Protist, Vol. 163, 91–104, January 2012
http://www.elsevier.de/protis
Published online date 15 April 2011

Protist

ORIGINAL PAPER

Lotharella reticulosa sp. nov.: A Highly Reticulated Network Forming Chlorarachniophyte from the Mediterranean Sea

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Submitted December 1, 2010; Accepted February 16, 2011
Monitoring Editor: Michael Melkonian

A new chlorarachniophyte *Lotharella reticulosa* sp. nov. is described from a culture isolated from the Mediterranean Sea. This strain is maintained as strain RCC375 at the Roscoff Culture Collection, France. This species presents a multiphasic life cycle: vegetative cells of this species were observed to be coccoid, but amoeboid cells with filopodia and globular suspended cells were also present in the life cycle, both of which were not dominant phases. Flagellate cells were also observed but remained very rare in culture. The vegetative cells were 9-16 μm in diameter and highly vacuolated, containing several green chloroplasts with a projecting pyrenoid, mitochondria, and a nucleus. The chloroplast was surrounded by four membranes possessing a nucleomorph in the periplastidial compartment near the pyrenoid base. According to ultrastructural observations of the pyrenoid and nucleomorph, the present species belongs to the genus *Lotharella* in the phylum Chlorarachniophyta. This taxonomic placement is consistent with the molecular phylogenetic trees of the 18S rRNA gene and ITS sequences. This species showed a unique colonization pattern. Clusters of cells extended cytoplasmic strands radially. Then, amoeboid cells being born proximately moved distally along the cytoplasmic strand like on a "railway track". Subsequently the amoeboid cell became coccoid near the strand. In this way, daughter cells were dispersed evenly on the substratum. We also observed that the present species regularly formed a structure of filopodial nodes in mid-stage and later-stage cultures, which is a novel phenotype in chlorarachniophytes. The unique colonization pattern and other unique features demonstrate that RCC375 is a new chlorarachniophyte belonging to genus *Lotharella*, which we describe as *Lotharella reticulosa* sp. nov.

Key words: chlorarachniophytes; *Lotharella*; Mediterranean Sea; RCC375; RCC376; taxonomy.

Introduction

Chlorarachniophytes are a small algal group of autotrophic or mixotrophic marine coccoid, flagellate, and amoeboid cells with green plastids (e.g.,

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doi:10.1016/j.protis.2011.02.004

Ishida et al. 2007). Under the current classification system, twelve species and eight genera have been described, all classified in the Chlorarachniophyceae (Calderon-Saenz and Schnetter 1987; Dietz et al. 2003; Geitler 1930; Hibberd and Norris 1984; Ishida et al. 1994, 1996, 2000, 2011; Moestrup and Sengco 2001; Ota et al. 2005, 2007a, 2007b, 2009a, 2009b), and a thirteenth species will be added soon (Ota et al. 2011). Molecular phylogenetic studies have shown that chlorarachniophytes belong to Rhizaria, which include foraminiferans, radiolarians, and cercozoans, most of which are heterotrophic (e.g., Bass et al. 2005; Burki et al. 2010; Cavalier-Smith and Chao 2003; Keeling 2001). Chlorarachniophytes, however, acquired photosynthesis secondarily and possess four-membrane chloroplasts with a nucleomorph, i.e. a reduced algal endosymbiont nucleus that resides in the periplastidial compartment (Hibberd and Norris 1984; Ludwig and Gibbs 1989; McFadden et al. 1994). Recent phylogenetic studies suggest that the host components of chlorarachniophytes are closely related to cercozoans, notably Metromonadea (Filosa) as suggested by Bass et al. (2009) and the secondary plastids originated from a green alga (e.g., Ishida et al. 1999; Rogers et al. 2007; Takahashi et al. 2007).

The generic delimitation of the chlorarachniophytes, except for *Cryptochlora* (Calderon-Saenz and Schnetter 1987) for which ultrastructural data are unavailable, is defined by a set of ultrastructural and life cycle characteristics as follows: (1) ultrastructure of pyrenoid, (2) nucleomorph position, and (3) dominant cell-type stage (Ishida et al. 1996, 2011; Ota et al. 2007b, 2009b). The life cycle patterns are mainly used as specific diagnostic features (Dietz et al. 2003; Ota et al. 2005, 2007a, 2009b).

The Roscoff Culture Collection (RCC) contains several chlorarachniophyte cultures (currently ca. 10 strains), most of which were collected from the Mediterranean Sea during the PROSOPE (PROductivité des Systèmes Océaniques PElagiques) cruise in 1999. These strains are mostly uncharacterized and their taxonomic positions are not known. Strain RCC365 was recently described as *Partenskyella glossopodia*, which is the first picoplanktonic chloarachniophyte (Ota et al. 2009b). Here we provide morphological and ultrastructural characterization of another strain, RCC375, using light and transmission electron microscopy, and formally describe it as a new chlorarachniophyte species, *Lotharella reticulosa* sp. nov. We also determined nucleomorph internal transcribed spacer (ITS) sequences of RCC375 and RCC376 as DNA barcoding in accordance with Gile et al. (2010).

Results

Morphology

Coccoid cells were the predominant life stage in the culture (Fig. 1A-C). The coccoid cells were 9-16 μm in diameter (mean = 13.2 μm, $n = 30$), settle onto the bottom of a culture vessel, and were not easily re-suspended after settlement. The coccoid cells usually possessed a single large vacuole, but non-vacuolated cells were also observed in culture (Fig. 1A). Each cell had two to eight, or sometimes more, green bilobed chloroplasts and each chloroplast possessed a projecting pyrenoid that was located between the chloroplast lobes (Fig. 1B). In early or old stage cultures, chloroplasts lacking pyrenoids were sometimes observed (Fig. 1A). The chloroplasts were located in the parietal region of the cell (Fig. 1B). Occasionally, an aperture in the lorica (cell wall) was observed through which some cells extended their filopodium (Fig. 1C). In middle or older aged cultures, the vegetative cell sometimes possessed a vacuole containing reddish particles (Supplementary Figure S1 for color micrograph). Some large cells (25-33 μm in diameter) occurred in old cultures; such cells always possessed red particles in a vacuole (Fig. 1E; Supplementary Figure S1 for color micrograph). Sometimes the large cells possessed one or more filopodia (Fig. 1F). Fluorescence microscopic observation showed that large cells usually possessed one nucleus and many chloroplasts (Fig. 1G, H). The nature of the cytoplasm in the large cells was similar to the normal vegetative cells or sometimes more granular. These large coccoid cells also settled onto the substratum like normal vegetative cells.

