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Partenskyella glossopodia gen. et sp. nov., the First Report of a Chlorarachniophyte that Lacks a Pyrenoid

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A new chlorarachniophyte, *Partenskyella glossopodia* gen. et sp. nov., is described from a culture isolated from the Mediterranean Sea pelagic waters and maintained as strain RCC365 at the Roscoff Culture Collection (France). Vegetative cells of *P. glossopodia* are non-motile naked spherical cells. However, flagellate and amoeboid stages are also present in its life cycle. The cells are 2—4 μm in diameter containing a pale-green, cup-shaped chloroplast, 1—2 mitochondria, a nucleus, and a Golgi apparatus. Vesicles containing storage product-like material are also present. The chloroplast is surrounded by four membranes possessing a nucleomorph in the periplastidal compartment. The minute cell size and the absence of a pyrenoid at any stage of the life cycle are unique characteristics among the chlorarachniophytes, which justifies our proposition for a new genus for strain RCC365.

Key words: Cercozoa; chlorarachniophytes; picoplankton; *Partenskyella glossopodia*; secondary endosymbiotic alga; taxonomy.

Introduction

Chlorarachniophytes are marine photosynthetic protists found widely distributed from temperate to tropical coastal environments as well as in the open ocean, whose cell forms are amoeboid, coccoid or flagellate. The typical amoeboid cells have several filopodia (pseudopodia) and the flagellate cells have a single flagellum (Hibberd and Norris 1984). Each cell possesses a nucleus and several green plastids bound by four membranes and containing chlorophylls *a* and *b* (Hibberd and Norris 1984). The plastid is accompanied by a vestigial nucleus of a green algal endosymbiont that was engulfed by the ancestral cercozoan protist (host component) and integrated as a plastid (Gilson and McFadden 1999; Ishida et al. 1999; Keeling et al. 1998; Ludwig and Gibbs 1989; McFadden et al. 1994; Van de Peer et al. 1996). This vestigial nucleus is called a nucleomorph and is located in the periplastidal compartment, a space between inner two and outer two of the four membranes that wrap the plastid (reviewed in Archibald 2007; Ishida et al. 2007).

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The phylum Chlorarachniophyta was established during a detailed reexamination of *Chlorarachnion reptans* Geitler by Hibberd and Norris (1984). To date, this phylum contains 10 species in six genera (Calderon-Saenz and Schnetter 1987; Dietz et al. 2003; Geitler 1930; Ishida and Hara 1994; Ishida et al. 1996, 2000; Moestrup and Sengco 2001; Ota et al. 2005, 2007a, b), and several unnamed species are waiting to be described. In the tree of life, this small algal group is placed within the Cercozoa that includes many amoebo-flagellates and filose amoebae such as euglyphinids and cercomonads (Bass et al. 2005; Cavalier-Smith and Chao 2003), and is a major component of the Rhizaria, one of the supergroups of eukaryotes (Adl et al. 2005; Cavalier-Smith 2002).

The Roscoff Culture Collection (RCC, Vaulot et al. 2004) maintains many pico/nano-planktonic cultures from various open ocean regions like the Mediterranean Sea and the Atlantic Ocean. It contains, in particular, several unidentified chlorarachniophytes from the Mediterranean Sea, one of which, RCC365, has a very small cell size. So far no pico-sized chlorarachniophyte has ever been described. In the present study, we provide morphological and ultrastructural characterization of strain RCC365 using light and transmission electron microscopy, as well as nuclear SSU/SSU+LSU rDNA phylogenetic analyses. Based on the results, we formally describe the strain as a new genus and species in the phylum Chlorarachniophyta. We also provide data on its putative life cycle.

Description

Partenskyella S. Ota, Vaulot et Ishida gen. nov.

Diagnosis: *Cellulae solitariae, amoeboideae; seu globosae seu flagellatae. Chloroplasti subvirides, pyrenoidibus carentia. Nucleomorphus in latere concavo chloroplasti in spatio periplasti.*

Cells solitary, coccoid, amoeboid, or flagellate. Chloroplast pale-green to green, lacking a pyrenoid. A nucleomorph is located at the concave side of the chloroplast in the periplastidal compartment.

Type species: *Partenskyella glossopodia* S. Ota, Vaulot et Ishida.

Etymology: The genus name *Partenskyella* is dedicated to Dr. F. Partensky who established strain RCC365.

Partenskyella glossopodia S. Ota, Vaulot et Ishida sp. nov.

Diagnosis: *Cellulae solitariae, nudae rotundatae vel flagellatae vel amoeboideae. Nucleus unicus, ad peripheriam. Chloroplastus unicus, subviridis, cupulatus, pyrenoidibus carentia. Cellulae nudae rotundatae, 2—4 μm diam., dominans in cultura. Propagatio vegetativa bifissione effecta. Cellulae amoeboideae, pyriformis ad ampulliformis, 2—3 μm diam., lobopodio. Cellulae flagellatae, c. 3 μm diam.; flagello unico, 2—8 μm longo. Cystae parietibus, c. 5 μm diam. Nucleomorphus in latere concavo chloroplasti in spatio periplasti. Cellulae planctonicae*.

Cells solitary, naked rounded, amoeboid, or flagellate. Single nucleus at cell periphery. Chloroplast single, pale green, cup-shaped, lacking pyrenoid. Naked rounded cells, 2—4 μm in diameter; dominant under the culture condition. Vegetative propagation by division into two. Amoeboid cells, pear- to flask-shaped, 2—3 μm in diameter, with a lobopodium. Flagellate cells, c. 3 μm in diameter; with a single flagellum, 2—8 μm in length. Cysts with cell wall, c. 5 μm in diameter. A nucleomorph is located at the concave side of the chloroplast in the periplastidal compartment. Cells planktonic.

Holotype: One microscope slide (TNS-AL-56389), deposited in the Department of Botany, the National Museum of Nature and Science (TNS), Tokyo.

Isotype: One EM block (TNS-AL-56390) in TNS. These cells are derived from the same sample as the holotype.

Type locality: Mediterranean Sea, Long: 15°37′E-Lat: 37°24′N, at about 5 m depth below, 26 September 1999, collected by Partensky F.

Habitat: Ocean surface water.

Distribution: Known only from the type locality.

Authentic culture: RCC365. This culture is maintained in the Roscoff Culture Collection at the Station Biologique, Roscoff, France.

Etymology: *glosso* (tongue-shaped)+*podia* (pseudopodium) refers to the shape of a pseudopodium in the amoeboid stage.

Results

Light Microscopical Morphology

Under the culture condition used, *Partenskyella glossopodia* showed four cell stages in its life cycle: naked coccoid (Fig. 1A, B), flagellate (Fig. 1C), amoeboid (Fig. 1D, E), and walled (cyst) (Fig. 1F). The coccoid cells were spherical, sometimes slightly ellipsoid or ovate, 2—4 μm in diameter (mean = 2.8 μm, $n = 30$) (Fig. 2A, B). The cells occasionally elongated a lobopodium (tongue shaped pseudopodium) unilaterally, such that the cells looked pear- or flask-shaped (Fig. 2C, D). The maximum length of the lobopodium was approximately 5 μm. Flagellate cells were spherical to cylindrical possessing a single flagellum inserted laterally at the mid region of the cell (Fig. 2E). Each stage of the cell possessed a single pale-green chloroplast which was ellipsoid or kidney shaped (Fig. 2B—E). Cyst-like cells that were somewhat larger than the vegetative cells (approximately 5 μm in diameter) were occasionally observed in old cultures (cf. Fig. 1F). The cyst-like cell had a cell wall and showed granular appearance in the cytoplasm (Fig. 1F). No pyrenoids, vacuoles or red droplets (e.g. Ota et al. 2005) were observed at any stage of the life cycle.

