Running title: *Minorisa minuta* and three novel species

Molecular and morphological characterization of three novel *Minorisa* species (Chlorarachnea) and proposal for an emended description of the *M. minuta*

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Summary

Minorisa is a group of tiny uniflagellates belonging to the Cercozoa. These flagellates are the closest heterotrophic relatives of photosynthetic chlorarachniophytes, and are one of the most abundant bacterivorous eukaryotes in coastal marine environments. Despite their evolutionary and ecological importance, taxonomic studies on *Minorisa* have not been conducted since the original description. In the present study, we isolated five *Minorisa* strains and performed molecular phylogenetic and microscopic analyses. Molecular phylogenetic analysis using small subunit ribosomal RNA gene sequences indicated that the strains form four different subclades in *Minorisa*. Microscopic observations revealed that these *Minorisa* strains possess an amoeboid stage that lacks a flagellum. In the amoeboid stage, cells possess lobose and/or extrusive pseudopodia. Based on the phylogenetic analysis and morphological observations, we revised the description of *Minorisa* and described the following three novel *Minorisa* species: *M. fusiformis* sp. nov., *M. magna* sp. nov., and *M. megafusiformis* sp. nov.

Key words: Cercozoa, Chlorarachniophyta, Chlorarachnida, heterotrophic flagellate, phylogenetic analysis

INTRODUCTION

Chlorarachniophytes is a group of marine unicellular amoeboids, coccoids, and flagellated photosynthetic algae. The first chlorarachniophyte, *Chlorarachnion reptans* Geitler, collected from the Canary Islands, was classified as a Heterokontae (Geitler 1930). More than half a century after the first report, Hibberd and Norris (1984) performed detailed microscopic observations and chlorophyll pigment analysis on *C. reptans* and reported that its zoospore had a single flagellum, the plastids contained chlorophyll *b* and were surrounded by four membranes, and a nucleomorph was placed in the space between the second and the third membrane in each plastid. Based on these characteristics, Chlorarachniophyta was established as a new division. Following molecular phylogenetic analyses, chlorarachniophytes were included in the phylum Cercozoa, a group of heterotrophic amoebae and flagellates (Cavalier-Smith & Chao 1997, 2003). At present, nine genera of photosynthetic chlorarachniophytes have been described in Chlorarachniales (Chlorarachnida) based on the ultrastructure of the pyrenoid, position of the nucleomorph, and dominant cell stage in the life cycle (Calderon-Saenz & Schnetter 1987; Cavalier-Smith et al. 2018; Ishida et al. 1996, 2011; Moestrup & Sengco 2001; Ota et al. 2007a, 2009; Shiratori et al. 2017).

Photosynthetic chlorarachniophytes were established via endosymbiosis between a host cercozoan and a green algal symbiont (Ishida et al., 1997, 1999; Ludwig & Gibbs, 1989;

McFadden et al., 1995; Van de Peer et al., 1996). Molecular phylogenetic studies using plastid genomes suggested that the green algal symbiont of chlorarachniophytes was derived from a green algal lineage closely related to Bryopsidales (Suzuki et al. 2016). On the other hand, del Campo et al. (2013) reported that *Minorisa minuta* del Campo is the heterotrophic species closest to photosynthetic chlorarachniophytes. *M. minuta* is a tiny uniflagellate and one of the most abundant bacterivorous eukaryotes in coastal marine environments (del Campo et al. 2013). The single flagellum of *M. minuta* wraps around the cell, corresponding to the flagellate stage of photosynthetic chlorarachniophytes (del Campo et al. 2013: Moestrup & Sengco 2001). Considering its morphological similarities and phylogenetic position, *M. minuta* is an important species for understanding the endosymbiotic evolution of photosynthetic chlorarachniophytes. However, this species has been observed exclusively using scanning electron microscopy, and its living cell behavior and intracellular ultrastructure remain uncertain.

Here, we performed microscopic observations and molecular phylogenetic analysis of five new strains of *Minorisa* spp. isolated from Japanese coastal seawater. Based on these analyses, we revised the description of *Minorisa* and established three new species. We also discuss the early evolution of chlorarachniophytes by comparing the morphology and ultrastructure of *Minorisa* with those of other chlorarachniophytes.

MATERIALS AND METHODS

Sample collection and culture establishment

Marine surface seawater samples were collected from Miyako Island (latitude = 24.8002°N, longitude = 125.2615°E; collection date: January 21, 2016), Tokyo Bay (latitude = 35.6392°N, longitude = 139.8578°E; collection date: March 8, 2016), Fukaya Channel (latitude = 34.2628°N, longitude = 136.8608°E; collection date: April 29, 2017), Azuri beach (latitude = 34.2606°N, longitude = 136.7791°E; collection date: April 29, 2017), and Yokkaichi Port (latitude = 35.0045°N, longitude = 136.6657°E; collection date: April 30, 2017) in Japan. The strains SRT609, SRT705, and SRT710 (from samples of Miyako Island, Fukaya Channel, and Azuri beach, respectively) were established as follows: seawater samples were filtered through a 5 μm-pore size Isopore Membrane Filter (Millipore Corporation, Billerica, MA, USA) and then incubated with ESM medium (Kasai et al. 2009) for two weeks. Cells in the incubated samples were then isolated by micropipetting. Strains Y-KSI-01 and Y-YKI-01 (from seawater samples of Tokyo bay and Yokkaichi Port, respectively) were established by filtering through a 3 μm-pore size Isopore Membrane Filter (Millipore Corporation) followed by incubation in ESM medium and isolation by the serial dilution method. All cultures were maintained in ESM medium at 18°C under a 14-h light/10-h dark cycle.

DNA extraction and polymerase chain reaction of SSU rDNA

Cells were collected by centrifugation, and total DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Small subunit ribosomal RNA (SSU rRNA) genes were amplified by polymerase chain reaction using SR1 and SR12 primer sets (Nakayama et al. 1998). The amplified DNA was gel-purified using the QIAquick Gel Extraction Kit (Qiagen) and cloned using the p-GEM® T-easy vector System (Promega KK, Tokyo, Japan). The plasmid was sequenced using a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The SSU rDNA sequences of the five *Minorisa* strains were deposited in GenBank (accession numbers: LC375243-LC375247).

