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Genetic differentiation among three colony-forming species of *Phaeocystis*: further evidence for the phylogeny of the Prymnesiophyta

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Sequence data from the 18S small subunit ribosomal RNA gene have been used to support the species status of three colony-forming species of *Phaeocystis* Lagerheim (Prymnesiophyta). Two of these correspond to *Phaeocystis globosa* Scherffel and *Phaeocystis pouchetii* (Hariot) Lagerheim. The third species originates from antarctic waters and is referred to *Phaeocystis antarctica*, described by Karsten at the turn of the century. Morphological and physiological data supporting the separation of the three species is compiled from the literature. Phylogenetic trees generated from the sequence data suggest that the warm-water species, *Phaeocystis globosa* diverged prior to the separation of the two cold-water forms. Tectonic events and climatic changes during the middle to late Cenozoic provide mechanisms by which speciation events could have occurred as both polar oceans were being formed.

INTRODUCTION

Phaeocystis Lagerheim is a cosmopolitan bloom-forming alga recognized both as a nuisance alga and an ecologically important member of the phytoplankton (Davidson 1985; Lancelot *et al.* 1987; Smith *et al.* 1991; Davidson & Marchant 1992; Baumann *et al.* 1994b). Its large-scale blooms are often avoided by fish (Savage 1930; Chang 1983) and appear detrimental to the growth and reproduction of shellfish and macrozooplankton (Davidson & Marchant 1992). Dissolved organic compounds released by *Phaeocystis* during bloom conditions can accumulate and foam, creating massive areas of pollution when washed onshore (Lancelot *et al.* 1987). *Phaeocystis* is also thought to be a major contributor to the global sulphur budget by releasing substantial quantities of dimethylsulphide (DMS) (Keller *et al.* 1989; Baumann *et al.* 1994a) and it may play yet another important ecological role with its production of UV-B absorbing compounds (Marchant *et al.* 1991; Davidson & Marchant 1992).

Phaeocystis has a polymorphic life cycle with both colonial and flagellated cells (Kørnmann 1955) but the colonial stage with cells embedded in a gelatinous matrix is most easily recognized. Thousands of cells can occur in a colony that may reach 2 cm in diameter (Jahnke & Baumann 1987; Verity *et al.* 1988b; Rousseau *et al.* 1990; Davidson & Marchant 1992). The difficulty in assigning a specific name to the colony stage has caused much taxonomic confusion.

The genus was erected by Lagerheim in 1893 to accommodate the colonial state of an alga originally described as *Tetraspora poucheti* by Hariot in Pouchet (1892). *Phaeocystis pouchetii* (its correct orthography) occurs in cold waters and forms globular, lobed colonies with cells arranged in packets of 4 (see Jahnke & Baumann 1987 for illustrations). *Phaeocystis globosa* was described by Scherffel (1900) from temperate waters, forming spherical colonies with cells arranged homogeneously within the gelatinous matrix (Jahnke & Baumann 1987), while older stages can assume distorted pear-shapes (Bätje & Michaelis 1986). Most early workers separated *Ph. pouchetii* and *Ph. globosa* based on different distributions and colonial morphologies until Kornmann (1955) expressed doubt on the differentiation between the two species. From his life cycle studies, he claimed that *Ph. globosa* cell types were juvenile forms of *Ph. pouchetii*. Since then, colony morphology has been judged an unreliable specific character.

Sournia (1988) reviewed the diagnostic features of *Phaeocystis*, and examined the validity of the 9 species described since the last century. Most were poorly described, often lacking essential ultrastructural features of the flagellated stage, hence Sournia recognized only 2 of the 9 as valid species: *Phaeocystis scrobiculata* Moestrup, known only from the flagellated state (Moestrup 1979), and *Ph. pouchetii*, which included *Ph. globosa*. Upon Sournia's (1988) recommendation, most marine ecologists report *Phaeocystis* colonies as *Ph. pouchetii* (the older name) or as *Phaeocystis* sp. to avoid confusion.

Recent studies have regarded this as over-simplification (Baumann & Jahnke 1986; Jahnke & Baumann 1986, 1987; Jahnke 1989). Observations on the maintenance of colony shapes in both juvenile and older stages of *Ph. globosa* and *Ph. pouchetii* have supported their recognition as separate species. Also, detailed studies of the temperature and light tolerances suggest separation at the species level. A third, unnamed colonial species from antarctic waters was recognized by Baumann *et al.* (1994b), which had a combination of features of *Ph. globosa* and *pouchetii*, as suggested earlier by Moestrup & Larsen (1992). The colonies resembled those of *Ph. globosa* (Larsen & Moestrup 1989), while temperature tolerances were similar to those of *Ph. pouchetii*. Notably, the strain had different pigment spectra (Buma *et al.* 1991; Vaulot *et al.* 1994) and DNA content (Vaulot *et al.* 1994).

We have investigated the validity of the three colony-forming species suggested as distinct species by Moestrup & Larsen

Table 1. Algal cultures analysed in this study

Species	Culture number	Origin	Maintenance temperature	Light/dark cycle
Phaeocystis globosa	SK 35	German Bight, water column, net haul (0–20 m)	20–22°C	12:12
Phaeocystis pouchetii	SK 34	Greenland Sea, East Greenland Current, water column, net haul (0–30 m)	0°C	18:6
Phaeocystis antarctica	SK 20	Weddell Sea, sea ice S 67°50′, W 20°51′	0°C	24:0
Phaeocystis antarctica	SK 21	Weddell Sea, sea ice S 65°12′, W 39°22′	0°C	24:0
Phaeocystis antarctica	SK 22	Weddell Sea, water column S 54°20′, W 03°20′	0°C	24:0
Phaeocystis antarctica	SK 23	Weddell Sea, water column S 63°15′, W 58°20′	0°C	24:0
Phaeocystis antarctica	CCMP 1374	McMurdo Sound, Antarctica	0°C	24:0

(1992) and Baumann *et al.* (1994b) using sequence data from the nuclear-encoded small subunit (ssu) ribosomal RNA gene. The data have also been used to assess the phylogenetic position of the Prymnesiophyta. The ssu rRNA gene has been used to infer phylogenetic relationships at various taxonomic levels in the algae (Gunderson *et al.* 1987; Bhattacharya *et al.* 1989, 1992; Medlin *et al.* 1991, 1993; Schlegel *et al.* 1991; Bird *et al.* 1992; Buchheim & Chapman 1992; Lewis *et al.* 1992; Saunders & Druehl 1992). It is considered an appropriate gene in determining close as well as distant phylogenetic relationships by possessing domains that exhibit varying degrees of conservation (Woese 1987). In addition we have supported our interpretation of species-level variation in the ssu rRNA molecule with morphological, ecological and physiological data from the literature.

