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Diplophrys mutabilis sp. nov., a New Member of Labyrinthulomycetes from Freshwater Habitats

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Abstract

Diplophrys is a ubiquitous protist genus belonging to the class Labyrinthulomycetes. Although most members of Labyrinthulomycetes prefer marine habitats, the genus *Diplophrys* exclusively consists of the freshwater species *Diplophrys archeri* and *Diplophrys parva*. To investigate the genus *Diplophrys*, several novel strains were isolated from Japanese freshwater environment, and cultures of the strains were established. Among the strains, an organism isolated from Lake Nojiri displayed some characteristic features different from that of both *D. archeri* and *D. parva*. Thus, we described this strain as a new species, *Diplophrys mutabilis. D. mutabilis* can be cultured using dried water flea as food. This species had an orbicular to fusiform shape, and it occasionally penetrated prey with prominent cytoplasm. From molecular phylogenetic analysis based on 18S rRNA sequences, *D. mutabilis* evidently belongs to Amphitremida, Labyrinthulomycetes. This study suggests that these species form a unique group in Labyrinthulomycetes.

Keywords

Amphitremida, *Diplophrys mutabilis*, lipid body, phylogeny, ultrastructure

Introduction

Labyrinthulomycetes is a heterotrophic protist group belonging to the protistan supergroup stramenopiles (Dick 2001 (as “Straminipila”); Patterson 1989), and the class is characterized by the following features: biflagellate zoospores possessing an anterior flagellum with tripartite tubular mastigonemes (Kazama 1973), rhizoid-like ectoplasmic net elements produced by a unique organelle, bothrosome (sagenogen, sagenogenetosome) (Moss 1980; Perkins 1972; Porter 1972), and multilamellate cell walls composed of Golgi body-derived scales (Alderman et al. 1974; Darley et al. 1973).Honda et al. 1999 classified Labyrinthulomycetes genus into two families: Thraustochytriaceae, characterized by globose cells forming ectoplasmic nets that are derived from a single bothrosome, and Labyrinthulaceae, spindle-shaped cells with gliding motility using the channels of ectoplasmic nets extending from a number of bothrosomes (Honda et al.1999). Thraustochytriaceae includes *Althornia, Aurantiochytrium, Botryochytrium, Japonochytrium, Oblongichytrium, Parietichytrium, Schizochytrium, Sicyoidochytrium, Thraustochytrium,* and *Ulkenia*, whereas Labyrinthulaceae includes only the genera *Aplanochytrium* and *Labyrinthula* (Anderson and Cavalier-Smith 2012; Yokoyama and Honda 2007; Yokoyama et al. 2007). Some genera such as *Diplophrys* and *Sorodiplophrys* are also included in Labyrinthulomycetes even though they are treated as *incertae sedis* (Dick 2001).

Labyrinthulomycetes species play ecological roles as decomposers or parasites. Naganuma et al. (1998) estimated the abundance of the Thraustochytriaceae in the Seto Inland Sea in Japan and demonstrated that their

biomass in coastal waters could reach 43% of the bacterial biomass. Some studies estimated the biomass of these organisms in the oceanic water column as being as high as 675 × 10^3 cell/L (Damare and Raghukumar 2008; Naganuma et al. 2006). Such high abundance and widespread occurrences indicate their ecological importance in coastal and oceanic environments. Conversely, the reality of Labyrinthulomycetes species in terrestrial water is poorly understood, and only a few freshwater genera have been described. Of these, the most common freshwater genus is *Diplophrys.*

Diplophrys was described with a type species *Diplophrys archeri* collected from a freshwater habitat in Great Britain (Barker 1868). This genus is characterized by the following features: nearly orbicular or broadly elliptical cells, a layer of scales covering the cell comprised of fine organic discs that can only be visualized by electron microscopy, a turf of filiform pseudopodia emanated from two opposite points, and an oil-like refractive orange-to-amber–colored globule immersed in the cytoplasm (Patterson 1996).

A new terrestrial species, *Diplophrys stercorea,* which possesses filopodia and a refractive granule, was added to the genus (Cienkowski, 1876). Although *D. stercorea* has a similar shape as *D. archeri*, it was moved to a separate genus, *Sorodiplophrys* L. Olive & M Dykstra (Dykstra and Olive 1975), based on its terrestrial habitat and aggregative behavior.

In addition, a marine protist having a prominent refractive granule, ectoplasmic elements, and gliding motility was isolated from both the Pacific and Atlantic coasts of the United States and named *Diplophrys marina* (Dykstra and Porter 1984). As a result of molecular phylogenetic analysis based on 18S rDNA

sequences, *D. marina* was classified into Thraustochytriaceae rather than into Labyrinthulaceae (Leander and Porter 2001). Although the phylogenetic position of *D. marina* appeared to be clarified, its gliding motility is characteristic of Labyrinthulaceae species. Recently, *D. marina* was transferred to the genus *Amphifila* upon the report of the novel species *Diplophrys parva* (Anderson and Cavalier-Smith 2012). In the paper, the authors proposed the reclassification of the entire class Labyrinthulomycetes, and the genus *Diplophrys* was classified into the order Thraustochytrida, family Diplophryidae. However, in the following year, Gomaa et al. described the new order Amphitremida, and *Diplophrys* members were transferred to this order together with testaceous amoeboid organisms with a bipolar symmetry (Gomaa et al. 2013). Based on these recent classifications, Labyrinthulomycetes should be composed of three orders: Thraustochytrida, Labyrinthulida, and Amphitremida including Diplophryidae. Though *Diplophrys* encountered unheralded testaceous neighbors, related uncultured organisms remain to be discovered, and the diversity of the genus itself is unclear.

In this study, we describe a new species in Diplophrys isolated from Lake Nojiri, Nagano, Japan using ultrastructural morphological features. The phylogenetic position of the new species is also consolidated using 18S rRNA sequence

Materials and Methods

Sample collection and cultivation

In November 2011, *D. mutabilis* was isolated from freshwater samples collected from Lake Nojiri, Nagano Pref., Japan. Surface water was collected in a sampling bottle.

Single-species cultures were established using the single-cell isolation technique with micropipettes. For feeding, autoclaved distilled water and commercially available dried water flea for aquarium fish were used. 5 – 10 of dried water fleas were added to 5 ml of distilled water and autoclaved at 120 °C for 20 min. The cultures were maintained in test tubes at room temperature in a shaded space. Another novel strain, Amphifilidae H-1, was isolated from freshwater samples collected from the surface layer water of Pond Hiuchi, Ibaraki Pref., Japan, in June 2011. The culture of strain H-1 was established and maintained using the same technique utilized for *D. mutabilis*. *Diplophrys* ATCC PRA-36 strain HAVA-2 was also obtained from the American Type Culture Collection for molecular phylogenetic analysis.

Morphological observations

For light microscopy, a Zeiss AX10 microscope (Carl Zeiss, Göttingen, Germany) and an Olympus IX71 microscope (Olympus, Tokyo, Japan) equipped with Nomarski differential interference contrast optics was used. IX71 was also used for fluorescent microscopy with Nile red-stained cells.

For scanning electron microscopy (SEM), cultured samples were mounted onto glass plates coated with poly L-lysine and fixed at 4°C for 2 h in 5%

glutaraldehyde. After rinsing with 0.2 M sodium cacodylate buffer (pH 7.2) several times, the prefixed samples were fixed in 1% osmium tetroxide for 30 min. These samples were then dehydrated through a graded ethanol series (50, 75, 90, 95, and 100%), keeping them in each concentration for 15 min, followed by substitution with dehydrated *t*-butyl alcohol. The specimens were freeze-dried using a VFD-21S freeze drier (SHINKU-DEVICE, Ibaraki, Japan) and mounted onto specimen stubs. These specimens were coated with platinum/palladium with an E102 ion-sputter (Hitachi, Tokyo, Japan) and observed using a JSM-6330F field emission scanning electron microscope (JEOL, Tokyo, Japan).

For whole-mount images, cells were exposed to 4% OsO_4 fumes for 5 min followed by washing in distilled water. Cells were stained for 3 min with 4% uranyl acetate. Cells were viewed with a Hitachi H-7650 (Hitachi) transmission electron microscope (TEM).

For thin sectioning, cells were fixed as follows. Vegetative cells were exposed to 1% OsO_4 fumes for 3 min. The cells were fixed in a solution containing 2.5% glutaraldehyde, 2% OsO_4, 4.5% sucrose, and 0.1 M cacodylate buffer at pH 7.0 for 90 min under refrigeration (4°C, in the dark) followed by washing in the same buffer thrice for 10 min each. The cells were successively dehydrated in 30, 50, 70, 90, 95, and 100% acetone for 10 min each under refrigeration, followed incubation in both acetone-propylene oxide (PO) mixtures and pure PO twice for 10 min. The dehydrated pellet was embedded in Agar low viscosity resin (LV Resin, VH1 and VH2 Hardener, and LV Accelerator, Agar Scientific, Essex, Great Britain), and a 1:1 mixture of PO and the resin was prepared. The resin

was polymerized for 12 h at 70°C.

Thin sections were cut on an ultramicrotome (Leica EM UC7, Leica Camera AG, Solms, Germany) and stained for 5 min with 4% uranyl acetate, followed by Sato's lead citrate (Sato 1968) for 5 min. The sections were viewed with a Hitachi H-7650 TEM.

