Eur. J. Phycol. (2015), 1–16

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Phaeocystis rex sp. nov. (Phaeocystales, Prymnesiophyceae): a new solitary species that produces a multilayered scale cell covering

ROBERT A. ANDERSEN[1], J. CRAIG BAILEY[2], JOHAN DECELLE[3,4] AND IAN PROBERT[5,6]

[1]*Friday Harbor Laboratories, University of Washington, 620 University Road, Friday Harbor, WA 98250 USA*
[2]*Center for Marine Science and Department of Biology and Marine Biology, UNC-Wilmington, 5600 MK Moss Lane, Wilmington, NC 28409 USA*
[3]*EPEP, Sorbonne Universités, UPMC Univ Paris 06, UMR 7144, Station Biologique de Roscoff, 29680 Roscoff, France*
[4]*CNRS, UMR 7144, Station Biologique de Roscoff, 29680 Roscoff, France*
[5]*Roscoff Culture Collection, Sorbonne Universités, UPMC Univ Paris 06, FR2424, Station Biologique de Roscoff, 29680 Roscoff, France*
[6]*CNRS, FR2424, Station Biologique de Roscoff, 29680 Roscoff, France*

(Received 22 September 2014; revised 24 November 2014; accepted 16 December 2014)

A morphologically distinct marine species, *Phaeocystis rex* sp. nov., was described on the basis of light microscopy, transmission electron microscopy and DNA sequence comparisons. Non-motile cells were solitary (non-colonial), 6–10 μm in diameter and 8–15 μm long, and possessed chloroplasts with distinctive finger-like lobes. TEM observations demonstrated the presence of two short flagella and a very short haptonema that arose from an invagination of the protoplast. Non-motile cells were surrounded by one to several dense layers composed of scales, presumably unmineralized, and an amorphous material. Phylogenetic analyses based upon combined partial nucleotide sequences for five nuclear- or plastid-encoded genes (18S rRNA, 28S rRNA, 16S rRNA, *psb*A and *rbc*L) from cultured strains and from uncharacterized acantharian symbionts confirmed that *P. rex* was a distinct species. These analyses implied that *P. rex* occupies an intermediate evolutionary position between solitary and colonial *Phaeocystis* species.

Key words: algae, *Phaeocystis rex*, Phaeocystales, Prymnesiophyceae, organic scales, systematics, ultrastructure

INTRODUCTION

The haptophyte microalga *Phaeocystis* Lagerheim is one of the most extensively studied genera of marine phytoplankton. Free-living *Phaeocystis* are ubiquitous from poles to tropics and from coastal to open ocean waters (Schoemann *et al.*, 2005). Colonial species of *Phaeocystis* rank among a handful of keystone eukaryotic phytoplankton taxa that shape the structure and functioning of marine ecosystems (Verity & Smetacek, 1996). *Phaeocystis* is not only a major contributor to global carbon cycling and export (Arrigo *et al.*, 1999; DiTullio *et al.*, 2000), but also impacts sulphur cycling by producing substantial amounts of dimethylsulphoniopropionate (DMSP) and its volatile catabolite dimethylsulphide (DMS), a climatically active trace gas emitted from the ocean (Stefels *et al.*, 2007). In coastal areas, blooms of *Phaeocystis*, which can contribute > 90% of total phytoplankton abundance, may be detrimental to the growth and reproduction of marine life, and strongly impact human activities such as fisheries, aquaculture and tourism (He *et al.*, 1999; Chen *et al.*, 2002; Schoemann *et al.*, 2005; Blauw *et al.*, 2010; Doan *et al.*, 2010). In oceanic regions from poles to tropics, several *Phaeocystis* species have recently been reported to form endosymbiotic associations with certain species of Acantharia, a widespread and abundant lineage of radiolarians (Decelle *et al.*, 2012).

The first report of *Phaeocystis* was by Pouchet (1892) who found large round gelatinous colonies in Norwegian marine waters (Lofoten Islands, Varanger) during summer 1882. Eight years later, he found the same alga in the North Atlantic Ocean (near Torshavn, Faroe Islands), and formally described it as *Tetraspora poucheti* Hariot (Pouchet, 1892) due to perceived similarity to another colony-forming alga, *Tetraspora giraudyi* Derbès & Solier (1851). Lagerheim (1893) found a similar alga in 1884 from the Koster Islands, Sweden, and argued that these two marine brownish-coloured algae should not be placed in the freshwater green algal genus *Tetraspora* Link ex Desvaux. Therefore, he proposed the new generic name *Phaeocystis* Lagerheim

Correspondence to: Robert A. Andersen. E-mail: raa48@uw.edu.

ISSN 0967-0262 (print)/ISSN 1469-4433 (online)/15/000001-16 © 2015 British Phycological Society
http://dx.doi.org/10.1080/09670262.2015.1024287

and designated *Phaeocystis pouchetii* (Hariot) Lagerheim as the type species. Hariot (1893) listed *Phaeocystis pouchetii* but did not describe it; he referred to Lagerheim's 1893 paper. Lagerheim (1893) also suggested that *Tetraspora fuscescens* Braun *ex* Kützing, a freshwater colonial alga, belonged to this small group of colonial brown-coloured algae. In 1895, DeToni recombined *T. giraudyi* and *T. fuscescens* as *Phaeocystis giraudyi* (Derbès & Solier) DeToni and *Phaeocystis fuscescens* (A. Braun *ex* Kützing) DeToni, respectively. Lagerheim (1896) made a more thorough study of *Phaeocystis pouchetii* and argued that *P. giraudyi* and *P. fuscescens* should be doubtful members of *Phaeocystis* until further detailed investigations were completed. In confirmation of Lagerheim's doubt, those two taxa are now known to be heterokont algae, not haptophytes. *Chrysoreinardia giraudyi* Billard (in Hoffmann *et al.*, 2000) is a member of the Pelagophyceae and *Tetrasporopsis fuscescens* (A. Braun *ex* Kützing) Lemmermann *ex* Schmidle belongs in Clade I of the heterokont algae (Entwisle & Andersen, 1990; Bailey *et al.*, 1998; Yang *et al.*, 2012).

A few new species of *Phaeocystis* were described during the next 25 years, beginning with *Phaeocystis globosa* Scherffel, which was collected in the North Sea, from Helgoland, Germany (Scherffel, 1899). Scherffel gave an exceptionally detailed description that included an illustration of the haptonema ('third flagellum') (Scherffel, 1900, plate 1, fig. 69). Soon after, Karsten (1905) described *P. antarctica* Karsten from the Southern Ocean. Although the alga was common and abundant, Karsten's description of individual cells and colonies was brief, and he was uncertain whether there was one deeply lobed chloroplast or two separate chloroplasts. He was convinced, however, that the cells were embedded in mucilage and specifically mentioned the resemblance of *P. antarctica* and *P. globosa*.

Phaeocystis sphaeroides Büttner and *P. amoeboidea* Büttner were then described from cultures established from the harbour of Kiel, Germany (Büttner, 1911). The two species were distinguished from each other based on the sizes of their swimming cells as well as the amoeboid nature of aflagellate cells. Büttner's descriptions were not as thorough as those of Scherffel, but he described brown gelatinous colonies visible to the naked eye, a single yellow-brown chloroplast per cell, and swimming cells with two equal flagella. Although Büttner did not report a 'third flagellum' (i.e. haptonema), he clearly stated that there was only a single chloroplast per cell except immediately before cell division when the chloroplast divides prior to cytokinesis. The gelatinous colony, the yellow-brown colour and the two equal flagella are consistent with *Phaeocytis*, but, as Medlin & Zingone (2007) point out, the single chloroplast per cell and metabolic nature of the cells casts some doubt on these algae belonging in *Phaeocystis*. Unfortunately, Büttner (1911) made no attempt to compare or distinguish among the colonial stages of his two new taxa or between them and earlier described *Phaeocystis* species.

Mangin (1922) described *Phaeocystis brucei* Mangin from Antarctica. The cells are 5–6 μm in diameter, each cell possesses two golden chloroplasts, and the colonies are 1–2 mm in diameter. Mangin suggested his new alga was different from *P. globosa* and *P. antarctica* because of the distinct form of the colonies: *P. brucei* colonies are characterized by the presence of separate clusters of cells that are interconnected by mucilaginous 'trails'.

Moestrup (1979) described a sixth species, *P. scrobiculata* Moestrup, from New Zealand coastal waters. Colonies are not known for this species and cells are *c.* 8 μm in size and are covered by a periplast consisting of two scale types. Cells also produce star-like arrays of filaments. Scales and the enigmatic star-like filaments were first discovered in *Phaeocystis* by Parke *et al.* (1971), and these scale and star-like features led Moestrup to conclude that *P. scrobiculata* belonged to *Phaeocystis* although it was a solitary (non-colonial) organism. Zingone *et al.* (1999) described two more solitary species, *P. cordata* Zingone & Chrétiennot-Dinet and *P. jahnii* Zingone from the Mediterranean Sea. Both species have small cells (3–4 μm and 3–5 μm, respectively) and bear flagella that are slightly unequal in length. In culture, *P. jahnii* also sometimes forms loose and small aggregations of immobile (non-flagellate) cells embedded in a gelatinous matrix, but these are different from classical *Phaeocystis* colonies in lacking a definite shape and a regular arrangement of cells as well as a visible external envelope (Zingone *et al.*, 1999).

