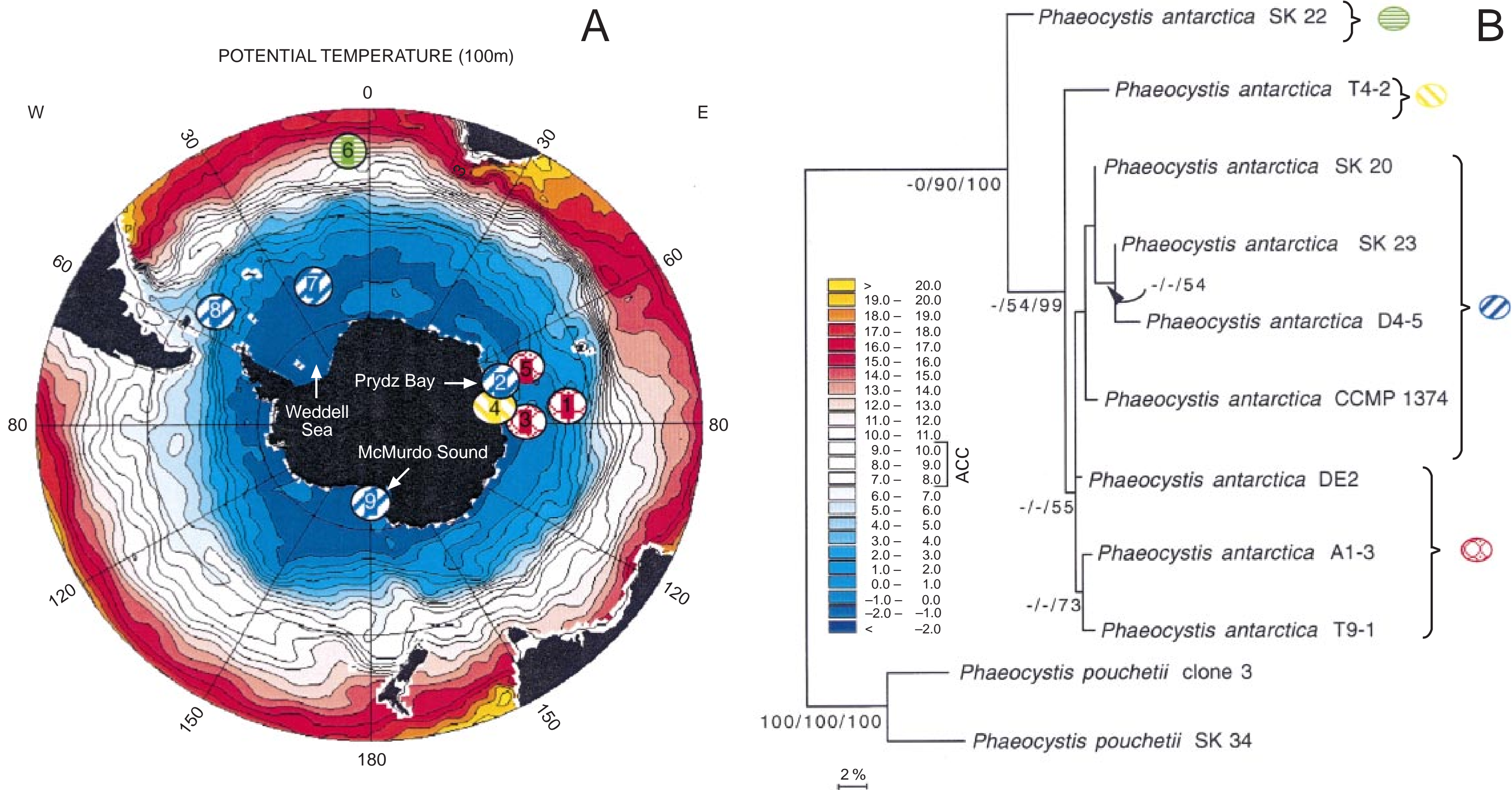

Fig. 4. (*A*) Locations of the strains of *P. antarctica* used in this study. Temperature isotherms at 100 m depth are shown on the map to indicate the position of the ACC (indicated on the temperature key) encircling the Antarctic (redrawn from plate 12 in Olbers *et al*., 1962). The location of different clades is indicated by the different patterns in the large circles and correspond to those clades in Fig. 4*B*; numbers indicate the strains for which the ITS1 spacer region was determined (Table 2). Prydz Bay locations in E. Antarctica are slightly displaced for visual clarity (see Table 2 for precise location). (*B*) Maximum likelihood tree inferred from ITS1 sequences from *P. antarctica* (strains SK 20, SK 22, SK 23, CCMP 1374, DE2, T9-1, D4-5, A1-3 and T4-2) with *P. pouchetii* (SK 34 and clone 3) as outgroup. Only sequence positions marked in the weighting mask 2 (Fig. 5) were used for this phylogenetic analysis. Bootstrap values are placed at the nodes from a maximum likelihood analysis (100 replicates) a neighbor-joining analysis (500 replicates) and a maximum parsimony analysis (500 replicates) Scale bar corresponds to 2 changes per 100 nucleotide positions. The patterned circles beside the strains refer to the haplotype designation in our analysis; for locations of the strains see Fig. 4*A* and Table 2.