Molecular phylogenetic analyses

To amplify the 18S rDNA of obtained strains, single cells were isolated again using micropipettes as mentioned above, and transferred into PCR tubes with autoclaved distilled water. Tubes were first stored overnight at room temperature to digest the feeds and then placed at −20°C in the freezer overnight to break membranes. The 18S rDNA was amplified by PCR with the primer pairs reported in Nakayama et al. (1998), namely SR1 and SR12. The first PCR products were amplified again using following primer pairs: SR1 and SR5, SR4 and SR9, and SR8 and SR12. Nonspecific PCR products were electrophoretically detected, and second PCR products were purified using the QIAquick® Gel Extraction Kit (Qiagen, Venlo, Netherlands). Purified products were sequenced with a BigDye Terminator V1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems 3130 genetic analyzer. Two sequences of 18S rDNA gene, namely of *D. mutabilis* (AB856527) and Amphifilidae H-1 (AB856528) were obtained. Other sequences of 18S rDNA were obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) and automatically aligned using CLUSTAL X version 1.81 (Thompson et al. 1997,

ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/). For phylogenetic analyses, ambiguously aligned regions were manually arranged or deleted using the BioEdit Sequence Alignment Editor version 7.0.9.0 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and finally, 1310 sites for 18S rDNA were used.

The phylogenetic trees were constructed using both maximum likelihood (ML) and Bayesian approaches based on a 1230-bp alignment using three sequences of Alveolata as the outgroup. We used Phylip ver. 3.69 (http://evolution.genetics.washington.edu/phylip.html) for ML and MrBayes 3.2.1 for Bayesian analysis. For Bayesian analysis, GTR+I+R model were selected using MrModeltest 2.3 (Nylander 2004, http://www.abc.se/~nylander/). The stability of relationships was assessed by performing bootstrap analyses based on 100 resamplings for ML. Bayesian analysis was run for 1,000,000 generations, with a sampling frequency of every 100th generation. All other settings were left at their default values.

Results

Taxonomic Treatments

Based on the morphological characteristics and the result of molecular phylogenetic analysis using SSU rDNA sequences, we describe a new species of the genus *Diplophrys*, *D*. *mutabilis* sp. nov.

Diplophrys mutabilis (ICBN)

Taxonomic Description

The cell shape of *D. mutabilis* was orbicular to fusiform, asymmetrical to the axis connecting the polar ends. The cells measured 3.1–8.3 × 3.4–10.3 μm in diameter, exhibiting an irregular gliding motility by means of fine filamentous, branching ectoplasmic elements extending up to 150 μm from both polar ends of the cell. The cells had hyaline cytoplasm containing one to several colorless, or yellow, orange, or amber-colored conspicuous refractive granules. The nucleus was located subcentrally with an evident nucleolus. One to several vacuoles were present, one of which was a contractile vacuole. Unidentified cytoplasmic membranes of various forms, including ring-like, single-helical, or double-helical structures, were present. The cell wall was composed of overlapping Golgi-derived circular scales (0.8–1.5 μm in diameter) displaying an incrassate rim. The cells grew by repeated binary fission. Sporangia, spores, and cysts were not observed. The species' SSU rDNA sequence places it in the *Diplophrys* clade, but it was separated from any known species.

Taxonomic summary: Chromalveolate, Stramenopiles, Labyrinthulomycetes (Labyrinthulea), Amphitremida, Diplophriidae.

Type material: Holotype: EM block (TNS-AL-57099).

Type strain: NIES-3361

Type habitat/locality: Nojiri Lake, Nagano Prefecture, Japan (36.830585N, 138.20848E).

Etymology: Specific epithet “mutabilis” means changeable cell shapes.

Gene sequence: AB856527

General morphology

Diplophrys mutabilis was orbicular or broadly elliptic in shape, and it contained refractive granules, a single nucleus, a contractile vacuole, and ectoplasmic elements emanating from the poles of the cells (Fig. 1). Cells changed their shape from orbicular (Fig. 1A, C, D) to fusiform (Fig. 1B). Gliding motility was observed, notably in fusiform cells. As many as 10 refractive bodies were observed in each cell. Using a Nile Red stain, refractive bodies were stained yellow and thus identified as lipid bodies containing neutral lipids (Fig. 1C, D).

Ectoplasmic elements were branching but not anastomosing, and one of the branching ectoplasmic elements for each pole was eminently longer than the others (Fig. 2A). The ectoplasmic elements were up to 150 μm in length. In the basal part of the ectoplasmic elements, ectoplasmic swelling was frequently observed (Fig. 2A, B). Distal ectoplasmic elements exhibited dichotomous branching (Fig. 2C).

The cell surface was covered with scales (Fig. 3A, B). The scales were round in shape with an incrassate rim but without palpable marking. They measured 0.8–1.5 μm in diameter and were extremely thin. Thus, overlapping of multiple scales was recognizable (Fig. 3B). These scales were Golgi-derived (see below). In the culture examined, bacteria were attached to the scale surface and ectoplasmic elements (Fig. 3A, C). No debris surrounding the cell was observed.

Ultrastructural observations

In thin-section observations using TEM, nucleus, mitochondria, lipid bodies, and Golgi bodies were observed (Fig. 4A). Ectoplasmic elements contained ribosome-free cytoplasm and tubular internal membrane system elements (Fig. 4B). Bothrosomes and bothrosome-like bodies were not observed. *D. mutabilis* possessed mitochondria containing distinctive cristae with short, stubby branches (Fig. 4C). Developed lipid bodies were observed in the cytoplasm. In these lipid bodies, mosaic patterns were occasionally observed (Fig. 4D). Many small vesicles were observed between the nucleus and Golgi body (Fig. 4A, E). Organic scales were formed in the dictyosomes near the cell surface (Fig. 4E, arrows).

In some cells, unidentified cytoplasmic membranes were observed (Fig. 5). These membranes displayed various forms, including concentric circles (Fig. 5A), a single helical form (Fig. 5B), and a double-helical form (Fig. 5C). These transverse and slant sections (Fig. 5D) suggested that these membrane systems are probably cylindrical in shape. Although the entire three-dimensional shape and the role of these membranes are unclear, some hypothetical functions are suggested on the basis of their location and neighboring organelles (described in Discussion).

Some unusual images were encountered in TEM observations (Fig. 6). In Fig. 6A, it is likely that *D. mutabilis* changes its cell shape and breaks into the feed body. This deformation was recognized only by TEM observations, and it has not been observed by light microscopy. The cells multiplied by repeated binary

fission (Fig. 6B). Some bacteria were present inside the scale layer of the parent cell. The scales of the parent cell were probably shed and discarded during cell division. It is unclear whether the scales of daughter cells are synthesized *de novo* or succeeded from the parent cell. Other types of multiplication, such as aplanosporogenesis or zoosporogenesis, were not observed.

Molecular phylogenetic analyses

Phylogenetic analyses based on the 18S rDNA gene sequence revealed that *D. mutabilis* was a new member of Labyrinthulomycetes (Fig. 7). The phylogenetic tree was similar to those reported previously (Anderson and Cavalier-Smith 2012; Leander and Porter 2001). Our analysis identified a close phylogenetic relationship between *D. mutabilis* and labyrinthuloid members, but it also revealed significant differences between them. It is known that Labyrinthulomycetes is divided into at least two phylogenetic groups, namely the labyrinthulid phylogenetic group (LPG) and thraustochytrid phylogenetic group (TPG) (Honda et al. 1999). ML algorithm and Bayesian analysis indicated that *Diplophrys* was not classified into either LPG or TPG, but it was included in Amphitremida. The branching orders were different, but this result was consistent with Gomaa et al. 2013. From the phylogenetic tree, there was no doubt that *D. mutabilis* belonged to order Amphitremida, family Diplophryidae. This clade contains *Diplophrys*, *Amphitrema*, *Archerella*, and many unidentified environmental clones from anoxic deep-sea samples reported by Edgcomb et al.

294 (2011). All identified members in this clade display a bipolar cell shape and are
295 unicellular, solitary organisms that do not form developed ectoplasmic networks.
296 They also share characteristic of having solid cell coverings; however,
297 *Amphitrema* and *Archerella* have monolithic testa, whereas *Diplophrys* have
298 layers of discrete scales.
299
300

301 Discussion

302 Concerning its appearance, *D. mutabilis* resembles *D. archeri*, *D. parva*,
303 *Amphifila marina*, and the vegetative cells of *Sorodiplophrys stercorea*
304 (Anderson and Cavalier-Smith 2012; Dykstra and Porter 1984; Dykstra and
305 Olive 1975). These organisms are nearly orbicular or broadly elliptic in shape
306 and contain refractive granules, a contractile vacuole, and ectoplasmic elements
307 emanating from the poles of the cells. *D. mutabilis* can change its cell shape, not
308 only from orbicular to fusiform (Fig. 1B) but also probably to a more plastic form
309 such as that penetrating to the substratum as observed by TEM (Fig. 6A). This
310 changeability of cell shape is one of the diagnostic characters of *D. mutabilis.*

311 Swelling in the basal part of the ectoplasmic elements was observed in *D.*
312 *mutabilis* (Fig. 2A, B). Similar swelling has been observed in *Af. marina* and *S.*
313 *stercorea*. However, their swellings occurred in the middle part of the
314 ectoplasmic elements (Porter 1984), not in the basal part as observed in *D.*
315 *mutabilis.* However, pseudopodial features are important morphological
316 characteristics of amoeboid organisms in general, but it is unclear whether this

317 difference reflects phylogenetic relationships in this group.