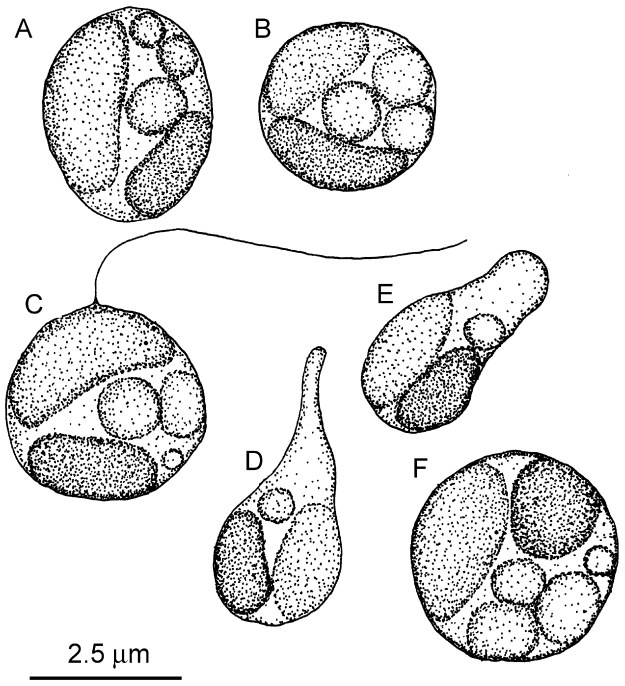


Figure 1. Line drawings of *Partenskyella glossopodia* gen. et sp. nov. **A, B.** Naked ellipsoid or spherical cells. **C.** Flagellated cells. **D, E.** Amoeboid cells with a lobopodium. **F.** Cyst-like cell.

Time-Lapse Video Observations and Life Cycle

Time-lapse video observations revealed that the vegetative cells reproduced by cell division, forming two identical daughter cells (Fig. 3). Cytokinesis occurred rapidly, within approximately 2—4 min (Fig. 3C—E). Whether the daughter cells possessed a flagellum could not be observed using the video system. Time-lapse video microscopy also showed that the coccoid cell could directly transform into an amoeboid cell (Fig. 4) by elongating their lobopodium unilaterally (Fig. 4A—C); then the cell showed a slight wriggling motion (Fig. 4D—F).

The putative life cycle of *Partenskyella glossopodia* is summarized in Figure 5. Naked coccoid cells were dominant in the culture. Coccoid cells (amoeboid cells) reproduced by cell division and were therefore vegetative cells. Some of the naked coccoid cells transformed into amoeboid cells with a lobopodium and vice versa. In older cultures (1—3 months old), some of the coccoid cells became cyst-like cells which possessed a cell wall. Flagellate cells were not dominant in cultures and perhaps do not reproduce vegetatively. Sexual reproduction was not observed.

Ultrastructure

Vegetative cells were naked and spherical (Fig. 6A). The nucleus, elliptical or kidney shaped, was always parietal in position (Fig. 6A). The chloroplast was kidney shaped in longitudinal section and surrounded by four membranes (Fig. 6A, B). The periplastidal compartment (the space between the inner two and outer two chloroplast membranes) was expanded on the inner chloroplast surface and it contained the nucleomorph (Fig. 6A, B). The chloroplast matrix was traversed by stacks of two to three thylakoids (Fig. 6B). One or two mitochondria with tubular cristae were observed in the cytoplasm (Fig. 6C). Serial sections revealed that the chloroplast was often cup-shaped and that the nucleomorph was sitting in the ‘cup’ (Fig. 6D—H). No pyrenoid was observed in the chloroplast.

The nucleomorph was almost spherical in shape and it was surrounded by double membranes that possessed several pores or gaps (Fig. 7A, B).

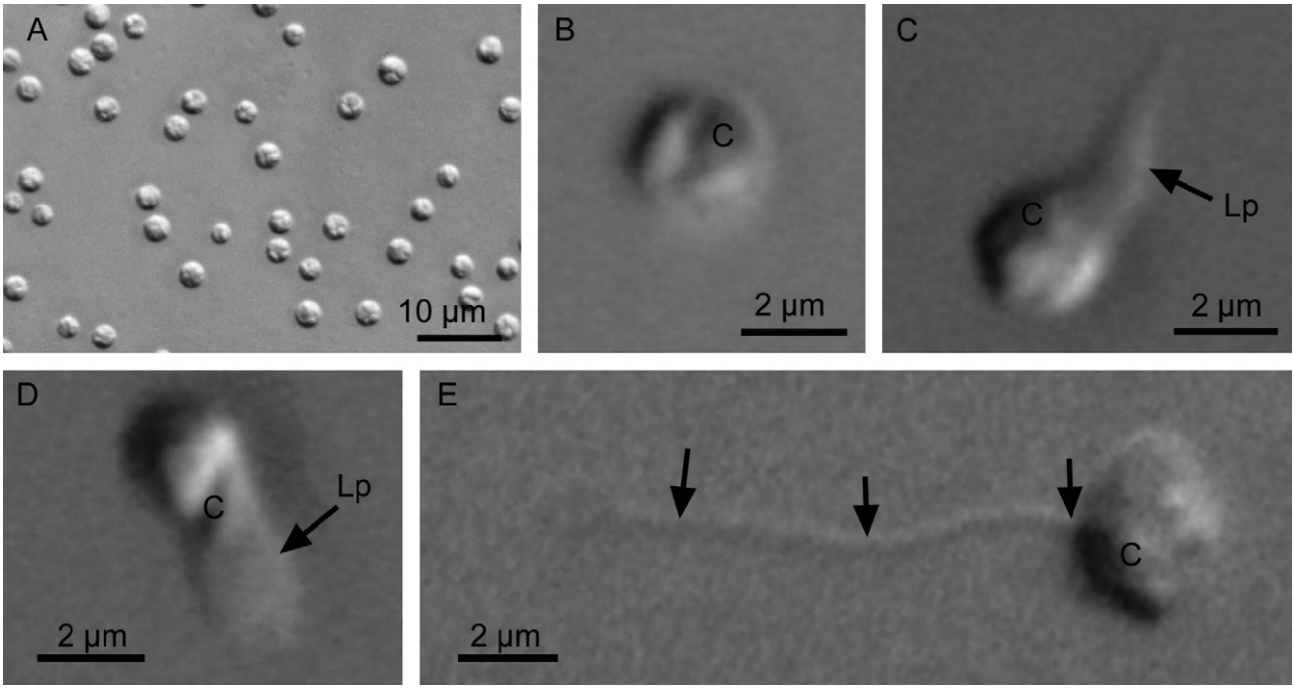


Figure 2. Light micrographs of *Partenskyella glossopodia* gen. et sp. nov. **A.** Low-magnification view of vegetative cells. **B.** Higher-magnification view of the naked coccoid cell. **C, D.** Typical amoeboid cell. Short lobopodium (Lp) is elongating unilaterally from the cell. **E.** Flagellate cell. A single flagellum (arrows) is inserted in the middle region of the cell. C = chloroplast.