Amoeboid cells were observed in early and mid-stage of cultures (Fig. 1I-N). The amoeboid cells were solitary, naked, and variable in form, possessing several fine filopodia of varying length. Amoeboid body (8-11 μm in diameter when rounded; mean = 9.4, $n = 10$) was lanceolate to narrowly elliptic (Fig. 1I, J) when cells were migrating along the filopodia, or slightly polygonal, circular to roughly elliptical (Fig. 1K–N). We occasionally observed suspended globular cells (11-25 μm in diameter; mean = 19.2, $n = 9$) in mid or later stage cultures, which were never dominant (Fig. 1O-R). This cytoplasmic structure was different from the normal vegetative cell; cell cytoplasm

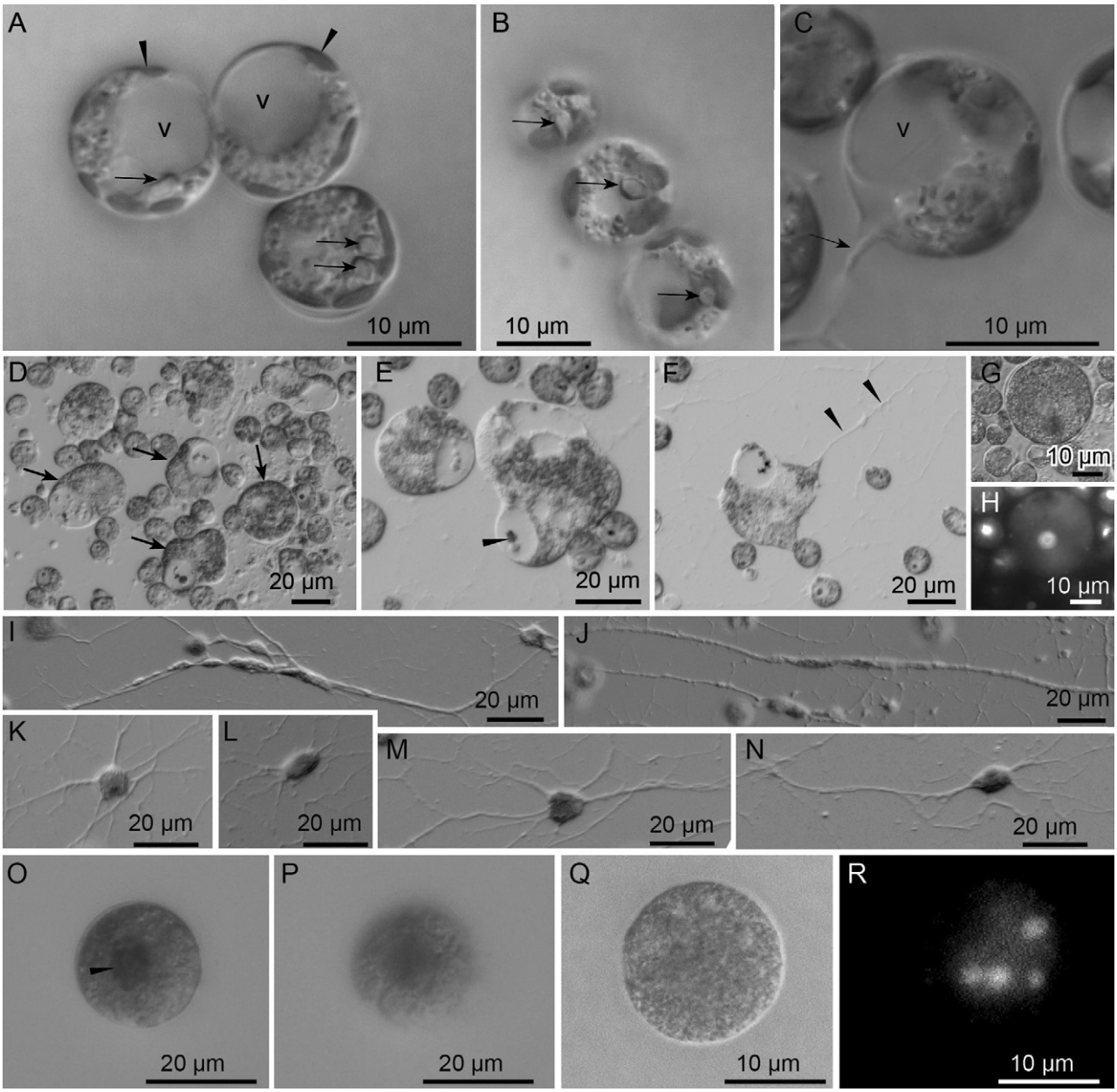

Figure 1. Light micrographs of *Lotharella reticulosa* sp. nov. **A**. Coccoid cells. Projecting pyrenoids (arrows) are visible. Arrowheads indicate chloroplasts lacking pyrenoids. **B**. Surface view of coccoid cells, showing bilobed chloroplasts possessing a projecting pyrenoid (arrows). **C**. Filopodium extending through an aperture of a lorica (arrow). **D**. Large cells (arrows). **E**. A large cell possessing a red particle (arrowhead). **F**. A large cell possessing a filopidium (arrowheads). **G, H**. Differential interference contrast and fluorescence microscopy images of the DAPI stained large cell. **I-N**. Amoeboid cells, showing varying shapes: narrowly elliptic/linear form (I, J) or slightly polygonal, circular to roughly elliptical (K–N). **O**. A globular cell. Arrowhead indicates reddish particles **P**. Surface view of the globular cell, showing cytoplasm is extremely granular and chloroplasts are located evenly. **Q, R**. Differential interference contrast and fluorescence microscopy images of the DAPI stained large coccoid cell, showing multinucleate. v = vacuole.