318 An internal membrane system in ectoplasmic elements is widely observed in
319 Labyrinthulomycetes species (Perkins 1972), and the system of *D. mutabilis* is
320 apparently more developed than those in other organisms. The system has been
321 observed in *S. stercorea* (Dykstra 1976a), but not in *Af. marina* and *D. parva*.
322 The ectoplasmic element of *Af. marina* appears to consist of fine fibrous
323 structures rather than a bundle of membranous tubes (Dykstra and Porter 1984).
324 Labyrinthulomycetes species have mitochondria with tubular cristae, which are
325 also observed in Stramenopiles, but *D. mutabilis* has mitochondria containing
326 distinctive cristae with short, stubby branches (Fig. 4C). This characteristic is
327 also recognized in *S. stercorea* (Dykstra 1976a, 1976b), *D. parva* (Anderson and
328 Cavalier-Smith 2012), and *Af. marina* (Porter 1984), but these mitochondrial
329 features have not been observed in other members of Labyrinthulomycetes. This
330 characteristic could be synapomorphic or apomorphic characteristics of the
331 genus *Diplophrys* and related lineages. Nevertheless it remain a matter of
332 debate because *Archerella flavum,* closely related to genus *Diplophrys*, have
333 mitochondria with tubular cristae (Bonnet 1981).

334 In TEM observation, unidentified concentric and helical cytoplasmic
335 membranes were observed (Fig. 5). Similar cytoplasmic membranes were
336 observed in *S. stercorea* (Dykstra et al. 1975) and *D. parva* (Anderson and
337 Cavalier-Smith 2012), but they have not been reported in other
338 Labyrinthulomycetes species including *Af. marina.* The function of these
339 organelles is unclear, and no particular explanation has been uncovered. One

possible insight is that these organelles appeared to be connected to lipid bodies and the outer membrane of mitochondria through the endoplasmic reticulum (Fig. 5B), so it is possible that this organelle plays some roles in development of lipid bodies. It means that this organelle will be an unusual type of smooth endoplasmic reticulum. Although further investigations are needed to answer the question, this organelle would be a key structure in the development of outstanding lipid bodies in *Diplophrys*.

Phylogenetic analyses demonstrated that the genus *Diplophrys,* including *D. mutabilis*, clearly belongs to Labyrinthulomycetes, Amphitremida, Diplophryidae. From our phylogenetic tree, *D. mutabilis* belongs to Amphitremida, and it exhibited a relationship with TPG rather than LPG (Fig. 7). This result is different from that of Gomaa et al. (2013), in which *Archerella, Amphitrema*, and *Diplophrys* formed a deep branching clade within all Labyrinthulomycetes. However being in progress, phylogenetic relationships in Labyrinthulomycetes remain controversial because of low bootstrap supports. More molecular data should be obtained to clarify ther relationships.

D. mutabilis resembles *D. archeri* in several manners. Based on the original description of *D. archeri* (Baker 1868), Anderson and Cavalier-Smith defined the average cell size of the species as 12.7 μm (Anderson and Cavalier-Smith 2012). This is approximately twice the size of *D. mutabilis* and *D. parva*. Concerning motility, no locomotion was mentioned in the original description of *D. archeri* (Barker 1868). In contrast, *D. mutabilis* possessed an ability of active gliding motility (Table 1). In addition, *D. archeri* has a few lipid bodies of an orange or

amber color, whereas *D. mutabilis* has 1–10 lipid bodies of a colorless or amber color. *D. archeri* was also reported to have a fixed shape because of its solid cell wall (Patterson 1996), whereas *D. mutabilis* can easily change its shape (Figs. 1A, 1B, 6A). These differences distinctly separate *D. mutabilis* from *D. archeri*.

D. parva appears to be the closest relative to *D. mutabilis*. The phylogenetic tree indicated that these species are closely related (Fig. 7). Moreover, their cell sizes are extremely similar. However, regarding motility, these species are different (Table 1). *D. parva* exhibits only minimal cell motility, if any at all, whereas *D. mutabilis* locomotes by active gliding with moving filopodia. Moreover, the inner structure of the ectoplasmic elements and their root morphology are different between these species. In *D. parva*, ectoplasmic elements emerge from the cell surface as electron dense conical projections, possibly sagenogens, and become longer tubular extensions (Andersen and Cavalier-Smith 2012). However, in *D. mutabilis*, sagenogen-like bodies were not observed, and the ectoplasmic elements contained ribosome-free cytoplasm and branching internal membrane system elements (Fig. 4B). Based on these differences concerning ectoplasmic elements, it is apparent that they are different species. In addition, whereas the scales of *D. parva* are slightly oval to elongated in shape, the scales of *D. mutabilis* are round. From this perspective, it is clear that they are separate species.

D. mutabilis has a different habitat from another morphologically similar species, *Af. marina*. Both species share a whole-cell morphology and thin, circular, simple scales. However, *Af. marina* lacks unidentified cytoplasmic membranes and an

386 internal membrane system of ectoplasmic elements (Dykstra and Porter 1984); it
387 is contrastingly well developed in *D. mutabilis* (Table 1). Furthermore, 18S rRNA
388 analysis (Fig. 7) demonstrated that they are distantly related within
389 Labyrinthulomycetes.

390 The vegetative cells of *S. stercorea* resemble *D. mutabilis* in light microscopic
391 morphology, gliding motility, and organelle structure such as unidentified
392 cytoplasmic membranes (Dykstra and Olive 1975). However, the aggregative
393 behavior, terrestrial habitat, and complicated life cycle including a sorocarp
394 would be sufficient to separate *Sorodiplophrys* from *Diplophrys* at the generic or
395 perhaps higher level (Table 1). This should be confirmed when the DNA
396 sequence of *Sorodiplophrys* becomes available. *Sorodiplophrys* may be related
397 to Amphifilidae because the latter possesses soil DNA (Fig. 7).

398 *Elaeorhanis cincta*, a filopodial amoeba with debris on its cell surface, has been
399 suggested to be closely related to *Diplophrys* species (Patterson 1996). They
400 share filopodia, an oil-like refractive body of an orange or amber color, and some
401 other features. Although *Diplophrys* and *Elaeorhanis* are easily distinguished
402 from one another by the presence or absence of debris layer, it is still possible
403 that they may be closely related species or simply different ecotypes of the same
404 organism. No *Elaeorhanis* strain or its sequence data are available at present
405 even though the genus is common in freshwater habitat. A detailed comparison
406 between these two organisms is required to settle this question.

407 From the phylogenetic tree, *D. mutabilis* clearly belonged to
408 Labyrinthulomycetes, Amphitremida, Diplophryidae, near *At. wrightianum* and *Ar.*

flavum. The two genera are very different from *Diplophrys* concerning cell size and presence of monolithic brown or hyaline testa and endosymbiotic algae. Endosymbionts, or preyed bacteria within *Diplophrys*, has never been reported despite the presence of attached bacteria to the scale surface and ectoplasmic elements (Fig. 2A, C). Thus, *Diplophrys* does not appear to display phagocytosis in our results. On the contrary, the ultrastructure of their ectoplasmic elements and roots is similar to that of *D. mutabilis*, including the absence of bothrosomes and presence of an internal membrane system (Table 1). *Diplophrys* is phylogenetically similar to these two genera, but it diverged before its species obtained endosymbiotic algae.

Concerning morphologically based aspects, Diplophryidae is more similar to Amphifilidae than to Amphitremidae, although Diplophryidae is closer to Amphitremidae than to Amphifilidae with respect to its molecular phylogeny. Interestingly, such discrepancies between morphology and molecular phylogeny are frequently observed in Labyrinthulomycetes. For example, *Oblongichytrium* species have morphological similarities to Thraustochytrida species (Yokoyama and Honda 2007); however, it was included in LPG in the molecular phylogenetic analysis (Gomaa et al. 2013; Yokoyama and Honda 2007). In terms of molecular phylogeny, *Diplophrys* tend to be related to TPG rather than LPG. Conversely, the ectoplasmic elements of the genera *Labyrinthula* and *Aplanochytrium,* which belong to LPG, support gliding motility as observed in *D. mutabilis, Af. marina*, and *S. stercorea*. However, the ectoplasmic elements of *Labyrinthula* species, e.g., *L. zosterae* (Muehlstein and Porter 1991), and *Aplanochytrium* species, e.g., *Ap. saliens* (Leander and Porter 2000; Quick 1974), are both branching and

anastomosing; therefore, they construct a highly developed ectoplasmic network. The ectoplasmic elements of *D. mutabilis* exhibited dichotomous branching (Fig. 2C), and an anastomosing network has never been observed. *D. mutabilis, Af. marina*, and *S. stercorea* lack bothrosomes, a shared characteristic of Labyrinthulomycetes species, but *D. parva* was reported to have bothrosome-like structures. More studies in both morphology and molecular phylogeny are required to establish a robust phylogenetic relationship between Labyrinthulomycetes species.