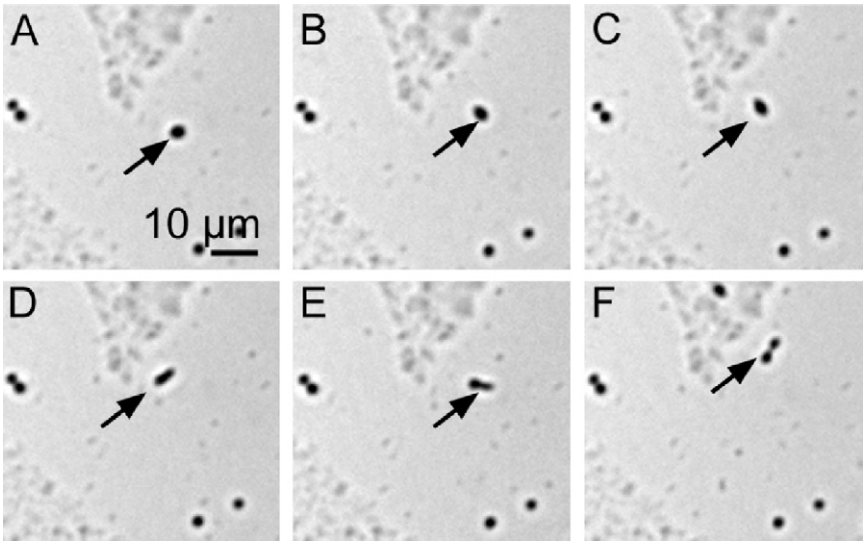


Figure 3. Time-lapse video sequence of cell division of *Partenskyella glossopodia* gen. et sp. nov. Note the cell (daughter cells) marked with arrows. The cell division takes approximately 2—4 min to complete. The time-lapse sequence is shown at 2 min intervals.

Several electron opaque globules were observed in the parietal region of the nucleomorph (Fig. 7B). The Golgi apparatus was often located near the chloroplast (Fig. 7C). Cyst-like or thicker walled cells were sometimes observed (Fig. 7D, E). The general cellular organization and ultrastructure of

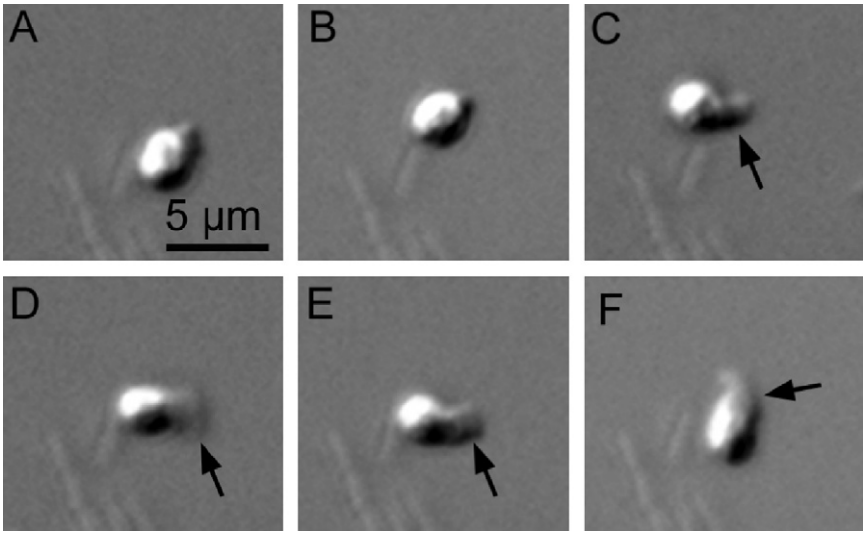


Figure 4. Time-lapse video sequence of coccoid/amoeboid cell of *Partenskyella glossopodia* gen. et sp. nov. The coccoid cell transformed into an amoeboid cell with lobopodium (arrows), and the amoeboid cell show a slight wriggling motion. The time-lapse sequence is shown at 6 s intervals.

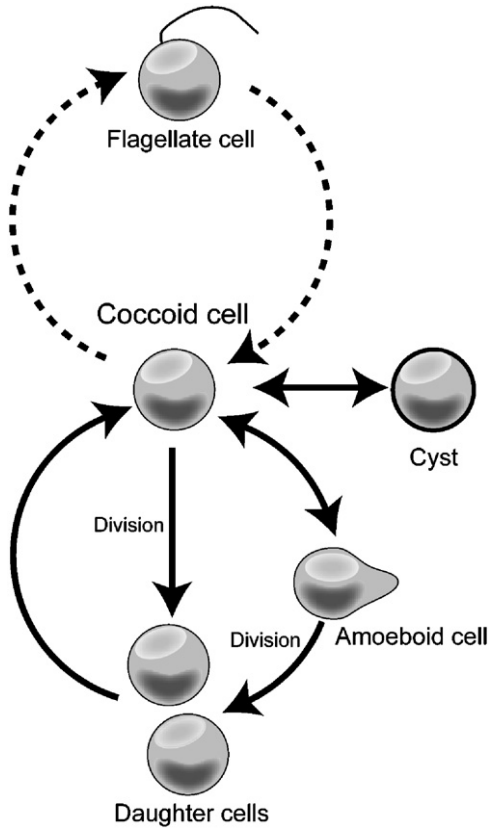


Figure 5. Diagram of the putative life cycle of *Partenskyella glossopodia* gen. et sp. nov. Solid-line arrows indicate the processes confirmed in the present study; broken-line arrows indicate predicted processes.

the cyst-like cells were basically similar to those of vegetative cells. Vesicles containing storage product-like materials were often observed in the parietal region of the vegetative or walled cells (Fig. 7D). Extrusome-like organelles were rarely observed covering the plasmalemma, and composed of a short, somewhat transparent, anterior and a long posterior cylinder in the longitudinal section (Fig. 7G). The flagellum consistently inserted close to the nucleus (Fig. 7H). The axonemal microtubules terminated in electron-opaque disks located at the level of the plasmalemma (Fig. 7H). In whole mount observations, flagellate cells were spherical and each cell had a single flagellum (Fig. 7I). The flagellum measured 2—8 µm in length (mean = 4.4 µm, $n = 18$), and terminated into a hair point (Fig. 7I).

Phylogenetic Analyses

The phylogenetic tree based on chlorarachniophyte SSU rDNA sequences showed that the six major clades previously known (*Gymnochlora*, *Lotharella*1, *Lotharella*2, *Chlorarachnion*, *Bigelowiella*, and *Norrisiella*) were recovered and supported by high bootstrap values (>98%, Fig. 8). However, RCC365 was excluded from these major clades and formed a distinct lineage, although the exact position of the RCC365 branch was not resolved (Fig. 8).