Six species have been examined using electron microscopy and/or DNA sequence analysis: *Phaeocystis pouchetii, P. antarctica, P. cordata, P. globosa, P. jahnii* and *P. scrobiculata*; the remaining three species (*P. amoeboidea, P. brucei, P. sphaeroides*) have not received modern study because they are unavailable in culture.

In this paper, we describe a new solitary species of *Phaeocystis* collected from the Arabian Sea. Unlike any previously described *Phaeocystis* species, the Arabian Sea isolate has chloroplasts with two elongate lobes and an unusual extracellular cell covering comprised of scales and other material. Electron microscope observations and five-gene phylogenetic analyses further demonstrate that this alga is a new *Phaeocystis* species.

MATERIALS AND METHODS

Culture origin and culture conditions

The alga was isolated from a sample collected by W. Balch on 12 November 1995 from the Arabian Sea (14.4490N,

64.9997E) using a Niskin bottle at 75 m depth (2% of surface light level). The first attempt produced a unialgal culture but a labyrinthulid-like parasite was present. A single-cell isolation (culture A9125) was established on 25 September 1998 by selecting a single swimming cell using a micropipette. The strain was deposited as CCMP2000 in the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (now the NCMA). Cells were grown in L1 or f/2 seawater culture medium enriched with soil extract (Guillard, 1975; Guillard & Hargraves, 1993). Cultures were maintained at *c.* 24ºC under a 13:11 h light:dark cycle with approximately 60–100 $\mu M\ s^{-1}\ m^{-2}$ of cool white fluorescent illumination.

Brightfield and transmission electron microscopy

Light microscope (LM) observations were made using a Zeiss Axio Imager (Z1) equipped with an Axio Cam HRm digital camera and Axio Vision 4.5 software (Carl Zeiss, Göttingen, Germany). For transmission electron microscopy (TEM), cells were gently pelleted, the supernatant was removed, and the cells were resuspended in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) containing 0.6 M sucrose. After 30 s, an equal volume of 2% osmium tetroxide in 0.01 M phosphate buffer with sucrose was added. After 50 min, cells were centrifuged, the fixative was discarded and the pellet was resuspended in 0.1 M phosphate buffer without sucrose. Cells were centrifuged again, the supernatant was discarded and the pellet was resuspended in 0.1 M phosphate buffer, and the solution was filtered onto a 13 mm Millipore filter (Merck-Millipore Corp., Billerica, Massachusetts, USA). The filter and cells were placed in a small plastic dish and enrobed with 1% low gelling point agar at 35ºC (Sigma-Aldrich, St. Louis, Missouri, USA). When the agar gelled, 0.5% uranyl acetate in de-ionized water was added, the dish was covered and then refrigerated at 4ºC overnight. The uranyl acetate was decanted and the agar-enrobed filter was dehydrated in an ethanol series (10%, 30%, 50%, 70%, 95%, 100%). Following two changes of 100% ethanol, the filter and cells were further dehydrated with two changes of 100% propylene oxide. Cells were infiltrated and embedded in Spurr's epoxy resin. Thin sections were examined on a Zeiss 902A transmission microscope (Carl Zeiss).

The culture strains for which new sequences were obtained in this study are listed in Table 1. Cultures were harvested in exponential growth phase and concentrated by centrifugation. Total nucleic acids were extracted using the Nucleospin RNA II kit (Macherey-Nagel GmbH, Düren, Germany) and quantified using a Nanodrop ND-1000 spectrophotometer (Labtech International Ltd, Uckfield, UK). In this study, we targeted the nuclear 18S and 28S rRNA, the plastid-encoded 16S rRNA, photosystem II D1 protein (*psb*A) and form-I ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L) genes using different primer sets (Decelle *et al.*, 2012). Amplifications were performed with Phusion high-fidelity DNA polymerase (Finnzymes; Thermo Fisher Scientific/Finnzymes Oy, Vantaa, Finland) in a 25 µL reaction volume, using the following PCR parameters: 30 s at 98°C; followed by 35 cycles of 10 s denaturation at 98°C, 30 s annealing at 50°C for the 18S, 28S and *psb*A genes and at 55°C for 16S and *rbc*L genes, and 30 s extension at 72°C; with a final elongation step of 10 min at 72°C. PCR products were then purified by EXOSAP-IT (GE

Table 1. *Phaeocystis* strains, geographic origin, and GenBank accession numbers for five nuclear and chloroplast genes.

Strain/ID number	*Phaeocystis* species	Isolation source	Geographic origin	plastid 16S rRNA	18S rRNA	28S rRNA	*psb*A	*rbc*L
P360	*Phaeocystis pouchetii*	Culture	Norwegian Sea	–	AF182114	–	–	–
P361	*Phaeocystis pouchetii*	Culture	Norwegian Sea	–	AJ278036	–	–	–
SK34	*Phaeocystis pouchetii*	Culture	Greenland Sea	–	X77475	–	–	–
CCMP1374 (RCC4023)	*Phaeocystis antarctica*	Culture	Antarctica (McMurdo Sound)	**KP144217**	**KP144246**	**KP144256**	**KP144270**	**KP144261**
CCMP1871 (RCC4024)	*Phaeocystis antarctica*	Culture	Antarctica (Arthur Harbour)	**KP144233**	**KP144253**	**KP144258**	**KP144272**	**KP144262**
RCC1383 (CCMP3104)	*Phaeocystis cordata*	Culture	Mediterranean Sea	EF051764	JX660992	JX660941	JX660950	JX660967
RCC1384 (CCMP2496)	*Phaeocystis jahnii*	Culture	Mediterranean Sea	**KP144232**	AF163148	**KP144257**	**KP144271**	**KP144263**
CCMP2000 (RCC4025)	***Phaeocystis rex***	**Culture**	**Arabian Sea (Indian Ocean)**	**KP144218**	**KP144247**	**KP144259**	**KP144273**	**KP144264**
K1321	*Phaeocystis globosa*	Culture	West Atlantic Ocean	–	JX660986	JX660935	JX660942	JX660982
K1398	*Phaeocystis globosa*	Culture	East China Sea	–	JX660987	JX660936	JX660943	JX660983
PCC540 (RCC3539)	*Phaeocystis globosa*	Culture	North East Atlantic Ocean	**KP144229**	AF182115	JX660930	JX660944	JX660978
PCC575	*Phaeocystis globosa*	Culture	North East Atlantic Ocean	**KP144231**	**KP144252**	JX660933	JX660946	JX660981
PCC64 (RCC3538)	*Phaeocystis globosa*	Culture	English Channel	–	JX660994	JX660934	JX660948	JX660977
RCC1719	*Phaeocystis globosa*	Culture	English Channel	–	–	JX660921	JX660951	JX660968
RCC739	*Phaeocystis globosa*	Culture	Pacific Ocean	**KP144234**	–	JX660924	JX660956	JX660971
RCC678	*Phaeocystis* sp. (undescribed)	Culture	North Sea	–	**KP144254**	JX660939	JX660955	**KP144266**

(*continued*)

Table 1. Continued.