MATERIALS AND METHODS

Cultures

The strains used are listed in Table 1. All isolates were grown in an enriched seawater medium (von Stosch & Drebes 1964) and stirred manually on a daily basis.

Isolation of DNA

Cultures were harvested during the log phase by centrifugation. They were immediately frozen in liquid nitrogen and kept at −70°C until needed. Cells were thawed in extraction buffer (100 mM Tris, pH 8.5; 100 mM NaCl, 50 mM EDTA) before extraction of total nucleic acids by vortexing the cells in the presence of 2% SDS and 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. Some extractions were performed using a 3% CTAB (hexadecyltrimethylammonium bromide) procedure (Doyle & Doyle 1990).

Amplification

Total nucleic acid preparations were used as templates for the amplification of the nuclear gene coding for the ssu rRNA molecule using polymerase chain reactions (PCR) as modified by Medlin *et al.* (1988). Oligonucleotide primers with multiple restriction endonuclease sites were used to permit directional cloning into single stranded M13 (Table 2). A minimum of five PCR reactions were performed and pooled for each species.

Cloning and sequencing

Amplification products were purified using BRL glass max spin columns following precipitation with ½ vol ammonium acetate and 2 vols 100% ethanol (5 min, room temperature). Purified gene products were ligated into the RF of M13 mp18 and M13 mp19 (Medlin *et al.* 1988) using a combination of PCR product cut with *Pst* I/*Bgl* II or *Bam* HI/*Sal* I and vector cut with *Pst* I/*Bam* HI or *Bam* HI/*Sal* I, respectively. Single stranded templates were prepared from as many as 4 pooled recombinant M13 phages in each orientation for each species. Internal oligonucleotide primers (Elwood *et al.* 1985) were used to initiate DNA synthesis in dideoxynucleotide chain-termination sequencing reactions (Sanger *et al.* 1977) of both the coding and non-coding strand.

Molecular character analysis

The sequences were aligned with small subunit ribosomal RNA genes from 150 eukaryotic organisms. *Acanthamoeba castellanii* (Douglas) Page was used as outgroup (Table 3). Secondary

Table 2. Primer nucleotide sequences used for PCR reactions in this study

5′ primer (35 bp)	5′ CCGAATTC GTCGACAACCTGGTTGATCCTGCCAGT 3′ (*EcoR* I, *Sal* I)
5′ primer (33 bp)	5′ CCCGGG AGATCTAACCTGGTTGATCCTGCCAGT 3′ (*Sma* I, *Bgl* II)
3′ primer (38 bp)	5′ CCCGGGATCCAAGCTTGATCCTTCTGCAGGTTCACTAC 3′ (*Sma* I, *Bam* HI, *Hind* III, *Pst* I)

Fig. 1. Alignment of 18S rRNA gene from the prymnesiophytes *Emiliania huxleyi, Phaeocystis antarctica* (strains CCMP 1374, SK 20, SK21, SK 22, SK 23), *Phaeocystis pouchetii* (SK34) and *Phaeocystis globosa* (SK35) with the dinoflagellate *Crypthecodinium cohnii,* the diatom *Skeletonema costatum* and the chrysophyte *Ochromonas danica.* Areas marked with a star indicate regions unique to the Prymnesiophyta.

structure of the rRNA molecule was used to aid the alignment on a VAX 6520 with the Olsen sequence editor (Olsen 1990). With this method 1644 of 3082 positions were used to infer the position of the Prymnesiophyta relative to other algae in the distance analysis. Of these, 531 were informative and used in the parsimony analysis. Gaps were treated as missing data. We compared the relationships among the strains of *Phaeocystis* with *Emiliania huxleyi* (Lohman) Hay et Mohler as outgroup, using the entire ssu rRNA sequence because prymnesiophyte signature sequences are eliminated from the analysis of the larger data set (Fig. 1).

Analytical methods

PARSIMONY ANALYSIS: Parsimony analysis of both data sets was performed using PAUP vers. 3.0L (Swofford 1991). Informative characters were treated as unordered multistate characters (Swofford 1991). The heuristic procedures using the TBR branch-swapping algorithm and the MULPARS option within PAUP were implemented for the larger data set, while an exhaustive search with these options was used with the smaller data set. A bootstrap analysis, using a 50% majority rule, was performed with 100 iterations from the larger data set (Felsenstein 1985). For those branch nodes supported by less than 50% in the bootstrap analysis, a decay study was performed to determine how many more steps must be added to the length of the minimal tree to collapse the branch (Mishler *et al.* 1991). The consistency index (CI) indicates the amount of homoplasy. The retention index (RI) expresses the amount of synapomorphy or group defining characters in the data set (Farris 1989).

DISTANCE ANALYSIS: Distance matrix methods fit a tree to a matrix of pairwise distances calculated between sequences (Felsenstein 1988). Pairwise comparison of sequences (taxa) were used to calculate similarity values (Fitch & Margoliash 1967) and converted to distance values using the Jukes & Cantor (1969) model, which assumes independent change at all sites, with equal probability of one base changing into another. Distance values were converted to phylogenetic trees as described by Olsen (1988). Distances were also calculated with the Kimura model (Kimura 1980), which allows for a difference between transversion and transition rates in base substitution, and converted into trees using the Neighbor program in Phylip (3.5; Felsentein 1992).

Fig. 1. Continued.