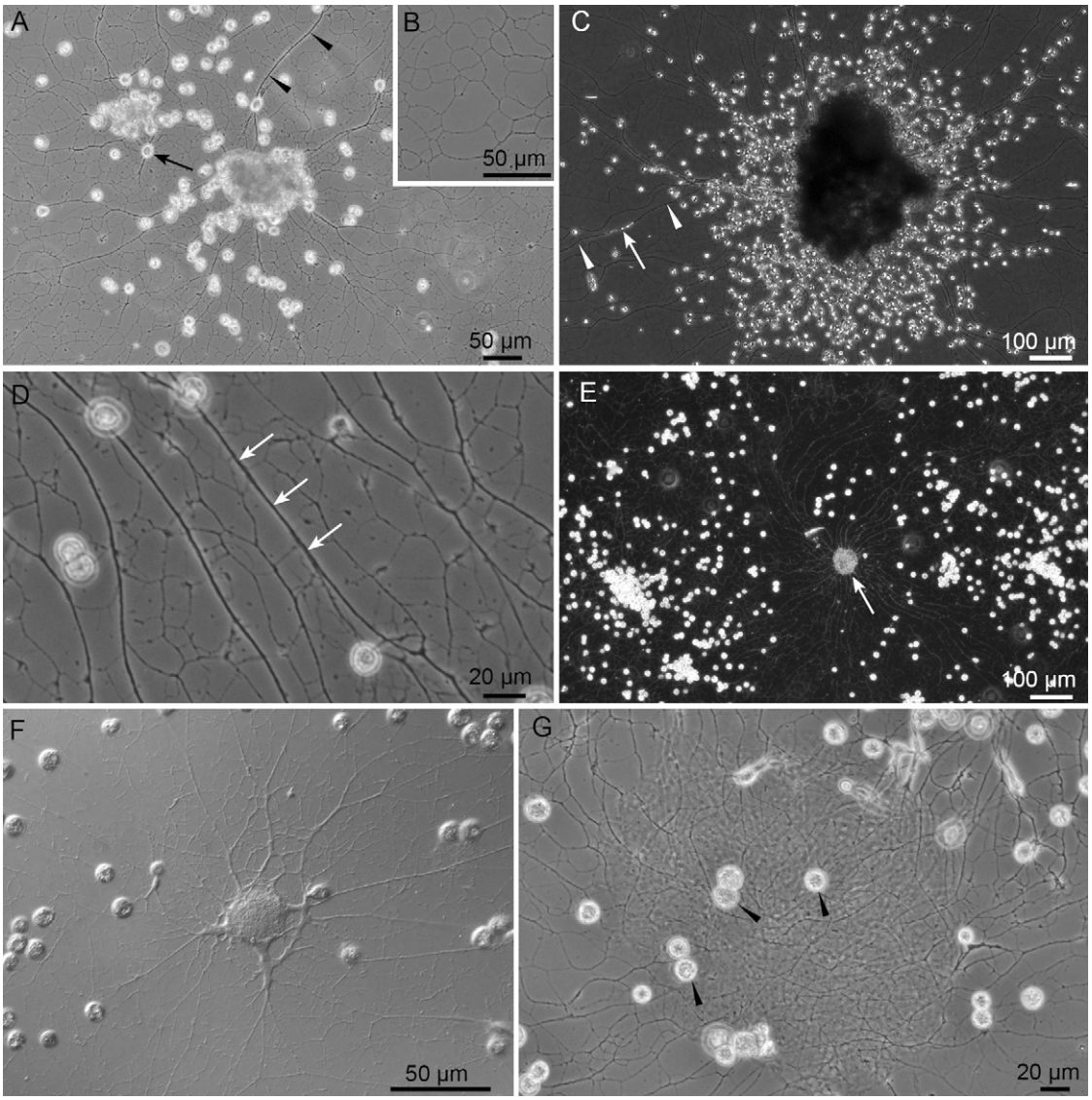

Figure 2. Light micrographs of *Lotharella reticulosa* sp. nov. **A**. Early-stage of cell cluster. Arrow indicates migrating amoeboid cell. Arrowheads indicate a cytoplasmic strand. **B**. High magnification view of reticulopodia. **C**. Mid-stage of cell cluster. Arrow indicates migrating amoeboid cell along the cytoplasmic strand (arrowheads). **D**. High magnification view of filopodia, showing a cytoplasmic strand (arrows). **E**. Low magnification image of a filopodial node (arrow). **F**. Mature filopodial node. **G**. High magnification view of a node, showing many filopodia and coccoid cells (arrowheads) in the node.

was extremely granular and the chloroplast was not located in the parietal regions, but distributed somewhat evenly throughout the cytoplasm, so that the cells were usually deep green (Fig. 1O, P; Supplementary Figure S1). We refer to this type of cell as "globular cells" in this article. Fluorescence microscopic observation showed that the cell was often multinucleate ($\leq$ 4 nuclei) (Fig. 1Q, R). The zoospore stage was very rare in the life cycle and could only be observed a few times during LM observations during the present work (Supplementary movie S2 represents a swimming zoospore.).

Lotharella reticulosa formed spider web-like colonies. A few days to one week after inoculation of an old culture into fresh medium, clusters of vegetative cells (colony) began to extend radially filopodia which strongly adhere to the substrate (Fig. 2A, B). The filopodia consisted of both radially extended pseudopodia originating from the cluster of coccoid cells (arrowheads in Fig. 2A, C) and fine reticulate pseudopodia (Fig. 2B). The radial

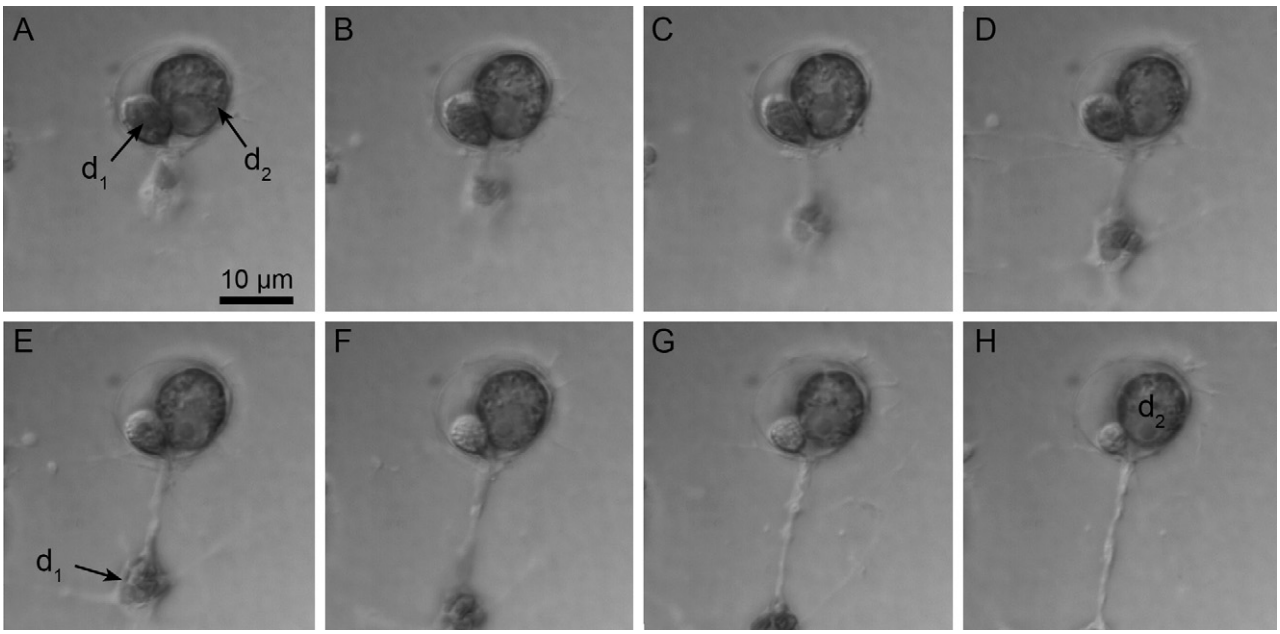

Figure 3. Time-lapse video sequence of post cell division of *Lotharella reticulosa* sp. nov. The time-lapse sequence is shown at 1 min intervals. One of the daughter cells (d_1) was transported outside the parental cell, and the other cell (d_2) remained in the parental lorica. Video movie of this sequence is available in the electronic Supplementary Material S3. d_1, d_2 = daughter cells.

extended filopodia were much thicker than the other reticulopodia and sometimes branched and anastomosed (Fig. 2D). We refer to the thick filopodia as "cytoplasmic strands" in this article.

At high cell density, cytoplasmic strands began to anastomose and converge, forming a filopodial node between the colonies (Fig. 2E-G). The filopodial node varied in size (25-140 µm in diameter) depending on the culture conditions and stages. Light and fluorescence microscopy observation showed that the node had neither nuclei nor chloroplasts (Fig. 2G; fluorescence micrograph not shown). The internal node was highly granular and its surface was covered by many filopodia (Fig. 2G). Some amoeboid cells (daughter cells) were carried along the filopodia and settled near the node

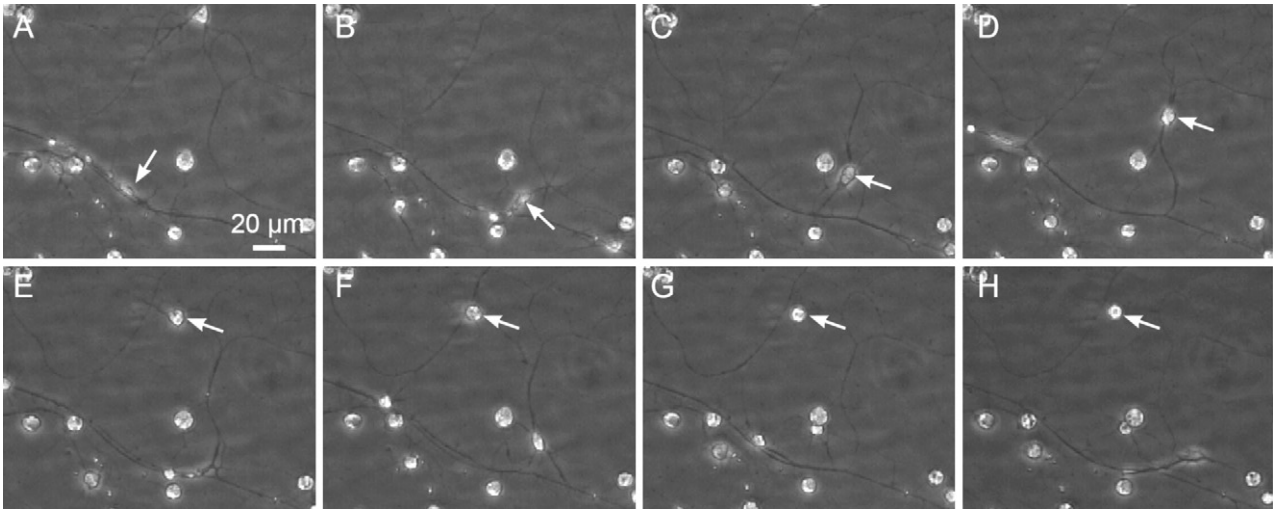

Figure 4. Time-lapse video sequence of *Lotharella reticulosa* sp. nov., showing that an amoeboid cell became a coccoid cell (arrows). The time-lapse sequence is shown at 8 min intervals and 16 min intervals in A-F and F-H, respectively. Video movie of this sequence is available in the electronic Supplementary Material S4.