Strain/ID number	*Phaeocystis* species	Isolation source	Geographic origin	plastid 16S rRNA	18S rRNA	28S rRNA	*psb*A	*rbc*L
PCC559 (RCC3541)	*Phaeocystis* sp. (undescribed)	Culture	North East Atlantic Ocean	**KP144230**	JX660995	JX660931	JX660945	JX660979
RCC876	*Phaeocystis* sp. (undescribed)	Culture	South East Pacific Ocean	**KP144235**	_	JX660928	JX660957	JX660975
RCC908	*Phaeocystis* sp. (undescribed)	Culture	South East Pacific Ocean	**KP144236**	EU106761/JX660990	JX660919	JX660959	JX660964
RCC935	*Phaeocystis* sp. (undescribed)	Culture	South East Pacific Ocean	**KP144237**	EU106782	JX660920	JX660960	JX660965
RCC942	*Phaeocystis* sp. (undescribed)	Culture	South East Pacific Ocean	**KP144238**	_	_	**KP144278**	_
RCC993	*Phaeocystis* sp. (undescribed)	Culture	South East Pacific Ocean	_	EU106820	JX660922	JX660961	JX660969
RCC994	*Phaeocystis* sp. (undescribed)	Culture	South East Pacific Ocean	**KP144239**	**KP144255**	**KP144260**	**KP144279**	**KP144265**
Oki29	*Phaeocystis* sp. (undescribed)	Symbiont	East Pacific Ocean	**KP144219**	**KP144248**	JX660792	JX660832	**KP144267**
Oki32	*Phaeocystis* sp. (undescribed)	Symbiont	East Pacific Ocean	_	**KP144249**	JX660793	**KP144274**	JX660892
Oki35	*Phaeocystis* sp. (undescribed)	Symbiont	East Pacific Ocean	_	**KP144250**	JX660794	JX660833	_
Oki36	*Phaeocystis* sp. (undescribed)	Symbiont	East Pacific Ocean	**KP144220**	**KP144251**	_	JX660834	JX660893
Oki43	*Phaeocystis* sp. (undescribed)	Symbiont	East Pacific Ocean	**KP144221**	JX660740	_	JX660835	JX660894
Oki44	*Phaeocystis* sp. (undescribed)	Symbiont	East Pacific Ocean	**KP144222**	JX660741	JX660795	JX660836	JX660895
Oki46	*Phaeocystis* sp. (undescribed)	Symbiont	East Pacific Ocean	**KP144223**	JX660742	JX660796	JX660837	JX660896
Oki57	*Phaeocystis* sp. (undescribed)	Symbiont	East Pacific Ocean	**KP144224**	JX660747	JX660797	**KP144275**	JX660898
Oki65	*Phaeocystis* sp. (undescribed)	Symbiont	East Pacific Ocean	**KP144225**	JX660749	_	**KP144276**	JX660900
Oki66	*Phaeocystis* sp. (undescribed)	Symbiont	East Pacific Ocean	**KP144226**	_	JX660798	**KP144277**	_
Oki74	*Phaeocystis* sp. (undescribed)	Symbiont	East Pacific Ocean	**KP144227**	JX660752	JX660800	JX660842	_
Oki85	*Phaeocystis* sp. (undescribed)	Symbiont	East Pacific Ocean	**KP144228**	JX660754	JX660801	JX660843	JX660903
Oki87	*Phaeocystis* sp. (undescribed)	Symbiont	East Pacific Ocean	_	JX660755	JX660802	JX660844	JX660904
Ros1	*Phaeocystis* sp. (undescribed)	Symbiont	English Channel	**KP144240**	JX660757	JX660804	JX660846	**KP144268**
Ros2	*Phaeocystis* sp. (undescribed)	Symbiont	English Channel	**KP144241**	JX660758	JX660805	JX660847	**KP144269**
Vil46	*Phaeocystis* sp. (undescribed)	Symbiont	Mediterranean Sea	**KP144242**	JX660766	JX660807	JX660852	JX660910
Vil84	*Phaeocystis* sp. (undescribed)	Symbiont	Mediterranean Sea	**KP144243**	JX660771	JX660809	JX660859	JX660914
Vil86	*Phaeocystis* sp. (undescribed)	Symbiont	Mediterranean Sea	**KP144244**	JX660772	JX660811	JX660861	JX660916
Vil96	*Phaeocystis* sp. (undescribed)	Symbiont	Mediterranean Sea	**KP144245**	JX660774	JX660813	JX660863	JX660918
Outgroup								
RCC1343	*Imantonia rotunda*	Culture		**KP144214**	AJ246267	EU729457	EU851963	AB043696
RCC1348	*Isochrysis galbana*	Culture		JF489944	AJ246266	EU729474	AJ575574	AB043693
RCC2476	*Pleurochrysis carterae*	Culture		**KP144215**	AJ246263	EU819084	AY119757	D11140
RCC1434	*Prymnesium parvum*	Culture		**KP144216**	AJ246269	EU729443	AY119758	AB043698

Healthcare, Little Chalfont, UK) and bidirectionally sequenced on an ABI3130*xl* automated DNA sequencer (Life Technologies Corporation, Carlsbad, California, USA) using the ABI-PRISM Big Dye Terminator Cycle Sequencing Kit (Life Technologies Corporation) according to the manufacturer's specifications. Raw sequences were edited and assembled with Chromas Pro v.1.5 (Technelysium Pty Ltd, South Brisbane, Australia). GenBank accession numbers for the sequences are shown in Table 1.

Phylogenetic analyses

To determine the phylogenetic affiliation of CCMP2000, a dataset for each of the five loci (16S rRNA, 18S rRNA, 28S rRNA, *psb*A and *rbc*L) was constructed including partial gene sequences from culture strains and from uncultured *Phaeocystis* living in symbiotic association with Acantharia (Decelle *et al.*, 2012). Four taxa from other haptophyte orders were included in each dataset as outgroups: *Isochrysis galbana* M. Parke, *Pleurochrysis carterae* (Braarud & Fagerland) Christensen [= *Chrysotila carterae* (Braarud & Fagerland) Andersen, Kim, Tittley & Yoon], *Prymnesium parvum* N. Carter and *Imantonia rotunda* Reynolds (Table 1).

For each locus, sequences were independently aligned with MUSCLE implemented in Seaview v4 (Gouy *et al.*, 2010). The length of aligned sequences was 734 bp for 16S rRNA, 465 bp for 18S rRNA, 836 bp for 28S rRNA, 572 bp for *psb*A and 479 bp for *rbc*L. Sequence divergence between the five genes was estimated by the p-distance (uniform rates among sites) with MEGA version 5 (Tamura *et al.*, 2011). The single-locus datasets were then separately subjected to maximum likelihood (ML) analyses with 100 bootstrap replicates using RAxML (Stamatakis *et al.*, 2008). Visual checking of the topology of each tree showed congruence for the phylogenetic placement of highly supported clades. A concatenation of the five datasets into a single partition (48 taxa, 3087 bp length, including gaps) was therefore performed with the FASconCAT program (Kück & Meusemann, 2010). Because the sequences of symbiotic *Phaeocystis* were short, a separate dataset (28 taxa, 4824 bp length, including gaps) was constructed without these sequences to increase the number of nucleotide positions.

For both concatenated datasets, the general-time-reversible model with gamma distributed rates (GTR+G) was selected by jModelTest v2 (Darriba *et al.*, 2012) as the favoured model of sequence evolution under the Akaike and Bayesian Information Criteria (AIC and BIC, respectively). Maximum likelihood topologies for the two concatenated datasets were inferred using RAxML with GTRGAMMA (GTR+Γ) model, and support for nodes was assessed by performing 2000 bootstrap replicates. The Bayesian inference was performed using MrBayes v3.2.1 (Ronquist & Huelsenbeck, 2003). Markov chain Monte Carlo (MCMC) analyses were run for two independent sets of four chains with a chain length of 5 million generations for both datasets, starting from a random tree with a sample frequency for trees of 1 in every 1000 generations. The average standard deviation of the split frequencies was < 0.01 and the effective sample size for all parameters was higher than 200, as recommended by Drummond & Rambaut (2007). Twenty-five per cent of the total trees were discarded as burn-in, and the remaining trees were used to build a consensus tree and to calculate posterior probabilities (PP) for each node. The final tree was visualized with FigTree v1.3.1.

RESULTS

Taxonomic description

***Phaeocystis rex* Andersen, Bailey, Decelle & Probert sp. nov. (Figs 1–17)**

DIAGNOSIS: Vegetative cells 6–10 µm in diameter and 8–15 µm long; cells surrounded by a wall-like covering comprised of multiple layers of organic scales and amorphous material; posterior end of the cell covering often, but not always, thickened forming a conspicuous knob; two chloroplasts per cell; each chloroplast with two anteriorly projecting finger-like lobes and one pyrenoid; non-motile cells with two short flagella and a short haptonema; free-swimming motile cells with two flagella and a haptonema; cell division inside the cell covering, with subsequent escape of one naked daughter cell; multiple wall-like layers accumulating with repeated cell divisions; nucleotide sequences distinct (GenBank accession numbers KP144218, KP144247, KP144259, KP144,273, KP144264).

HOLOTYPE HERE DESIGNATED: NY02026433, plastic TEM block RAA-665, deposited in the New York Botanical Garden, New York, USA.