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Figure Legends

Figure 1. Light micrographs of *Diplophrys mutabilis*

A. Colonial cells connected through ectoplasmic elements (white arrowheads). This culture is not axenic, and thus, bacterial contaminations are present (arrowheads). Arrows denote the contractile vacuoles in cells.

B. Elongated fusiform (spindle-shaped) cell. Ectoplasmic elements (white arrowheads) and a contractile vacuole (arrow) are also recognizable.

C, D. Spherical cell of *Diplophrys mutabilis* containing oil droplets.

C. Differential interference contrast.

D. Fluorescent micrograph of a Nile Red-stained cell. Neutral lipid emits yellow fluorescence. Red fluorescence is derived from polar lipids such as phospholipid.

Figure 2. Whole-mounted cells of *Diplophrys mutabilis*

A. Cell projecting ectoplasmic elements. Swelling is observed in the basal part of ectoplasmic elements (arrows). Many bacteria (arrowheads) are also contained.

B. Close-up image of the swelling with an inhomogeneous texture.

C. Close-up image of the distal part of ectoplasmic elements exhibiting dichotomous branching.

Figure 3. SEM images of *Diplophrys mutabilis*

A. Lyophilized cell. Some bacteria (arrowheads) are attached to the surface of the cell.

B. Close-up image of a scale. The scale is round and displays an incrassate

margin (arrows). Scales are very thin, and thus, overlapping of multiple scales is recognizable (arrowheads).

C. Ectoplasmic elements projecting from cells (white arrowheads).

Figure 4. TEM images of *Diplophrys mutabilis*

A. Spherical cell. G: Golgi body, L: lipid body, M: mitochondria, N: nucleus, B: bacteria.

B. Longitudinal section of the basal part of the ectoplasmic element. Internal membranous tubes are observed (arrowhead).

C. Mitochondria with inflated finger-like tubular cristae.

D. Lipid body.

E. Golgi body. Two developing scales are inside (arrows). Some vesicles (arrowhead) are observed between the nucleus and cis-side of the Golgi body.

Figure 5. TEM images of unidentified cytoplasmic membranes revealing different topologies

A. Concentric ring form.

B. Single helical form. L: lipid body, M; mitochondria. Arrowhead denotes the inner end of the helix.

C. Double-helical form. Arrowheads denote two inner ends of helixes.

D. Slanted transverse section.

Figure 6. TEM images of *Diplophrys mutabilis*

A. Plastic cell penetrating the substratum. S: Substratum

B. Dividing cell. Two daughter cells are recognizable. Some bacteria (arrowheads) are located inside the cell wall of the parent cell. The ectoplasmic element (white arrowheads) elongates via the cleft of the parent scale layer. D: daughter cell.

Figure 7. Phylogenetic tree based on the 18S rDNA sequences and constructed using the maximum likelihood method based on a 1230-bp alignment. Bayesian approach also estimated the same topology of the tree (not shown). Support values at each node are presented for ML/Bayes. Bootstrap values larger than 50 and posterior probabilities larger than 0.80 are shown. Smaller values are represented by "–."

Table 1. A comparative table of *Diplophrys mutabilis* and related organisms

?: Question mark indicates that the corresponding organ-like microstructures are observed but less certain.

＊: The character is unspecified but determined from the information of other species of the same genus.

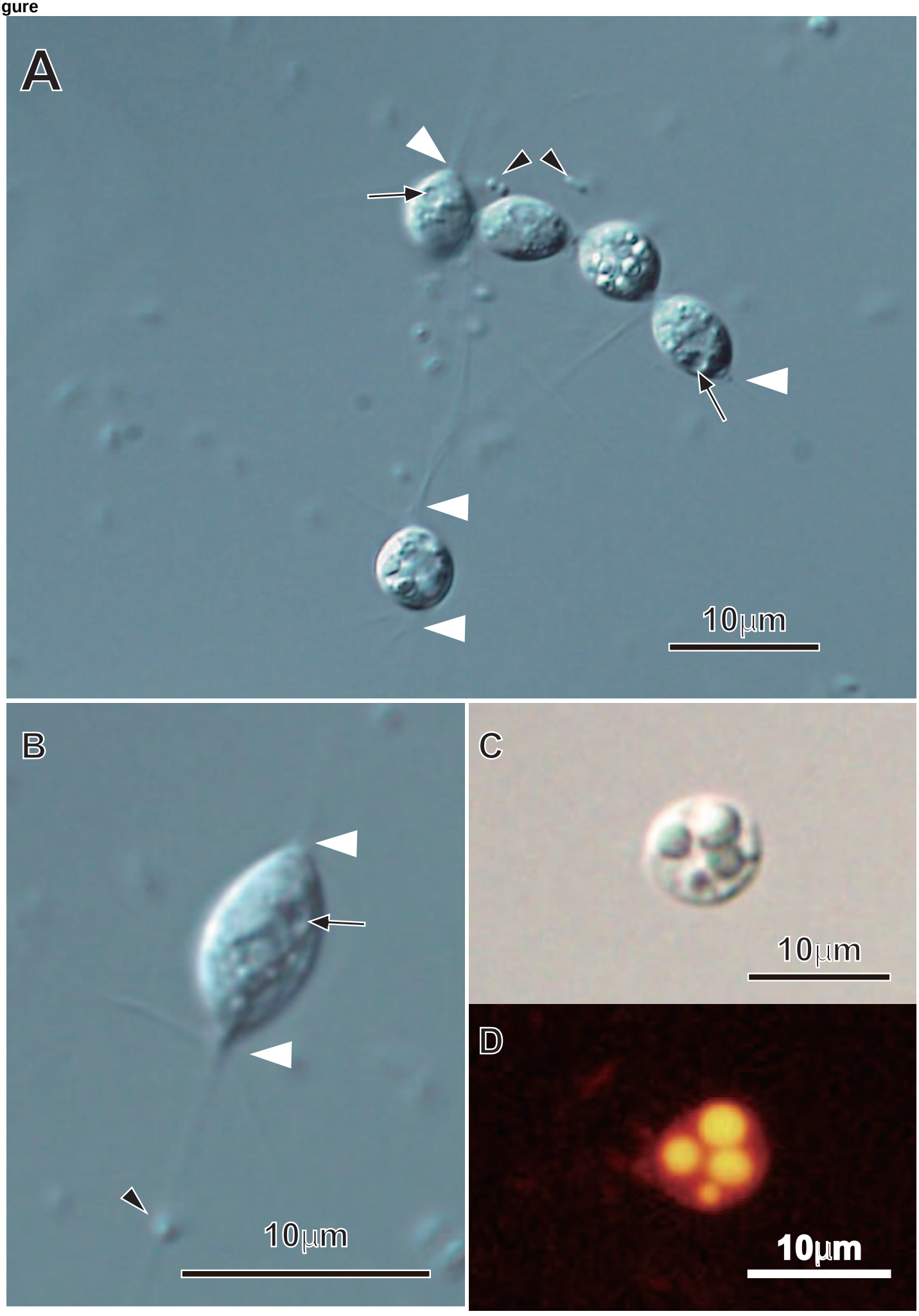
＊＊: In this table, "aggregation" refers to active aggregation of free-moving individuals. Aggregation as a result of cell division of aplanatic or sluggish cells observed in some species is treated as incapable ("–") in this table.

Table

Table 1

Genus/species (sources)	Internl membrane system	unidentified cytoplasmic membranes	shape of mitochondrial cristae	Cell size (μm)	gliding motility	sagenogenetosome (= bothrosome, sagenogen)	habitat	aggregation (**)	endosymbiotic algae
Diplophrys mutabilis （This study）	＋＋	＋＋	short, stubby branches	3.1 - 8.3 × 3.4 - 10.3	＋	－	freshwater	－	－
Diplophrys archeri Barker, 1868 (Anderson and Cavalier-smith 2012, Barker 1868, Patterson 1996)	No data	No data	No data	12.7	－	No data	freshwater	－	－
Diplophrys parva Anderson et Cavarier-smith, 2012 (Anderson and Cavarier-smith 2012)	－	＋＋	short, stubby branches	6.5 ± 0.08 × 5.5 ± 0.06; mean ± SE	－	＋?	freshwater	－	－
Amphifila marina Dykstra et Porter, 1984 （Dykstra And Porter 1984）	－	－	short, stubby branches	3.7 - 5.9 × 5.1 - 8.5	＋	－	marine	－	－
Sorodiplophrys stercorea (Cienkowski) Olive et Dykstra, 1975 （Dykstra And Olive 1975）	＋	＋	short, stubby branches	2.4 - 4.8 × 4.8 - 9.6	＋	－	terrestrial	＋	－
Elaeorhanis cincta Greeff, 1873 （Lee 2000, Patterson 1996）	No data	No data	No data	10 - 20 in diameter	No data	No data	freshwater	No data	－
Amphitrema wrightianum Archer, 1869 （Edmondson1959, Gomma 2013）	No data	No data	No data	61 - 95 in diameter	＋	No data	freshwater	－	＋
Archerella flavum Loeclich et Tappan, 1961 （Bonnet et al. 1981,	＋?	－	tubular cristae	45 - 77 in diameter	＋	－	freshwater	－	＋
Labyrinthula zosterae Muehlstein et Porter, 1991 (Muehlstein and Porter 1991)	＋* （Perkins 1972）	－	tubular cristae	15.5 - 19.5 × 3.5 - 5.0	＋	＋	marine	＋	－
Aplanochytrium stocchinoi Morro et al. 2003 (Morro et al. 2003)	No data	－	tubular cristae	4 - 8 in diameter	＋	＋* (Watanabe 2012)	marine	－	－
Schizochytrium aggregatum Goldstein et Belsky, 1964 (Goldstein and Belsky 1964)	＋* （Perkins 1972）	－	tubular cristae	6 - 12 in diameter	－	＋	marine	－	－