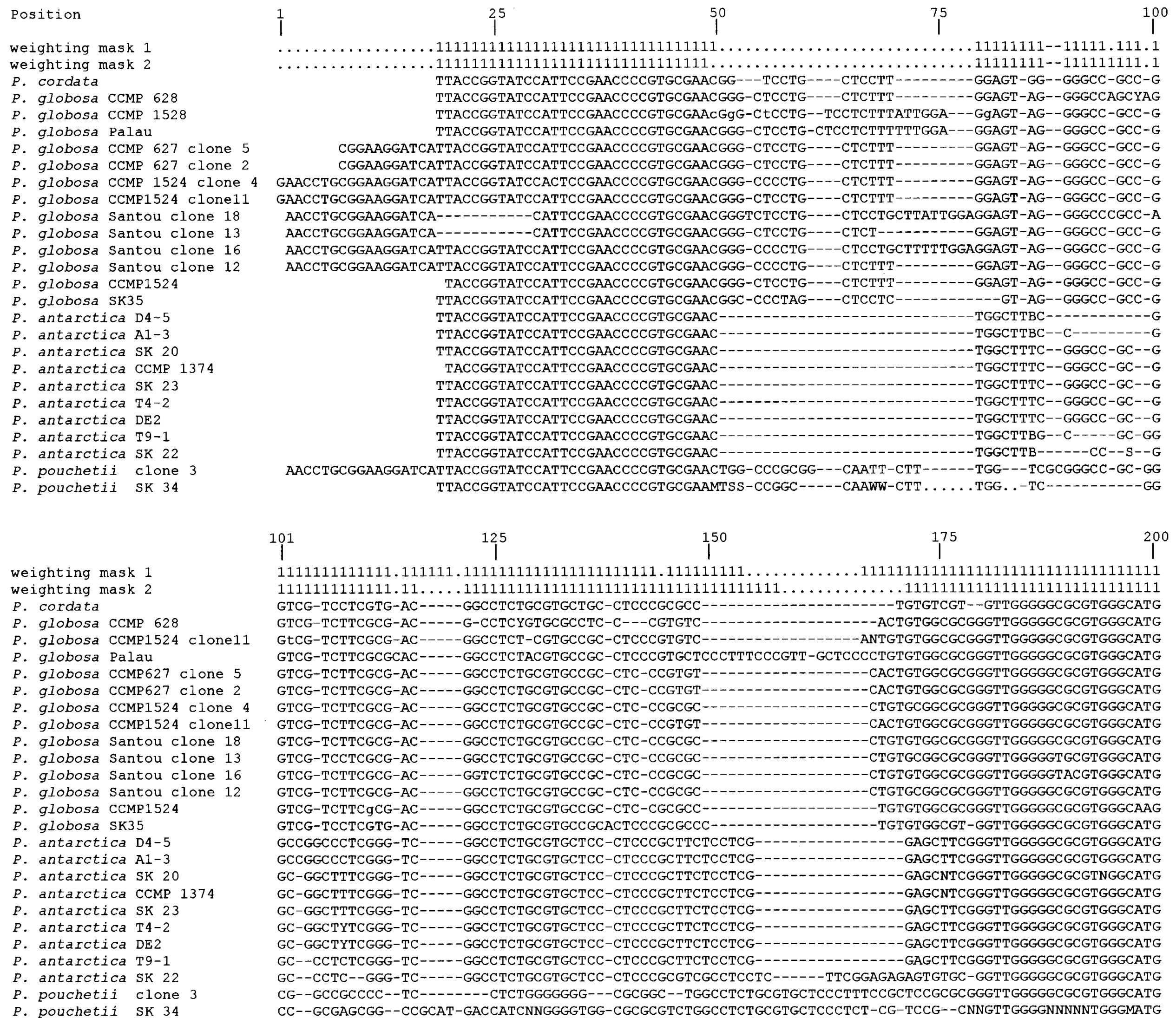

Fig. 5. Alignment of ITS1 sequences from *P. globosa* (strains CCMP 628, CCMP 627, CCMP 1528, CCMP 1524, SK 35, Palau, Santou (China)), *P. pouchetii* (SK 34 and SK 34 clone 3), *P. cordata* and *P. antarctica* (strains SK 20, SK 22, SK 23, CCMP 1374, DE2, T9-1, D4-5, A1-3 and T4-2). Only sequence positions marked in the weighting mask 1 were used for phylogenetic analysis in Fig. 6.

Some problems were encountered during the direct sequencing of ITS1. A clear sequence from warm-water *Phaeocystis* spp. was difficult to obtain because many ambiguities were generated in the sequencing reactions despite the use of amplification and sequencing primers specific for colonial *Phaeocystis* species. Notably, ambiguities were found only in the ITS1 region and not in the 3′ end of the 18S rRNA gene in warm-water *Phaeocystis* species. This suggests that different ITS1 copies may exist within a culture and were amplified and subsequently sequenced, thus causing the sequence to become unreadable once the multiple copies coincided in the sequence run. Sequences obtained from clone libraries made from a single strain of *P. globosa* from China were clearly different, documenting unequivocally that there is variation in the copies of the ITS in *P. globosa* and also *P. pouchetii* (Fig. 4*B*). In one strain of *P. antarctica* approximately 17 nucleotides at the 3′ end of ITS1 region could not be determined (Fig. 5).

Based on our 18S rDNA analysis, we used *P. cordata* as outgroup to examine relationships among the colonial *Phaeocystis* spp. (Fig. 6) in the analysis of the ITS1 region using the positions marked by the weighting mask 1. All three colonial species are recovered in the three analyses. The clade containing all the cold-water isolates (*P. antarctica* and *P. pouchetii*) had strong bootstrap support (92–100% in the three analyses). The strains of *P. globosa* are very divergent from one another and the sequences from one strain did not fall together (Figs 5, 6). The warm-water clade (isolates of *P. globosa*) was only weakly supported in the bootstrap analyses (<50, 50 and 94), with the highest bootstrap support coming from the maximum parsimony analysis in which the data were weighted by a re-scaled consistency index. Within