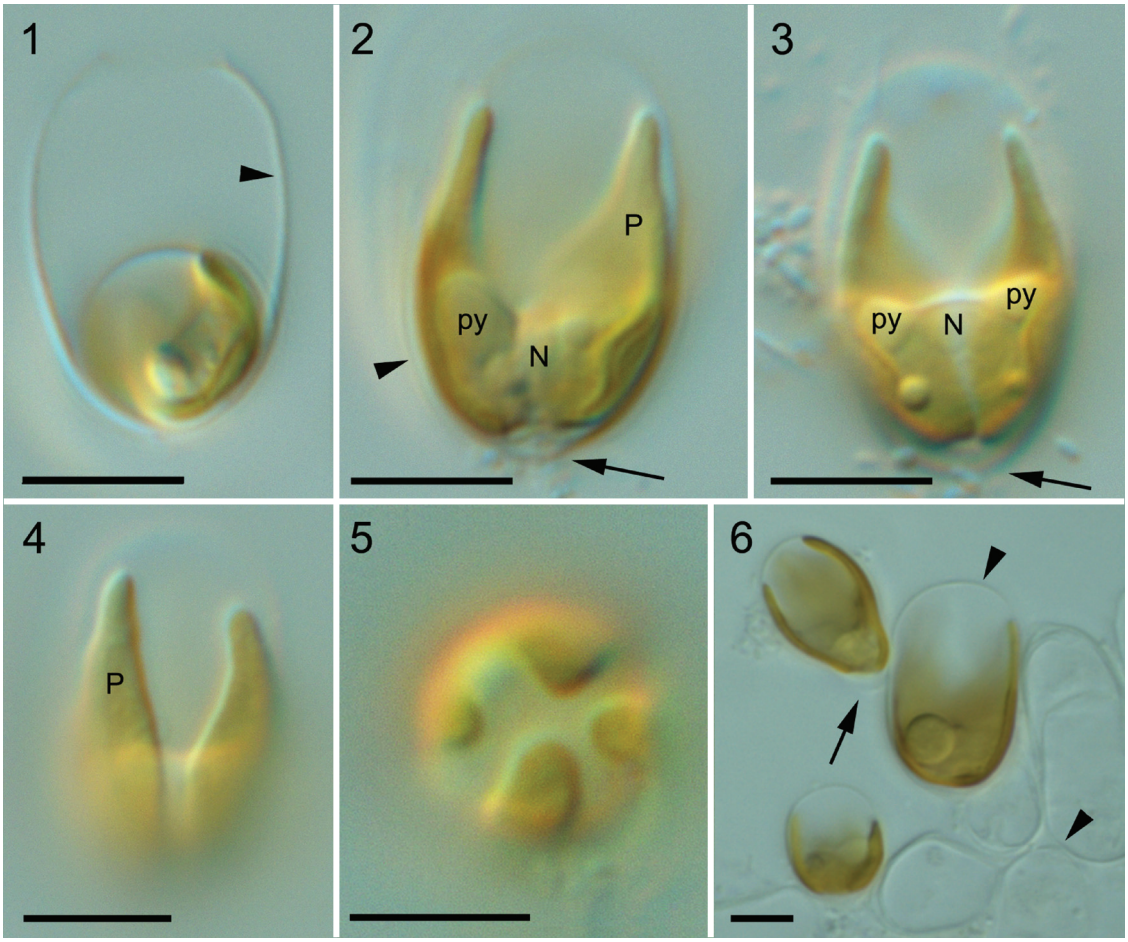
ISOTYPE HERE DESIGNATED: Cryopreserved culture strain CCMP2000 deposited in the Provasoli-Guillard National Center for Marine Algae and Microbiota, Bigelow Laboratory for Ocean Sciences, East Boothbay, Maine, USA.

TYPE LOCALITY: Arabian Sea (14.4490N, 64.9997E) at a depth of 75 m.

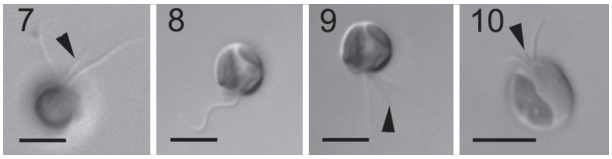
ETYMOLOGY: Latin, *rex* = king; refers to the crown-like appearance imparted by the four lobes of the two plastids.

Description: light microscopy

Cells were typically ellipsoid, 6–10 µm in diameter and 8–15 µm long, although round and oval cells were also observed immediately after cell division (Figs 1–6). A conspicuous thin, translucent cell covering surrounded the cell (arrowheads, Figs 1, 2, 6). Two chloroplasts occurred in each mature cell and were pressed together forming a cup-like base (Figs 2, 3). Each plastid typically had two long, tapering finger-like lobes that extended towards the (apparent) anterior end of the cell, although only two lobes were visible in most LM images taken at high magnification (Figs 2–4). When viewed from the tip of the cell, the four lobes were evident (Fig. 5). Each chloroplast had



Figs 1–6. Differential interference contrast light micrographs of *Phaeocystis rex* non-motile cells. **Fig. 1.** Newly formed non-motile cell with rounded protoplasm and oval wall-like covering (arrowhead). **Figs 2–4.** Mature non-motile cells, more-or-less elliptical in shape; cells with a central nucleus (N), two lobed chloroplasts (P) and a conspicuous pyrenoid (py) in each chloroplast. Arrow indicates thickening at one end of the wall-like covering. **Fig. 5.** Cell viewed from the tip showing the four chloroplast lobes. **Fig. 6.** Living and dead cells weakly attached. The wall-like covering (arrrowheads) and thickening at one end of the wall-like covering (arrow) are indicated. Scale bars = 5 µm.

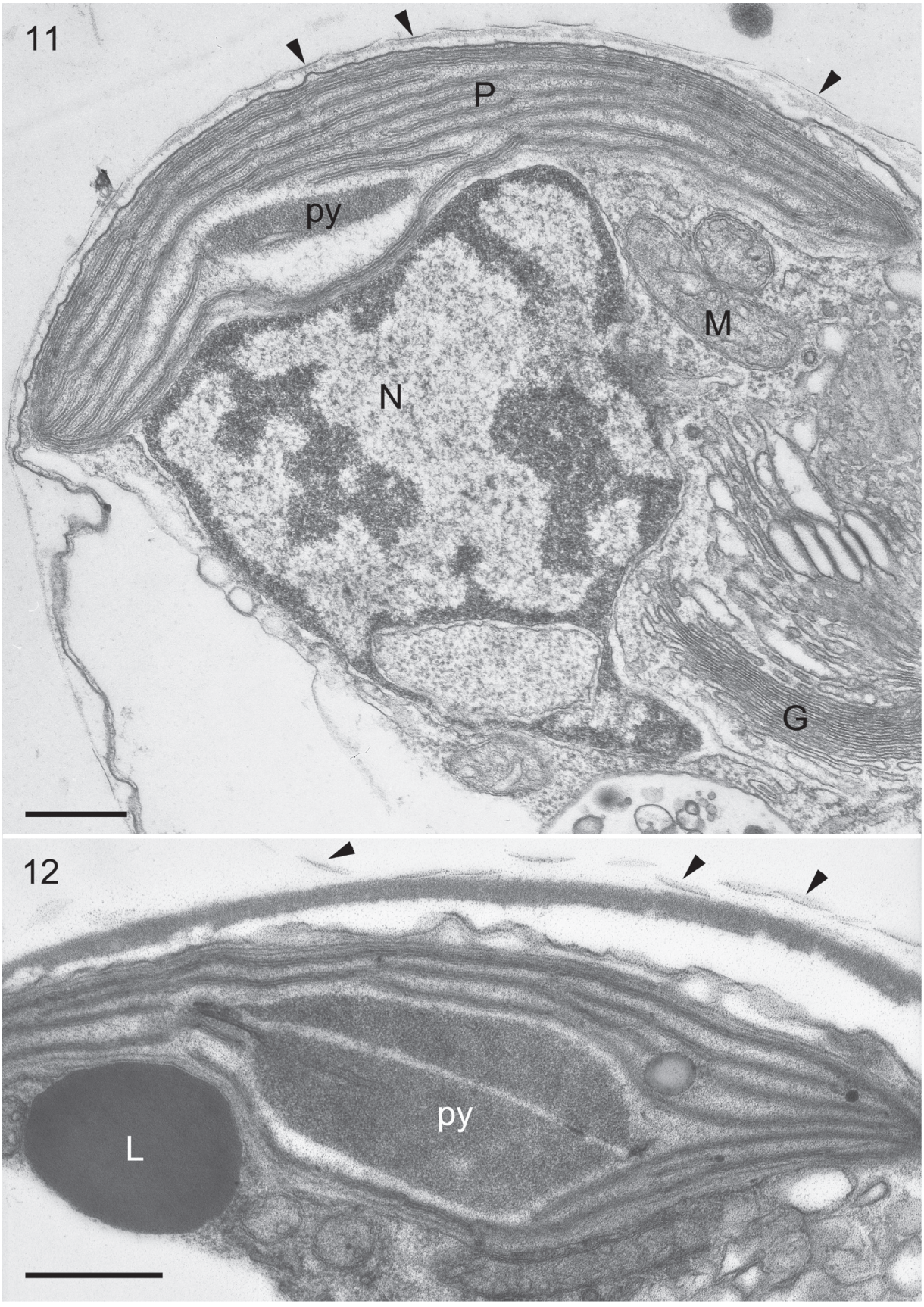


Figs 7–10. Differential interference contrast light micrographs of *Phaeocystis rex*. Motile cells showing one or two flagella and a haptonema (arrowhead). Scale bars = 5 µm.