Sequence alignment and phylogenetic analysis

For molecular phylogenetic analysis, we collected cercozoan SSU rRNA gene sequences, representing all major lineages of chlorarachniophytes and several *Minorisa*-related environmental sequences, from the NCBI database (https://www.ncbi.nlm.nih.gov). The SSU rRNA gene dataset comprised 23 SSU rDNA sequences, including those from the five *Minorisa* strains established in this study. The dataset was aligned using MAFFT v7.520 (Katoh & Standley 2013) and edited manually using SeaView version 5.0.5 (Gouy et al. 2010). Ambiguously aligned regions were deleted. The final dataset comprised 1622 nucleotide positions. The maximum likelihood tree was constructed using IQ-Tree v.1.6.12 (Nguyen et al.,

2015) with the TIM+F+I+G4 model. Branch supports were obtained using an ultrafast bootstrap (bp, 1,000 replicates) with an IQ-Tree (Hoang et al., 2018). Bayesian analysis was carried out using MrBayes v.3.2.6 (Ronquist et al. 2012) with the GTR+Γ model. One cold and three heated Markov chain Monte Carlo simulations with default chain temperatures were run for 2×10^6 generations, sampling log likelihood values and trees at 100-generation intervals. Convergence was assessed using the average standard deviation of split frequencies, and the first 25% of the total generations in each analysis were discarded as "burn-in." Bayesian posterior probabilities (bpp) and branch lengths were calculated from the remaining trees.

Light microscopy

Living cells were observed in glass-bottomed dishes using an Olympus IX71 inverted microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP73 CCD camera (Olympus) or on microscope slides using a Zeiss Axio Imager A2 microscope (Zeiss, Oberkochen, Germany) equipped with an Olympus DP71 CCD camera (Olympus).

Electron microscopy

Cells were collected by centrifugation and fixed with 0.1 M sodium cacodylate buffer (SCB; pH 7.2), containing 2.5% (v/v) glutaraldehyde and 0.25 M sucrose, for 45 min at room temperature. The fixed cells were washed with SCB and post-fixed with 1% (v/v) osmium tetroxide in 0.2M SCB for 1 h at room temperature. Thereafter, the cells were washed twice with SCB and

dehydrated using 30%, 50%, 75%, 90%, and 100% ethanol. After dehydration, the cells were placed in a mixture of 100% Ethanol : 100% acetone (1 : 1) for 10 min, then in 100% acetone for 10 min, and finally in a mixture of 100% acetone : Agar Low Viscosity Resin R1078 (Agar Scientific Ltd., Stansted, England) (1 : 1) for 30 min. Cells were embedded in the resin for 2 h and then polymerized by incubating at 70°C for 12 h. Ultrathin sections were prepared using a Reichert Ultracut S ultramicrotome (Leica, Vienna, Austria), stained with 2% uranyl acetate and lead citrate, and observed using a Hitachi H-7650 electron microscope (Hitachi High-Technologies Corp., Tokyo, Japan) equipped with a Veleta TEM CCD camera (Olympus Soft Imaging System, Münster, Germany).

RESULTS

Molecular phylogenetic analysis

Molecular phylogenetic analysis using SSU rRNA gene sequences showed that the five *Minorisa* strains, SRT609, SRT705, SRT710, Y-KSI-01, and Y-YKI-01, formed a robust clade with *M. minuta* (bp = 100%, bpp = 1; Fig. 1). This clade was positioned as a sister lineage of photosynthetic chlorarachniophytes, with strong statistical support (bp = 83%, bpp = 1). *Minorisa minuta* and the five strains were separated into two subclades, one consisting of *M. minuta* and SRT609, and the other consisting of SRT705, SRT710, Y-KSI-01, and Y-YKI-01. *Minorisa minuta* and SRT609 shared identical 18S rRNA gene sequences, except for a single

deletion/insertion. The monophyly of the clade comprising the other four strains was robustly supported (bp = 96%, bpp = 1). Among the four strains, Y-KSI-01 and Y-YKI-01 shared almost identical sequences (single substitution), while the others exhibited 2.7-3.2% difference in their sequences.

Light microscopy

Cells of the strain SRT609 were 2.6 (2.2–2.9) μm in length and 2.1 (1.8–2.3) μm in width (n = 30), spherical, ellipsoidal, or pyriform shape without any cell wall or scales (Fig. 2a−c). The cells were granular and had a single flagellum that was inserted anteriorly and directed posteriorly and wrapped the cell in a clockwise manner (Fig. 2b). The cells either rotated in place or swam along their longitudinal axis. Some cells did not swim and were attached to the substrate. These cells glided on the substrate by trailing their flagellum. Occasionally, they detached from the substrate and swam again. The flagellated cells exhibited binary fission. Aside from flagellated cells, amoeboid cells without flagella were occasionally observed (Fig. 2d–f). These amoeboid cells moved on the substrate by extending a lobose pseudopodium anteriorly (Fig. 2e, f). Immotile amoeboid cells with one or two extrusive pseudopodia were rarely observed (Fig. 2d). Amoeboid cells that detach from substrate were occasionally observed. Some amoeboid cells formed a flagellum and became amoeboflagellates possessing lobose or extrusive pseudopodia. They occasionally lost their pseudopodia to change into flagellate cells.

The cells of strains Y-KSI-01 and Y-YKI-01 exhibited similar morphological characteristics. Cells of both these strains were granular, uniflagellate, and exhibited a spherical, ellipsoidal, or pyriform shape without any cell walls or scales (Fig. 2g, h, k). Cells of strain Y-KSI-01 were 3.2 (2.8–3.5) μm in length and 2.6 (2.2–3.2) μm in width (n = 31), and cells of strain Y-YKI-01 were 3.0 (2.8–3.2) μm in length and 2.6 (2.4–2.8) μm in width (n = 42). For cells of both these strains, the flagellum was inserted anteriorly and directed posteriorly, and wrapped the cell in a clockwise manner. The cells either rotated in place or swam along their longitudinal axis. Some cells did not swim and were attached to the substrate. These cells glided on the substrate by trailing their flagellum. Occasionally, they detached from the substrate and swam again. The flagellated cells exhibited binary fission. Amoeboid cells without flagella were occasionally observed (Fig. 2i, j, l). These amoeboid cells were immotile and extended one or two extrusive pseudopodia (Fig. 2i, j, l). Amoeboid cells with lobose pseudopodia were not observed. Amoeboid cells that detach from substrate were occasionally observed. Some amoeboid cells formed a flagellum and became amoeboflagellates possessing extrusive pseudopodia. They occasionally lost their pseudopodia to change into flagellate cells.