Figure



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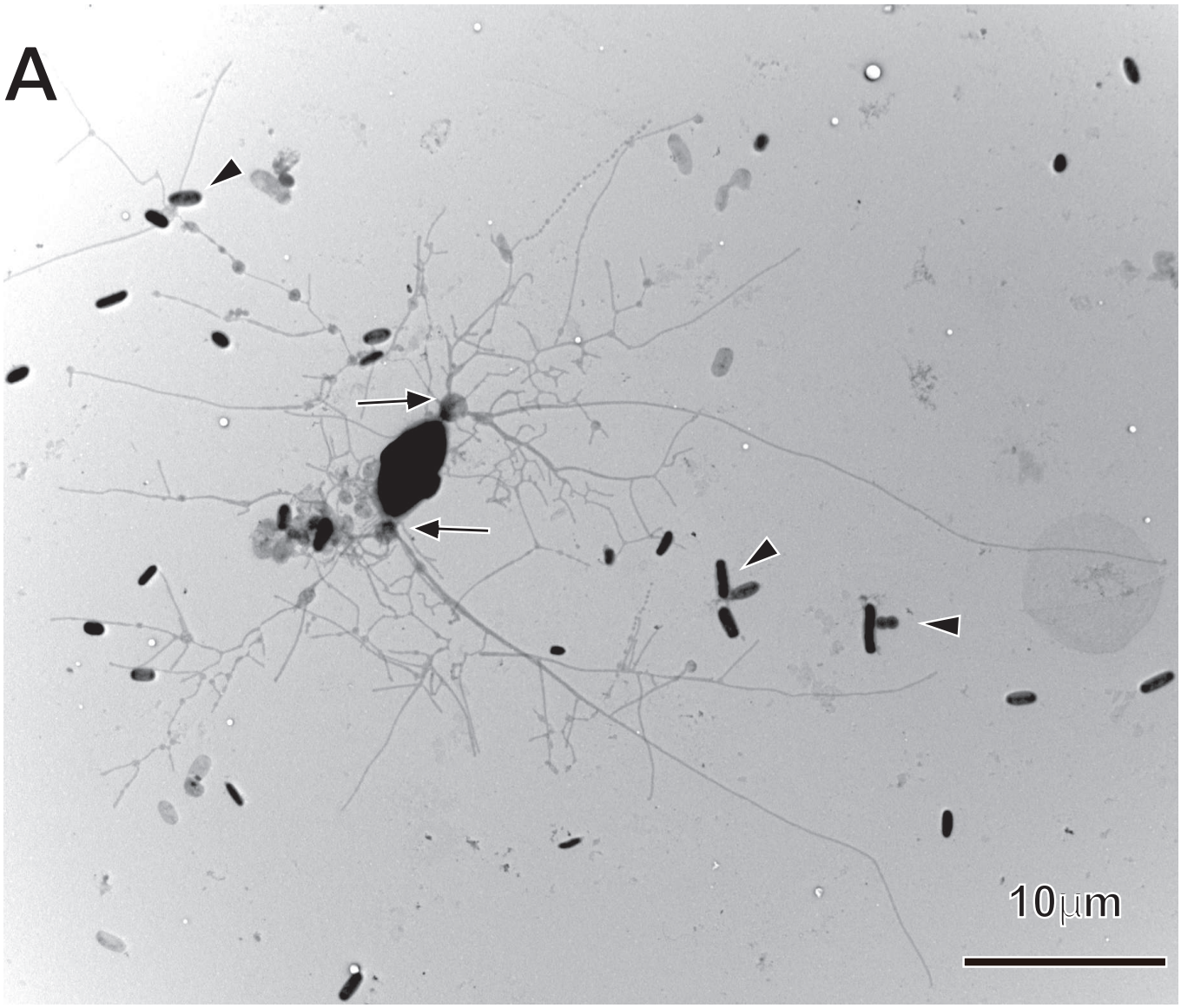