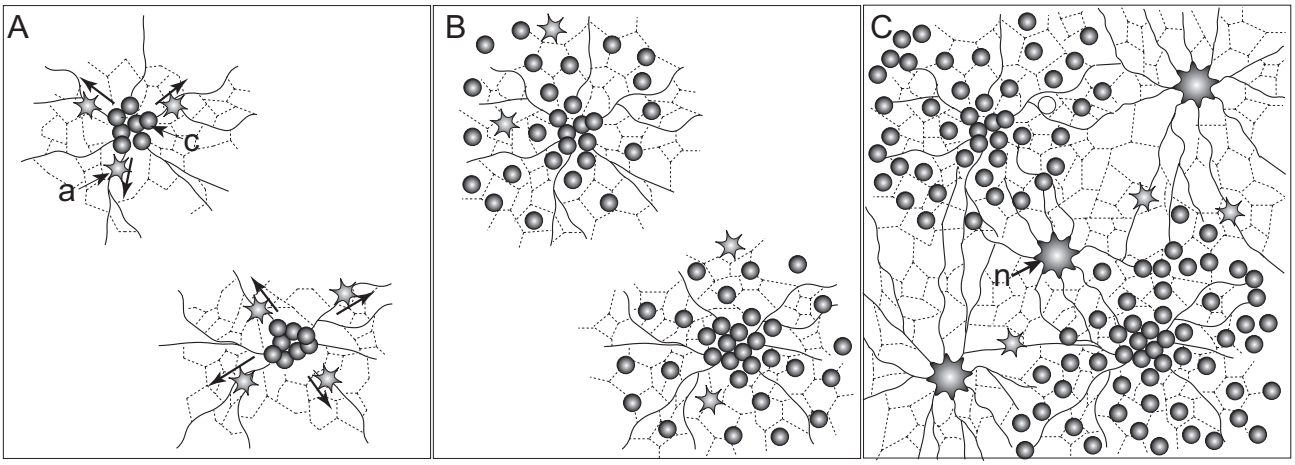

Figure 5. Illustration of the colonization behaviour in *Lotharella reticulosa* sp. nov. **A**. Early-stage of cell cluster. a = amoeboid cell, c = coccoid cell. Arrows indicate the direction of amoeboid movement. **B**. Mid-stage of cell clusters developing reticulate filopodia. Daughter cells were dispersed concentrically. Note that newly formed coccoid cells also produced amoeboid cells. **C**. Later-stage of cell cluster and nods (n) between the clusters.

as coccoid cells, when cell density became high (Fig. 2G).

Life Cycle

Time-lapse video-microscopy revealed that vegetative cells (coccoid cells) produced two different types of daughter cells: one coccoid cell and one amoeboid cell. After cytokinesis occurred within the lorica of a coccoid cell, one of the daughter cells extended a filopodium through an aperture of the lorica (Fig. 3A). Subsequently, organelles such as nucleus or chloroplasts, began to pass out of the parental lorica by transporting through a filopodium (Fig. 3A-D). After the transportation, a solitary amoeboid cell had therefore been produced which was then separated from the parental cell (Fig. 3E, F). The other daughter cell remained within the parental lorica (Fig. 3G, H). The amoeboid cell had several filopodia and displayed amoeboid locomotion on a substratum or along cytoplasmic strands.

After a few hours, the amoeboid cell withdraws its filopodia and became a coccoid cell identical to the normal vegetative cell (Fig. 4). This type of reproduction was always observed in the present species.

Large coccoid cells (Fig. 1D) were likely to be a stage equivalent to the vegetative cells. We could not observe directly switching between the vegetative and globular cell stages.

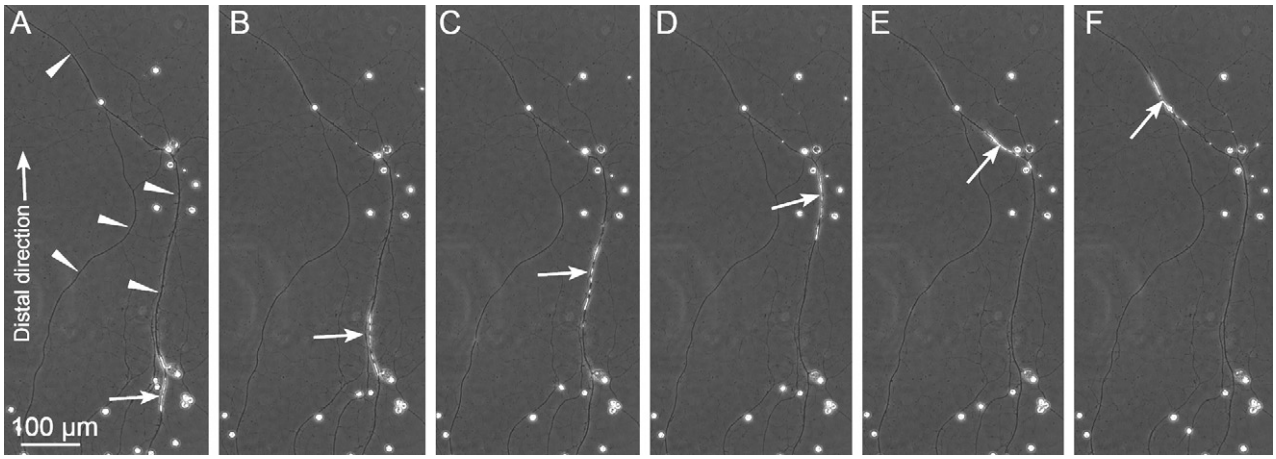

Figure 6. Time-lapse video sequence of a migrating amoeboid cell of *Lotharella reticulosa* sp. nov. Cytoplasmic strands are shown in arrowheads. The amoeboid cell (arrows) was migrating along the cytoplasmic strand toward the distal end. Video movie of this sequence is available in the electronic Supplementary Material S5.