Cells of the strain SRT705 cells were granular, uniflagellate, and exhibited a spherical, ellipsoidal, or pyriform shape without any cell wall or scales (Fig. 2m–o). These cells were 2.3 (2.0–2.6) μm in length and 1.9 (1.7–2.2) μm in width (n = 54). The flagellum was inserted

anteriorly and directed posteriorly, and wrapped the cell in a clockwise manner (Fig. 2n, o). The cells rotated in place or swam along their longitudinal axis. Some cells did not swim and attached to the substrate. These cells glided on the substrate by trailing their flagellum. Occasionally, they detached from the substrate and swam again. The flagellated cells exhibited binary fission. Amoeboid cells without flagella were also occasionally observed (Fig. 2p). These amoeboid cells were immotile and extended one or two extrusive pseudopodia (Fig. 2p). Amoeboid cells with lobose pseudopodia were not observed. Amoeboid cells that detach from substrate were occasionally observed. Some amoeboid cells formed a flagellum and became amoeboflagellates possessing extrusive pseudopodia. They occasionally lost their pseudopodia to change into flagellate cells.

Cells of the strain SRT710 were granular, uniflagellate, and spherical, ellipsoidal, or pyriform in shape without any cell wall or scales (Fig. 2q–s). Cells were 4.0 (3.4–4.3) μm in length and 3.1 (2.6–3.5) μm in width (n = 33). The flagellum was inserted anteriorly and posteriorly, and wrapped the cell in a clockwise manner (Fig. 2r, s). The cells either rotated in place or swam along their longitudinal axis. Some cells did not swim and attached to the substrate. These cells glided on the substrate by trailing their flagellum. Occasionally, they detached from the substrate and swam again. The flagellated cells exhibited binary fission. Amoeboid cells without flagella were occasionally observed (Figures 2t, u). These amoeboid

cells moved on the substrate by extending a lobose pseudopodium anteriorly (Fig. 2u). immotile amoeboid cells with one or two extrusive pseudopodia were rarely observed (Fig. 2t). Amoeboid cells that detach from substrate were occasionally observed. Some amoeboid cells formed a flagellum and became amoeboflagellates possessing lobose or extrusive pseudopodia. They occasionally lost their pseudopodia to change into flagellate cells.

Electron microscopy of strain SRT609

We observed the strain SRT609 under a transmission electron microscope. Cells of the strain SRT609 exhibited a nucleus, which contained a nucleolus at the center (Fig. 3a). The mitochondria were spherical with tubular cristae (Fig. 3a). The Golgi apparatus was located near the nucleus (Fig. 3a). The cells occasionally contained multiple large food vacuoles with digested bacteria and numerous small vesicles (Fig. 3a). Microbodies were also observed (Fig. 3b). No extrusomes were observed. In each cell, one long and one short basal body was observed located near the nucleus (Fig. 3a–c). The long basal body lied along the nucleus and had a flagellum, whereas the short basal body lacked a flagellum and formed a right angle with the long basal body (Fig. 3c). Long basal bodies without flagella, which could represent amoeboid cells, were occasionally observed in some sections (Fig 3a).

DISCUSSION

Molecular phylogenetic analysis using the SSU rRNA gene sequences showed that the five

Minorisa strains, SRT609, SRT705, SRT710, Y-KSI-01, and Y-YKI-01, formed a robust clade with *Minorisa minuta*. Although the phylogenetic tree indicates that except for SRT609, the remaining four strains are genetically distant from *M. minuta*, these strains are small uniflagellates that feed on bacteria, a trait similar to that of *M. minuta* (del Campo et al. 2013). Based on phylogenetic relationships and morphological similarities, we concluded that the five strains belonged to the genus *Minorisa*. Compared with photosynthetic chlorarachniophytes, which comprise nine genera with different morphologies and life cycles, *Minorisa* is conserved in morphology relative to genetic differences.

The morphological characteristics of *M. minuta* and the five strains of *Minorisa* are summarized in Table 1. Strains SRT609 and *M. minuta* share almost identical SSU rRNA gene sequences but differ in cell size and in the presence or absence of amoeboid cells. The cell size of *M. minuta* in the original description, 1.0–2.1 μm in length and 0.8–2 μm in width (del Campo et al. 2013), is smaller than that of the strain SRT609 (2.2–2.9 μm in length and 1.8–2.3 μm in width). We observed that strain SRT609 has an amoeboid stage with lobose or extrusive pseudopodia, which was not reported in the original description of *M. minuta*. We suspect that these differences between strain SRT609 and the original description were caused by different observation methods. In the original description, the cells were observed under a scanning electron microscope (SEM), and the specimen possibly shrank during the fixation and

dehydration processes. Because pseudopodia were observed for all five strains, these are probably common to *Minorisa*. Pseudopodia may have been lost during the SEM specimen preparation or overlooked in the original description. Therefore, we considered SRT609 to be *M. minuta* and revised the diagnosis of *M. minuta* based on the observation of strain SRT609.

The SSU rRNA gene phylogeny showed that, except for SRT609, the remaining four strains form a robust clade. These strains exhibit a difference of 2.7–3.2% in their sequence, except for the strains Y-KSI-01 and Y-YKI-01, which share almost identical sequences. Further, the strains Y-KSI-01 and Y-YKI-01 exhibit no significant differences in cell size or morphology. The strains SRT609 and SRT705 share similar cell sizes; however, SRT705 lacks the lobose pseudopodia observed in SRT609. The other pairs, SRT705 vs. other strains and SRT705 vs. Y-KSI-01 (Y-YKI-01), were distinguished based on their cell sizes (Table 1). Although all strains exhibit extrusive pseudopodia, lobose pseudopodia were exclusively observed in the strains SRT609 and SRT710. Except for strains Y-KSI-01 and Y-YKI-01, all the strains could be distinguished by the 18S rRNA gene sequence, cell size, and morphology of pseudopodia. Therefore, we propose the following three new species of *Minorisa*: *M. fusiformis* sp. nov. (SRT705), *M. magna* sp. nov. (SRT710), and *M. megafusiformis* sp. nov. (Y-KSI-01 and Y-YKI-01). Note that species identification based on pseudopodia and cell size may require careful, culture-based observations and therefore molecular data is preferable for practical identification.

TAXONOMY

Rhizaria, Cercozoa, Chlorarachnea, Minorisida, Minorisidae

Minorisa J. del Campo 2013, emend. Shiratori, Kato et Ishida

Emended diagnosis: Naked bacterivorous flagellates. Flagellate cells ellipsoid, spherical, or ovoid, with a single flagellum wrapped around the cell. Flagellates swim by rotating along their longitudinal axis. Amoeboid stage without flagella. Mitochondria exhibit tubular cristae. Plastids and extrusomes absent.

Type species: *M. minuta*

Minorisa minuta J. del Campo 2013, emend. Shiratori, Kato et Ishida

Emended diagnosis: Flagellated cells 1.0–2.9 μm in length and 0.8–2.3 μm in width. Amoeboid cells with lobose and extrusive pseudopodia.