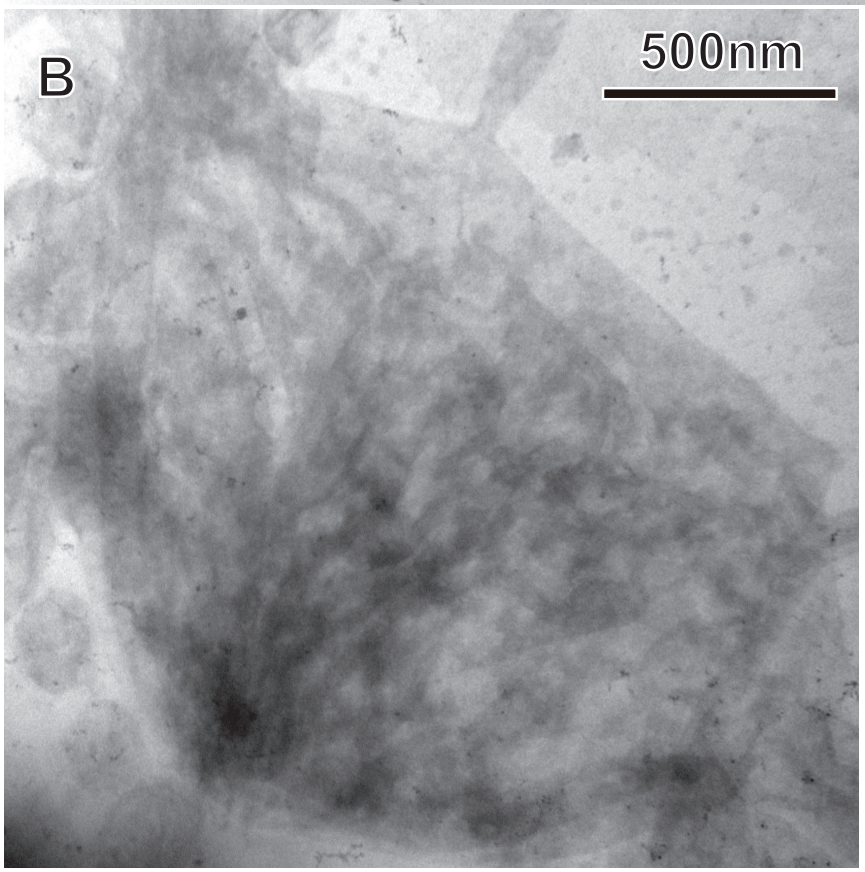

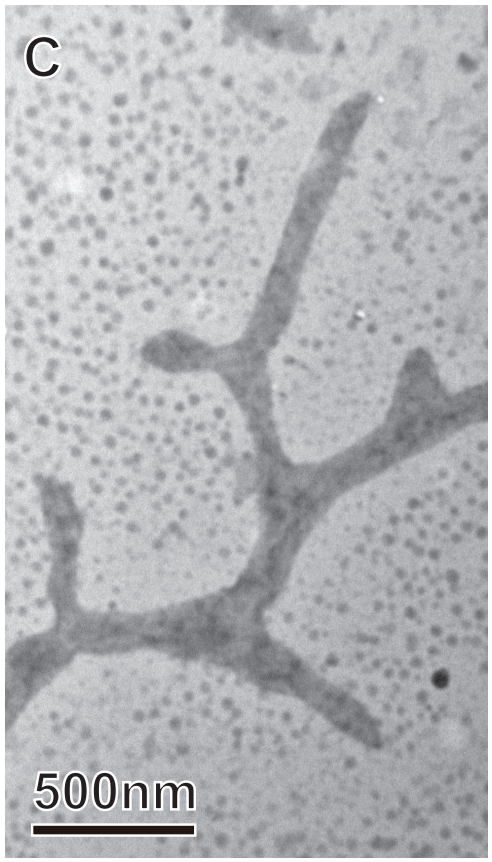

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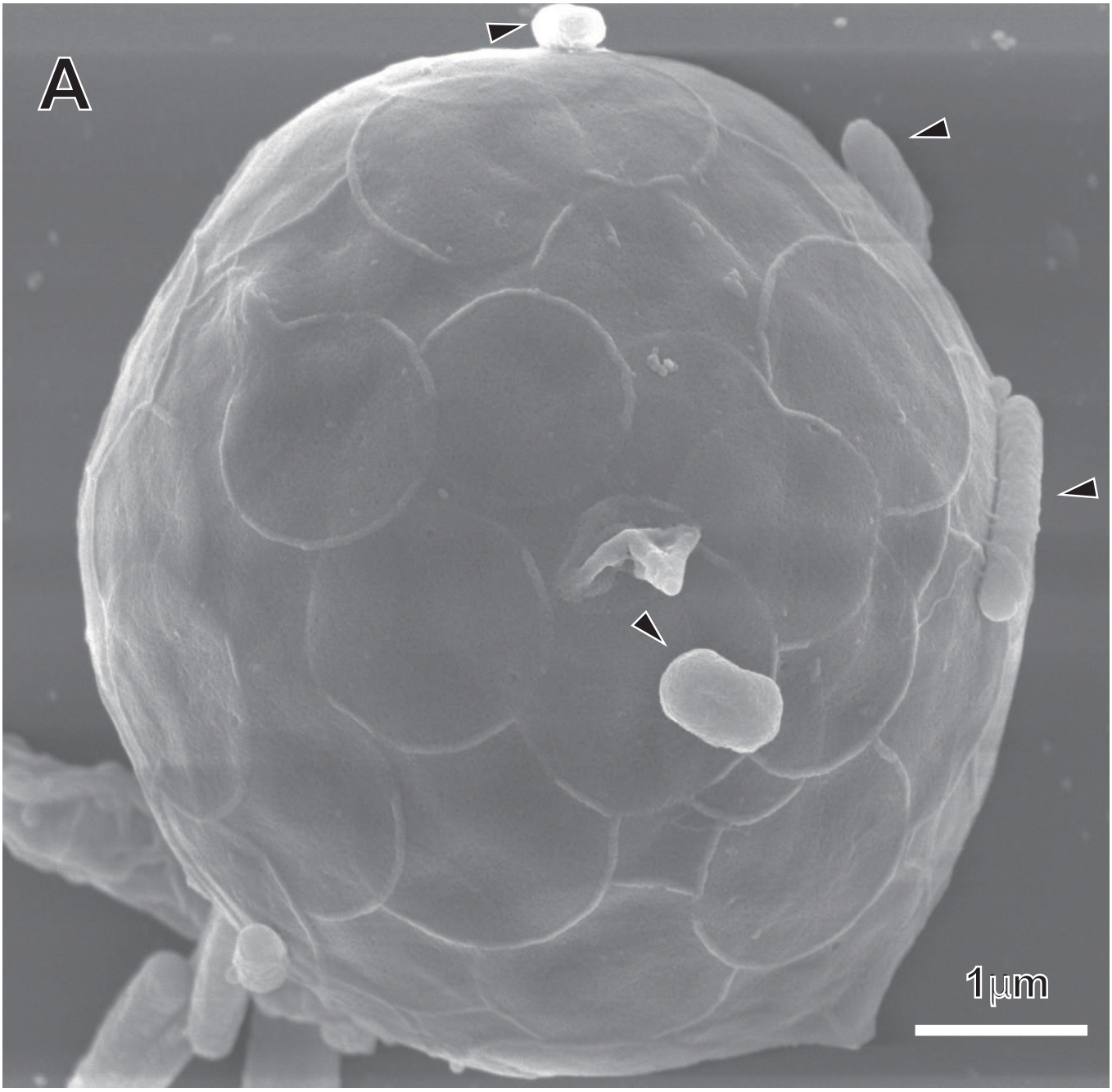

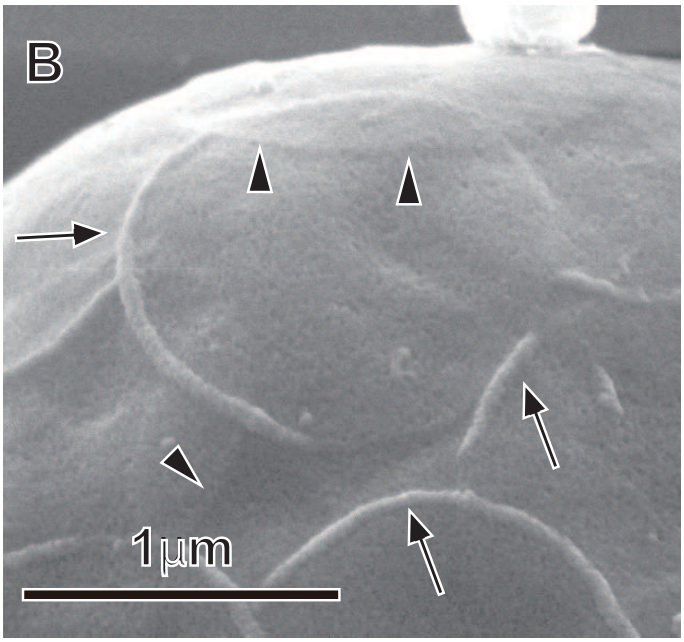

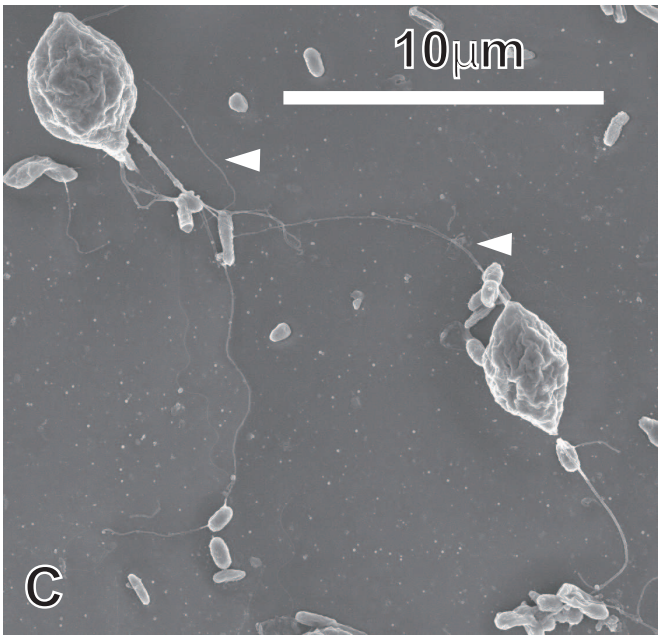

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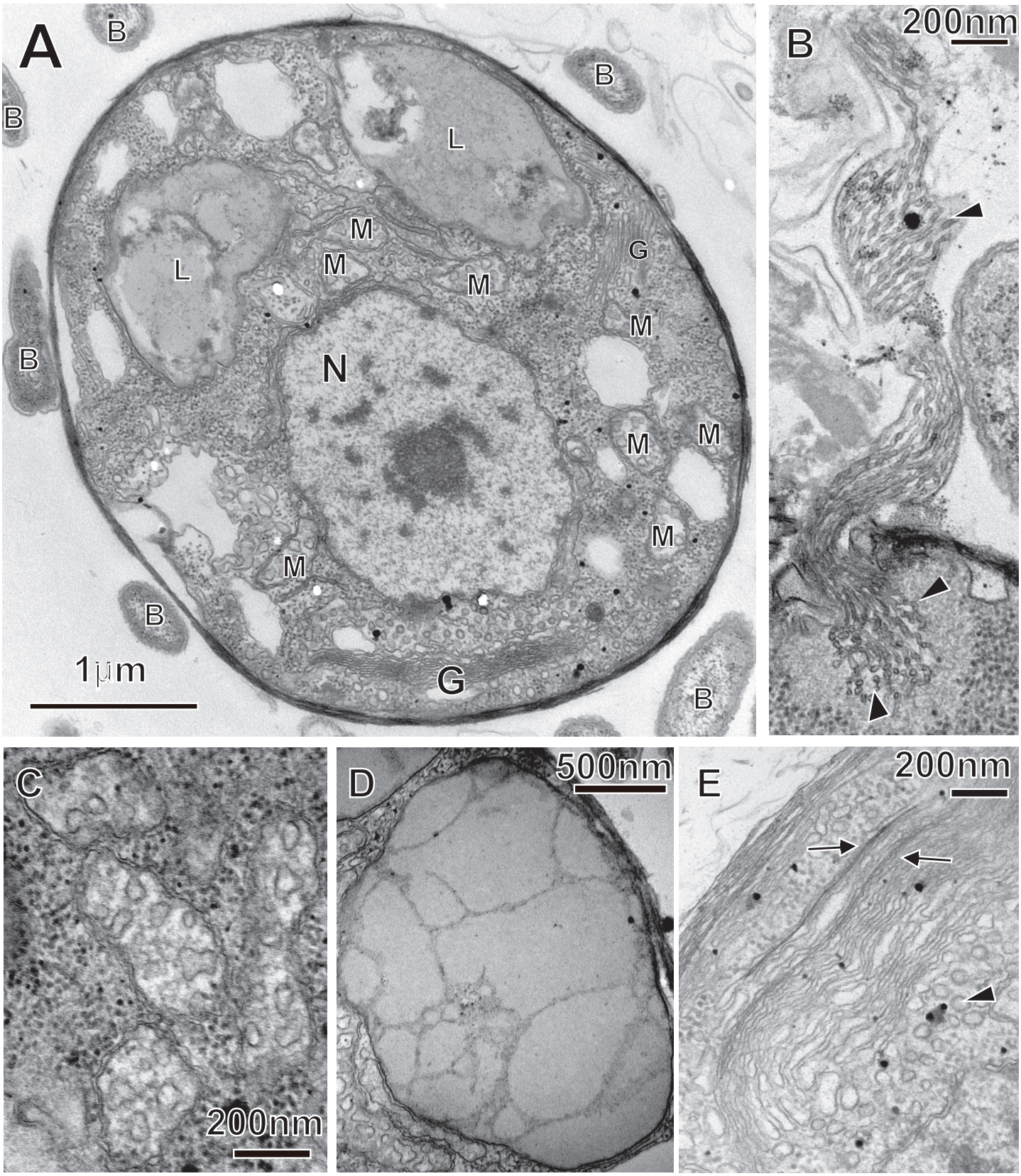