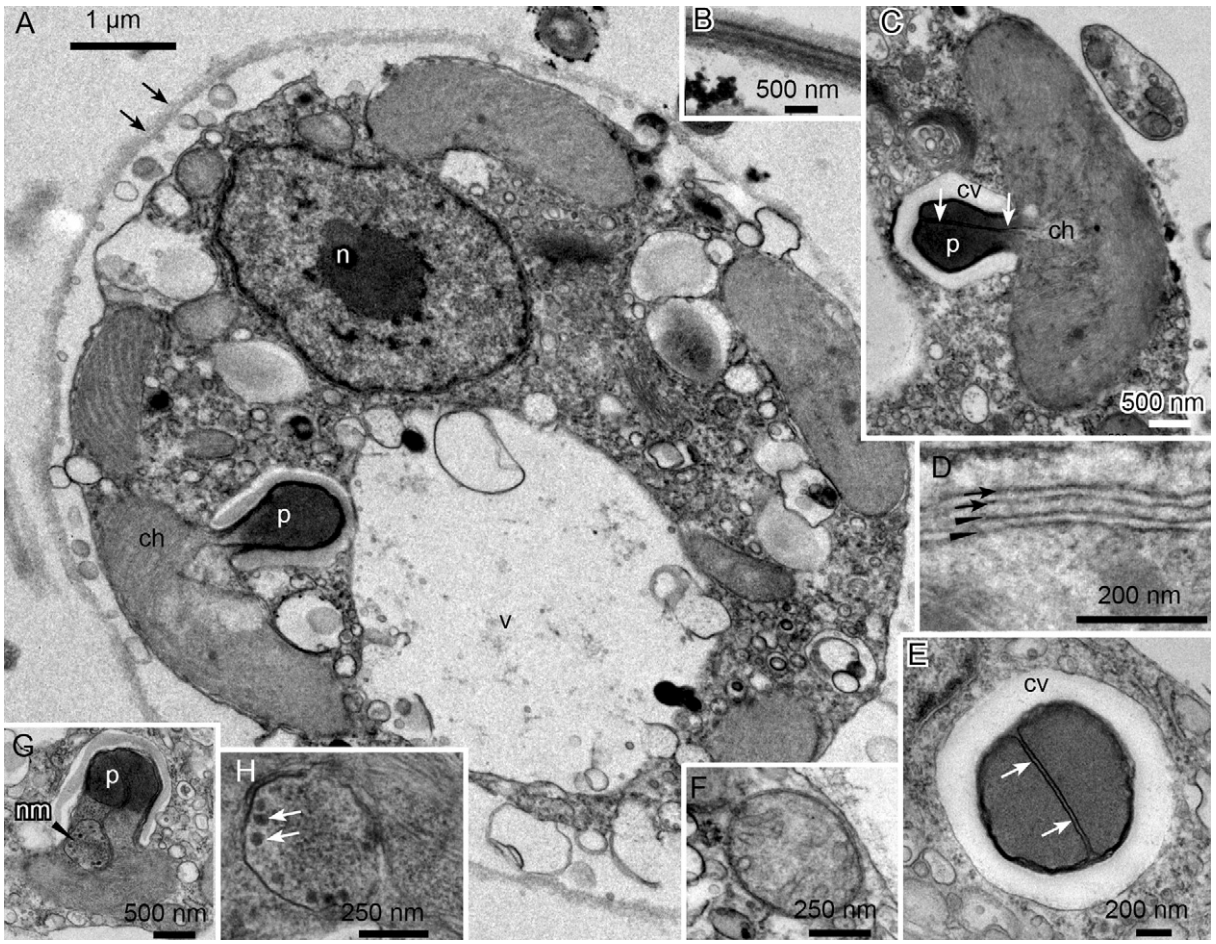

Figure 7. Transmission electron micrographs of *Lotharella reticulosa* sp. nov. **A**. General ultrastructure, showing nucleus (n), chloroplasts (ch) with a pyrenoid (p), lorica (arrows), and vacuole (v). **B**. Multi-layered loricae. **C**. Longitudinal section of chloroplast and pyrenoid (p), showing a deep slit of the periplastidial compartment (arrows). cv = capping vesicle. **D**. High magnification of chloroplast membranes showing two outer membranes (arrows) and two inner membranes (arrowheads). **E**. Transverse section of pyrenoid, showing a slit of the periplastidial compartment (arrows). The pyrenoid is covered by a capping vesicle (cv). **F**. Mitochondria with tubular cristae. **G**. Longitudinal section of chloroplast and pyrenoid (p), showing a nucleomorph (nm) located near the pyrenoid base. **H**. High magnification of the nucleomorph (nm). Small electron-dense globules (arrows) are visible in the parietal position of the nucleomorph.

Colonization Behaviour

The colonization behaviour is schematically illustrated in Figure 5. At the early stage of the culture, clusters of vegetative cells began to extend cytoplasmic strands radially (Fig. 5A). At the same time, an amoeboid daughter cell born from a coccoid cell began to migrate along the cytoplasmic strands toward the distal end of the strands (Fig. 6; Supplementary movie S5). The amoeboid cell got off the strand randomly, and migrated around the strand and became a coccoid cell near where it got off (Figs 4, 5B). The newly formed coccoid cells also produced amoeboid cells after cytokinesis. This colonization behavior caused daughter cells to be dispersed in a roughly concentric pattern (Fig. 5B). When cells density became high, cytoplasmic strands began to anastomose, forming filopodial nodes between the colonies (Fig. 5C).

Ultrastructure

General ultrastructure of a typical vegetative cell is shown in Figure 7A. A lorica was observed in many cases, which was either single-layered or multi-layered (Fig. 7A, B). Vegetative cells possessed a nucleus, chloroplasts, a few mitochondria, and a single large conspicuous vacuole in most cases