Minorisa fusiformis Shiratori, Kato et Ishida, sp. nov.

Diagnosis: Flagellated cells 2.0–2.6 μm in length and 1.7–2.2 μm in width. Amoeboid cells with lobose and extrusive pseudopodia.

Holotype: One microscope slide (TNS-AL-58958s), deposited in the herbarium of the National Museum of Nature and Science (TNS), Tsukuba.

Paratype: One EM block (TNS-AL-58958tb), deposited in TNS. These cells were derived from the same sample as the hapantotype.

Type locality: Surface seawater from Fukaya Channel (latitude 34.2628°N, longitude 136.8608°E), Mie, Japan.

DNA sequence: Small subunit ribosomal RNA gene, LC375246

Collection date: April 29, 2017

Etymology: "*fusiformis*" (spindle shape) refers to the shape of the amoeboid cell of the species.

Type culture: Strain SRT705 was used to describe this species, and was deposited in and maintained by the National Institute for Environmental Sciences, Tsukuba, Japan as NIES-4229.

Zoobank Registration: urn:lsid:zoobank.org:act:D9464150-5F8C-44B3-9D04-DF35BC7B4AC6

Minorisa magna Shiratori, Kato et Ishida, sp. nov.

Diagnosis: Flagellated cells 3.4–4.3 μm in length and 2.6–3.5 μm in width. Amoeboid cells with lobose and extrusive pseudopodia.

Holotype: One microscope slide (TNS-AL-58959s), deposited in TNS.

Paratype: One EM block (TNS-AL-58959tb) was deposited in TNS. These cells were derived from the same sample as the hapantotype.

Type locality: Surface seawater from Azuri Beach (latitude 34.2606°N, longitude 136.7791°E), Mie, Japan.

DNA sequence: Small subunit ribosomal RNA gene, LC375247

Collection date: April 29, 2017

Etymology: Specific epithet "*magnus*" (large, great) refers to the cell size of the species.

Type culture: Strain SRT710 was used to describe this species, and was deposited in and maintained by the National Institute for Environmental Sciences, Tsukuba, Japan as NIES-4230.

Zoobank Registration: urn:lsid:zoobank.org:act:C6CE4EB5-5B0D-40DC-A14D-4FC950516BAE

Minorisa megafusiformis Shiratori, Kato et Ishida, sp. nov.

Diagnosis: Flagellated cells 2.8–3.5 μm in length and 2.2–3.2 μm in width. Amoeboid Cells with extrusive pseudopodia.

Holotype: One microscope slide (TNS-AL-58960s), deposited in TNS.

Paratype: One EM block (TNS-AL-58960tb), deposited in TNS. These cells were derived from the same sample as the hapantotype.

Type locality: Surface seawater from Tokyo Bay (latitude = 35.6392°N, longitude = 139.8578°E), Tokyo, Japan.

DNA sequence: Small subunit ribosomal RNA gene, LC375243

Collection date: March 8, 2016

Etymology: "*Mega*" (large) + "*fusiformis*" (spindle shape) refers to the shape of the amoeboid cell and size of the species.

Type culture: The strain Y-KSI-01 was used to describe this species, and was deposited in and maintained by the National Institute for Environmental Sciences, Tsukuba, Japan as NIES-4231.

Zoobank Registration: urn:lsid:zoobank.org:act:34250D98-F6D7-4AB2-834B-04FCAAE6E57E

Morphological and ultrastructural evolution of Chlorarachnea

Plastids of photosynthetic chlorarachniophytes are surrounded by four membranes and contain a nucleomorph, which is a remnant of the green algal nucleus, in the space between outer two and inner two membranes (Ishida et al. 1999; Ludwig and Gibbs, 1989). Recent plastid genome-based phylogenetic analysis has shown that the plastids of chlorarachniophytes are derived from a green algal lineage that is closely related to Bryopsidales (Suzuki et al., 2016). The first heterotrophic chlorarachnean *M. minuta* was discovered by del Campo (2013), and molecular phylogenetic analysis showed that it branches as a sister group to photosynthetic chlorarachniophytes. This suggests that *M. minuta* branched from photosynthetic

chlorarachniophytes before acquiring its plastids or lost the plastid secondarily. Our TEM observations did not reveal plastids or remnant organelles in *M. minuta*. Because complete loss of the plastid structure has rarely been reported (Toso and Omoto 2007), and *Minorisa* is not branched within photosynthetic chlorarachniophytes, *Minorisa* possibly branched from photosynthetic chlorarachniophytes prior to plastid acquisition. Future genomic studies on *Minorisa* strains are required to confirm traces of plastids for understanding their evolution in Chlorarachnea.

Photosynthetic chlorarachniophytes have a complex life cycle consisting of amoeboid cells, walled or naked coccid cells, and flagellated cells (Keeling 2017; Ishida et al. 2007), which vary among genera and are considered as important taxonomic traits. Our study showed that *Minorisa* has the flagellate cell as the main vegetative stage. The flagellate cells of *Minorisa* possess a single flagellum that wraps around the cell. The cells swim by rotating along their longitudinal axis. Photosynthetic chlorarachniophyte genera, except for *Gymnochlora*, exhibit flagellate stages, and the morphology and swimming behavior of the flagellate cells are similar to those of *Minorisa* (Calderon-Saenz & Schnetter 1987; Ishida et al. 1996, 2011; Moestrup & Sengco 2001; Ota et al. 2007a, 2009; Shiratori et al. 2017). The flagellate cells of both *Minorisa* and photosynthetic chlorarachniophytes possess long flagellated and short non-flagellated basal bodies located at approximately right angles. These morphological and ultrastructural similarities

between flagellate cells of *Minorisa* and photosynthetic chlorarachniophytes indicate that a common ancestor of Chlorarachnea possessed similar flagellate cells. Although flagellate cells are common in Chlorarachnea, these are present in only two species of *Bigelowiella* have it as a main vegetative stage. The similarity between *Minorisa* and *Bigelowiella natans* Moestrup suggests that flagellated cells, representing the main vegetative stage, can be considered as the ancestral state of Chlorarachnea. However, the phylogenetic placement of *Bigelowiella* is not basal in Chlorarachnea and the life cycles of chlorarachneans differ even within the same genus. Additional taxon sampling around the Chlorarachnean lineages is required to unveil the ancestral state of the life cycle and the main vegetative stage.