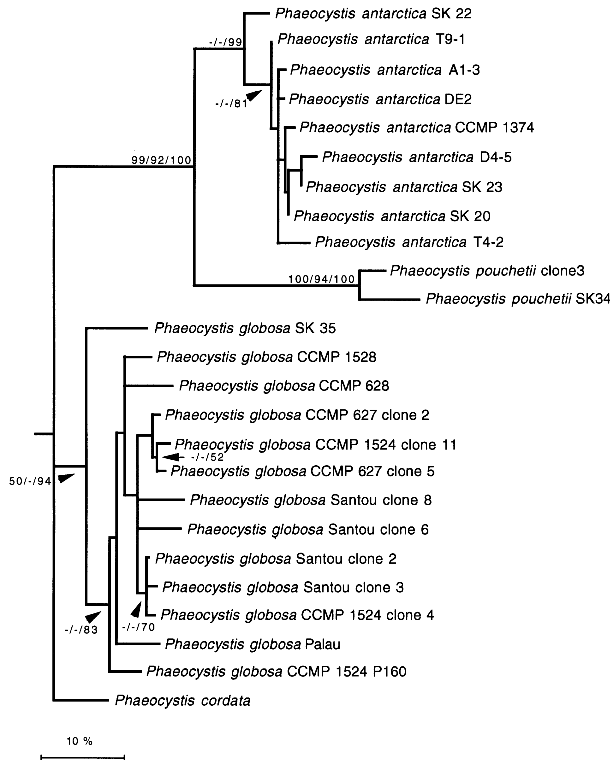

Fig. 6. Maximum likelihood tree inferred from ITS1 sequences from *P. globosa* (strains CCMP 628, CCMP 627 (multiple clones), CCMP 1528, CCMP 1524 (multiple clones), SK 35, Palau, Santou (multiple clones from China), *P. pouchetii* (SK 34 and SK 34 clone 3), *P. antarctica* (strains SK 20, SK 22, SK 23, CCMP 1374, DE2, T9-1, D4-5, A1-3 and T4-2) and *P. cordata* as outgroup. Only sequence positions marked in the weighting mask 1 (Fig. 5) were used for this phylogenetic analysis. Bootstrap values are placed at the nodes from a maximum likelihood analysis (100 replicates) a neighbor-joining analysis (500 replicates) and a maximum parsimony analysis (500 replicates). Scale bar corresponds to 10 changes per 100 nucleotide positions.

the cold-water complex, the two sequences of the single strain of *Phaeocystis pouchetii* fall together and are distinctly supported by the bootstrap analyses.

In contrast, we obtained only one sequence from each of the eight *P. antarctica* strains isolated from the water masses defined by the Antarctic continental boundary currents and they show a high degree of similarity (Table 2; Figs 5, 6) . We feel that they can be separated into at least four different lineages if we analyse the Antarctic strains alone using nearly all ITS1 sequence data (see weighting mask 2) and *P. pouchetii* as an outgroup (Figs 4*B*, 6). The first divergence among the Antarctic clade is strain SK 22, which was isolated from the ACC. The second divergence is a strain from Prydz Bay (T4-2) and this is then followed by a nearly simultaneous divergence of the remaining strains. One divergence includes a cluster of strains from Prydz Bay (DE2, A1-3 and T9-1). The branching order of this cluster of strains from Prydz Bay is slightly different in Figs 4*B* and 6. The other divergence includes strain CCMP 1374 from the Ross Sea and two *Phaeocystis* strains from the Weddell Sea (SK 20, SK 23) (Figs 5, 6; Table 3) and finally strain D4-5 from Prydz Bay, which shares a last common ancestor with the Weddell Sea strain SK 23, as the final cluster to diverge among the Antarctic strains. Our initial PAUP analysis produced 42 equally parsimonious trees (CI = 0·94), which could not resolve any differences within the nine strains of *P. antarctica* because there were too few informative sites unless the data were weighted, in which case only one tree was produced. It did, however, consistently separate *P. antarctica* SK 22 and *P. pouchetii* as earlier divergences sister to the

Table 3. Description of sequence attributes in the ITS1 sequences of *Phaeocystis* species

