Phylogenetic Systematics of Freshwater Labyrinthulomycetes

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LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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 environments, and cultures of the strains were established. Among the strains, it revealed that one strain, isolated from Lake Nojiri, obviously belonged to genus *Diplophrys* from a molecular phylogenetic analysis based on 18S rRNA sequences. This strain 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*. All other strains were clearly distinct from *Diplophrys* on the basis of 18S rDNA sequences. Molecular phylogenetic analysis showed a close relationship of these strains with *Amphifila marina*, and its sequence is similar to many environmental stramenopile sequences. We also studied the ultrastructure of these strains and revealed these strains were clearly distinct from *Af.marina*. Thus, we described one of these strain as a new genus and species, *Fibrophrys columna*. This study suggests that these species form a unique group in Labyrinthulomycetes

Keywords: Amphitremida ;Amphifilidae; *Diplophrys mutabilis* sp.nov.; *Fibrophrys* gen. nov.; *Fibrophrys columna* sp. nov.; Phylogeny; ultrastructure.

CHAPTER 1: INTRODUCTION

1.1 General introduction

The Labyrinthulomycetes, a class of mainly marine protists, is a member of the stramenopiles [Dick 2001 (as"Straminipila"); Patterson 1989] and is characterized by the following features: rhizoid-like ectoplasmic net elements produced by a unique organelle, the bothrosome (sagenogen, sagenogenetosome) (Moss 1980; Perkins 1972; Porter 1972), biflagellate zoospores possessing an anterior flagellum with tripartite tubular mastigonemes (Kazama 1973), and multil-amellate cell walls composed of Golgi body-derived scales (Alderman et al. 1974; Darley et al. 1973). This class includes three orders and one superfamily: Thraustochytrida Sparrow1973, Labyrinthulida Doflein 1901, Amphitremida Poche1913, and Amphifiloidea Cavalier-Smith 2012. Thraustochytrida, characterized by globose cells forming ectoplasmic nets that are derived from a single bothrosome, includes *Althornia*, *Aurantiochytrium*, *Botryochytrium*, *Japonochytrium*,*Oblongichytrium*, *Parietichytrium*, *Schizochytrium*, *Sicyoidochytrium*, *Thraustochytrium*, and *Ulkenia* (Tsui 2009). Labyrinthulida, characterized by spindle-shaped cells with gliding motility using the channels of ectoplasmic nets extending from a number of bothrosomes (Honda et al.1999), includes only the genera *Aplanochytrium* and *Labyrinthula* (Andersonand Cavalier-Smith 2012; Yokoyama and Honda2007; Yokoyama et al. 2007). Amphitremida includes *Amphitrema*, *Archerella*, and *Diplophrys*. The Amphifiloidea superfamily comprises two

families: Amphifilidae Cavalier-Smith 2012, including only *Amphifila*, and Sorodiplophryidae Cavalier-Smith2012, including only *Sorodiplophrys*.

Labyrinthulomycetes species play ecological roles as decomposers or parasites. Naganuma et al. (1998) estimated the abundance of the Thraustochytridae 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 675×10^3cells/L (Damare and Raghukumar 2008; Naganuma et al. 2006). Such high abundance and widespread occurrence indicate their ecological importance in coastal and oceanic environments. Conversely, the distribution of Labyrinthulomycetes species in freshwater water is poorly understood, and only a few freshwater genera have been described. Of these, the most common freshwater genus is *Diplophrys*. Similarly, many environment sequences detected from freshwater habitat were included in Amphifiloidea, but details are unclear.

1.2 *Diplophrys*

Diplophrys was described with the 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 emanating 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 that displays 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 Thraustochytrida rather than Labyrinthulida (Leander and Porter 2001).At first, 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 features. The phylogenetic position of the new species is also consolidated using 18S rRNA sequence comparisons.