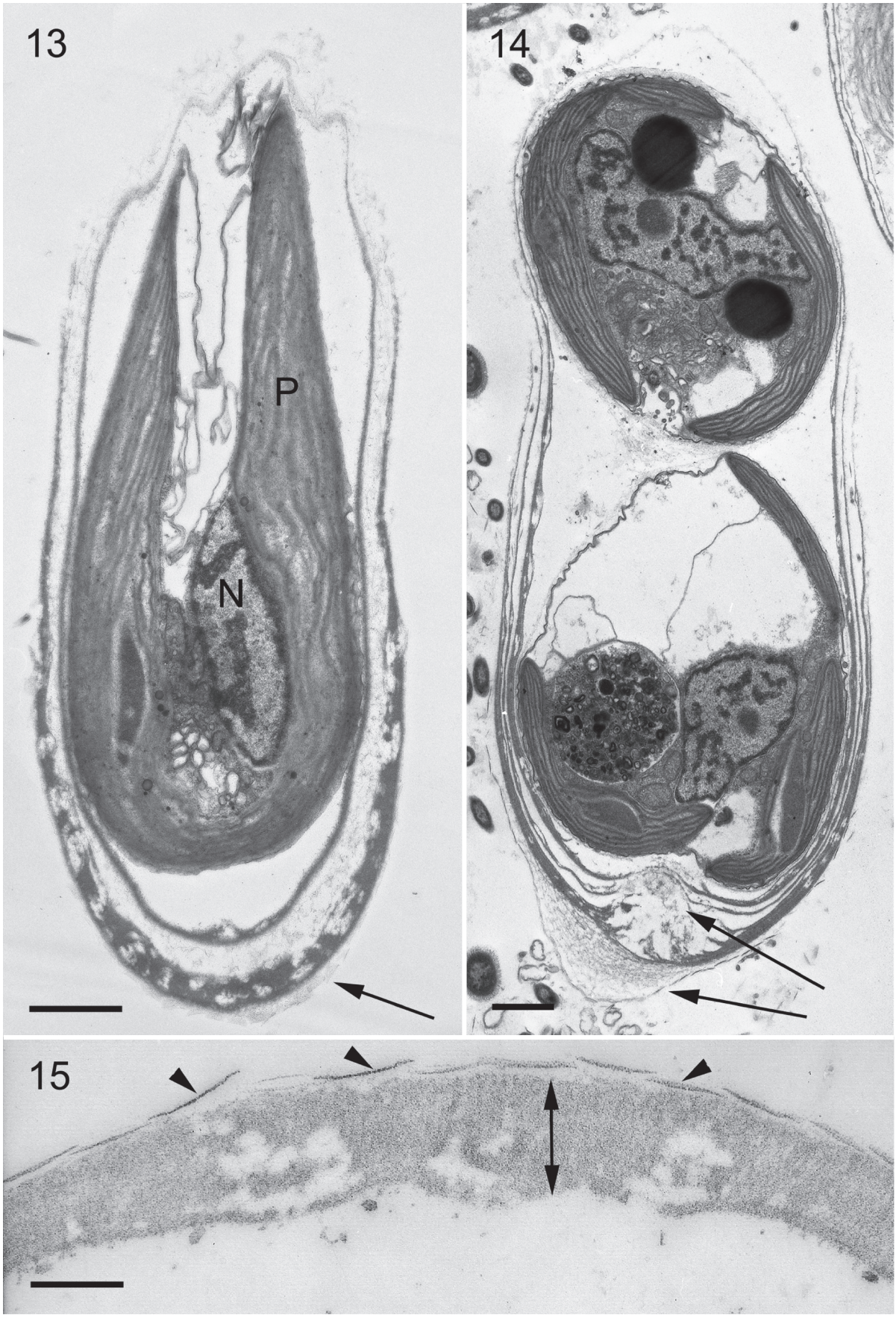
an immersed, bulging pyrenoid (Figs 2, 3). The cells were sometimes attached to older walls or debris (Fig. 6), and the attached region was often thickened (arrows, Figs 2, 3, 6). Plastid-bearing motile cells were spheroid, 5–7 µm in diameter, and had two emergent flagella as well as a short, variable-length haptonema (Figs 7–10).

Electron microscopy

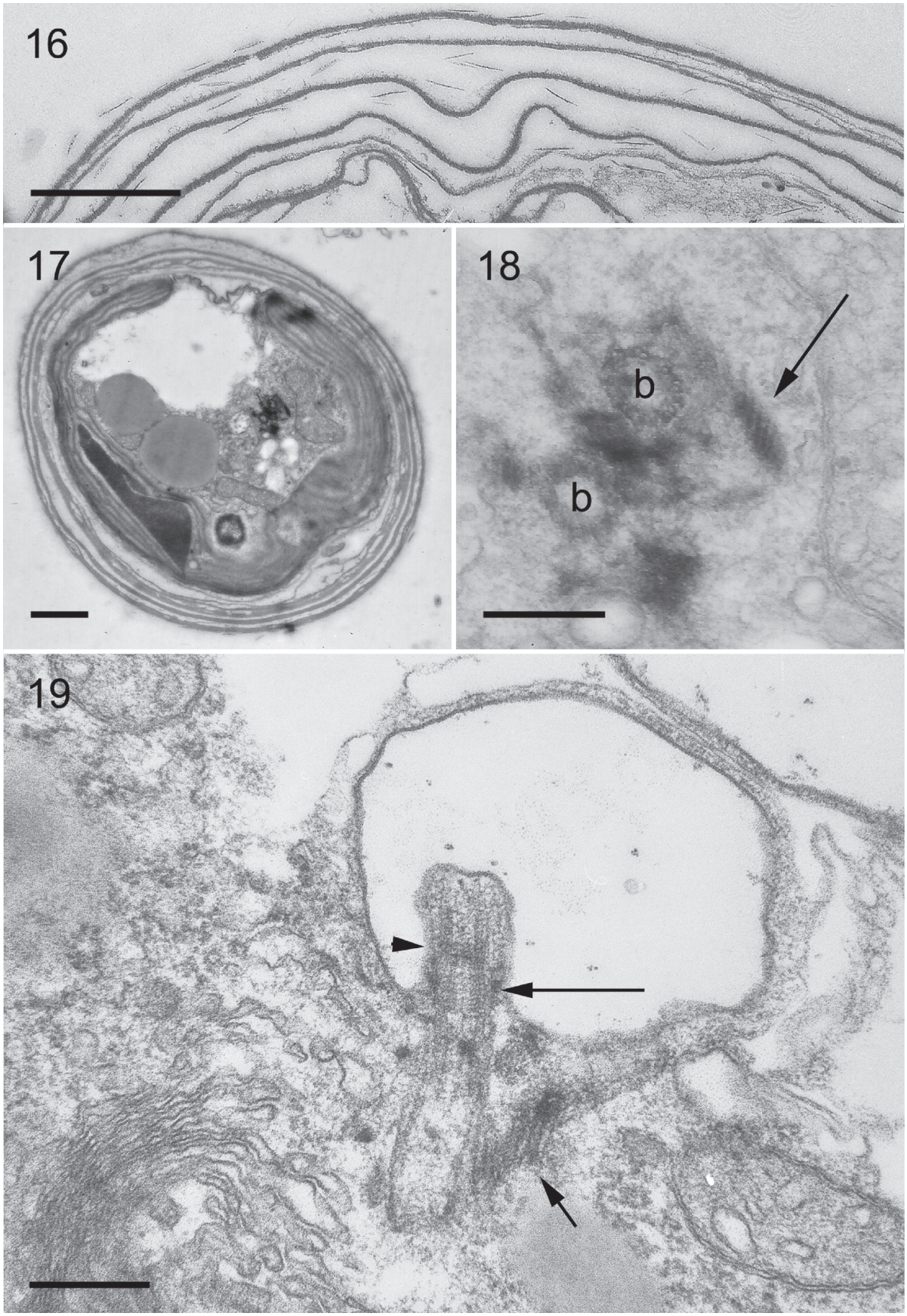
Non-motile cells had a single nucleus with darkly staining nucleolar RNA and mitochondrial profiles showed the presence of tubular cristae (Fig. 11). A single Golgi body was adjacent to the nucleus and contained 15 or more individual cisternae. Each chloroplast had lamellae consisting of three adpressed thylakoids and an embedded pyrenoid was located near the nucleus (Figs 11–13). The pyrenoid was traversed by a single thylakoid (Fig. 12). Cells sectioned longitudinally showed the extended chloroplast lobes (Fig. 13). The wall-like structure observed by LM was more complex when observed with TEM (Figs 13, 14). There were alternating electron-dense and transparent layers. The electron-dense layers included scales (Figs 11, 12, 15, 16) that are presumably organic (unmineralized), but the majority of the dense layer was composed of an amorphous material. The precise pattern on the scales was not determined, but the scales do lack a folded margin (Figs 11, 12, 15, 16). An enlarged image of the dense layer showed that the amorphous layer was distinct from the scales (Fig. 15). There was always a layer of scales outside the thicker amorphous layer, suggesting that after each cell division, daughter cells first deposited scales and then deposited the amorphous