Table 3. Source of rRNA sequences analysed in this study

Zea mays Linnaeus	Neefs *et al.* 1991
Chlorella vulgaris Beijerinck	Neefs *et al.* 1991
Chlorella ellipsoidea Gerneck	EMBL X63520
Cryptomonas phi nucleus	Douglas *et al.* 1991
Rhodomonas salina (Wisłouch) Hill et Wetherbee	Eschback *et al.* 1991 (as *Pyrenomonas salina* (Wisłouch) Santore)
Acanthamoeba castellanii (Douglas) Page	Neefs *et al.* 1991
Palmaria palmata (Linnaeus) Kuntze	Neefs *et al.* 1991 (as *Porphyra umbilicalis* (Linnaeus) J. Agardh)
Gracilaria tikvahiae McLachlan	Neefs *et al.* 1991
Cryptomonas phi nucleomorph	Douglas *et al.* 1991
Oxytricha nova D. Prescott	Neefs *et al.* 1991
Sarcocystis muris (Railliet) Labbe	EMBL M34846
Prorocentrum micans Ehrenberg	Neefs *et al.* 1991
Crypthecodinium cohnii (Seligo) Chatton	EMBL M34847
Emiliania huxleyi (Lohman) Hay et Mohler	Bhattacharya *et al.* 1992
Phaeocystis pouchetii (Hariot) Lagerheim	This study
Phaeocystis globosa Scherffel	This study
Phaeocystis antarctica Karsten	This study
Achlya bisexualis Coker	Neefs *et al.* 1991
Skeletonema costatum (Greville) Cleve	Neefs *et al.* 1991
Ochromonas danica Pringsheim	Neefs *et al.* 1991
Mallomonas papillosa Harris et Bradley	Bhattacharya *et al.* 1992
Synura spinosa Korschikov	Bhattacharya *et al.* 1992
Tribonema aequale Pascher	Bhattacharya *et al.* 1992
Fucus distichus Linnaeus	Bhattacharya *et al.* 1992

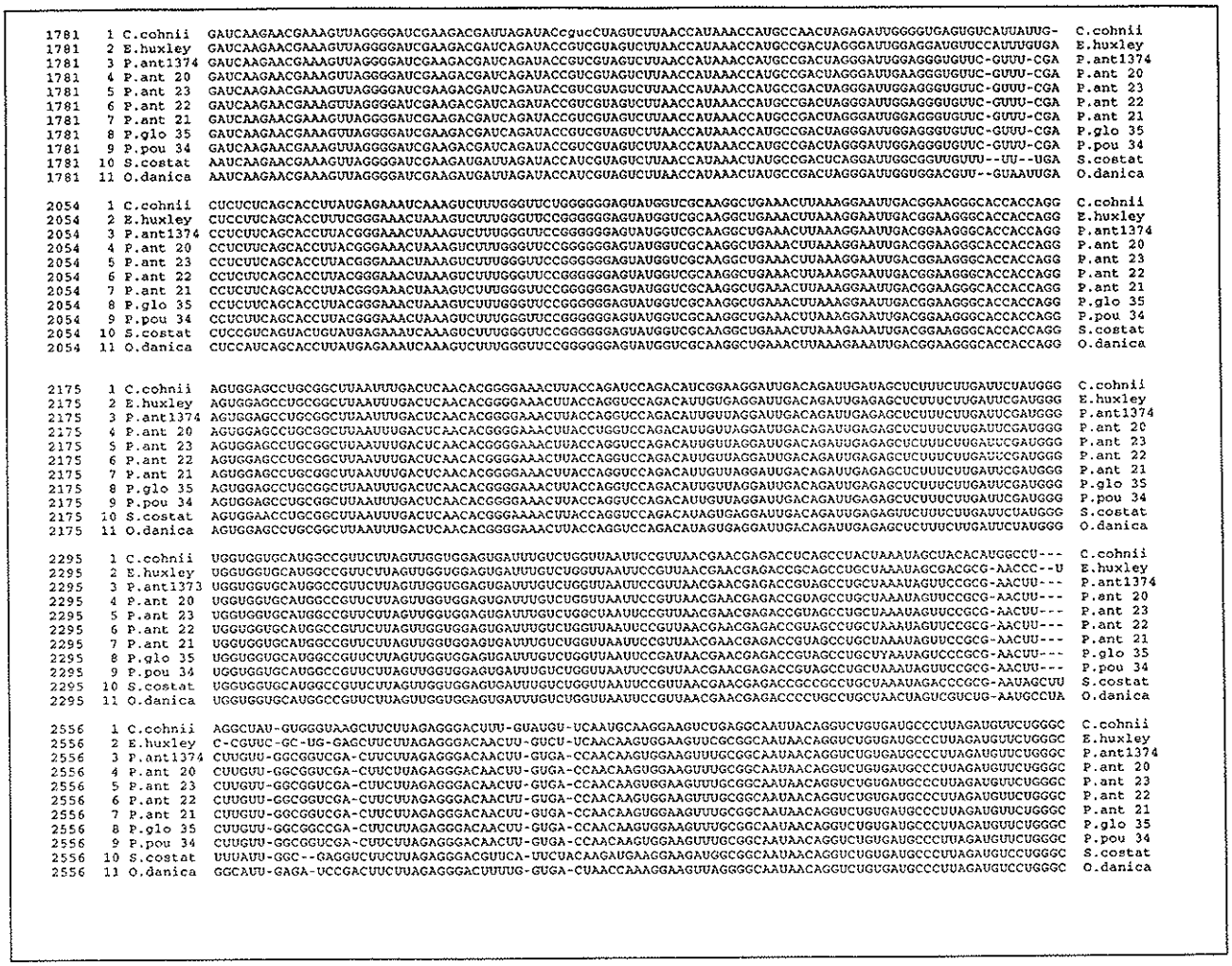

Fig. 1. Continued.

RATE CONSTANCY TESTS: The relative rate of evolution was calculated to determine if the cold- and warm-water *Phaeocystis* strains differed significantly from *E. huxleyi* (Li & Graur 1991). The variance in the number of substitutions per lineage was compared to the mean to determine if these rates were significantly different (Ochman & Wilson 1987).

RESULTS

Molecular analysis

Complete 18S rRNA sequences were determined for the *Phaeocystis* strains and deposited in Genbank (accession nos X77475 and X77481). The 18S ssu rRNA gene consists of 1803 nucleotides. The alignment with *E. huxleyi*, *Skeletonema costatum* (Greville) Cleve, *Ochromonas danica* Pringsheim and *Cryptheco dinium cohnii* (Seligo) Chatton is presented in Fig. 1, while an alignment of *E. huxleyi* with many other chlorophyll *a* & *c* algae can be found in Bhattacharya *et al.* 1992. Ambiguities were noted in each strain.