The concatenated phylogenetic analysis of SSU+LSU rDNA sequences showed better resolution in terms of the position of the strain

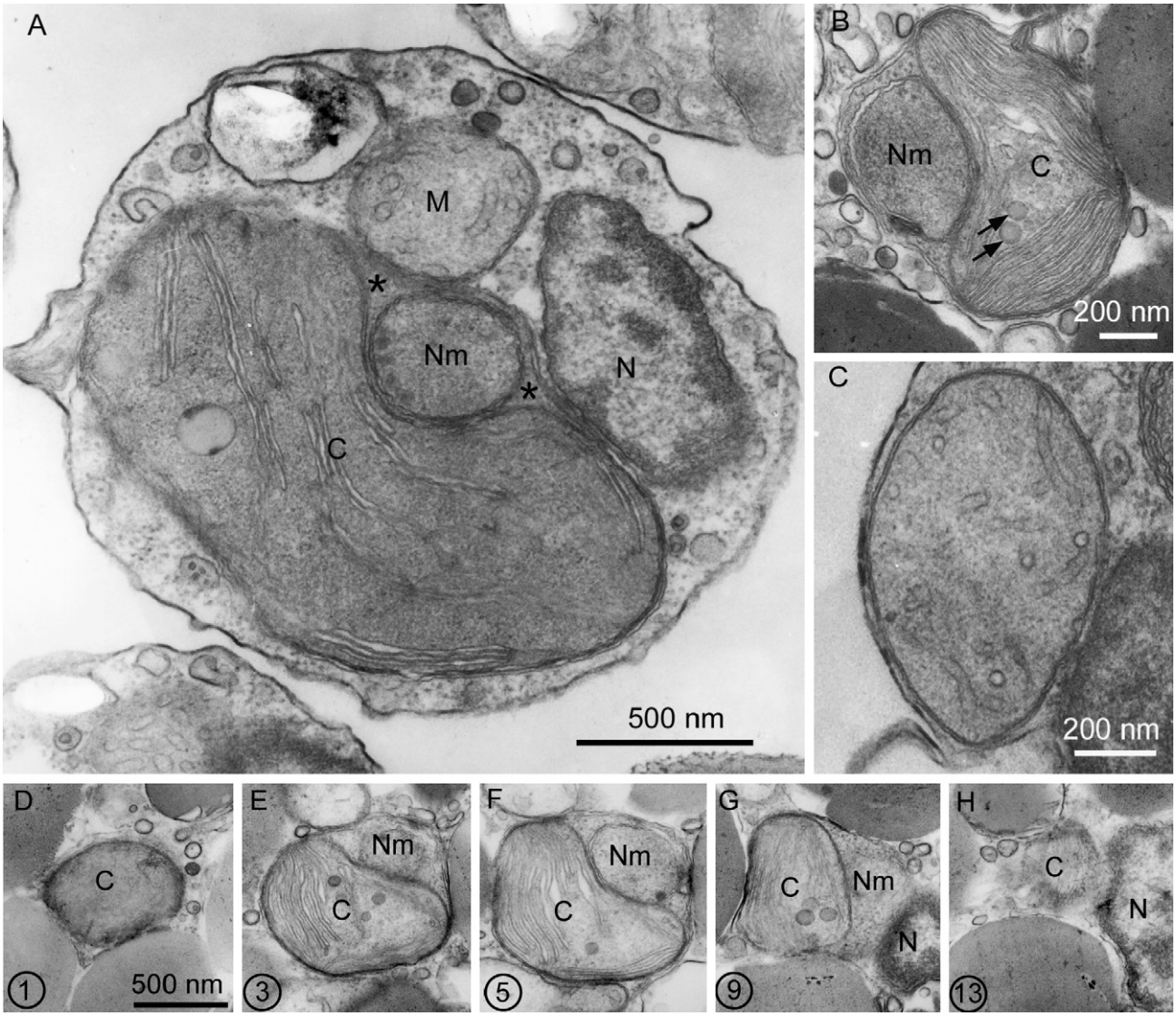


Figure 6. Transmission electron micrographs of *Partenskyella glossopodia* gen. et sp. nov. **A.** General ultrastructure showing a parietal nucleus (N), mitochondrion (M), chloroplast (C) with four surrounding membranes. The nucleomorph (Nm) is in the periplastidal compartment (*). **B.** Transverse section of the chloroplast showing lamellae with stacks of 1—3 thylakoids; the chloroplast is surrounded by the inner two and the outer two membranes. Nucleomorph (Nm) located at the concave side of the chloroplast in the periplastidal compartment. Some plastgranules (arrows) are present in the stroma. **C.** A mitochondrion with tubular cristae. **D—H.** Selected sections from a series of 14 consecutive sections (section numbers are indicated inside circles). The reconstructed cell did not possess any pyrenoids in the cup-shaped chloroplast.

RCC365. The RCC 365 branch was grouped with three clades (*Bigelowiella*, *Norrisiella*, and *Chlorarachnion*) with relatively good bootstrap support (92%, Fig. 9). Within this monophyletic group, the RCC365 branch clustered with the *Chlorarachnion* clade, although the bootstrap support was not high (Fig. 9).

Discussion

Partenskyella glossopodia belongs to the phylum Chlorarachniophyta because of several morphological features, i.e. a green chloroplast with four membranes, a single flagellum, a nucleomorph in the periplastidal compartment, a mitochondrion

with tubular cristae, and the absence of an eyespot. Phylogenetic analysis also supports the placement of *P. glossopodia* in this phylum. Table 1 shows a comparison of morphological, ultrastructural and life cycle characteristics among chlorarachniophyte genera. For generic-level classification, Ishida et al. (1996) proposed the pyrenoid ultrastructure and nucleomorph position to be the main diagnostic characters. Moestrup and Sengco (2001) used a different diagnostic characteristic for establishing the genus, *Bigelowiella*, which was defined by the fact that the flagellate form is the dominant vegetative stage. Ota et al. (2007b) recognized all the described genera, except for *Cryptochlora* for which ultrastructural information is unavailable, using a combination of electron microscopic and life cycle characteristics: (1) pyrenoid ultrastructure, (2) nucleomorph position, and (3) the type of cells in dominant vegetative stage.