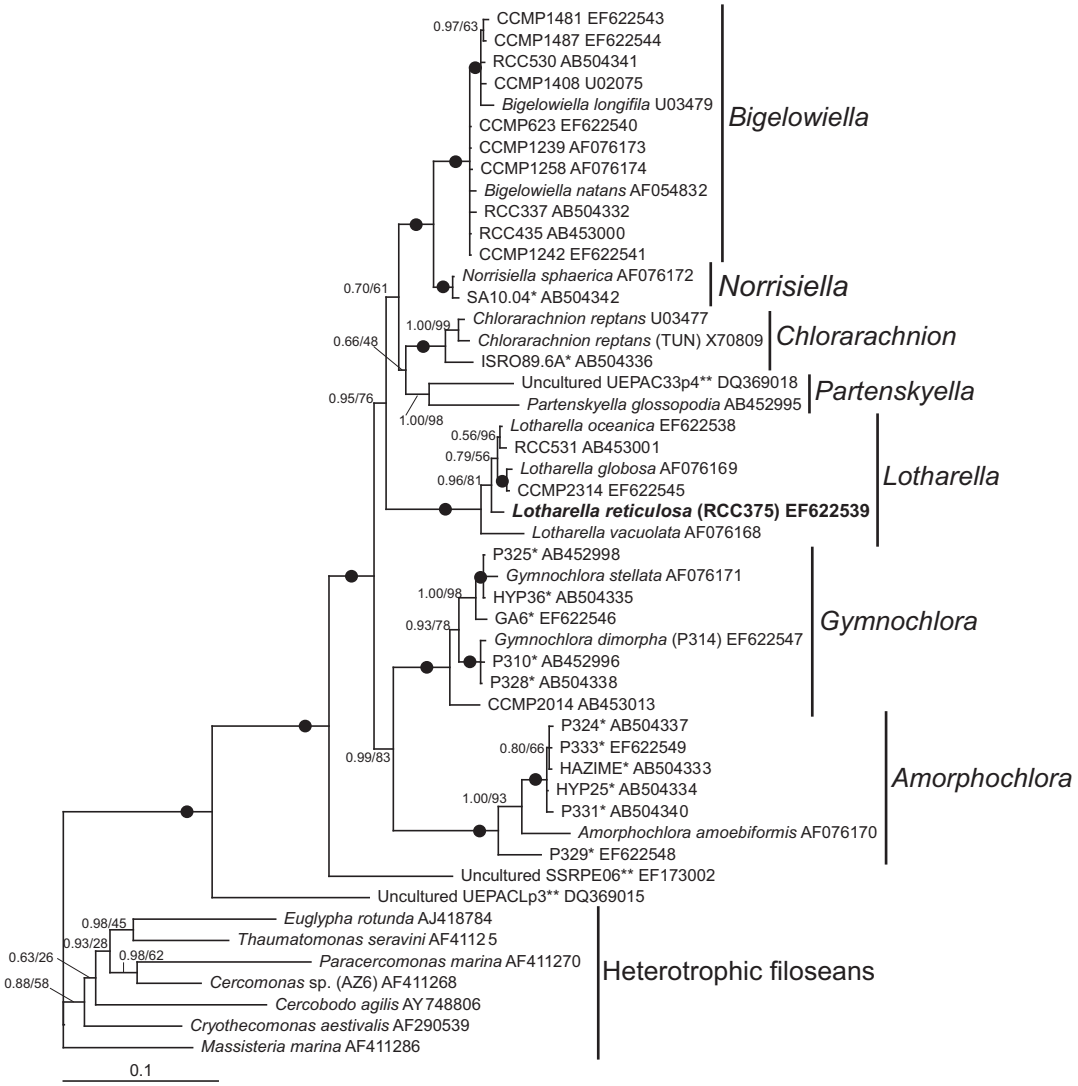

Figure 8. Bayesian phylogenetic tree of chlorarachniophytes based on 18S rRNA gene sequences (49 OTUs and 1,571 nucleotide positions). Support values are given as Bayesian posterior probabilities/ML bootstrap supports. Black dots represent support value of 1.00/100. Accession numbers are given after the species/strain names. The present species is shown in bold. An asterisk indicates strains from non-public cultures; double asterisks indicate environmental sequences.

(Fig. 7A). The nucleus was nearly spherical, positioned near the plasma membrane (Fig. 7A). The chloroplasts were located in the parietal region and surrounded by four membranes (Fig. 7A, C, D). The chloroplast possessed a projecting pyrenoid usually filled with electron dense material and covered by a capping vesicle (Fig. 7C, E). In transverse sections, the pyrenoid matrix was divided into two halves by a slit of the periplastidial compartment (i.e., the space between the second and the third chloroplast membrane) (Fig. 7E). A longitudinal section through the pyrenoid demonstrated that the slit reached near the pyrenoid base and this pyrenoid was thus referred as “a deep slit type” (Fig. 7C). Several mitochondria with tubular cristae were observed in the cytoplasm (Fig. 7F). A nucleomorph was located in the periplastidial compartment near the base of the pyrenoid (Fig. 7G). It was surrounded by double membranes and possessed a nucleolus-like region and several small electron-dense globules (Fig. 7H).

Molecular Phylogeny

Figure 8 represents an 18S rRNA phylogenetic tree of chlorarachniophytes including three related envi-

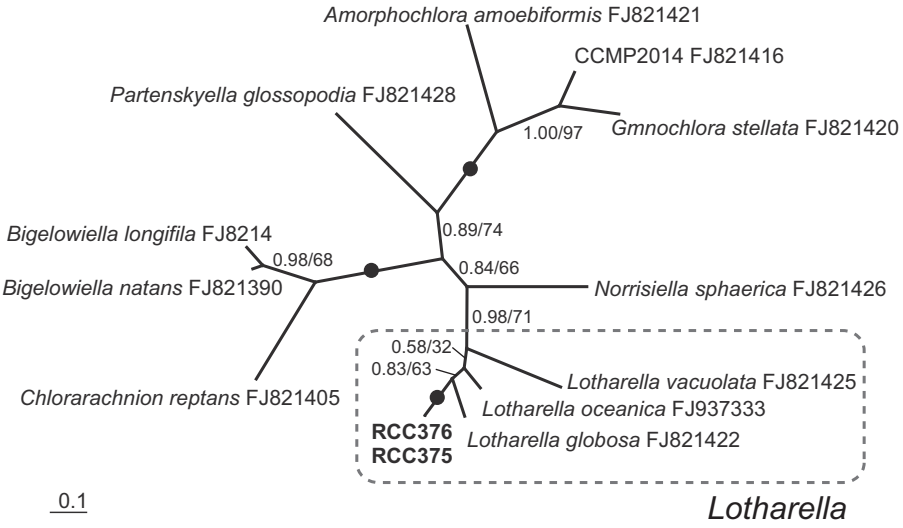

Figure 9. Unrooted Bayesian phylogenetic tree of chlorarachniophytes based on nucleomorph-encoded internal transcribed spacer sequences (13 OTUs and 342 nucleotide positions). Support values are given as Bayesian posterior probabilities/ML bootstrap supports. Black dots represent support value of 1.00/100. Accession numbers are given after the species/strain names.

ronmental sequences. In both maximum likelihood (ML) and Bayesian inferences, seven major clades corresponding to the known genera were recognized with high statistical supports (1.00 Bayesian posterior probabilities (PP); $\geq$ 98% ML bootstrap values). The present strain RCC375 was positioned within the *Lotharella* clade which was robustly recovered as a monophyletic group (1.00 PP; 100% ML bootstrap values).

Figure 9 represents a phylogenetic tree of chlorarachniophyte nucleomorph encoded ITS sequences. Strains RCC375/RCC376 had 100% identical nucleomorph encoded ITS sequence. In the present analyses, the monophyly of *Lotharella* clade was moderately supported (0.98 PP; 71% ML bootstrap values). Strains RCC375 and RCC376 were positioned within the *Lotharella* clade, and these strains were sister to *L. globosa*.

Discussion

Taxonomy

Based on the observation of morphology and ultrastructure, strain RCC375 should be classified in the genus *Lotharella*, as it shares diagnostic features that are specific to the genus, i.e.: (1) the chloroplast possessed a deep slit type pyrenoid, (2) the nucleomorph was located in the periplastidial compartment near the pyrenoid base, and (3) the vegetative cells were coccoid. This is consistent with the phylogenetic analyses of chlorarachniophytes; the strain RCC375 and close relatives, including the type species of the *Lotharella* (*L. globosa*), formed a monophyletic as *Lotharella* clade with high statistical support (1.00 PP; 100% ML bootstrap values in this 18S rRNA analyses).

At the specific level, however, *L. reticulosa* can be distinguished from all other *Lotharella* species (Table 1). Although the present species possessed a zoospore stage, zoospores were very rare in its life cycle. For the other *Lotharella* species, however, zoospores are more frequent in their life cycle. For example, zoospores of *L. oceanica* are definitely present at all cultures stages, but the amoeboid stage was never observed (Ota et al. 2009a), whereas in *L. vacuolata* or *L. globosa*, the zoospore stage occurs more rarely during their life cycle, but can always be observed in early stage cultures (Dietz et al. 2003; Ota et al. 2005). *L. reticulosa* seemed to be closely related to *L. globosa* or *L. oceanica* rather than *L. vacuolata* in the present phylogenetic analyses with statistically good support for 18S rRNA (0.96 PP; 81% ML bootstrap values) and moderate supports for ITS (0.83 PP; 61% ML bootstrap values). Taking into account the differences in the life cycle pattern and the phylogenetic positions, the proportion of the zoospore stage in the life cycle could be an apomorphic feature among the *Lotharella* species, implying that this life cycle phenotype might be used for species identification.

Furthermore, *L. reticulosa* regularly formed holoplasmodium-like nodes in mid- and later-

Table 1. Comparison of life cycle stages and ultrastructure in *Lotharella* species.