Our study revealed *that Minorisa* has a nonvegetative amoeboid stage. The amoeboid cells of *Minorisa* possess unbranched short lobose or extrusive pseudopodia. Amoeboid stages have been reported in most genera of photosynthetic chlorarachniophytes, with the exception of *Norrisiella* and *Viridiuvalis* (Calderon-Saenz & Schnetter 1987; Ishida et al. 1996, 2000; Moestrup & Sengco 2001; Ota et al. 2005, 2007b, 2009). Amoeboid chlorarachneans generally develop filose or reticulose pseudopodia, which occasionally form intercellular reticulopodial networks (Meroplasmodium) (Hibberd & Norris 1984; Ota & Vaulot 2009). *Chlorarachnion reptans* and *Cryptochlora perforans* Calderon-Saenz et Schnetter possess lobate or blunt pseudopodia, as well as filose or reticulose pseudopodia (Beutlich & Schnette 1993; Hibberd &

Norris 1984). The planktonic chlorarachneans *Partenskyella glossopodia* Ota, Vaulot et Ishida and *B. natans* lack filopodia but possess lobose or finger-like pseudopodia (Moestrup & Sengco 2001 Ota et al. 2009). In addition, *B. natans* possesses pseudopodia-like extrusions similar to the extrusive pseudopodia of *Minorisa*. The wide distribution of lobose and filose or reticulose pseudopodia in chlorarachneans suggests that these are derived from a common ancestor, and *Minorisa* may have lost its filose pseudopodia secondarily.

Cell walls are also observed in photosynthetic chlorarachniophytes; some species such as *B. natans* and *C. perforans* exhibit walled coccid cells as cyst (Calderon-Saenz & Schnetter, 1987; Hibberd & Norris, 1984; Moestrup & Sengco, 2001; Ota et al., 2009). In contrast, *Lotharella*, *Norrisiella*, and *Viridiuvalis* exhibit walled coccoid cells as main vegetative stage (Dietz et al., 2003; Ishida & Hara, 1994; Ota et al. 2005, 2007a; Shiratori et al., 2017). Although no walled coccoid cells or cysts have been observed in *Minorisa*, cell walls are widely distributed in Cercozoa as cysts (Brabender et al. 2012; Cavalier-Smith et al. 2009; Howe et al. 2009) or as extracellular thecae (Shiratori & Ishida 2016; Shiratori et al. 2020; Thomsen et al. 1991). Therefore, *Minorisa* may lack walled cells secondarily.

This study revealed the species diversity and ultrastructure of *Minorisa* spp. In contrast to photosynthetic chlorarachniophyte genera, *Minorisa* exhibits more conserved morphologies and lifecycles than genetic diversity. The common ancestor of Chlorarachnea is suggested to

have had uniflagellated cells, in which the flagellum was wrapped around the cell. The ultrastructural study suggested that *Minorisa* branched from photosynthetic chlorarachniophytes before acquiring plastids. Future genomic studies using the *Minorisa* strains identified in this study will provide further insights into the origin of Chlorarachnea and the process of secondary endosymbiosis.

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REFERENCES

Bass D., Chao E. E., Nikolaev S., et al. 2009. Phylogeny of novel naked filose and reticulose Cercozoa: Granofilosea cl. n. and Proteomyxidea revised. *Protist*, **160**:75–109.

Beutlich A. and Schnetter R. 1993. The life cycle of *Cryptochlora perforans* (Chlorarachniophyta). *Bot. Acta*, **106**:441–47.

Brabender M., Kiss Á. K., Domonell A., Nitsche F. and Arndt H. 2012. Phylogenetic and morphological diversity of novel soil cercomonad species with a description of two new genera (*Nucleocercomonas* and *Metabolomonas*). *Protist*, **163**:495–528.

Cavalier-Smith T. and Chao E. E. 2003. Phylogeny and classification of phylum Cercozoa (Protozoa). *Protist*, **154**:341–58.

Cavalier-Smith T. and Chao E. E. 1997. Sarcomonad ribosomal RNA sequences, rhizopod phylogeny, and the origin of euglyphid amoebae. *Arch. für Protistenkd*., **147**:227–36.

Cavalier-Smith T., Chao E. E. and Lewis R. 2018. Multigene phylogeny and cell evolution of chromist infrakingdom Rhizaria: contrasting cell organisation of sister phyla Cercozoa and Retaria. *Protoplasma*, **255**:1517–74.

Cavalier-Smith T., Lewis R., Chao E. E., Oates B. and Bass D. 2009. *Helkesimastix marina* n. sp. (Cercozoa: Sainouroidea superfam. n.) a gliding zooflagellate of novel ultrastructure and unusual ciliary behaviour. *Protist*, **160**: 452–479.

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

Chantangsi C. and Leander B. S. 2010. An SSU rDNA barcoding approach to the diversity of marine interstitial cercozoans, including descriptions of four novel genera and nine novel species. *Int. J. Syst. Evol. Microbiol.*, **60**:1962–77.

del Campo J., Not F., Forn I., Sieracki M. E. and Massana R. 2013. Taming the smallest predators of the oceans. *ISME J.*, **7**:351–8.

Dietz C., Ehlers K., Wilhelm C., Gil-Rodríguez M. C. and Schnetter R. 2003. *Lotharella polymorpha* sp. nov. (Chlorarachniophyta) from the coast of Portugal. *Phycologia*, **42**:582–93.

Geitler L. 1930. Ein grünes Filarplasmodium und andere neue Protisten. *Arch. für Protistenkd*., **69**:615–36.

Gouy M., Guindon S. and Gascuel O. 2010. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol. Biol. Evol.* **27**:221–24

Hibberd D. J. and Norris R. E. 1984. Cytology and ultrastructure of *Chlorarachnion reptans* (Chlorarachniophyta divisio nova, Chlorarachniophyceae classis nova). *J. Phycol*., **20**:310‑30.

Hoang D. T., Chernomor O., von Haeseler A., Minh B. Q. and Vinh L. S. 2018. UFBoot2: improving the ultrafast bootstrap approximation. *Mol Biol Evol.* **35**:518–22.

Howe A. T., Bass D., Scoble J. M., et al. 2011. Novel cultured protists identify deep-branching environmental DNA clades of Cercozoa: new genera *Tremula*, *Micrometopion*, *Minimassisteria*, *Nudifila*, *Peregrinia*. *Protist*, **162**:332–72.

Howe A. T., Bass D., Vickerman K., Chao E. E., and Cavalier-Smith, T. 2009. Phylogeny, taxonomy, and astounding genetic diversity of Glissomonadida ord. nov., the dominant gliding zooflagellates in soil (Protozoa: Cercozoa). *Protist*, **160**:159–189.