The relationship between the Prymnesiophyta, represented in our study by *Ph. pouchetii* and *E. huxleyi*, and other algal groups were determined by both distance and parsimony analyses. For the distance analysis, only the tree generated by the Kimura model is shown (Fig. 2). In this tree, the Prymnesiophyta do not share a recent history with the stramenopiles (heterokont flagellates, Patterson 1989) and are the first algal group to emerge in the major radiation giving rise to all of the non-green algae, except for the cryptomonads which are related to *Acanthamoeba*. The tree generated by the Jukes and Cantor model (tree not shown) is identical to the single most parsimonious tree (2550 steps) obtained using the heuristic search within PAUP (Fig. 3) and places the Prymnesiophyta as the second major algal group to emerge after the Rhodophyta. In the trees based on the Kimura model and the Jukes/Cantor model, the emergence of the Prymnesiophyta and the Rhodophyta are interchanged, with the prymnesiophytes being more distantly related to the stramenopiles (heterokont flagellates) in the Kimura model. With two additional steps added to the length of the parsimony minimum tree, the two branch nodes with a less than 50% majority rule in the bootstrap analysis collapsed, but six additional steps were necessary to collapse nodes supported by 61–62% of the resampled trees. Of these nodes, that leading to prymnesiophytes is the least repeatable. Those internal nodes supporting ≥ 70% of the

Fig. 1. Continued.

bootstrap proportions probably do reflect accurate clades (Hillis & Bull 1993). Nevertheless, the Prymnesiophyta appear monophyletic in all trees (see bootstrap value, Fig. 3) and are equally similar to all major algal groups (Fig. 2).

The relationship among seven strains of *Phaeocystis* was examined using the complete 18S rRNA sequences and analysed with both distance and parsimony methods. Similarity values among the strains of *Phaeocystis* and *E. huxleyi*, which was used as outgroup, range from 94% to 100% (Table 4). The absolute number of nucleotide differences separating the strains of *Phaeocystis* (Table 4) are comparable to species differences within the protozoan *Tetrahymena* (0–33) (Sogin *et al.* 1986) and the diatom *Skeletonema* (11) (Medlin *et al.* 1991). Similar differences separating *Phaeocystis* and *Emiliania* were found between distantly related genera within the Chlorococcales (Huss & Sogin 1991) and Volvocales (Larson *et al.* 1992), while more closely related taxa were separated by base differences comparable to the differences between our *Phaeocystis* strains corresponding to distinct species.

The number of nucleotides separating well-established species in these studies suggests that there are sufficient numbers of nucleotide differences to separate these colony-forming *Phaeocystis* strains into three separate species in agreement with the recommendations of Moestrup & Larsen (1992) and Baumann *et al.* (1994b). We recognize the antarctic cold-water forms as *Phaeocystis antarctica* (Karsten 1905) along with the two resurrected species, *Ph. globosa* and *Ph. pouchetii*, the latter occurring in the Arctic Ocean and in sub-polar regions of the North Atlantic. Variation in the 18S ssu rRNA molecule within *Ph. antarctica* show an intraspecific variation from 0 to 5 bases. Four isolates of *Ph. antarctica* are remarkably identical (0–2 bases), despite originating from both open water and ice samples and from both sides of the antarctic continent (Fig. 4). Only strain SK 20 is separated from the other strains by more base substitutions (Table 4). Unfortunately, this culture has been lost and we are unable to re-assess its identity (see discussion below).

Both the distance and the parsimony trees indicate that the warm-water form, *Ph. globosa*, diverged prior to the divergence of the two cold-water species (Fig. 5). The distance tree resolves the relationship of all of the strains analysed to date and places strain SK 20 of *Ph. antarctica* as the most derived strain within this species (Fig. 5a). However, if all strains are included in the parsimony analysis, the five strains of *Ph. antarctica* cannot be separated from one another because they share only two informative sites. 150 equally parsimonious

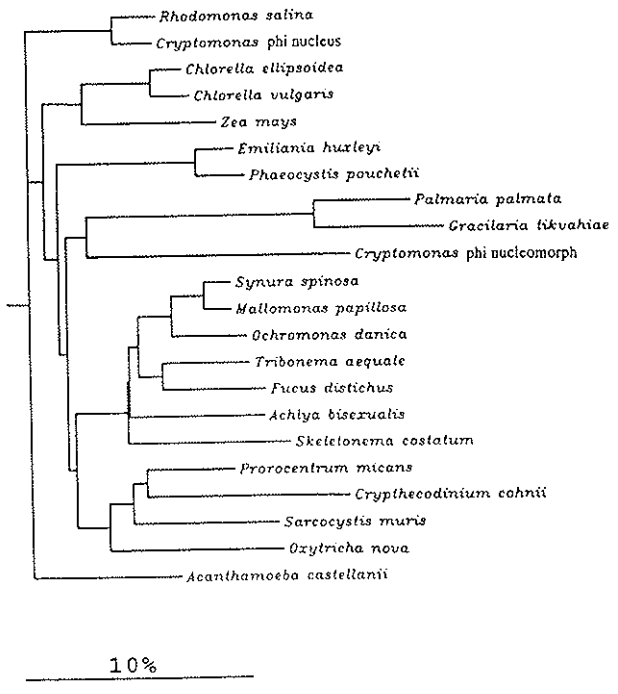

Fig. 2. Phylogenetic representation of the position of the ssu rRNA gene from the prymnesiophyte nucleus; Kimura model using the neighbour joining algorithm (Phylip 3.5). The distance corresponding to 10 changes per 100 nucleotide positions is placed below the distance tree. It is reflected in the horizontal separation of taxa in the tree.