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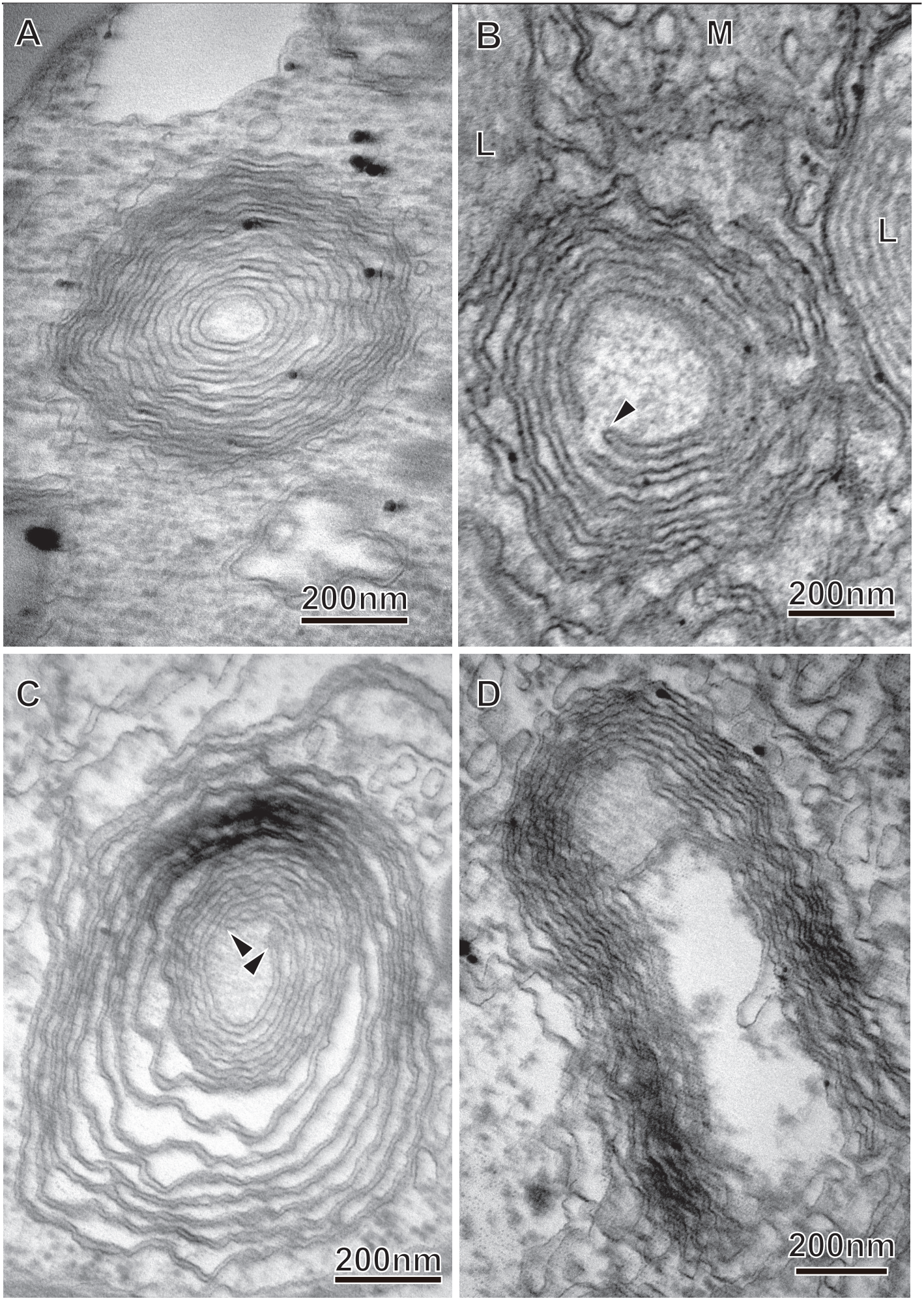

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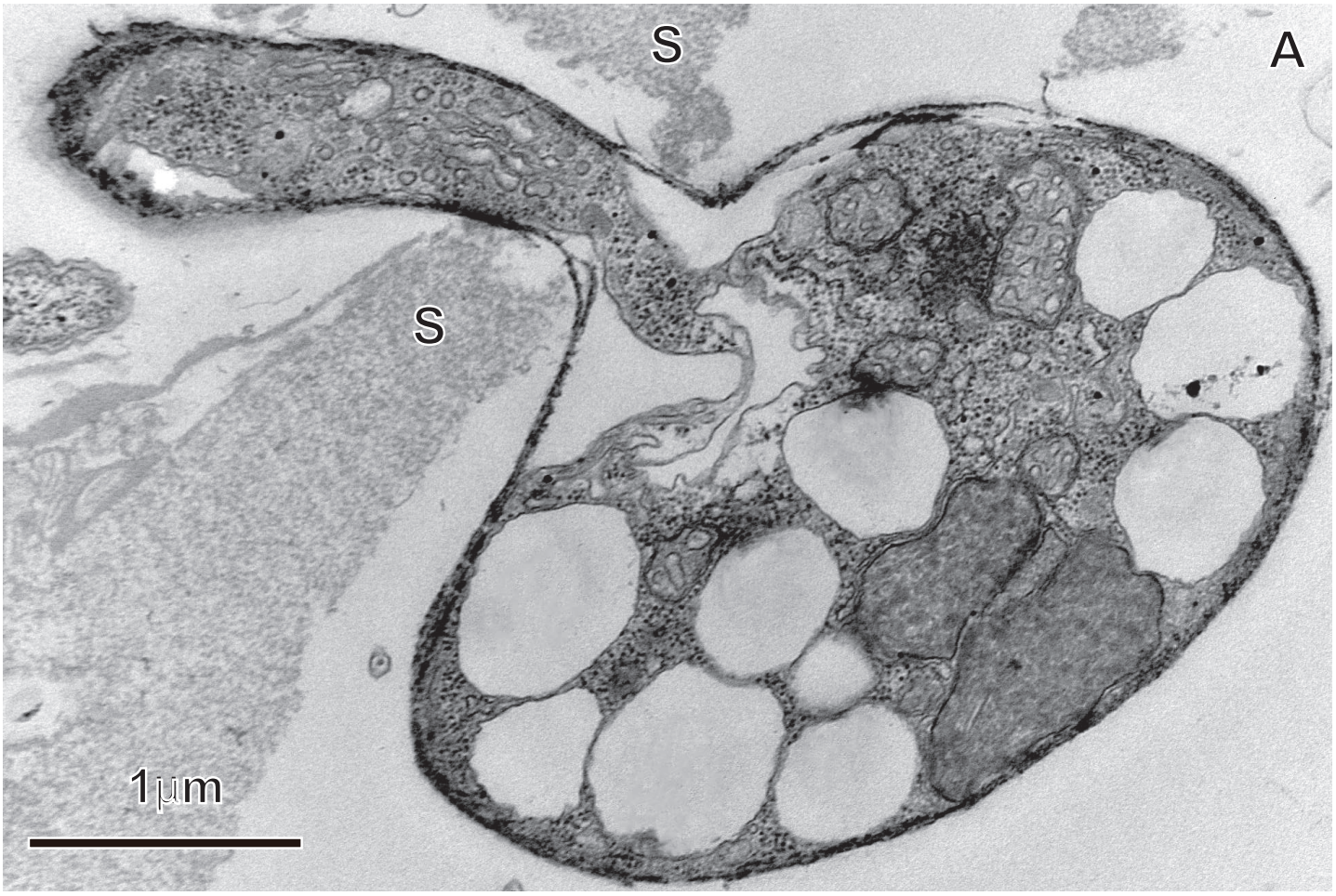