Species and strains	Total length of ITS1	% G+C and (absolute no. of nucleotides)	No. of ambiguities
Phaeocystis antarctica SK 20	267	65·17 (174)	6
Phaeocystis antarctica SK 23	267	67·04 (179)	0
Phaeocystis antarctica CCMP 1374	267	65·92 (176)	2
Phaeocystis antarctica DE2	266	66·17 (176)	2
Phaeocystis antarctica T4-2	268	66·42 (178)	2
Phaeocystis antarctica T9-1	259	66·80 (173)	1
Phaeocystis antarctica D4-5	247[a]	68·83 (170)	3
Phaeocystis antarctica A1-3	263	66·92 (176)	2
Phaeocystis antarctica SK 22	273	65·93 (180)	4
Phaeocystis pouchetii SK 34	n.d.[b]	– (199)	24
Phaeocystis globosa CCMP 628	279	65·23 (182)	4
Phaeocystis globosa SK 35	288	68·40 (197)	0
Phaeocystis globosa P277	301	68·77 (207)	0
Phaeocystis globosa CCMP 1528	290	68·62 (199)	1
Phaeocystis globosa CCMP 1524	289	65·74 (190)	0
Phaeocystis cordata	256	60·55 (155)	9

[a] Seventeen nucleotides at the 3′ end could not be determined.
[b] The 3′ end was highly variable and not alignable.

remaining Antarctic strains. In the short fragment of the *rbc*S gene, strain SK 22 was more closely related to *P. pouchetii* with a single base substitution shared by *P. antarctica* strain (SK 22) and *Phaeocystis pouchetii*.

Discussion

Morphological features

A summary of the salient morphological features of the *Phaeocystis* species used in this study is presented in Table 1. Species can be separated by DNA genome size as well as by features and size of the colonies and by size of the scales on the flagellated stage and length of the flagella.

18S rRNA

The phylogenetic relationships inferred from the analysis of the 18S rRNA data set continue to support the differentiation of the three major colonial forms of *Phaeocystis* at the species level (Medlin *et al*., 1994). Each of these three major species (*P. antarctica*, *P. pouchetii* and *P. globosa*) is monophyletic in 18S rRNA analyses. Taken together with the morphological and genome size differences shown in Table 1, it seems unwise to lump them into a single taxon as has been suggested in the past based on morphological evidence (Sournia, 1988) and more recently by Gallagher (1998), based on a more conservative opinion of species boundaries as inferred from molecular data. Unfortunately, some morphological data overlap and other clear morphological support is needed. Perhaps ultrastructural detail, such as pyrenoid structure or flagellar roots, may be helpful in this respect. Zingone *et al*. (1999) have provided some morphological characters differentiating the unicellular species *P. jahnii* from the colonial ones. It can be inferred from the branching order in the rRNA tree that the colonial form is derived. Because viruses are known only to attack the flagellated, unicellular stages of *Phaeocystis* (Jacobsen *et al*., 1996), it is not unreasonable to hypothesize that the evolution of the colonial stage has resulted in prevention of viral control of bloom populations (see discussions in Hamm *et al*., 1999) until the colony matrix is broken down, presumably by bacterial activity.

RUBISCO

In comparison with the 18S rDNA data set for these species, the relationships recovered by the RUBISCO spacer showed less resolution (Fig. 3). There are more base substitutions separating the major clades in the rRNA tree than in the RUBISCO spacer tree but this represents a smaller fraction of the total bases compared. For example, in the rRNA tree, one of the Mediterranean isolates, *P. cordata*, is separated from the warm-water *P. globosa* complex by 24–38 nucleotides and from the cold-water *P. antarctica*/*P. pouchetii* complex by 17–22 nucleotides (Medlin *et al*., 1994; Lange, 1997). In the RUBISCO analysis, these same taxa are separated by 24 nucleotides, respectively representing a 2% difference in the rRNA gene and a 24% difference in the RUBISCO spacer. Variation in the 18S rRNA gene ranged from 6 to 12 base substitutions between *P. globosa* strains and *P. antarctica*/*P. pouchetii* strains, whereas in the RUBISCO spacer these taxa are identical. If we were to base our species level determination on the RUBISCO spacer alone, we would lump all the colonial species into one taxon.