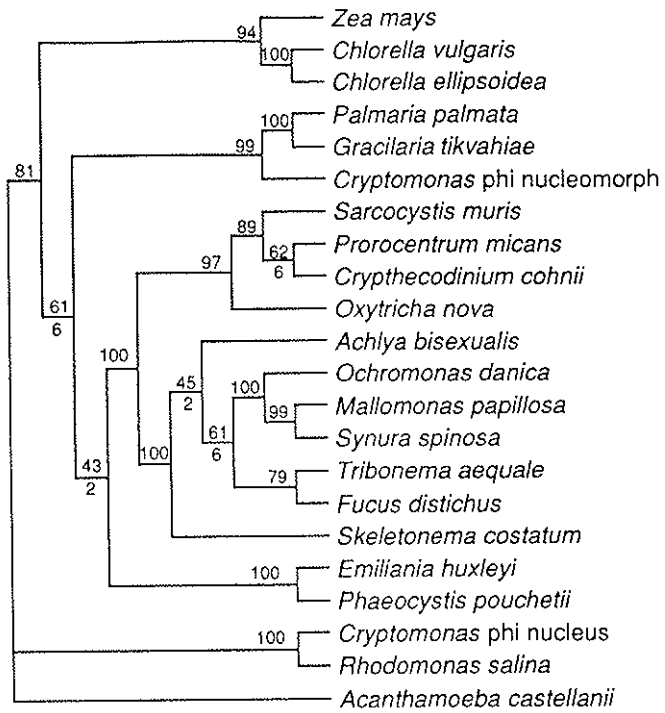

Fig. 3. Phylogenetic representation of the position of the ssu rRNA gene from the prymnesiophyte nucleus from the single most parsimonious tree using the heuristic branch swapping algorithm (PAUP). (Tree length 2550 steps, CI excluding uninformative characters = 0.506, RI = 0.494). Values placed above the nodes indicate the bootstrap value from the consensus tree using a 50% majority rule. Those nodes where the bootstrap value is less than 50 were collapsed and unresolved in the bootstrap consensus tree. Values placed under the nodes refer to the decay index or the number of steps beyond that in the minimal tree at which the branch collapses. The decay index was calculated only for those nodes where the bootstrap value was under 70%.

trees are generated, each showing different recombinations of the antarctic strains (trees not shown). A consensus tree shows the polar taxa unresolved. If only two strains (SK 20 and CCMP 1374) are chosen to represent *Ph. antarctica* in our parsimony analysis, then three equally parsimonious trees are generated (trees not shown). Two interchange the earlier divergence of the arctic species with the antarctic strains, while one shows an unresolved trichotomy between the three cold-water strains analysed. Parsimony analyses can fail to find the shortest tree if there are too few phylogenetically informative sites relative to the number of taxa included (Stewart 1993). Therefore the only parsimony tree that can resolve the branching order is one with only a single strain representing the taxon *Phaeocystis antarctica* (Fig. 5b). The branching order is identical to that found in the distance analysis.

The rate of evolution of the warm- and cold-water taxa relative to *E. huxleyi* was calculated with the relative rate test using the number of base substitutions in Table 4. The two cold-water species, *Ph. antarctica* and *Ph. pouchetii* are evolving slightly slower (.7 and .5 times respectively) than *Ph. globosa* relative to *E. huxleyi*, but this rate is not significant. If rates are relatively constant in any two lineages, then the ratio of the variance in the number of substitutions to the mean (R) is 1 (Ochman & Wilson 1987). Our calculated R value is .2, which indicates that the rate of evolution in both lineages is constant.

Morphological and physiological analysis

Previously published morphological and physiological features of *Phaeocystis* cells undoubtedly conflict because of taxonomic confusion surrounding the identity of the colony-forming stage of *Phaeocystis*. We have critically reviewed published observations on *Phaeocystis* spp. and amassed a summary of features, which reflect our interpretation of the taxa.

Features of colony morphology that can be used to differentiate the three species as circumscribed in this study are presented in Table 5. There is an obvious identity in the morphological features of *Ph. globosa* and *Ph. antarctica*, but the two taxa exhibit distinctly different temperature tolerances and growth optima (Table 5, Fig. 6). At present, their geographic separation represents the only reliable feature upon which to assign a specific name, although differences in pigment spectra and DNA content (Vaulot *et al.* 1994) and in the ultrastructure of the colonial and flagellated stages (Table 6 and Chrétiennot-Dinet, personal communication) support the genetic separation of *Ph. antarctica* from *Ph. globosa*. In contrast, *Ph. pouchetii* has more morphological features that identify its colonial stage. It is smaller and the cells are in groups of four situated primarily in the curves of the lobes of more delicate colonies.

Previously, only observations on the flagellated stage of *Ph. scrobiculata* and *Ph. pouchetii* (including both *Ph. pouchetii* and *globosa*) have been compared (Davidson & Marchant 1992). We present a summary of the morphological features of the flagellated stages of *Ph. globosa*, *Ph. scrobiculata*, *Ph. pouchetii*, and *Ph. antarctica* based on published and unpublished observations (Table 6). Features assignable to *Ph. glo-*

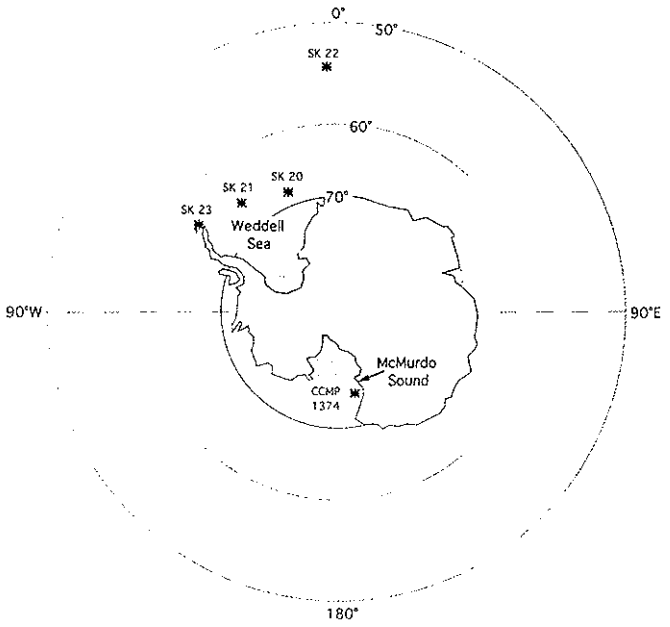

Fig. 4. Location of the strains of *Phaeocystis antarctica* analysed in this study.