Ishida K., Cao Y., Hasegawa M., Okada N. and Hara Y. 1997. The origin of chlorarachniophyte plastids, as inferred from phylogenetic comparisons of amino acid sequences of EF-Tu. *J. Mol. Evol.*, **45**:682–7.

Ishida K., Green B. R. and 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. and Hara Y. 1994. Taxonomic studies on the Chlorarachniophyta. I. *Chlorarachnion globosum* sp. nov. *Phycologia*, **33**:351–58.

Ishida K., Ishida N. and Hara Y. 2000. *Lotharella amoeboformis* sp. nov.: A new species of chlorarachniophytes from Japan. *Phycol. Res.*, **48**:221–29.

Ishida K., Nakayama T. and 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. and Ota S. 2007. The chlorarachniophytes: evolution and classification. In: Brodie J. and Lewis J. (eds) Unravelling the algae: the past, present, and future of algal systematics. CRC Press, Taylor & Francis Group, Boca Raton, 171–82

Ishida K., Yabuki A. and Ota S. 2011. Research note: *Amorphochlora amoebiformis* gen. et comb. nov. (Chlorarachniophyceae). *Phycol. Res.*, **59**:52–3.

Katoh K. and Standley D. M. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol*. **30**:772–80.

Keeling P. J. 2017. Chlorarachniophytes. In: Archibald J. M., Simpson A. G. B. and Slamovits C. (eds) *Handbook of the protists*, 2nd edition. Cham, Switzerland:Springer International Publishing. 765–81.

Ludwig M. and Gibbs S. P. 1989. Evidence that the nucleomorphs of *Chlorarachnion reptans* (Chlorarachniophyceae) are vestigial nuclei: morphology, division and DNA-DAPI fluorescence. *J. Phycol.*, **25**:385–94.

McFadden G. I., Gilson P. R. and Waller R. F. 1995. Molecular phylogeny of chlorarachniophytes based on plastid rRNA and rbcL sequences. *Arch. für Protistenkd.*, **145**:231–39.

Moestrup Ø. and Sengco M. 2001. Ultrastructural studies on *Bigelowiella natans*, Gen. et sp. nov., a chlorarachniophytes flagellate. *J. Phycol.*, **37**:624–46.

Nakayama T., Marin B., Kranz H. D., Surek B., Huss V. A. R., Inoue I. and Melkonian M. 1998.

The basal position of scaly green flagellates among the green algae (Chlorophyta) is revealed by analyses of nuclear-encoded SSU rRNA sequences. *Protist*. **149**:367–80

Nguyen L.-T., Schmidt H. A., von Haeseler A. and Minh, B. Q. 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol. Biol. Evol.* **32**:268–74

Ota S., Ueda K. and 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**:275–86.

Ota S., Ueda K. and Ishida K. 2007a. *Norrisiella sphaerica* gen. et sp. nov., a new coccoid chlorarachniophyte from Baja California, Mexico. *J. Plant Res.*, **120**:661–70.

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

Ota S., Vaulot D., Gall F. Le, Yabuki A. and Ishida K., 2009. *Partenskyella glossopodia* gen. et sp. nov., the first report of a Chlorarachniophyte that lacks a pyrenoid. *Protist*, **160**:137–50.

Ronquist F., Teslenko M., von der Mark P., et al. 2012. MrBayes 3.2: efficient Bayesian

phylogenetic inference and model choice across a large model space. *Syst. Biol.*, **61**:539–42.

Shiratori T., Fujita S., Shimizu T., Nakayama T. and Ishida K. 2017. *Viridiuvalis adhaerens* gen. et sp. nov., a novel colony-forming chlorarachniophyte. *J. Plant Res.*, **130**:999–1012.

Shiratori T., and Ishida, K. 2016. *Trachyrhizium urniformis* n. g., n. sp., a novel marine filose thecate amoeba related to a Cercozoan environmental clade (Novel Clade 4). *J. Eukaryot. Microbiol.*, **63**:722–731.

Shiratori T., Yabuki A. and Ishida K. 2020. Morphology, ultrastructure, and phylogeny of two novel species of *Ventrifissura* (*V. oblonga* n. sp. and *V. velata* n. sp., Thecofilosea, Cercozoa). *Protist*, **171**:125731

Suzuki S., Hirakawa Y., Kofuji R., Sugita M. and Ishida K. 2016. Plastid genome sequences of *Gymnochlora stellata*, *Lotharella vacuolata*, and *Partenskyella glossopodia* reveal remarkable structural conservation among chlorarachniophyte species. *J. Plant Res.*, **129**:581–90.

Thomsen H. A., Buck K. R., Bolt P. A. and Garrison D. L. 1991. Fine structure and biology of *Cryothecomonas* gen. nov. (Protista incertae sedis) from the ice biota. *Can. J. Zool.*, **69**:1048–1070.

Toso M. A. and Omoto C. K. 2007. Gregarina niphandrodes may lack both a plastid genome and

organelle. *J. Eukaryot. Microbiol.*, **54**:66–72.