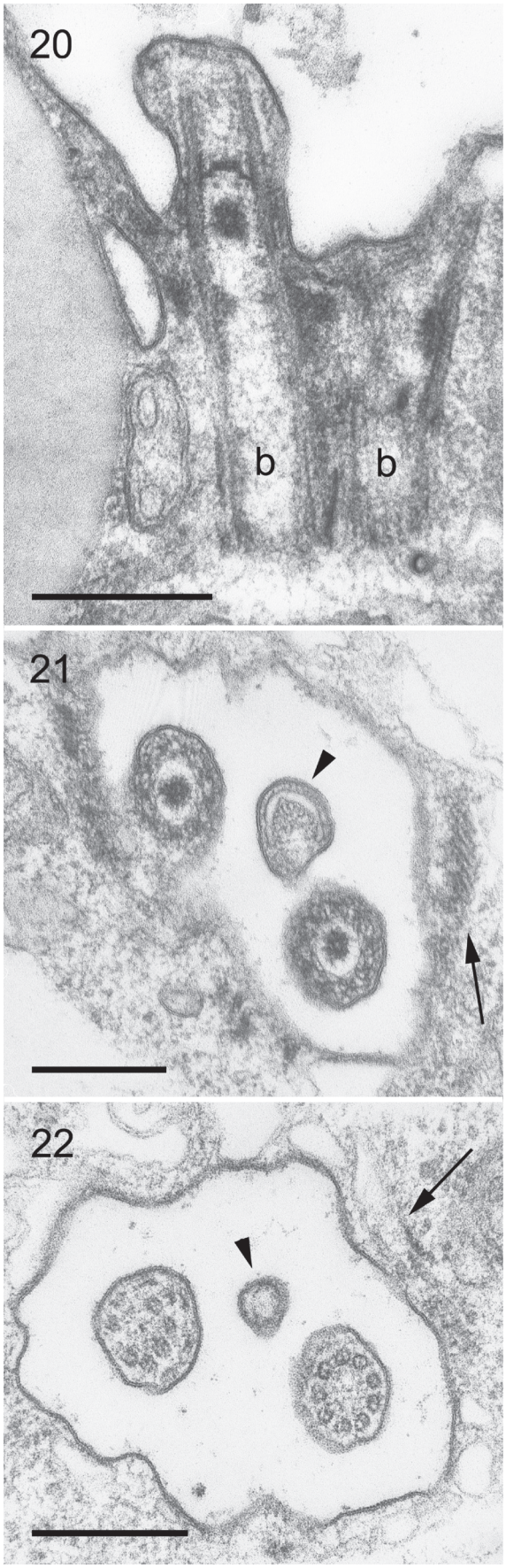
Figs 11–12. TEM micrographs of *Phaeocystis rex* non-motile cells. **Fig. 11.** Section depicting typical cellular features including the nucleus (N), Golgi body (G), mitochondrial profiles (M), one chloroplast (P) and its pyrenoid (py). Note the densely staining chromatin in the nucleus and the presence of the scales (arrowheads). **Fig. 12.** Section showing the pyrenoid (py) transversed by a single thylakoid and a lipid body (L). Note the scales (arrowheads). Scale bars = 500 nm.



Figs 13–15. TEM micrographs of *Phaeocystis rex* non-motile cells. **Fig. 13.** Longitudinal section showing the elongated chloroplast lobe (P) and the nucleus (N). Note the thickened wall-like layers at one end (arrow). **Fig. 14.** Two daughter cells shortly after cell division. Note the thickened wall-like layers at one end (arrows). **Fig. 15.** Enlarged view of a wall-like layer showing the scales (arrowheads) and the electron dense amorphous layer (arrow). Scale bars: Figs 13, 14 = 1 µm; Fig. 15 = 200 nm.



Figs 16–19. TEM micrographs of *Phaeocystis rex* non-motile cells. **Fig. 16.** Alternating wall-like layers with electron-dense amorphous materials and electron-transparent zones with scales. **Fig. 17.** Vegetative cell with wall-like layers. Note the basal bodies in the cell centre (arrow). **Fig. 18.** Enlarged view of the basal bodies (b), mitochondrion (M) and a microtubular root (arrow). **Fig. 19.** Section of a flagellum emerging in a flagellar depression. Note the transitional plate (arrowhead), the proximal transitional helix (long arrow) and the microtubular root (short arrow). Scale bars: Figs 16, 17 = 1 μm; Figs 18, 19 = 300 nm.



Figs 20–22. TEM micrographs of *Phaeocystis rex* non-motile cells. **Fig. 20.** Rudimentary flagellum for a vegetative wall-like cell showing the two basal bodies (b). **Figs 21-22.** Cross-sections of two emergent rudimentary flagella and the haptonema (arrowhead). Note the microtubular root (arrow). Scale bars = 300 nm.

layer (Figs 15, 16). Furthermore, adjacent to the nucleus-Golgi-chloroplasts complex, the electron-dense layers were thicker and swollen (Figs 13–14). The swollen region was also visible by LM (Figs 2, 3, 5). The ornamentation of scales was not studied in detail.

Two rudimentary flagella (*c.* 500–1000 nm long), a short haptonema, and at least one nascent microtubular root were present in non-motile cells with the wall-like layers (Figs 17–22). The flagella appeared to have a transitional helix located on the proximal side of the transitional plate (Figs 19, 20). The basal bodies were anchored in the cell near the Golgi body, mitochondrion and nucleus (Figs 17–20). The flagella and haptonema emerged from a shallow depression and a microtubular root ran along the margin of the invagination (Figs 19, 21, 22). Despite numerous efforts during the past 15 years, we were unable to successfully fix swimming cells for TEM.

A morphological comparison of *Phaeocystis rex* to the type descriptions for other currently recognized *Phaeocystis* species is presented in Table 2. The presence of the wall-like layers and the morphology of the chloroplasts distinguished *P. rex* from all previously described *Phaeocystis* spp.

Phylogenetic analyses

Phylogenetic analyses based on concatenated datasets of partial DNA sequences from five nuclear and plastidial genes were carried out to determine the relationships of *Phaeocystis rex* to other *Phaeocystis* species. Two phylogenetic trees with and without sequences of the symbiotic *Phaeocystis* of Acantharia were constructed (Figs 23, 24, respectively). The phylogenetic tree involving only the described species (Fig. 24, dataset containing fewer taxa but longer sequences) displayed stronger overall statistical supports for deep nodes. In both trees, *P. rex* held the same phylogenetic position with strong support in both Bayesian and ML analyses, branching between the solitary *Phaeocystis* species (*P. jahnii* and *P. cordata*) and the colony-forming species (*P. globosa, P. antarctica* and *P. pouchetii*). Among these *Phaeocystis* species, sequence divergence estimates (p-distance) varied according to the gene, but on average, *P. rex* was genetically closer to *P. antarctica* (0.018% of dissimilarity) (Table 3). Phylogenetic analyses also demonstrated that the undescribed *Phaeocystis* clades represented by symbionts of Acantharia were the earliest diverging lineages, followed by *P. jahnii, P. cordata, P. rex, P. globosa, P. antarctica* and *P. pouchetii*. Significantly, we confirm here that PLY559 is closely related to *P. jahnii*, and that *P. globosa* includes several cryptic species that require definition. One taxon identified as *P. pouchetii* (sequence AB280613) clearly grouped in the *P. globosa* clade, indicating that this is probably a mis-identification.

Table 2. Morphological features for *Phaeocystis rex* and other *Phaeocystis* species as determined from their type descriptions.

Species	Dominate gross morphology	Colony size	Cell size	Flagellate cell size	Flagellar length	Plastid no.	Pyrenoids	Scales
P. amoeboidea[1]	sphaeroidal colonies	??	6 × 10 µm	6 × 10 µm	equal, 10 µm	one	unknown	unknown
P. antarctica[2]	sphaeroidal colonies	??	??	??	unknown	two	unknown	unknown
P. brucei[3]	irregular colonies, gelatinous trails	1–2 mm	5–6 µm	unknown	unknown	two	unknown	unknown
P. cordata[4]	single cell flagellate	??	3–4 µm	3–4 µm	unequal	two	immersed	present, dimorphic
P. globosa[5]	sphaeroidal colonies	2–3 mm	7–15 µm	4–6 µm	equal, subequal	two	none	unknown[9]
P. jahnii[4]	single cell flagellate	??	3–5 µm	3–5 µm	unequal	two	immersed	present, dimorphic
P. pouchetii[6]	irregular colonies	1–2 mm	6–8 µm	6–8 µm	unequal, ~ 10–20 µm	two	unknown	unknown
P. scrobiculata[7]	single cell flagellate	??	8 µm	8 µm	subequal, 23–30 µm	two	unknown	present, dimorphic; star-like thread
***P. rex*[8]**	**single-celled with wall-like covering**	??	**6–10 × 8–15 µm**	**6–8 µm**	**unequal**	**two**	**immersed**	**present**
P. sphaeroides[1]	sphaeroidal colonies	??	5–6 µm	5–6 µm	equal, 5–6 µm	one	unknown	unknown

[1] Büttner (1911), [2] Karsten (1905), [3] Mangin (1922), [4] Zingone *et al.* (1999), [5] Scherffel (1900), [6] Pouchet (1892), [7] Moestrup (1979), [8] Present study.