The lack of variation in the RUBISCO spacer among well-defined *Phaeocystis* species was surprising in view of the amount of inter- and intraspecific variation reported in red algae (Maggs *et al*., 1992; Goff *et al*., 1994; Brodie *et al*., 1998; Zuccarello *et al*., 2000). The RUBISCO spacer was too highly conserved in *Phaeocystis* to be used for population-level studies or even to infer phylogenetic relationships between closely related colonial species because these sequences were identical. Even more surprising was the fact that 105 nucleotides in the *rbc*S gene were more informative than the spacer region and able to resolve differences among the colonial strains. The Prymnesiophyceae are a much younger group than the red algae, which may be an important factor in the lack of variation detected in this non-coding region among *Phaeocystis* species as was postulated for global isolates of the young species *Emiliania huxleyi* (250000 years) (Medlin *et al*., 1996). However, the high similarity among the four diatom taxa for which RUBISCO sequence data are available (Fig. 2) suggests that the RUBISCO spacer is also highly conserved among diatom genera as well. These diatom genera are very different morphologically and phylogenetically, ranging from the advanced nitzschioid *Cylindrotheca* to bipolar centrics, yet the RUBISCO spacer region among these taxa is very similar. Although we cannot be sure of the identification of the contaminant that we picked up in our RUBISCO sequencing, we suspect that it is the genus *Chaetoceros*. In field samples, small, unicellular *Chaetoceros* spp. often reside in or around *Phaeocystis* colonies and among the known diatom genera here *Chaetoceros* would be basal to the other three in a molecular analysis (Chesnick *et al*., 1997). An undetected diatom in the colony could explain the diatom-like pigment signature found in some *Phaeocystis* strains (Vaulot *et al*., 1994). Stache-Crain *et al*. (1997) also found the RUBISCO spacer

useful only at the generic level in the Ectocarpales of the Phaeophyceae, another younger algal lineage. It may also be that, in these groups with little variation in the spacer, this region may be under some type of functional constraint (as yet unknown) that prevents it from evolving at a higher rate. Bailey & Andersen (1999) found no variation among multiple isolates of two pelagophytes and they interpreted this to infer that they had not uncovered any hidden varieties or cryptic species in the algae they examined.

ITS regions

The ITS regions in the warm-water species are highly variable and we have documented that there is variation in the copies of the ITS regions within a single strain. These non-homogeneous copies make interpretation of phylogenetic relationships among the warm water strains impossible. One possible explanation for the variable copies is that *P. globosa* is indeed a multi-species complex as indicated by the variation in DNA content among several strains and these species have hybridized with one another yielding heterozygous alleles for the ITS regions. In addition, the large divergence in the ITS region and the low to moderate bootstrap support among the strains of *P. globosa* support the earlier hypothesis (Vaulot *et al.*, 1994) that *P. globosa* is actually a species complex (Table 2). Variable copies of the second intron region of the mini-collagen gene in mass spawning corals was taken as genetic evidence of recent hybridizations between coral species (Hatta *et al.*, 1999) and variable copies of the ITS region have also been reported in the green algae *Caulerpa taxifolia* and *C. racemosa* (Jousson *et al.*, 2000; Famà *et al.*, 2000).

Interpretation of the phylogenetic relationships in the cold-water species was easier because only *P. pouchetii* exhibited variable copies of the ITS region within one strain. *P. antarctica* thus appears, based on clear ITS sequence, to contain only strains which have homogeneous ITS regions and we interpret this as support for a single species in Antarctic waters. It appears from our limited sampling around the Antarctic continent that the ITS1 region is suitable for resolving the biogeographic history of *Phaeocystis*, especially in the Antarctic. Populations of *P. antarctica* within the continental boundary water masses appear to be well mixed because currents around the Antarctic continent move rather quickly and may effectively act as a barrier to significant population structure. Strain SK 22 isolated within the ACC is clearly different. Our earlier hypothesis, proposed from rDNA data (Medlin *et al.*, 1994), that ancestral populations in the Antarctic gave rise to present-day *P. antarctica* and *P. pouchetii* populations appears to be supported by our ITS1 analysis of the cold-water *Phaeocystis* strains. *P. antarctica* and *P. pouchetii*, both polar, are more closely related to one another than either is to the cold and warm-temperate to tropical populations of present-day *P. globosa* as supported in all our analyses from the two different regions/genes. This suggests that dispersal did not occur from present-day warm-water populations into present-day cold-water populations and also that gene flow has occurred from pole to pole across tropical oceans. Arctic *P. pouchetii* populations thus probably arose by a dispersal event from the south to the north during colder climate periods that allowed populations to survive the crossing of equatorial waters, as has been documented for other organisms (Crame, 1993; Darling *et al.*, 2000).