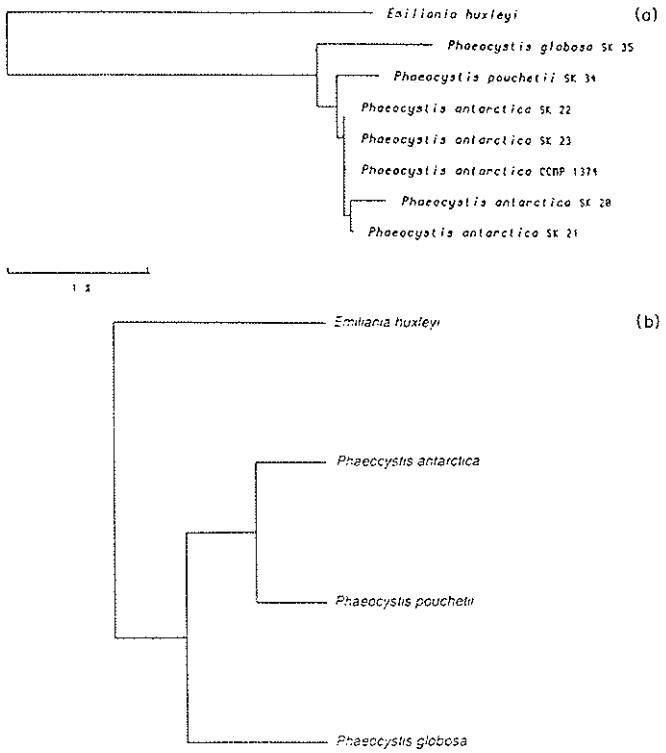

Fig. 5. Separation of *Phaeocystis* spp. using distance matrix (a) and parsimony methods (b). The distance corresponding to 1 change per 100 nucleotide positions is placed below the distance tree. It is reflected in the horizontal separation of taxa in the tree. The parsimony tree is the most parsimonious one found using the exhaustive branch swapping algorithm (PAUP), tree length 105, CI = 1.0, RI = 1.0.

bosa in our table were based on published descriptions of *Ph. pouchetii* and we have used the expected biogeographic distribution of *Ph. globosa* as a deciding factor in determining the specific epithet examined in these studies Although some features remain to be investigated, the size of the cells, the length of the flagella and the structure of the threads, all of which can be seen in field samples, separate the four taxa. Differences between flagellated stages of *Ph. antarctica* and *Ph. pouchetii* are very slight, but the geographic separation of the taxa is substantial. These ultrastructural differences are supported by our rDNA analysis.

Physiological observations on *Phaeocystis* sp. undoubtedly represent a major part of the published literature on this genus. We have replotted published data on the maximum specific growth rates of *Phaeocystis* spp. in those cases where we were certain of the identity of the species investigated (Fig. 6) and calculated the doublings per day. Although each of the three species illustrated has a different optimum growth rate, all three exhibit the same doublings per day (Fig. 6). Our relative rate test substantiates that the warm- and cold-water species are evolving at the same rate, a reflection of their similar generation times.

DISCUSSION

Phylogeny of the Prymnesiophyta

The phylogenetic relationships of the Prymnesiophyta have been the subject of several morphological and cladistic investigations (Cavalier-Smith 1986, 1994; Andersen 1991). Although it was removed from the Class Chrysophyceae by Christensen (1962), most recent workers still believe that the group is closely related to the Chromophyta/Oomycota (Cavalier-Smith 1986, 1994; Andersen 1991). Sequence data from the large (lsu) (Perasso *et al.* 1989) and small (ssu) subunits of the ribosomal RNA (rRNA) molecules (this study, Bhattacharya *et al.* 1992) have been used to provide an independent

Table 4. Per cent similarity (× 100) between small subunit ribosomal RNA sequences of *Emiliania huxleyi* and *Phaeocystis* spp. (upper triangle) and absolute number of nucleotide differences between these sequences excluding gaps and ambiguous nucleotides (lower triangle)

		Similarity to							
Organism		*E. hux*	*CCMP 1374*	*SK 20*	*SK 23*	*SK 22*	*SK 21*	*SK 35*	*SK 34*
E. huxleyi			0,950	0,949	0,951	0,951	0,951	0,945	0,948
P. antarctica	CCMP 1374	77		0,997	0,999	0,999	1,000	0,989	0,996
P. antarctica	SK 20	81	4		0,997	0,997	0,998	0,988	0,994
P. antarctica	SK 23	78	1	5		0,999	0,999	0,990	0,996
P. antarctica	SK 22	77	1	5	2		0,999	0,990	0,996
P. antarctica	SK 21	77	0	4	1	1		0,990	0,996
P. globosa	SK 35	88	17	22	18	18	18		0,987
P. pouchetii	SK 34	82	6	10	7	7	6	22	

Table 5. Colony morphology and temperature tolerance of *Phaeocystis globosa*, *Ph. antarctica* and *Ph. pouchetii*, after Jahnke & Baumann (1987) and Baumann *et al.* (1994b)

Criteria	*Ph. globosa*	*Ph. antarctica*[1]	*Ph. pouchetii*
Colony morphology			
Maximum size	*c.* 8–9 mm	At least 9 mm?	1.5–2 mm
Shape	Spherical and numerous derived forms		Spherical up to a colony diameter of 0.1 mm, lobed above 0.3 mm
Cell distribution	Evenly along the periphery	Evenly along the periphery	Only in the curves of the lobes of larger colonies, and mostly regular: 4 cells form a square, cell free mucilage in between
Mucilage	Solid	Solid	Delicate
Physiology			
Growth range	4 to 22°C	−1.6[2] to 14°C	−2 to 12°C
Growth optimum	16°C	4.5°C	8°C
Temperature tolerance	−0.6[3] to 22°C	< −2 to 14°C	< −2 to 14°C

[1] Baumann *et al.* 1993b.
[2] No lower temperature tested so far.
[3] Cadée 1992.

assessment of the phylogenetic relationship of the Prymnesiophyta as have an analysis of the genes coding for the large and small subunits of Rubisco (Fujiwara *et al.* 1993). All indicate that the Prymnesiophyta are a distinct eukaryotic lineage that does not share a recent evolutionary history with the stramenopiles (heterokont flagellates). The Cryptophyta and Dinophyta are also separate lineages. In both our analysis and in that of Bhattacharya *et al.* (1992) the dinoflagellates and their heterotrophic relatives emerge between the Prymnesiophyta and the stramenopiles. The bootstrap analysis demonstrates the high degree of repeatability in the branching order of the dinoflagellates and stramenopiles but the exact position of the prymnesiophytes is not strongly supported. A study of tree decay indicates that only two steps are needed to change the position of the prymnesiophytes. This further supports the hypothesis that branching orders are difficult to resolve during this period of rapid radiation in the eukaryotic lineage, despite using a closely related eukaryote, *Acanthamoeba*, as outgroup. It is clear, however, that the prymnesiophytes are not a sister taxon to the stramenopiles and perhaps the idea of the kingdom Chromista should be redefined.