	Main vegetative stage	Multi-layered loricae	Amoeboid stage	Zoospore stage	Reticulopodial network	Filopodial nodes	References
L. globosa	Coccoid	Present	Absent	Present (+)	Absent	Absent	Ishida and Hara (1994)
L. polymorpha	Coccoid	Absent	Present*	Present (+)	Present	Absent	Ditz et al. (2003)
L. vacuolata	Coccoid	Present	Present	Present (+)	Present	Absent	Ota et al. (2005)
L. oceanica	Spherical**	Absent	Absent	Present (++)	Absent	Absent	Ota et al. (2009a)
L. reticulosa sp. nov.	Coccoid	Present	Present	Present (r)	Present	Present	This study

* *L. polymorpha* has two types of naked amoeboid cells; one is "migrating amoebae" like *L. amoebiformis* cells, and the other is "heliozoan-like cells". ** "Spherical cells" means non-walled and non-flagellated cells. "+" means that zoospores are observed only in early stage cultures; "++" means that zoospores are observed at almost all stages of cultures, "r" means that the zoospore stage is very rare.

stage cultures, which is a novel phenotype in chlorarachniophytes. Bass et al. (2009) describe haloplasmodium for cercozoans, which is similar to the structure observed in the present species. Based on fluorescence microscopy, the holoplasmodium-like structure was formed mainly of filopodia, since neither nuclei, chloroplasts, nor bacteria could be observed in the main body of the node. However Bass et al. (2009) reported that a holoplasmodium of *Filoreta* species (cercozoans) is multinucleate, suggesting that the node in this species may not be a homologous stage to those of cercozoans. Here we thus simply refer to the structure as "filopodial nodes". The nodes occurr only in *L. reticulosa*, so this feature might be a good taxonomic marker to distinguish it from other *Lotharella* species.

Based on the morphological and ultrastructural observations and the molecular phylogenetic analyses, strain RCC375 should be described as a new chlorarachniophyte in the genus *Lotharella*. We thus propose a new species, *Lotharella reticulosa*. We also examined another strain RCC376 and identified it as *L. reticulosa*, because ITS sequences of strains RCC375 and RCC376 are 100% identical as well as because they are morphologically similar.

Molecular Phylogeny

For the nuclear 18S rRNA gene, tree topology was almost similar to that of the previously published trees by Silver et al. (2007) and Ota et al. (2009b). In the present analyses, however, *Partenskyella glossopodia* and the closest environmental sequence (UEPAC33p4; Worden 2006) were weakly sister to the *Chlorarachnion* clade (0.66 Bayesian posterior probabilities (PP), 48% ML bootstrap values). We identified seven major clades corresponding to the known chlorarachniophyte genera with high statistical supports (1.00 PP; $\geq$98% ML bootstrap values). Recently, Ishida et al. (2011) proposed a new combination, *Amorphochlora amoebiformis* for *Lotharella amoebiformis* (Ishida et al. 2000) and established the genus *Amorphochlora*. This is because *L. amoebiformis* clearly differs morphologically and phylogenetically from *Lotharella* (sensu stricto). Here, we use the term "*Amorphochlora* clade" instead of "*Lotharella* 2 clade" or "*Lotharella amoebiforms* clade" (Ota et al. 2009b; Silver et al. 2007).

In the present analyses, three environmental sequences (Not et al. 2007; Worden 2006) were included in the dataset, two of which (SSRPE06 and UEPACLp3) represent the basal branches of chlorarachniophytes. The monophyly of the chlorarachniophytes and the two environmental sequences were robustly recovered (1.00 PP, 100% ML bootstrap values).

The present topology of nucleomorph-encoded ITS sequence phylogeny was roughly identical to the tree shown by Gile et al. (2010). In the present analysis, however, the positions of *Norrisiella sphaerica* and *Partenskyella glossopodia* were different from the tree of Gile et al. (2010). Since they were long branches, we could not

deduce their phylogenetic positions based only on the ITS region. What is clear in the present analysis is that strain RCC375/376 was positioned in the *Lotharella* clade. This is consistent with the molecular phylogenetic trees of the 18S rRNA gene. In addition, the present tree showed some genetic differentiation in *Lotharella* species (e.g., about 78% similarities between *L. globosa* and *L. reticulosa*), supporting our taxonomic conclusion that the strains RCC375/RCC376 should be regarded as a new *Lotharella* species.

Life Cycle and Highly Reticulated Network

In *L. reticulosa*, deep green globular cells were usually present in mid or older cultures and often suspended in the water. Unfortunately, we could not trace the switch between life cycle stages by time-lapse microscopy. However their cytoplasmic nature was very different from that of vegetative cells, raising the possibility that the globular cells are either cysts or zygotes. Further examination of the life cycle is needed to know whether sexual reproduction is occurring in this species.

As mentioned above, *L. reticulosa* formed a filopodial node. The exact role of the node is not clear, but given that it was always observed where cell density was low, it could play a role as a matrix to which daughter cells settle. As a result, the daughter cells cover the substratum evenly. The highly-reticulated filopodia seem to be related to the unique colonization behaviour observed in *L. reticulosa*.

Not only *L. reticulosa* but also many other chlorarachniophytes are typically possessing filopodia in coccoid and amoeboid stages. It could be inferred that cytoplasmic strands play roles in (1) adhering to the substrate, (2) migrating onto the substrate, (3) preying food particles, and (4) carrying granules (e.g., food particles) and/or organelles. In some species, filopodia anastomose and form a network (an interconnected meroplasmodium) as in *Chlorarachnion reptans* initially described by Geitler (1930), whereas in other species, filopodia do not anastomose as in *Gymnochlora stellata* (Ishida et al. 1996). Among the *Lotharella* species, *L. polymorpha*, *L. vacuolata,* and the present species possess anastomosing filopodia and form networks. Notably the new species, *L. reticulosa*, built a highly reticulated network, implying that a cluster of cells formed roughly symmetrical cytoplasmic strands resembling a spider web. In this study we found a fifth role for the cytoplasmic strand in chlorarachniophytes which acted as a "rail" along which amoeboid daughter cells migrate distally. As a result, the daughter cells were dispersed concentrically and covered the substratum evenly. So far, only intracellular migration of daughter cells has been known in chlorarachniophytes (e.g., Ota et al. 2005, 2007a); intercellular migration found in *L. reticulosa* constitutes a first report in the chlorarachniophytes. It would be interesting to understand the mechanism and evolution of intercellular communication between parent and daughter cells.

Taxonomic Treatment

Lotharella reticulosa S. Ota sp. nov.

Cellulae globosae parietibus, vel amoeboideae, vel fllagelliformis. Chloroplasti virides, 2-8 vel plures. Cellulae globosae (9–16 μm diam.) dominans in cultura, saepe folopodiis; filopodia reticulata maxime. Cellulae amoeboideae (8–11 μm diam.), solitariae, nudae, polymorphae; circulares ad polygonies, vel lanceolatae ad ellipticae peranguste, folopodiis. Cellulae fllagelliformis (zoospore) raro observatae. Cellulae globosae suspensae (11–25 μm diam.), interdum observatae. Coloniae (fasciculi cellularum) extensae filopodia radiala. Filopodia concenturiata, formantia nodum (25–140 μm diam.). Pyrenoides dimidiata longitudinaliter tenuistrato spati periplasti. Nucleomorphus locates prope basim pyrenoidim in spatio periplasti. Holotypus PC0078363; isotypi TNS-AL-56974, TNS-AL-56975.