Table 3. Genetic distances (p-distances) between *Phaeocystis* species calculated using partial sequences for each of the five genes examined in this study.

		P. jahnii (CCMP 1384)	*P. cordata* (CCMP 1383)	*P. pouchetii*	*P. antarctica* (CCMP 1374)	*P. globosa* (RCC 1719)	*P. globosa* (PCC 540)
***Phaeocystis rex* sp. nov. (CCMP 2000)**	**16S rRNA (734 bp)**	0.030	0.009	_	0.008	0.009	0.009
	18S rRNA (1069 bp)	0.053	0.004	0.015	0.004	0.015	0.015
	28S rRNA (820 bp)	0.113	0.032	_	0.012	0.069	0.020
	***rbc*L (1061 bp)**	0.103	0.071	0.051	0.043	0.044	0.043
	***psb*A (569 bp)**	0.027	0.027	_	0.023	0.021	0.021
	p-distance mean	0.065	0.029	0.033	0.018	0.032	0.022

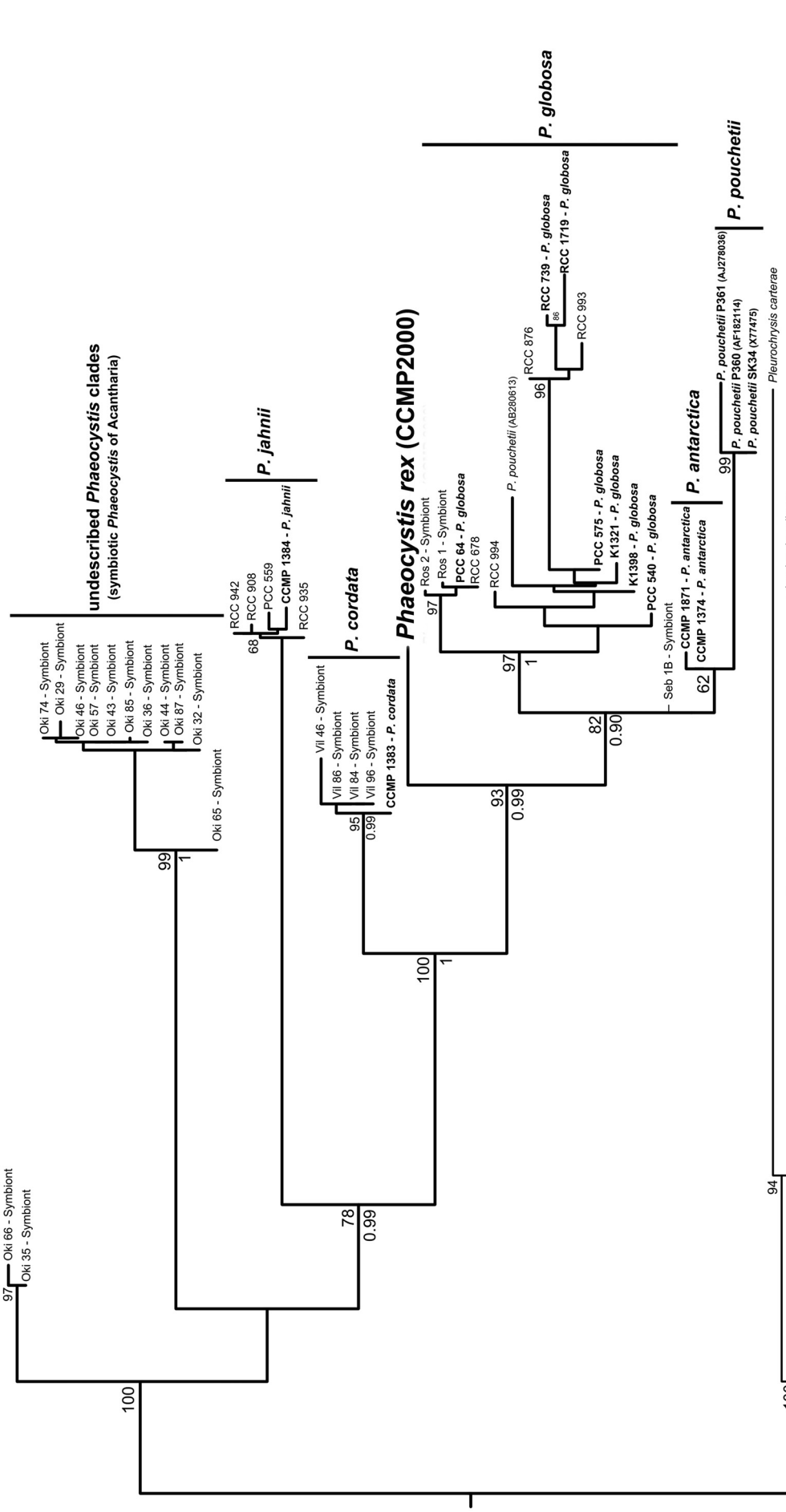


Fig. 23. Phylogenetic tree of *Phaeocystis* inferred from a concatenated alignment of five genes (the ribosomal 16S rRNA, 18S rRNA and 28S rRNA, and the plastidial *psb*A and *rbc*L genes) using RAxML (48 taxa with 3087 aligned positions). Sequences come from different culture strains and uncultured *Phaeocystis* that live in symbiotic association with acantharian radiolarians. RAxML bootstrap percentages based on 2000 pseudoreplicates and Bayesian posterior probabilities (PP) are indicated at each node when support values are higher than 60% and 0.8, respectively. The tree is rooted with sequences of four haptophyte species that do not belong to the order Phaeocystales.

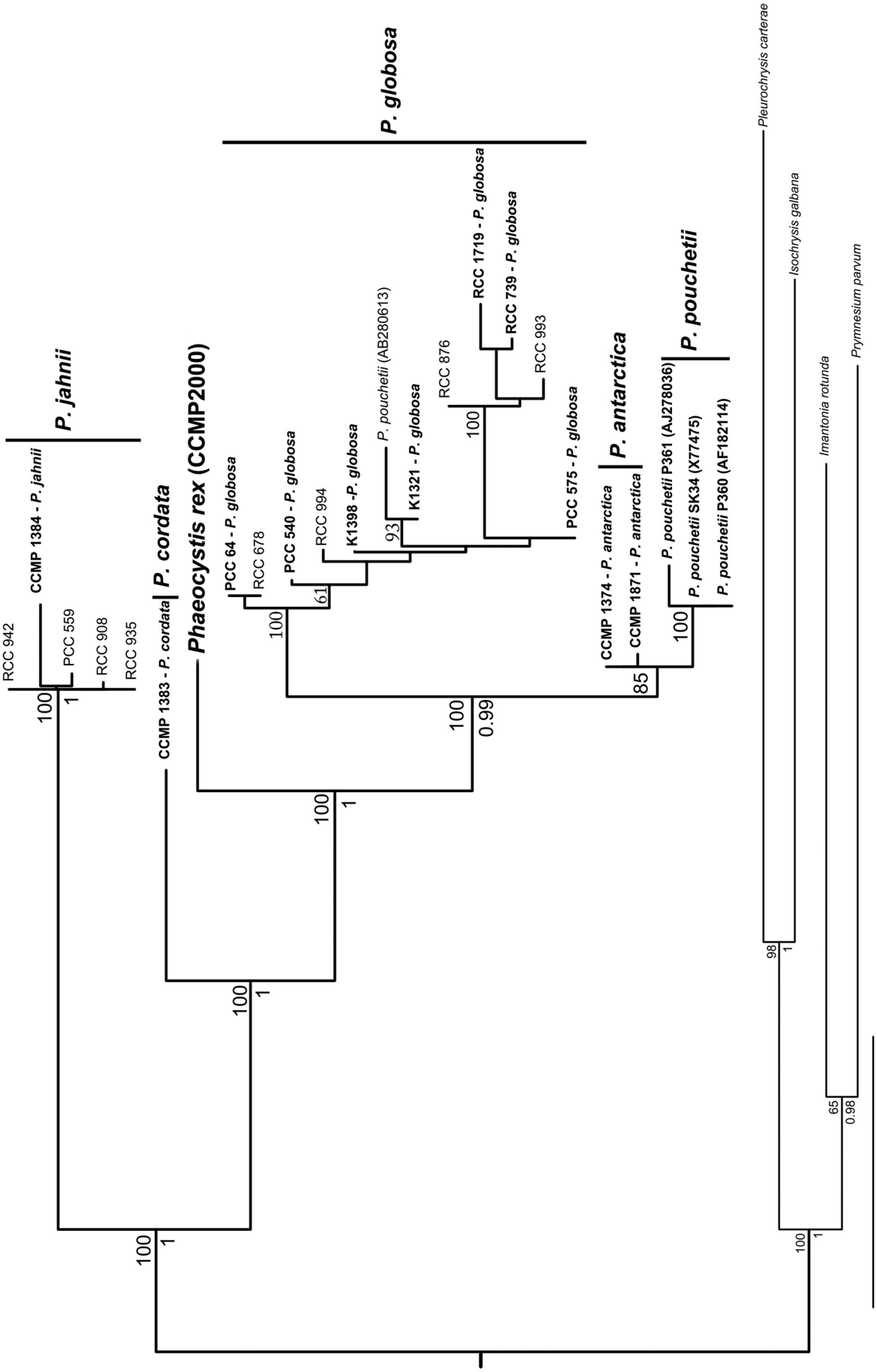


Fig. 24. Phylogenetic tree of *Phaeocystis* inferred from a concatenated alignment of five genes (the ribosomal 16S rRNA, 18S rRNA and 28S rRNA, and the plastidial *psb*A and *rbc*L genes) using RAxML (28 taxa with 4824 aligned positions). Only sequences from *Phaeocystis* cultures were included in this analysis. RAxML bootstrap percentages based on 2000 pseudoreplicates and bayesian posterior probabilities (PP) are indicated at each node when support values are higher than 60% and 0.8, respectively. The tree is rooted with sequences of four haptophyte species that do not belong to the Phaeocystales.