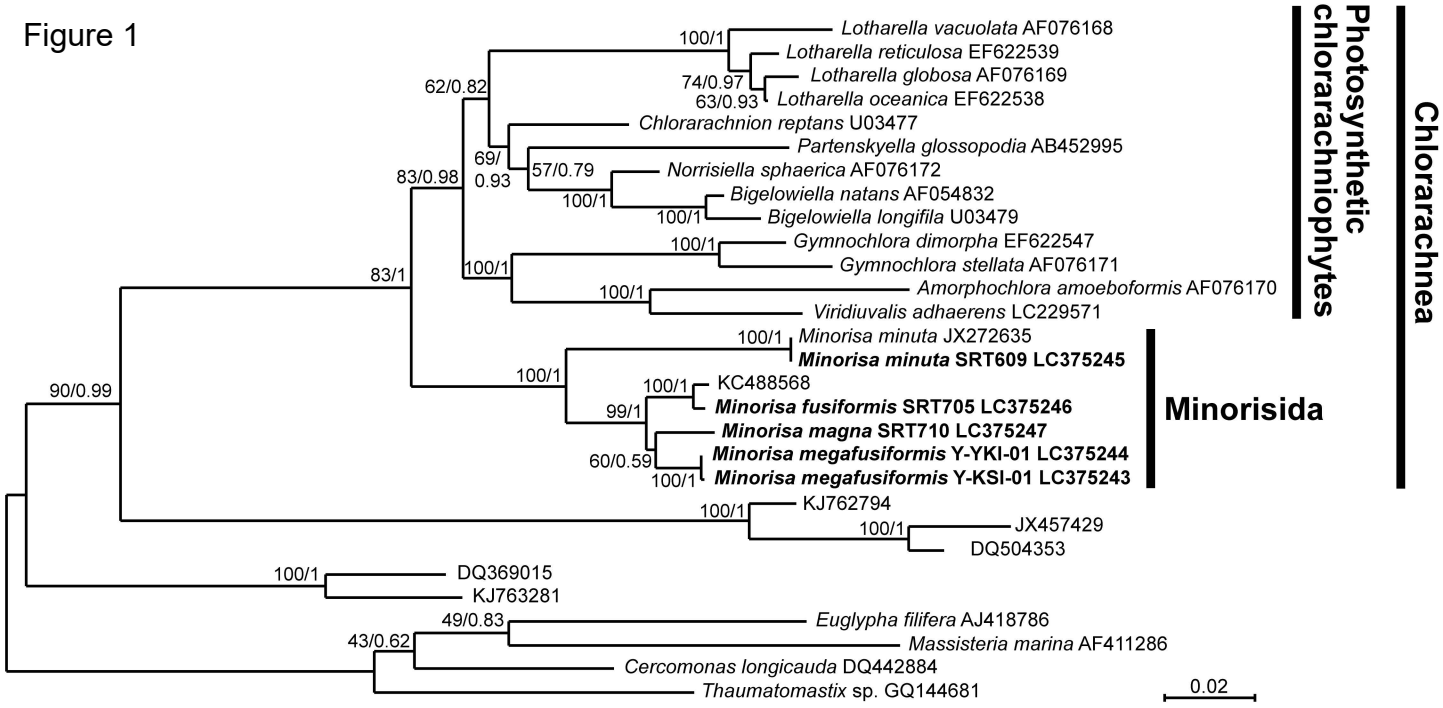
Van de Peer Y., Rensing S. A., Maier U. G. and De Wachter R. 1996. Substitution rate calibration of small subunit ribosomal RNA identifies chlorarachniophyte endosymbionts as remnants of green algae. *Proc. Natl. Acad. Sci.*, **93**:7732–36.

Table 1. Comparison of the morphological characteristics of *Minorisa* spp.

	M. minuta[*]	*M. minuta*	*M. megafusiformis*		*M. fusiformis*	*M. magna*
		(SRT609)	(Y-KSI-01)	(Y-YKI-01)	(SRT705)	(SRT710)
Cell length (μm)	1.0–2.1	2.2–2.9	2.8–3.5	2.8–3.2	2.0–2.6	3.4–4.3
Cell width (μm)	0.8–2	1.8–2.3	2.2–3.2	2.4–2.8	1.7–2.2	2.6–3.5
Cell shape	not reported	spherical, ellipsoidal, or pyriform	spherical, ellipsoidal, or pyriform	spherical, ellipsoidal, or pyriform	spherical, ellipsoidal, or pyriform	spherical, ellipsoidal, or pyriform
Number of flagella	1	1	1	1	1	1
Prey	bacteria	bacteria	bacteria	bacteria	bacteria	bacteria
Pseudopodia	not reported	lobose, extrusive	extrusive	extrusive	extrusive	lobose, extrusive

* del Campo et al. (2013)

Figure 1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Lotharella globosa AF076169
Lotharella oceanica EF622538
Chlorarachnion reptans U03477
Partenskyella glossopodia AB452995
Norrisiella sphaerica AF076172
Bigelowiella natans AF054832
Bigelowiella longifila U03479
Gymnochlora dimorpha EF622547
Gymnochlora stellata AF076171
Amorphochlora amoeboformis AF076170
Viridiuvalis adhaerens LC229571
Minorisa minuta JX272635
Minorisa minuta SRT609 LC375245
KC488568
Minorisa fusiformis SRT705 LC375246
Minorisa magna SRT710 LC375247
Minorisa megafusiformis Y-YKI-01 LC375244
Minorisa megafusiformis Y-KSI-01 LC375243
KJ762794
JX457429
DQ504353
DQ369015
KJ763281
Euglypha filifera AJ418786
Massisteria marina AF411286
Cercomonas longicauda DQ442884
Thaumatomastix sp. GQ144681
100/1
74/0.97
63/0.93
62/0.82
69/
0.93
57/0.79
83/0.98
100/1
100/1
100/1
100/1
83/1
100/1
100/1
100/1
100/1
99/1
60/0.59
100/1
90/0.99
100/1
100/1
100/1
49/0.83
43/0.62
0.02
Photosynthetic chlorarachniophytes
Chlorarachnea
Minorisida