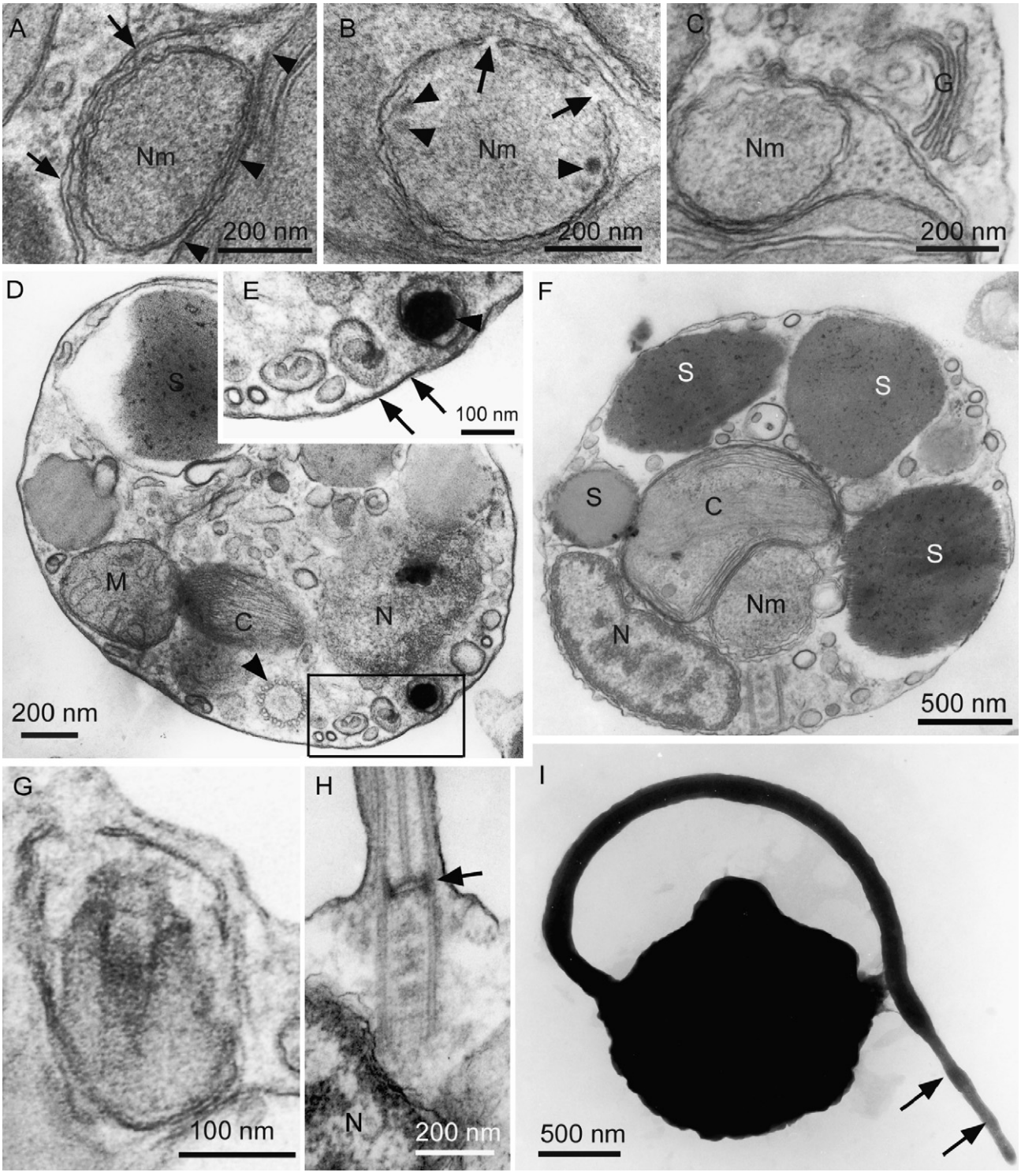
Based upon observations of strain RCC365, *Partenskyella grossopodia* is characterized by (1) a single, cup-shaped chloroplast that lacks a pyrenoid, (2) a nucleomorph located in the cup of the plastid, and (3) a main life cycle stage consisting of naked and coccoid cells. This unique set of characteristics readily distinguishes it from all other chlorarachniophyte genera. Therefore, we propose the establishment of a new genus *Partenskyella* for strain RCC365. The simple internal cellular organization and lack of pyrenoid may be good taxonomic markers to distinguish the genus *Partenskyella*, because all chlorarachniophytes described to date possess a projecting pyrenoid (Calderon-Saenz and Schnetter 1987; Dietz et al. 2003; Hibberd and Norris 1984; Ishida and Hara 1994; Ishida et al. 1996, 2000; Moestrup and Sengco 2001; Ota et al. 2005, 2007a, b).

Molecular phylogenetic trees based on the SSU rDNA and the SSU+LSU rDNA concatenated datasets display six major clades corresponding to each described genus that were well supported by high bootstrap values ($>98\%$), suggesting that *P. glossopodia* does not belong to any of the major clades, and may form a new lineage. This molecular phylogenetic evidence also supports the establishment of a new genus for strain RCC365. In the SSU+LSU rDNA tree, *P. glossopodia*, together with the *Bigelowiella*, *Norrisiella* and *Chlorarachnion* clades, formed a monophyletic clade with high bootstrap value. This suggests that *P. glossopodia* is not an ancestral lineage within the chlorarachniophytes, and therefore, the character state of *P. glossopodia* (i.e. reduced cell size and the absence of pyrenoid) is not plesiomorphic but apomorphic. However, the branching order among the lineages in this monophyletic clade is still unresolved. Taxon sampling for the present concatenated data analysis is probably still not sufficient. The precise phylogenetic position of *P. glossopodia* will require more molecular data and taxon sampling.

Picoplanktonic members of several major algal groups, such as heterokonts, haptophytes, and prasinophytes, have been reported from oceanic environments (e.g. Booth and Marchant 1987; Guillou et al. 1999, 2004). The present study demonstrates for the first time that chlorarachniophytes are present in pelagic picoplankton, suggesting that parallel evolution of cell volume-reduction has occurred recurrently in distantly related lineages.

Benthic members of chlorarachniophytes are known to be distributed mainly in the temperate and tropical coastal regions (e.g. Calderon-Saenz and Schnetter 1987; Hibberd and Norris 1984; Ishida and Hara 1994; Ishida et al. 2007). However, little is known about the distribution of planktonic members. Planktonic taxa have only been described to date the Sargasso Sea and the Mediterranean Sea (Moestrup and Sengco 2001; Ota et al. 2007b; this study). Recently, several chlorarachniophyte SSU rDNA sequences have also been detected from picoplanktonic size fractions of natural sea water samples (e.g. Massana et al. 2004; Medlin et al. 2006; Not et al. 2007). These recent studies suggested that picoplanktonic chlorarachniophytes are distributed at least in three oceanic regions (the Mediterranean, Sargasso and North Seas). In Blanes Bay (NW Mediterranean), two possible chlorarachniophyte SSU rDNA sequences (BL000921.31 and BL010625.12) were identified (Massana et al. 2004). These chlorarachniophyte sequences shared more than 99.5% identity with a SSU rDNA sequence from the Sargasso Sea (SSRPE06) (Not et al. 2007). Those sequences were, however, distinct from known chlorarachniophyte sequences from cultured strains, including strain RCC365 (RCC365 and the Mediterranean sequences share only 87% identity as determined by BLAST search). In addition to the Mediterranean and the Sargasso Sea sequences, Medlin et al. (2006) found two chlorarachniophyte-like sequences from picoplankton samples at Helgoland (German Bight, North Sea). These sequences (env.part.He000803.100 and env.part. He000803.100) are relatively distinct from each other (90% identities), so that they might correspond to different taxa. During the PROSOPE

cruise where RCC365 was isolated, a large number of chlorarachniophyte sequences have been recovered from picoplankton samples taken at different stations (M. Viprey, pers. comm.). The diversity of these chlorarachniophyte environmental sequences suggests that more undescribed picoplanktonic chlorarachniophytes do exist in marine environments.