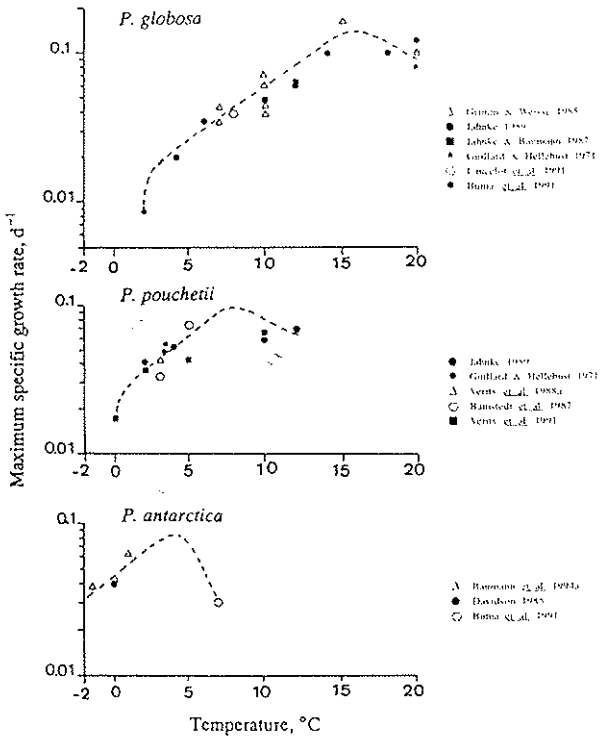

Fig. 6. Summary of maximum specific growth rates of three species of *Phaeocystis*, taken from Baumann *et al.* (1994b).

Phylogeny and biogeographic distribution of *Phaeocystis*

Our analysis of the smaller data set using *E. huxleyi* as outgroup to examine the relationships among the *Phaeocystis* strains substantiates the separation of the colony-forming *Phaeocystis* strains into three separate species following the interpretation of Moestrup & Larsen (1992) and Baumann *et al.* (1994a, 1994b). Our preliminary results, including four other temperate/tropical strains, indicate that *Phaeocystis* originated as a warm-water genus; the cold-water forms evolved more recently. *Ph. antarctica* retained the morphology of the warm-water ancestor, while *Ph. pouchetii*'s morphology diverged. Since their separation from their last common ancestor, both the cold-water and the warm-water species have been evolving at the same rate, indicated by their nearly identical generation times.

Morphological separation can be achieved using a combination of colony morphology and features of the flagellated stages. *Phaeocystis globosa* and *Ph. antarctica* are difficult to separate using colony morphology alone and their distribution may overlap in the Southern Hemisphere. Although features of the flagellated stages of *Ph. antarctica* and *Ph. pouchetii* are nearly identical, their colonial stages are not, and their distribution does not overlap. Clearly, more features are needed to discriminate between stages of the life cycle at the species level.

One of the most interesting topics to emerge from the use of molecular data in assessing ecological/taxonomic problems is that these types of data can be used not only to infer the evolutionary history within a group of organisms but also to test theories of biogeographical distribution of taxa through

Table 6. Features of the motile cell of *Phaeocystis* commonly assumed to be species specific (from Baumann *et al.* 1994b)

	Ph. globosa	*Ph. scrobiculata*	*Ph. pouchetii*	*Ph. antarctica*[5]
Size	3–8 μm[1]	8 μm[2]	Diam. *c.* 5 μm[4]	3–8 μm[5]
Threads[1,2,3]	Pentagonal figure (length up to 20 μm, diam. 0.05 μm)[1]	Nine ray figure (four pairs plus one) (length can exceed 50 μm, diam. 0.1 μm)[2]	Pentagonal figure[6]	Pentagonal figure[8] (length: 46 μm, diam. 0.1 μm)[5]
Scales[1,2,3]	Two different scale types: both show a pattern of radiating ridges, visible on both surfaces	Two different scale types: both show on the ventral side a pattern of ridges which radiate from a plain centre, the dorsal side is without visible patterning	Two different scale types present, but not characterized[6]	Two different scale types present, but not characterized[7]
	– larger scales are most circular flat plates with vertically upstanding rims, usually exactly 48 ridges, radiating from an approximately rectangular plain centre (0.18 × 0.19 μm)[1] (diam.: 0.25 μm)[3]	– larger scales are oval with a peripheral upstanding rim which shows no distinct pattern (0.60 × 0.45 μm)[2] (0.41 × 0.3 μm)[3]		
	– smaller scales are oval plates with strongly inflexed rims, 30 ridges, radiating from an oval plain centre (0.10 × 0.13 μm)[1] (diam.: 0.12 μm)[3]	– smaller scales are circular oval with a dorsal patternless rim (0.19–0.21 μm)[2] (0.1 μm)[3]		
Flagella[2]	*c.* 10–15 μm long[2]	23–30 μm long[2]	8 μm long[4]	*c.* 12 μm long[5]
Haptonema[2]	Non-coiling type, slightly shorter than that of *Ph. scrobiculata*[2]	Non-coiling type, 5 μm long[2]	3 μm long[4]	Not investigated

[1] Parke *et al.* (1971) (In their introduction the authors declare (p. 927) that referring to the requirement of the International Code of Botanical Nomenclature (Article 73, recommendation 73c) the species name *Ph. pouchetii* was used, although they admit 'With respect to material from British waters, there is some observational and experimental evidence in favour of treating *Ph. pouchetii* and *Ph. globosa* as different forms of a single taxon. . . This evidence is, however, by no means conclusive either with respect to the Northern Hemisphere or the world as a whole'. From their sampling site it must be concluded that the strains the authors isolated were *Ph. globosa*.
[2] Moestrup (1979) (features which were described here for *Ph. pouchetii* have been attributed to *Ph. globosa*).
[3] Hallegraeff (1983) (features which were described here for *Ph. pouchetii* have been attributed to *Ph. globosa*).
[4] Baumann & Jahnke (1986).
[5] Baumann, own unpublished observations.
[6] H.A. Thomsen, personal communication.
[7] Larsen & Moestrup (1989).
[8] Moestrup & Larsen (1992).

either vicariance or dispersal events (Bakker *et al.* 1992; van Oppen *et al.* 1993). Known divergence times can be correlated with such events. Although it has been demonstrated that a molecular clock does exist in a variety of genes and can be calibrated using known divergence times from the fossil record (see Ochman & Wilson 1987), caution has been exercised in extrapolating from one group of organisms to another where insufficient evidence occurs, i.e. the lack of a fossil record. This is certainly the case for these prymnesiophytes, which have no fossil record. However, divergence times for bacteria have been estimated using large-scale ecological events (Ochman & Wilson 1987) and, more recently, divergence times for endosymbiont bacteria estimated using fossil host phylogenies (see review in Harvey & May 1993). In all studies where a molecular clock has been calibrated for the rRNA molecule, a 1% difference in base composition in the rRNA gene equates to a 50–60 my divergence in animals (Ochman & Wilson 1987; Wilson *et al.* 1987), to a 25–50 my divergence in prokaryotes (Ochman & Wilson 1987; Wilson *et al.* 1987; Moran *et al.* 1993), and to a 25 my divergence in higher plants (Wilson *et al.* 1987). Such estimates provide a speculative starting-point at which the upper limits of the divergence of the *Phaeocystis* spp. in this study can be estimated. *Phaeocystis globosa* differs from the two cold-water species by 17–22 bases or, using the Wilson estimate, a separation from their last common ancestor by no more than 50 my.