Figure 2

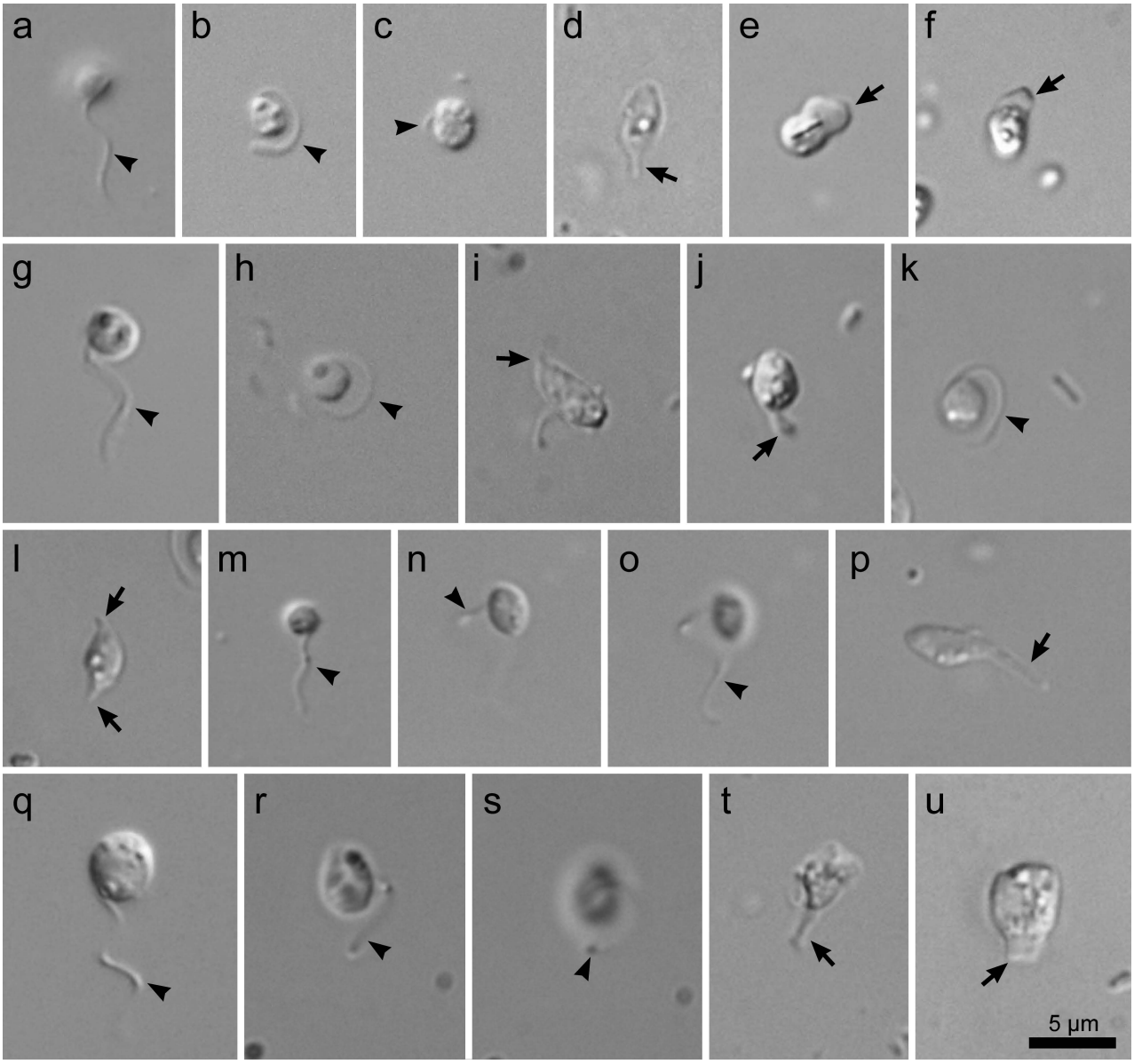

Figure 3

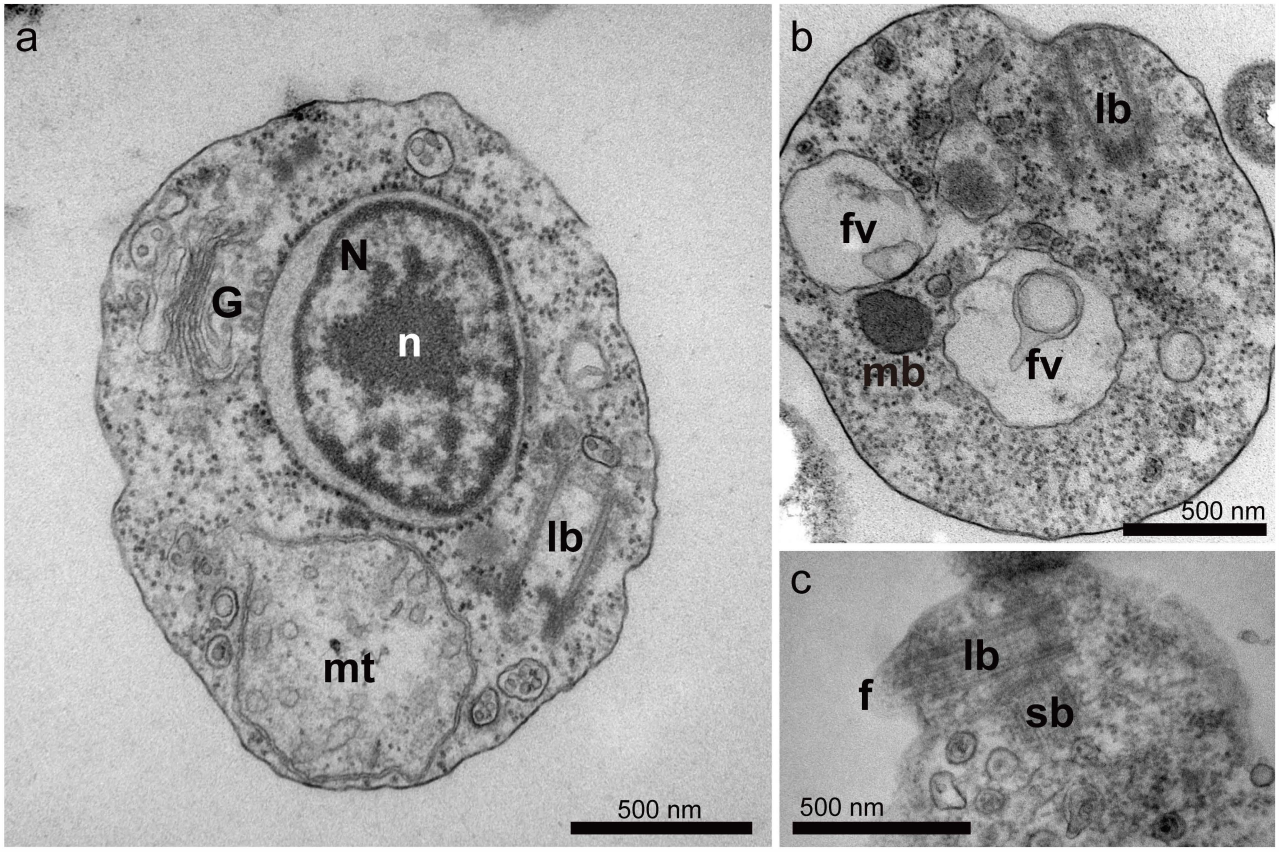

FIGURE LEGEND

Fig. 1 Maximum likelihood tree of chlorarachniophytes using 1622 positions from the SSU rRNA gene. Environmental sequences are labeled only with accession numbers. Values on each node are bootstrap percentages (left) and Bayesian posterior probabilities (right).

Fig. 2 Differential interference contrast micrographs of *Minorisa* spp. Arrows indicate pseudopodia. Arrowheads indicate flagella. **a–f.** Strain SRT609 (*M. minuta*). **g–j.** Strain Y-KSI-01 (*M. megafusiformis* sp. nov.). **k–l.** Strain Y-YKI-01 (*M. megafusiformis* sp. nov.). **m–p.** Strain SRT705 (*M. fusiformis* sp. nov.). **q–u.** Strain SRT710 (*M. magna* sp. nov.).

Fig. 3 Transmission electron micrographs of strain SRT609 (*Minorisa minuta*). f, flagellum; fv, food vacuole; G, Golgi apparatus; lb, long basal body; mb, microbody; mt, mitochondria; N, nucleus; n, nucleolus; sb, short basal body. **a, b.** cell of strain SRT609. **c.** basal bodies of strain SRT609.