If we follow the branching order in Figs 1, 4*B* and 6, we can hypothesize the following scenario. It is clear from Fig. 1 that the ancestral populations in the Antarctic were derived from ancestors of the present-day warm-water species. Our own molecular clock calculations based on 18S rRNA sequence data calibrated with the fossil record from haptophyte coccolithophorid species indicate that the warm-water *Phaeocystis* species diverged from the cold water species approximately 30 Mya, which coincides with the time that the Drake passage opened and the ACC system was formed (Medlin *et al.*, 1994). This would have effectively isolated ancestral populations in the Antarctic sufficiently to allow them to speciate from their warm-water ancestors. It can be inferred from Figs 4*B* and 6 that presumed descendants of these warm-water ancestors were first entrained in the ACC. Some of these ancestral populations must have been transported northward and across the Equator shortly after the Drake passage opened because the *P. pouchettii* populations are sister to the *P. antarctica* populations. The ACC today encircles the Antarctic continent every 1–2 years. Water is entrained from this current into the major gyres of the continental water masses (Treshnikov, 1964). Using the branching order in Fig. 4*B* we can trace the dispersal of the clones from the ACC, although the bootstrap support for the branching order among the clades is weak and even then only with the weighted maximum parsimony analysis. From Fig. 4*B*, the first entrainment with a bootstrap support of 99% appears to be into Prydz Bay, because strain T4-2 isolated from this bay is the first divergence in our tree. These populations then established themselves in the eastern Antarctic in Prydz Bay. Subsequent divergences in the tree indicate that populations were then entrained into the Ross Sea and almost simultaneously they were entrained into the Weddell Sea (bootstrap support 54%). Both isolates from

the Weddell Sea were the last to diverge before the populations were again entrained back into Prydz Bay from populations in the Weddell Sea, because isolates from this bay are some of the last divergences in the tree (bootstrap support 54%). The distribution of these isolates in this fashion follows the predominant current patterns of surface waters in the Antarctic today. What we do not know is how different the surface-water circulation was 30 Mya ago before the ACC was established. What is clear from these data is that *P. antarctica* does not have variable copies of the ITS as do *P. globosa* and *P. pouchetii*. We infer from these data that the populations of the latter two may in fact represent more than one species, whereas *P. antarctica* is only one species because the Antarctic continental water masses have homogenized the populations.

Other studies have also shown the effect of mixing on the homogenization of the genetic structure of Antarctic populations. Antarctic krill species within the Antarctic continental water masses are very similar as documented by both mitochondrial DNA (T. Patarnello *et al.*, unpublished) and isozyme analysis (Fevolden & Schneppenheim, 1989). The mitochondrial DNA study also suggested that the formation of the ACC effectively isolated krill species in Antarctic water masses from those north of the ACC. Calculation of the time of divergence between species groups found either side of the ACC coincided with the timing of the ACC, approximately 30 Mya.

It is clear from our analyses of the ITS1 region from *P. antarctica* populations that modern-day dispersal of these populations in Antarctic continental waters follows the current circulation pattern of the ACC around the Antarctic and cladogenesis from warm-water ancestors was coincident with the opening of the Drake passage and the establishment of the ACC. Both polar populations are descended from a last common ancestor and we believe the dispersal from pole to pole occurred from south to north.

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