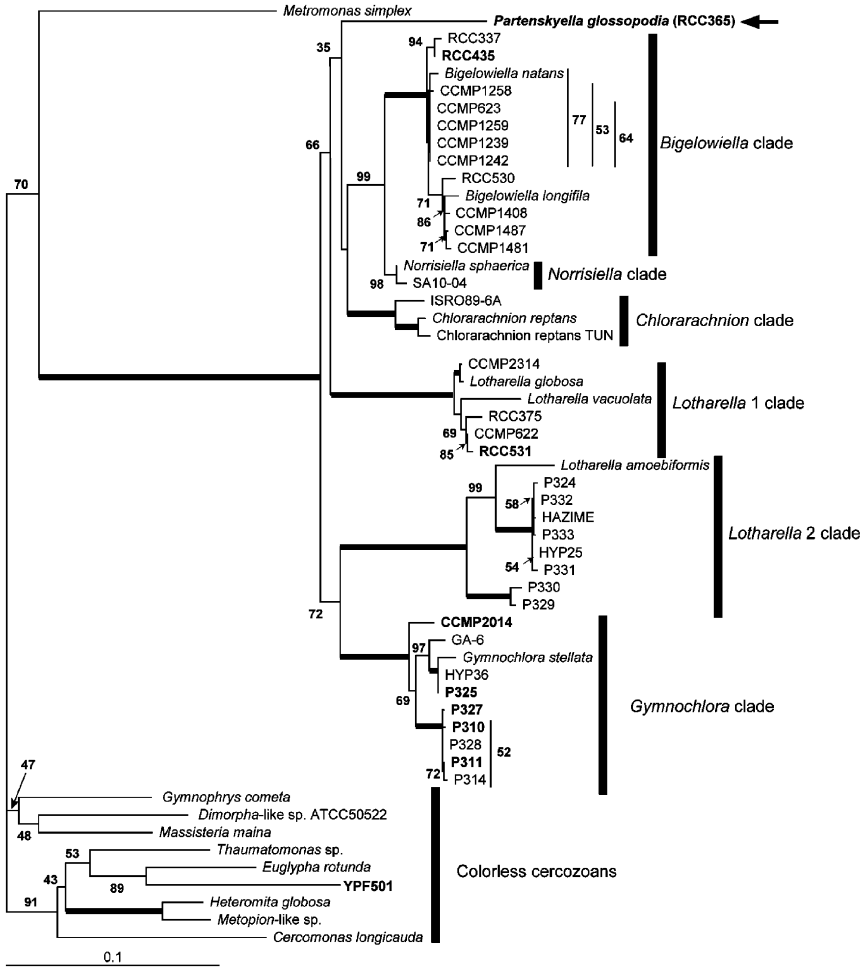


Figure 8. Maximum likelihood tree inferred using SSU rDNA sequences of chlorarachniophytes and colorless cercozoans. The bootstrap values (>50%) obtained by 100 pseudo-replicates re-sampled from the original data are shown at nodes or right of OTUs. Branches with 100% bootstrap supports are shown in thick lines. New sequences are shown in bold. Sequence for *P. glossopodia* was indicated by an arrow.

Methods

Sampling and culture condition: Strain RCC365 was isolated by F. Partensky from $<0.6\,\mu m$ filtered seawater collected at about 5 m depth in the Mediterranean Sea (Long. 15°37′E; Lat. 37°24′N) on 26 September 1999 during the PROSOPE cruise. The strain was purified using a dilution method and deposited to the RCC (http://www.sb-roscoff.fr/Phyto/RCC/). The culture was grown in glass tubes or 50 mL polystyrene tissue culture flasks (Asahi Technoglass, Tokyo,

Figure 7. Transmission electron micrographs of *Partenskyella glossopodia* gen. et sp. nov. **A.** Detailed profile of a nucleomorph (Nm), surrounded by double-membranes. The arrows indicate the outer two chloroplast membranes and the arrowheads indicate the inner two chloroplast membranes. **B.** Nucleomorph (Nm) having several electron-opaque globules (arrowheads). The arrows indicate pores of the nucleomorph membranes. **C.** A single Golgi body (G) is present near the chloroplast. **D.** Section of a walled spherical cell, showing the cell wall. The arrowhead indicates the transverse section of a basal body near the nucleus. The boxed area is enlarged in E. **E.** Higher magnification of cell wall (arrows). Arrowhead indicates a vesicle containing electron-opaque material just beneath the cell wall. **F.** Typical vegetative cell having several vesicles containing storage product-like materials (S) in parietal region of the cell. **G.** Longitudinal section of an extrusome-like organelle. **H.** The flagellar transition region in longitudinal section. Arrow indicates the electron-opaque plates located near the proximal of the axoneme microtubules. **I.** Whole-mount flagellate cell, showing a terminal hair point (arrows).

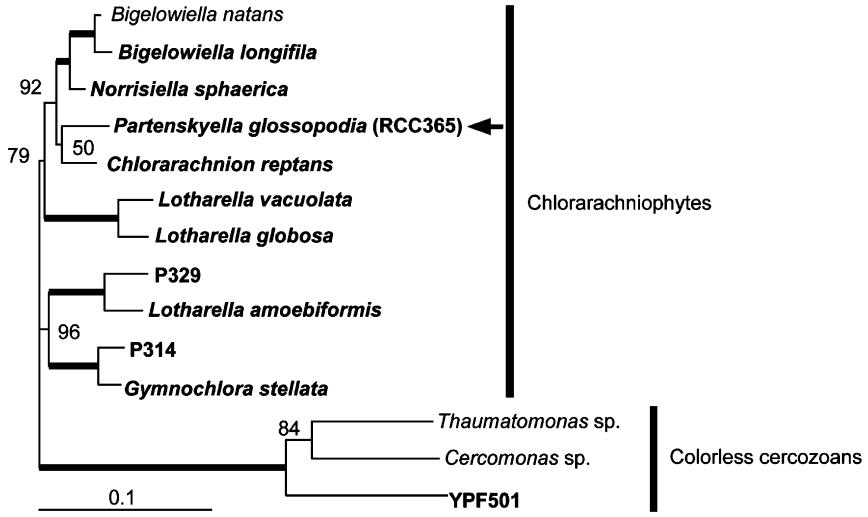


Figure 9. Maximum likelihood tree inferred using concatenated data set (SSU+LSU rDNA sequences) of chlorarachniophytes and colorless cercozoans. The bootstrap values (>50%) obtained by 100 pseudo-replicates re-sampled from the original data are shown at nodes. Branches with 100% bootstrap supports are shown in thick lines. New sequences (LSU DNA) are shown in bold. Sequence for *P. glossopodia* is indicated by an arrow.

Japan) containing 10—20 mL ESM (Kasai et al. 2004), K (Keller et al. 1987) or IMK (Wako Pure Chemical Industries, Ltd., Osaka, Japan) media, and incubated at 20 °C with 14:10 light:dark cycle under 20—40 μmol photons $m^{-2} s^{-1}$ cool-white illumination.

Light- and time-lapse video microscopy: For light microscopy, living or fixed cells were observed under Nomarski differential interference contrast optics using a Leica DMR microscope (Leica, Wetzlar, Germany). For time-lapse video microscopy, cells were grown for several days on a small, sterile coverslip (e.g., 18 × 18 mm) placed in a culture dish. Just before observation, the coverslip was placed on a slide grass and a large coverslip (e.g., 24 × 24 mm) was placed over the small coverslip. To reduce evaporation, the edges of the large coverslip were sealed with 'VALAP', a 1:1:1 mixture of paraffin wax, lanolin and Vaseline (Pickett-Heaps and West 1998). The cells were examined with Nomarski differential interference contrast optics using the Leica DMLB microscope or a Nikon Optiphot microscope (Nikon, Tokyo, Japan). Video images were taken with a color 3CCD camera (model:QIC-CLR-12, QImaging, British Columbia, Canada).

Transmission electron microscopy: Cells were slowly mixed with a fixative solution containing 2.5% glutaraldehyde and 0.25 M sucrose in 0.1 M sodium cacodylate buffer (pH 7.2). The cells were fixed 2 h at room temperature, followed by centrifugation to concentrate the cells into a pellet. The supernatant was removed, and the cells were washed six times (5 min each) with 0.1 M sodium cacodylate buffer with reduced sucrose (0.25, 0.1, and 0.05 M). After removal of the supernatant, the pellet was postfixed in 0.5 or 1% osmium tetroxide for 2 h at 4 °C. The pellet was rinsed twice with the same buffer (0.1 and 0.05 M sodium cacodylate), and then rinsed once with Milli-Q water, and dehydrated through a graded ethanol series (20%, 40%, 60%, 80%, 95%, and 100% × 4; 15 min each) on ice. The cells were infiltrated with 1:2, 1:1, and 2:1 Spurr:ethanol resin (Polysciences, Inc., Warrington, PA, USA) for 1 h each, followed by incubation in 100% Spurr resin overnight at room temperature. The pellet was allowed to sink in a fresh resin and was then polymerized at 70 °C for 8—12 h. Ultrathin sections were cut on a Reichert Ultracut S ultramicrotome (Leica, Wien, Austria) using a diamond knife, and the sections were mounted on one-slot or mesh copper grids coated with polyvinyl Formvar films, and stained with uranyl acetate and lead citrate (Reynolds 1963). For whole mount preparations, cells were fixed for a few minutes with glutaraldehyde (1% final conc.) in 0.25 M sucrose/0.2 M cacodylate buffer (pH 7.2). Then the cells were mounted on copper mesh grids coated with polyvinyl Formvar films, and washed with the Milli-Q water. The cells were stained with uranyl acetate for about 30 s. Sections and whole mount preparations were observed using a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan) at 80 kV.