Our rRNA analysis suggests that the direction of change in *Phaeocystis* is from warm to cold water. If the time divergence estimate of 25 to 50 ma (million years ago) is correct, then the ancestors of modern *Phaeocystis* spp. were probably warm-water cosmopolitan species, occurring in all ocean basins of the Eocene. This interpretation is supported by the Eocene thermal maximum 55–50 ma when mean annual ocean temperatures were 30°C (Crowley & North 1991). This is the warmest time during the Cenozoic and a period when sea levels were at their highest (Crowley & North 1991), oceans more temperate and more mixed (Johnson 1990), and floras and faunas more cosmopolitan (Baldauf & Barron 1990) with estimates of poleward intrusions of tropical taxa as far as between 45 and 78°N and S (Crowley & North 1991).

Since then there has been an increased global cooling that has enhanced latitudinal temperature differences (Baldauf & Barron 1990). These extraordinarily rapid and extreme climatic changes have occurred in fluctuating stages (Johnson 1990). During colder periods a partitioning of surface waters occurs, which effectively isolates water masses and in turn increases floral/fauna provincialism (Baldauf & Barron 1990). One particularly abrupt and dramatic cooling event at 38–40

my has been correlated with increased changes in land and sea distributions and variation in atmospheric CO_2 and may have contributed to a subsequent major faunal overturn when warmth-loving species were replaced by cold-tolerant ones (Crowley & North 1991; Frakes *et al.* 1992). The beginning of a more vigorous, colder, deep-water circulation is also associated with this climatic event (Crowley & North 1991). A second major ice volume increase and concomitant sea-level drop is at 12–14 ma when there is an abrupt increase in the $\sigma^{18}O$ (Frakes *et al.* 1992). These two major cooling events could well be correlated (1) with the separation of the *Phaeocystis* cold-water forms from their warm-water ancestors and (2) with the divergence of the two polar *Phaeocystis* species from their common ancestor. It equates with the Wilson estimate of a 1% divergence for 25 my, as in the flowering plants.

Of the major late Cenozoic tectonic events that strongly influenced the formation of both polar oceans, two involved the opening of ocean gateways and the development of new ocean currents (Crowley & North 1991). First, the Arctic Basin was isolated from the rest of the world's oceans from 100–60 ma until 15–10 ma when the Svalbard-Greenland Sea opened (Briggs 1987; Lawver *et al.* 1990). Then, a shallow-water connection between the Arctic Ocean and the North Atlantic opened to a more deep-water connection. Second, the antarctic seas, consisting of the southernmost portions of the Atlantic, Pacific and Indian Oceans, were formed by 82 ma after the breakup of Gondwanaland (Johnson 1990). By the end of the Oligocene (30 ma) the Drake passage opened, and the circum-Antarctic circulation commenced, which drastically changed paleoceanographic regimes (Barker & Burrell 1977). This effectively isolated the floras and faunas present in the antarctic seas during cooler climatic periods and provides a vicariant mechanism by which the speciation events could have occurred.

Given the direction of change implied in our rRNA tree, the last common ancestor of both *Ph. antarctica* and *Ph. pouchetii* must have been present in both polar regions prior to their speciation. The second major cooling event at 12–14 my coincides with our separation of the two cold-water species from their last common ancestor as determined by rRNA analysis using a 1% divergence for 25 my. The commencement of the circum-Antarctic circulation, a vicariant event, would have already isolated the ancestors of *Ph. antarctica* in the southern oceans prior to this second major cooling event. Because of the long period in which the biota of the Arctic Basin was isolated, it seems more likely that the ancestors of *Ph. pouchetii* were introduced into the Arctic Ocean from the North Atlantic, a dispersal event. This interpretation is supported by an incongruence in area and taxa cladograms. In an area cladogram, the antarctic and arctic regions do not share a recent geological history, while in our taxa cladogram the antarctic and arctic species do. Such inconsistencies between area and taxa cladograms strengthen the hypothesis that a dispersal event accounts for the present-day distribution of *Ph. pouchetii* (Brooks 1990).

Thus, the tectonic events during the middle to late Cenozoic provide mechanisms for (1) the probable introduction of *Phaeocystis* into the Arctic Ocean from the North Atlantic and (2) its isolation in the antarctic seas. Both events could lead to speciation during cooler climate periods when these water masses were more effectively isolated from others. The presence of a cosmopolitan ancestor provides the necessary requirement in which the ancestral population can be fragmented (Platnick & Nelson 1978). We interpret the speciation of *Ph. antarctica* to be a vicariant event, resulting from the establishment of the circum-polar current, while that of *Ph. pouchetii* is a biotic dispersal event, resulting from the opening of the Svalbard–Greenland Sea. This interpretation of the historical biogeography of *Phaeocystis* is supported by the present-day distribution of the taxa, the phylogenetic history inferred from our rRNA data and the incongruence of our area and taxa cladograms.

We expect further species of *Phaeocystis* to be erected based on analysis of their ssu rRNA genes (work in progress) and on their DNA content as revealed by flow cytometry (Vaulot *et al.* 1994); preliminary analysis including these species in our rRNA analysis suggests that our interpretation of the biogeographic history of the genus will not change. Other previously described species, such as *Phaeocystis brucei* Manguin from antarctic waters, may in fact be valid and simply disregarded because of the oversimplification of the genus. Similarly, many records of *Phaeocystis* may not belong to *Phaeocystis* because colonies with a *Phaeocystis*-like colonial stage release flagellated stages that can be assigned to other genera (Marchant & Thomsen 1994). The perplexing problem will be whether sufficient morphological features can be identified as specific markers to aid ecologists in their routine identification in areas where different genotypes are known to overlap in their distribution.

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