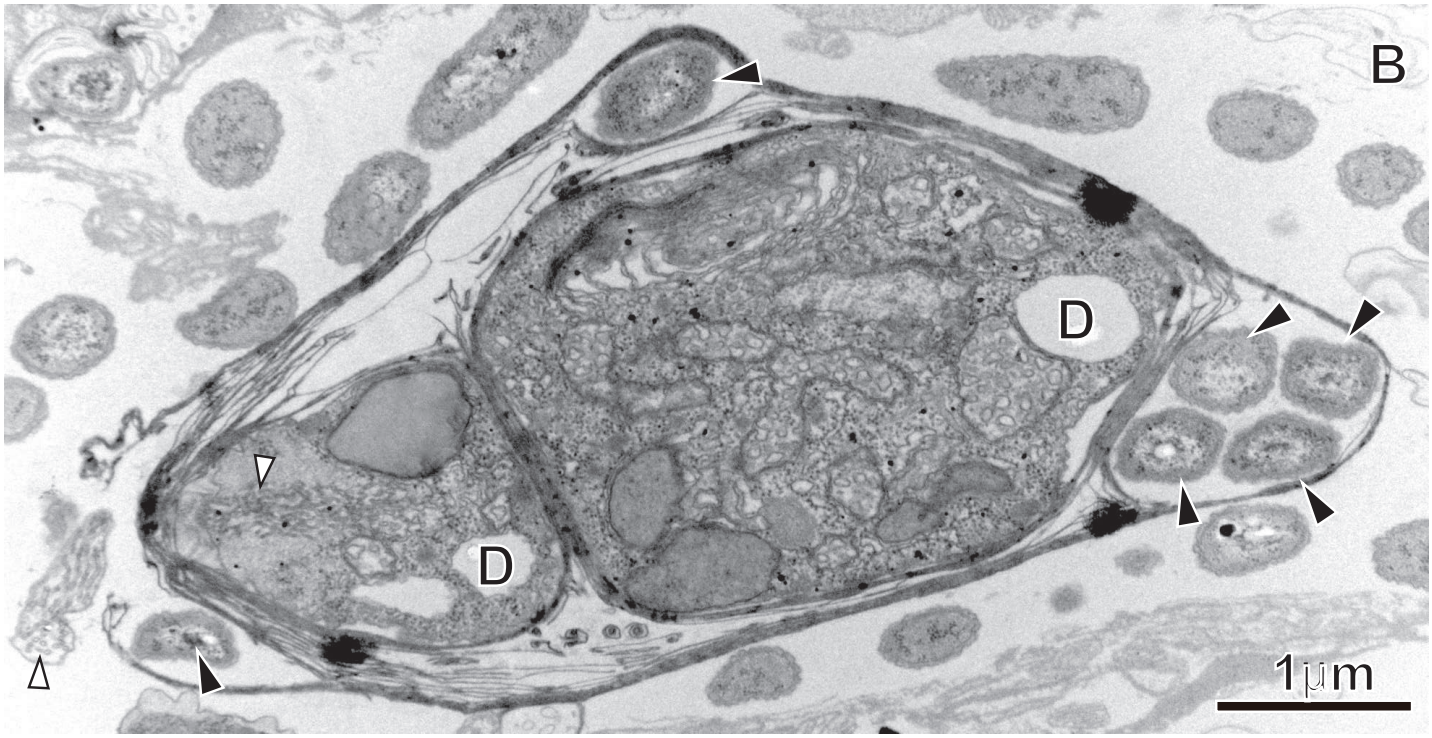

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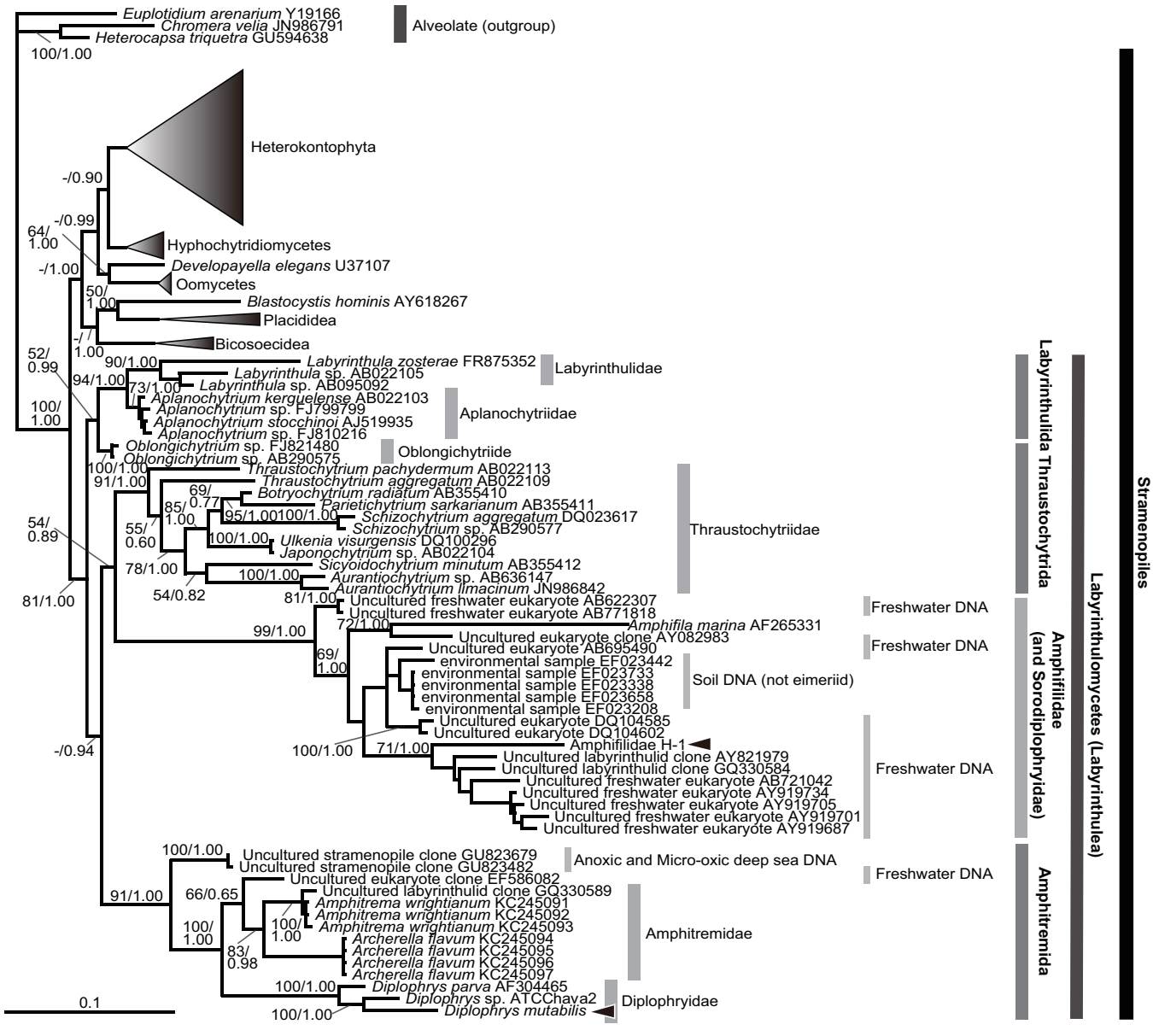
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-/0.90
-/0.99
64/
1.00
Hyphochytridiomycetes
-/1.00
Developayella elegans U37107
Oomycetes
50/
1.00
Blastocystis hominis AY618267
Placididea
-/
1.00
Bicosoecidea
52/
0.99
90/1.00
Labyrinthula zosterae FR875352
Labyrinthulidae
94/1.00
Labyrinthula sp. AB022105
73/1.00
Labyrinthula sp. AB095092
100/
1.00
Aplanochytrium kerguelense AB022103
Aplanochytrium sp. FJ799799
Aplanochytrium stocchinoi AJ519535
Aplanochytrium sp. FJ810216
Aplanochytriidae
Oblongichytrium sp. FJ821480
Oblongichytrium sp. AB290575
Oblongichytriide
100/1.00
91/1.00
Thraustochytrium pachydermum AB022113
Thraustochytrium aggregatum AB022109
69/
0.77
Botryochytrium radiatum AB355410
85/
1.00
Parietichytrium sarkarianum AB355411
95/1.00100/1.00
Schizochytrium aggregatum DQ023617
Schizochytrium sp. AB290577
54/
0.89
55/
0.60
100/1.00
Ulkenia visurgensis DQ100296
Japonochytrium sp. AB022104
Thraustochytriidae
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Sicyoidochytrium minutum AB355412
100/1.00
Aurantiochytrium sp. AB636147
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81/1.00
81/1.00
Uncultured freshwater eukaryote AB622307
Uncultured freshwater eukaryote AB771818
Freshwater DNA
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Amphifila marina AF265331
99/1.00
Uncultured eukaryote clone AY082983
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Freshwater DNA
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1.00
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environmental sample EF023733
environmental sample EF023338
environmental sample EF023658
Soil DNA (not eimeriid)
environmental sample EF023208
Uncultured eukaryote DQ104585
Uncultured eukaryote DQ104602
-/0.94
100/1.00
71/1.00
Amphifilidae H-1
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Uncultured labyrinthulid clone GQ330584
Uncultured freshwater eukaryote AB721042
Uncultured freshwater eukaryote AY919734
Uncultured freshwater eukaryote AY919705
Uncultured freshwater eukaryote AY919701
Uncultured freshwater eukaryote AY919687
Freshwater DNA
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Uncultured stramenopile clone GU823679
Uncultured stramenopile clone GU823482
Anoxic and Micro-oxic deep sea DNA
Uncultured eukaryote clone EF586082
Freshwater DNA
91/1.00
66/0.65
Uncultured labyrinthulid clone GQ330589
Amphitrema wrightianum KC245091
Amphitrema wrightianum KC245092
100/
1.00
100/
1.00
Amphitrema wrightianum KC245093
Amphitremidae
Archerella flavum KC245094
83/
0.98
Archerella flavum KC245095
Archerella flavum KC245096
Archerella flavum KC245097
100/1.00
Diplophrys parva AF304465
Diplophrys sp. ATCChaya2
Diplophryidae
100/1.00
Diplophrys mutabilis
0.1
Labyrinthulida Thraustochytrida
Amphifilidae (and Sorodiplophryidae)
Amphitremida
Labyrinthulomycetes (Labyrinthulea)
Stramenopiles