DNA extraction, PCR, and sequencing: The cells were collected from 300 mL of 2-weeks-old culture and total DNA was extracted using a CTAB protocol as described by Ishida et al. (1999). For polymerase chain reaction (PCR), approximately 50 ng of the total DNA was used as template. The conditions of PCR reactions were as described in Silver et al. (2007). The oligonucleotide primers used to amplify the SSU and LSU rDNA were Nu-SSU-F (5′-AACCTGGTTGATCCTGCCAG-3′) and Nu-SSU-R (5′-CYGCAGGTTCACCTACGGAA-3′) and Nu-LSU-F (5′-ACCCGCTGAAYTTAAGCATAT-3′) and Nu-LSU-R (5′-GGCTKAATCTCARYRGATCG-3′). Amplified products were gel-purified, cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) or the p-GEM® T-easy vector System (Promega KK, Tokyo, Japan) and sequenced with a DNA sequencer (ABI Prism 3137, Perkin Elmer Corp., UK), using the dye terminator method according to the manufacturer's instructions (Dye-Terminator Cycle sequencing Core Kit, Perkin Elmer Corp., UK).

Phylogenetic analyses: Accession numbers of SSU and LSU rDNA sequences used for the present phylogenetic analyses are listed in Table 2. The sequences were aligned

Table 1. Comparison of life cycle stages, morphology and ultrastructure among chlorarachniophyte genera.

	Chlorarachnion[a]	*Cryptochlora*[b]	*Lotharella*[c]	*Gymnochlora*[d]	*Bigelowiella*[e]	*Norrisiella*[f]	*Partenskyella*
Dominant vegetative stage	Amoeboid	Coccoid	Amoeboid/coccoid[a]	Amoeboid	Flagellate/amoeboid	Coccoid	Coccoid
Flagellate cell (zoospore)	Present	Present	Present	Absent	Present	Present	Present
Stage after flagellate stage	Amoeboid	Coccoid	Coccoid	—	Flagellate	Coccoid	Coccoid?
Reticulopodial colony	Present	Absent	Present or absent[g]	Absent	Absent	Absent	Absent
Pyrenoid	Present	Present	Present	Present	Present	Present	Absent
Pyrenoid ultrastructure	Nm-containing type[h]	Unknown	Deep slit type[i]	tubular invagination type[j]	Shallow slit type[k]	Shallow slit type[k]	—
Location of a nucleomorph	In the pyrenoid	Unknown	Near the pyrenoid base	Near the pyrenoid base	Near the pyrenoid base	Near the pyrenoid base	Inside base of the cup-like chloroplast

[a]Hibberd and Norris (1984).
[b]Beutlich and Schnetter (1993), Calderon-Saenz and Schnetter (1987).
[c]Dietz et al. (2003), Ishida and Hara (1994), Ishida et al. (2000), Ota et al. (2005).
[d]Ishida et al. (1996).
[e]Moestrup and Sengco (2001), Ota et al. (2007a).
[f]Ota et al. (2007b).
[g]*L. globosa* and *L. amoebiformis* do not form the reticulopodial colony.
[h]Pyrenoid matrix is invaded deeply by periplastidial compartment, and a nucleomorph is located in the invagination of the pyrenoid.
[i]Pyrenoid matrix is divided longitudinally into two halves by an invagination of the inner two of four chloroplast membranes.
[j]Pyrenoid matrix possesses many tubular invaginations of an innermost chloroplast membrane.
[k]Pyrenoid matrix is invaded longitudinally by shallow plate-like periplastidial compartment.

Table 2. GenBank accession numbers used for phylogenetic analyses of nuclear (Nu) SSU rDNA and LSU rDNA.

Species/strains	Nu-SSU	Nu-LSU
Norrisiella sphaerica	AF076172	AB453003
Bigelowiella longifila	U03479	AB453004
Bigelowiella natans	AF054832	AF289036
CCMP1239	AF076173	—
CCMP1242	EF622541	—
CCMP1258	AF076174	—
CCMP1259	EF622542	—
CCMP1408	U02075	—
CCMP1481	EF622543	—
CCMP1487	EF622544	—
CCMP2014	AB453013	—
CCMP2314	EF622545	—
CCMP622	EF622538	—
CCMP623	EF622540	—
Chlorarachnion reptans	U03477	AB453005
Chlorarachnion reptans TUN	X70809	—
GA-6	EF622546	—
Gymnochlora stellata	AF076171	AB453006
Lotharella amoebiformis	AF176170	AB453007
Lotharella globosa	AF076169	AB453008
Lotharella vacuolata	AF076168	AB453009
P310	AB452996	—
P311	AB452997	—
P314	EF622547	AB453014
P325	AB452998	—
P327	AB452999	—
P329	EF622548	AB453010
P333	EF622549	—
RCC435	AB453000	—
RCC337	EF622558	—
RCC365 (*Partenskyella glossopodia*)	AB452995	AB453011
RCC375	EF622539	—
RCC531	AB453001	—
YFP501	AB453002	AB453012
Cercomonas longicauda	AF411270	—
Cercomonas sp.	—	DQ386165
Euglypha rotunda	X77692	—
Gymnophrys cometa	AF411284	—
Heteromita globosa	U42447	—
Massisteria marina	AF411286	—
Metromonas simplex	AY620254	—
Paracercomonas marina	—	DQ386164
Thaumatomonas sp.	AF411260	DQ980477
Dimorpha-like sp. ATCC50522	AF411283	—
Metopion-like sp.	AF411278	—

automatically using CLUSTAL X version 1.83 (Thompson et al. 1997). The alignments were manually edited using the MacClade 4.01 (Maddison and Maddison 2003). The concatenated data set of SSU and LSU rDNA sequences was created manually. For the analyses of SSU and SSU+LSU rDNA sequences, 1595 and 4372 unambiguously aligned nucleotide positions were used, respectively. Because the LSU rDNA sequence of *Cercomonas longicauda* was not available, we alternatively used the LSU rDNA sequence of *Cercomonas* sp. (DQ386165) in the concatenated analysis. Maximum Likelihood (ML) analyses were conducted on the both datasets using RAxML (Stamatakis 2006) with GTRMIX model. For the RAxML analyses, the numbers of rate categories was set to 25, and the best tree was searched for from ten randomized maximum parsimony trees, and the rapid hill-climbing algorithm were used. Statistical support for the resulting topologies was assessed by bootstrapping with 100 pseudo-replicates re-sampled from the original data. In the bootstrap analyses, GTRCAT model with 25 categories was applied.