Cells coccoid, amoeboid or flagellate. Chloroplasts green, 2-8 or more. The coccoid cells (9–16 μm in diameter) dominant in culture, often with filopodia; filopodia highly reticulate. The amoeboid cells (8–11 μm in diameter), solitary, naked, very variable in form; circular to polygonal or lanceolate to very narrowly elliptic, with filopodia. Flagellate cells (zoospores) rarely observed. Sometimes suspended globular cells (11–25 μm in diameter) observed. Colony (cell cluster) extending filopodia radially. Filopodia concentrated, forming nodes (25–140 μm in diameter). Pyrenoid divided into two halves longitudinally by a thin layer of periplastidial compartment. A nucleomorph is located at the pyrenoid base.

HOLOTYPE: One microscope slide (PC0078363), deposited in the Muséum National d'Histoire Naturelle, Paris (PC). ISOTYPE: One microscope slide (TNS-AL-56974) and TEM blocks (TNS-AL-56975), deposited in the National Museum of Nature and Science, Department of Botany, Tsukuba, Japan (TNS).

AUTHENTIC CULTURE: RCC375. This culture is maintained in the Roscoff Culture Collection at the Station Biologique, Roscoff, France.

DNA SEQUENCE INFORMATION: The accession number EF622539 is a nuclear 18S rDNA sequence of RCC375 (*Lotharella reticulosa* sp. nov.) (Silver et al. 2007). Barcode sequences (Nm ITS) was deposited with the accession number AB610593.

TYPE LOCALITY: Mediterranean Sea (approximate GPS position: 37° 24' N, 15° 37' E), Depth 5 m, 26 September 1999, isolated by Dr F. Partensky and purified by F. Le Gall.

HABITAT: marine.

ETYMOLOGY: The species epithet emphasizes the highly reticulate network formed by this species.

OTHER CULTURE STRAIN EXAMINED: Strain RCC376, 26 September 1999, Mediterranean Sea, approximate GPS position: 37° 24' N, 15° 37' E, 5 m depth, leg. F. Partensky, accession number of Nm ITS: AB610594.

Methods

Isolation and maintenance of culture: Strain RCC375 was isolated by F. Partensky from seawater sample collected at 5 m in the Mediterranean Sea near Sicily (approximate GPS position: 37° 24' N, 15° 37' E) during the PROSOPE cruise in 1999. The strain is maintained with K medium (Keller et al. 1987) in the Roscoff Culture Collection (http://www.sb-roscoff.fr/Phyto/RCC/). For observation, the culture was grown in 50 mL polystyrene culture flasks or polystyrene culture dishes (Asahi Technoglass, Tokyo, Japan) containing ESM (Kasai et al. 2009; http://mcc.nies.go.jp/) or K medium, and incubated at 17-20 °C under a 14:10 light:dark cycle.

Light- and time-lapse video microscopy: For light microscopy (LM) preparation, cells were grown for 1-7 day(s) on a sterile coverslip in a culture dish filled with K or ESM medium. Living cells on the coverslip were observed using an Olympus BX51 microscope (Olympus, Tokyo, Japan) and a Zeiss Axio Scope.A1 (Carl Zeiss, Oberkochen, Germany) equipped with Nomarski differential interference contrast and phase contract optics. Light micrographs and time-lapse sequential images were taken with a SPOT RT-slider digital camera (Diagnostics Instruments, Sterling Heights, MI) and a Nikon D5000 digital camera (Nikon, Tokyo, Japan). Sequential images were edited and assembled in movie files (avi) using ImageJ v.1.43u (National Institutes of Health, Bethesda, MD, USA). For fluorescence microscopy, cells were fixed with glutaraldehyde (1% final concentration) and stained with 5 μg/ml of 4', 6-diamidino-2-phenylindole (DAPI) dissolved in filtered seawater.

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 M, 0.1 M, and 0.05 M). After removal of the supernatant, the pellet was post-fixed in 0.5% osmium tetroxide for 2 h at 4 °C. The pellet was rinsed twice with 0.1 M sodium cacodylate buffer followed by 0.05 M sodium cacodylate buffer (pH 7.2), and then rinsed once with Milli-Q water. After dehydration in 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 copper grids coated with polyvinyl formvar films, and stained with uranyl acetate and lead citrate (Reynolds 1963). Sections and whole mount preparations were observed using a JEM-1400 transmission electron microscope (JEOL, Tokyo, Japan) at 80 kV.

DNA extraction, polymerase chain reaction (PCR), and sequencing: The cells were collected from about 10 mL of 5-days-old culture growing in a 25 cm^2 culture flasks (Nunc, Roskilde, Denmark) containing 10-20 ml medium and total DNA was extracted using the E.Z.N.A.™ SP Plant DNA kit (Omega-Bio-Tek, Norcross, GA, USA) according to the manufacturer's protocol. ITS (ITS1-5.8S-ITS2) was amplified using chlorarachniophyte nucleomorph-specific primers nmITSF and ITS4 as described by Gile et al. (2010). PCR condition was as follows: initial denaturation at 94 °C for 3 min, 35 cycles (denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 68 °C for 1 min), and final extension at 72 °C for 10 min. PCR products were run on a 0.8% agarose gel and checked for purity and correct fragment length. The PCR products were purified using ExoSAP-IT® (USB Corp., Cleveland, OH, USA) and bidirectionally sequenced using an Applied Biosystems 3730 analyzer (Applied Biosystems, CA, USA) sequencing device at the Department of Biology, University of Oslo.

Phylogenetic analyses: Sequences of 18S rRNA gene were aligned using ClustalX v. 2.0 (Larkin et al. 2007) and manually edited using BioEdit v. 7.0.5.3 (Hall 1999). For the ITS dataset, sequences were aligned using ClustalX v. 2.0 (Larkin et al. 2007) with the alignment parameter setting as described Gile et al. (2010), and ambiguously aligned regions were removed by GBlocks 0.91b (Castresana 2000). Maximum likelihood (ML) analyses of all datasets were carried out using the PhyML v. 3.0 (Guindon and Gascuel 2003) with a BioNJ starting tree. The general time reversible model with parameters accounting for invariable sites (I) and gamma-distributed (G) rate variation across sites with four discrete rate categories and HKY+I+G were used for 18S rRNA and ITS datasets, respectively. The bootstrap analyses were done in 100 replicates for the ML analyses. Bayesian inference under the same evolutional model was preformed with MrBayes v. 3.1.2 (Ronquist and Huelsenbeck 2003). Two Markov Chain Monte Carlo (MCMC) runs each with four chains were performed for 5,000,000 and 1,000,000 generations for 18S rRNA and ITS datasets, respectively, where the average standard deviation of split frequencies were below 0.01. Trees were sampled every 100 generations. Bayesian posterior probabilities were calculated from the majority rule consensus of the tree sampled after the initial burn-in phase. The PhyML bootstrap values were annotated onto the Bayesian trees. The 18S rRNA gene phylogenetic tree was rooted with heterotrophic filoseans as shown by the phylogenetic trees with close outgroups in Bass et al. (2009). All phylogenetic analyses were carried out on the University of Oslo Bioportal (www.bioportal.uio.no).

Acknowledgements

We are grateful to all participants of the PROSOPE cruise as well as to the curators of the Roscoff Culture Collection, in particular F. Le Gall. We are also grateful to Sophie Le Panse and Jean Sourimant from Roscoff for their kind help. This work was supported by the Contrat de Projet Etat-Région "Souchothèque de Bretagne", and by the ASSEMBLE EU FP7 research infrastructure initiative (EU-RI-227799). SO postdoctoral stay in the Station Biologique de Roscoff and the University of Oslo was supported by the French embassy in Japan and the EU BiodivERsA project BioMarKs (2008-29765-62739-34), respectively.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.protis.2011.02.004.

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