DISCUSSION

Systematics of *Phaeocystis rex*

Phaeocystis rex is morphologically distinct from all currently recognized *Phaeocystis* species. When the alga was first isolated and examined by TEM it was initially presumed to be a new prymnesiophyte genus. However, our DNA sequence analyses clearly demonstrate that this alga is a close relative of other *Phaeocystis* species for which comparable molecular data are available. Like most other *Phaeocystis* species, *P. rex* cells possess two plastids with immersed pyrenoids, motile cells with two flagella and a haptonema, and the characteristic arrangement of subcellular organelles (particularly the nucleus, Golgi body and flagellar apparatus) common to haptophytes in general (Parke *et al.*, 1971; Chang, 1984; Inouye & Pienaar, 1985). However, *P. rex* differs from all other described *Phaeocystis* species in two principal respects (Table 2). First, the external cell covering, in mature non-motile cells, is comprised of alternating layers of presumably inorganic scales and an underlying layer of amorphous wall-like material. Second, the finger-like chloroplast lobes are unlike those described for any other *Phaeocystis* species. Additionally, the sizes of *P. rex* cells, the ellipsoidal outline of mature cells, and the uneven posterior thickening of the extracellular covering are useful for identifying the alga, but their possible taxonomic and/or phylogenetic importance is unclear at this time. Importantly, we demonstrated for the *P. rex* non-motile stage the presence of (1) scales, and also (2) two rudimentary flagella and a haptonema inside (beneath) the extracellular cell covering. These observations for *P. rex* are not completely in agreement with the emended generic description for *Phaeocystis* presented in Medlin & Zingone (2007). We failed in attempts to determine the exact pattern on the scales of *P. rex*. A comparative study of the composition and architecture of body scales found on cells of *P. rex* and other *Phaeocystis* species would be helpful (Parke *et al.*, 1971; Moestrup, 1979; Lange *et al.*, 1996; Zingone *et al.*, 1999).

In this study we specifically compared morphological data for *P. rex* to information only found in the original descriptions for other *Phaeocystis* species, and for *P. rex* we designated a cryopreserved culture (CCMP2000) as the isotype that provides a dependable source for future research on the species. Authentic cultures of *P. cordata* and *P. jahnii* are also available for study (Zingone *et al.*, 1999). However, such biological resources (cultured strains designated as epi- or isotypes), which are associated with publicly archived DNA sequences of importance for systematics and other investigations, are not available for any other species of *Phaeocystis*. Thus, connections between their original descriptions and publicly available DNA sequence data are much more tenuous for the many culture stains identified as *P. antarctica, P. globosa* and *P. pouchetii* (the type species). We recommend designating a single cryopreserved culture strain collected from the type locality as a nomenclatural epitype to anchor the names *P. pouchetii, P. globosa, P. antarctica, P. brucei* and *P. scrobiculata*. Until epitypic strains are designated for all *Phaeocystis* species, morphological studies will be hampered and nomenclatural uncertainty will prevail. For example, early studies of *Phaeocystis* species did not include ultrastructural data or information on scales because electron microscopes had not been invented. Thus, for half of all *Phaeocystis* species this information is lacking and is, obviously, not found in the original species description. Therefore, when extant or newly derived cultures are examined by TEM there is uncertainty regarding their identity. For example, Parke *et al.* (1971) studied two strains that they implicitly, if not explicitly, identified as *Phaeocystis pouchetii*, largely following the reasoning provided by Kornmann (1955) that there was only one highly variable species and that the name should be based upon priority (i.e. *P. pouchetii*). Twenty-five years later, we find that one of the cultures (Clone 64 = PLY64) used by Parke *et al*. (1971) was identified using DNA sequence analysis as *P. globosa* (Lange *et al.*, 1996), and this same strain appears in our trees as *P. globosa* PCC64. Even now, can we accurately interpret the TEM observations of Parke *et al.* (e.g. scale morphologies, flagellar transitional region, embedded pyrenoid) as belonging to *P. pouchetii* or *P. globosa* – or some new species in a cryptic species complex? The situation becomes even more tenuous for ecological studies where identifications have often been based upon LM observations. In summary, morphological comparisons (e.g. Medlin & Zingone, 2007) are not entirely stable without nomenclatural anchoring of names (e.g. by epitype). While an epitype may not actually represent the original taxon, the firmly anchored names will at least allow for a way forward in a meaningful manner.

Phylogenetics, evolution and ecology

Phylogenetic analyses of combined DNA sequence data from the five nuclear- or plastid-encoded genes provided solid support for the conclusion, based on our microscope observations, that *Phaeocystis rex* is a new *Phaeocystis* species. Additionally, the consistent and highly supported topologies obtained provide insights into the evolutionary history of not only the species *P. rex*, but also more broadly on the genus *Phaeocystis*. *Phaeocystis rex* occupies an intermediate position between the solitary species (*P. jahnii* and *P. cordata*) and the colony-forming species (*P. globosa, P. antarctica* and *P. pouchetii*). *Phaeocystis jahnii* has been described as forming small, loose, occasionally palmelloid aggregations of cells (Zingone *et al.*,

1999), but these do not seem to be colonies in the same sense as those produced by *P. globosa, P. antarctica* and *P. pouchetii*. *Phaeocystis rex* has not been observed to form colonies in culture, and therefore is presumed not to form colonies in the natural environment. To date, *P. cordata* and *P. jahnii* have only been reported from the Mediterranean Sea, whereas *P. globosa* has been collected from both temperate and tropical waters worldwide (Schoemann *et al.*, 2005). The polar species *P. pouchetii* and *P. antartica* are better adapted to cold temperatures and are mainly restricted to Arctic and Antarctic waters, respectively (Schoemann *et al.*, 2005). The phylogenetic position of *P. rex* therefore suggests that it may represent an evolutionary transition between the solitary and colonial lifestyles, and between distinct ecological preferences. Our phylogenetic analyses provide rigorous support for the hypothesis that *Phaeocystis* likely originated in warm waters and that the colonial lifestyle is a derived character (Medlin & Zingone, 2007). A wider diversity of *Phaeocystis* need to be morphologically characterized, particularly from the *P. globosa* clade, as well as from the clades that form symbiotic relationships with the Acantharia. These symbiont sequences that form the basal clades in the *Phaeocystis* phylogeny were retrieved from the subtropical Pacific ocean (Okinawa Islands, Japan), providing additional evidence for an evolutionary origin in warm waters.

From a biodiversity perspective it is interesting to recall that the first five species assigned to *Phaeocystis* are in nature predominately colonial organisms (Pouchet, 1892; Lagerheim, 1896; Scherffel, 1899, 1900; Karsten, 1905; Büttner, 1911; Mangin, 1922). These organisms exhibit other life forms in the course of their life histories (e.g. motile flagellate and/or benthic palmelloid life forms) but most often take the form of colonies of cells in mucilage (e.g. Rousseau *et al.*, 2007; Gaebler-Schwarz *et al.*, 2010). The last four species of *Phaeocystis* described, including *P. rex*, are predominately solitary organisms (Moestrup, 1979; Zingone *et al.*, 1999). Following this trend, the majority of undiscovered *Phaeocystis* species in nature are liable to be predominately solitary organisms. Environmental metabarcoding may help to constrain the extent of undiscovered biodiversity in this cosmopolitan genus and improve our understanding of the ecological and biogeochemical significance of different *Phaeocystis* species in coastal and open ocean regions.

FUNDING

R.A.A. was supported by a USA National Science Foundation [grant number 0951733]. J.D. was supported by the project OCEANOMICS, that has received funding from the French government, managed by the Agence Nationale de la Recherche, under the grant agreement 'Investissement d'Avenir' [ANR-11-BTBR-0008].

AUTHOR CONTRIBUTIONS

R. A. Andersen: strain isolation, USA algal culture, TEM and writing; J. C. Bailey: LM, DNA sequencing and writing; J. Decelle: DNA sequencing, phylogenetic analysis and writing; I. Probert: France algal culture and writing.

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