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References

Adl SM, Simpson AGB, Farmer MA, Andersen RA, Anderson OR, Barta JR, Bowser SS, Brugerolle G, Fensome RA, Fredericq S, James TY, Karpov S, Kugrens P, Krug J, Lane CE, Lewis LA, Lodge J, Lynn DH, Mann DG, McCourt RM, Mendoza L, Moestrup Ø, Mozley-Standridge SE, Nerad TA, Shearer CA, Smirnov AV, Spiegel FW, Taylor FJR (2005) The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. J Eukaryot Microbiol **52**: 399—451

Archibald JM (2007) Nucleomorph genomes: structure, function, origin and evolution. BioEssays **29**: 392—402

Bass D, Moreira D, López-García P, Polet S, Chao EE, von der Heyden S, Pawlowski J, Cavalier-Smith T (2005)

Polyubiquitin insertions and the phylogeny of Cercozoa and Rhizaria. Protist **156**: 149—161

Beutlich A, Schnetter R (1993) The life cycle of *Cryptochlora perforans* (Chlorarachniophyta). Bot Acta **106**: 441—447

Booth BC, Marchant HJ (1987) Parmales, a new order of marine chrysophytes, with descriptions of three new genera and seven new species. J Phycol **23**: 245—260

Calderon-Saenz E, Schnetter R (1987) *Cryptochlora perforans*, a new genus and species of algae (Chlorarachniophyta), capable of penetrating dead algal filaments. Pl Syst Evol **158**: 69—71

Cavalier-Smith T (2002) The phagotrophic origin of eukaryotes and phylogenetic classification of Protozoa. Int J Syst Evol Microbiol **52**: 297—354

Cavalier-Smith T, Chao EE (2003) Phylogeny and classification of phylum Cercozoa (Protozoa). Protist **154**: 341—358

Dietz C, Ehlers K, Wilhelm C, Gil-Rodríguez MC, Schnetter R (2003) *Lotharella polymorpha* sp. nov. (Chlorarachniophyta) from the coast of Portugal. Phycologia **42**: 582—593

Geitler L (1930) Ein grünes Filarplasmodium und andere neue Protisten. Arch Protistenkd **69**: 615—636

Gilson PR, McFadden GI (1999) Molecular, morphological and phylogenetic characterization of six chlorarachniophyte strains. Phycol Res **47**: 7—19

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

Guillou L, Eikrem W, Chrétiennot-Dinet M-J, Gall FL, Massana R, Romari K, Pedrós-Alió D, Vaulot D (2004) Diversity of picoplanktonic prasinophytes assessed by direct nuclear SSU rDNA sequencing of environmental samples and novel isolates retrieved from oceanic and coastal marine ecosystems. Protist **155**: 193—214

Hibberd DJ, Norris RE (1984) Cytology and ultrastructure of *Chlorarachnion reptans* (Chlorarachniophyta divisio nova, Chlorarachniophyceae classis nova). J Phycol **20**: 310—330

Ishida K, Hara Y (1994) Taxonomic studies on the Chlorarachniophyta. I. *Chlorarachnion globosum* sp. nov. Phycologia **33**: 351—358

Ishida K, Green BR, Cavalier-Smith T (1999) Diversification of a chimaeric algal group, the chlorarachniophytes: phylogeny of nuclear and nucleomorph small-subunit rRNA genes. Mol Biol Evol **16**: 321—331

Ishida K, Ishida N, Hara Y (2000) *Lotharella amoeboformis* sp. nov.: a new species of chlorarachniophytes from Japan. Phycol Res **48**: 221—229

Ishida K, Nakayama T, Hara Y (1996) Taxonomic studies on the Chlorarachniophyta. II. Generic delimitation of the chlorarachniophytes and description of *Gymnochlora stellata* gen. et sp. nov. and *Lotharella* gen. nov. Phycol Res **44**: 37—45

Ishida K, Yabuki A, Ota S (2007) The Chlorarachniophytes: Evolution and Classification. In Brodie J, Lewis J (eds) Unravelling the Algae the Past, Present, and Future of Algal Systematics. CRC Press, Taylor & Francis Group, Boca Raton, FL, pp 171—182

Kasai F, Kawachi M, Erata M, Watanabe MM (2004) NIES-Collection List of Strains: Microalgae and Protozoa, 7th Ed. The Microbial Culture Collection, The National Institute for Environmental Studies, Tsukuba, p 54

Keeling PJ, Deane JA, McFadden GI (1998) The phylogenetic position of alpha- and beta-tubulins from the *Chlorarachnion* host and *Cercomonas* (Cercozoa). J Eukaryot Microbiol **45**: 561—570

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

Ludwig M, Gibbs SP (1989) Evidence that the nucleomorphs of *Chlorarachnion reptans* (Chlorarachniophyceae) are vestigial nuclei: morphology, division and DNA-DAPI fluorescence. J Phycol **25**: 385—394

Maddison DR, Maddison WP (2003) MacClade 4. Sinauer Associates, Inc. Publishers, Sunderland, MA

Massana R, Balagué V, Guillou L, Pedrós-Alió C (2004) Picoeukaryotic diversity in an oligotrophic coastal site studied by molecular and culturing approaches. FEMS Microbiol Ecol **50**: 231—243

McFadden GI, Gilson PR, Hofmann CJB, Adcock GJ, Maier U-G (1994) Evidence that an amoeba acquired a chloroplast by relating part of an engulfed eukaryotic alga. Proc Natl Acad Sci USA **91**: 3690—3694

Medlin LK, Metfies K, Mehl H, Wiltshire K, Valentin K (2006) Picoeukaryotic plankton diversity at the Helgoland time series site as assessed by three molecular methods. Microb Ecol **52**: 53—71

Moestrup Ø, Sengco M (2001) Ultrastructural studies on *Bigelowiella natans*, gen. et sp. nov., a chlorarachniophyte flagellate. J Phycol **37**: 624—646

Not F, Gausling R, Azam F, Heidelberg JF, Worden AZ (2007) Vertical distribution of picoeukaryotic diversity in the Sargasso Sea. Environ Microbiol **9**: 1233—1252

Ota S, Ueda K, Ishida K (2005) *Lotharella vacuolata* sp. nov., a new species of chlorarachniophyte algae, and time-lapse video observations on its unique post-cell division behavior. Phycol Res **53**: 275—286

Ota S, Ueda K, Ishida K (2007a) Taxonomic study of *Bigelowiella longifila* sp. nov. (Chlorarachniophyta) and a time-lapse video observation on the unique migration of amoeboid cells. J Phycol **43**: 333—343

Ota S, Ueda K, Ishida K (2007b) *Norrisiella sphaerica* gen. et sp. nov., a new coccoid chlorarachniophyte from Baja California, Mexico. J Plant Res **120**: 661—670

Pickett-Heaps JD, West J (1998) Time-lapse video observations on sexual plasmogamy in the red alga *Bostrychia*. Eur J Phycol **33**: 43—56

Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol **17**: 208—212

Silver TD, Koike S, Yabuki A, Kofuji R, Archibald JM, Ishida K (2007) Phylogeny and nucleomorph karyotype diversity of chlorarachniophyte algae. J Eukaryot Microbiol **54**: 403—410

Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics **21**: 2688—2690

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res **25**: 4876—4882

Van de Peer Y, Rensing SA, Maier U-G, De Wachter R (1996) Substitution rate calibration of small subunit ribosomal RNA identifies chlorarachniophyte endosymbionts as remnants of green algae. Proc Natl Acad Sci USA **93**: 7732—7736

Vaulot D, Gall FL, Marie D, Guillou L, Partensky F (2004) The Roscoff Culture Collection (RCC): a collection dedicated to marine picoplankton. Nova Hedwigia **79**: 49—70