1.3 *Fibrophrys*

As previously described, *Amphifila marina*, the type species of Amphifilidae which was first described as *Diplophrys marina* (Dykstra and Porter 1984), was transferred to a new genus, *Amphifila*, Amphifilidae (Andersonand Cavalier-Smith 2012). The Amphifilidae is currently composed of only one marine species, *Af. marina*. However, based on molecular phylogeny, many environmental DNA sequences obtained from freshwater and terrestrial sampling sites across several regions, including Asia (Kojima et al.2009), Europe (Lara et al. 2011; Slapeta et al. 2005; Zettleret al. 2002), America (Richards et al. 2005), and Antarctica (Nakai et al. 2012), have been found to belong to this family. Furthermore, related sequences were obtained from extreme environments such as suboxic ponds (Slapeta et al.2005), rivers with a low pH and high concentrations of heavy metals (Zettler et al. 2002), and glacial ponds in Antarctica (Nakai et al. 2012). These reports have revealed wide ecological distribution of related organisms. However, because there are no reports of successful isolation or available cultures for the members of this family, except *Af. marina*, their morphological features remain unclear.

This study is the first report of the isolation and establishment of a stable culture of members of the Amphifilidae family obtained from freshwater habitats. We describe a new genus and a new species isolated from Hiuchigaike Pond, Ibaraki Prefecture, Japan,

and specify the morphological characteristics and molecular phylogenetic position of this new genus based on microscopy and 18S rDNA sequence comparisons.

CHAPTER 2: MATERIALS AND METHODS

2.1 Sample collection and cultivation

D.mutabilis was isolated from freshwater samples collected from Lake Nojiri, Nagano Pref., Japan. *Fibrophrys columna* was isolated from freshwater samples collected from Hiuchigaike Pond, Ibaraki Prefecture, Japan in July 2011. Another strain, *Fibrophrys* sp. E-1, was isolated from the freshwater samples collected from Lake Echigo, Hokkaido Prefecture, Japan in July 2012. All samples were collected from surface water using a sampling bottle. A clonal culture of each strain was established using a single-cell isolation technique with micropipettes. Autoclaved distilled water and commercially available dried water fleas for aquarium fish were used as the growth medium. We added 5–10 individual dried water fleas to 5 ml of distilled water and autoclaved the mixture at 120◦C for 20 min. The cultures were maintained in test tubes at room temperature under a shade.

2.2 Morphological observations

For light microscopy, a Leica DM2500 microscope (Leica Microsystems KK, Tokyo, Japan) and an Olympus IX71microscope (Olympus Corporation, Tokyo, Japan) equipped with Nomarski differential interference contrast optics were used. For scanning electron microscopy, cultured samples were mounted on 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 then fixed in

1% osmium tetroxide (OsO_4) for30 min. The samples were then dehydrated through a graded ethanol series (50, 75, 90, 95, and 100%) by incubating the samples at each concentration for 15 min, followed by the substitution of 100% ethanol with dehydrated t-butyl alcohol. The specimens were then freeze-dried using a VFD-21Sfreeze drier (Shinku Device Co., Ltd., Ibaraki, Japan) and mounted on specimen stubs. Next, the specimens were coated with platinum/palladium using an E102 ion sputter (Hitachi, Ltd., Tokyo, Japan) and observed using a JSM-6330F field emission scanning electron microscope (JEOL, Ltd., Tokyo, Japan).

For whole-mount images, the cells were exposed to 4%OsO_4 fumes for 5 min, followed by washing in distilled water. The cells were stained for 3 min with 4% uranyl acetate and then viewed using a Hitachi H-7650 transmission electron microscope (TEM; Hitachi, Ltd., Tokyo, Japan).For thin sectioning, vegetative cells were exposed to 1%OsO4fumes for 3 min. The cells were then fixed in a solution containing 2.5% glutaraldehyde, 2% OsO4, 4.5% sucrose, and 0.1 M cacodylate buffer at pH 7.0 for 90 min under refrigeration (4◦C, in darkness), followed by washing in the same buffer three times for 10 min each. The cells were successively dehydrated in 30, 50, 70, 90, 95, and 100%acetone by incubating the cells at each concentration for10 min under refrigeration, followed by incubating twice in an acetone–propylene oxide (PO) mixture and pure PO for10 min each. The dehydrated pellet was embedded in an agar low-viscosity resin (LV Resin, VH1 and VH2 Hardener, and LV Accelerator; Agar Scientific, Stansted, Essex, UK), and a1:1 mixture of PO and the resin was prepared. The resin was polymerized for 12 h at 70◦C.Thin sections were cut with an ultramicrotome (EM UC7;Leica Camera AG, Wetzlar, Germany) and stained for 5 min with 4% uranyl acetate, followed by incubation with Sato's lead citrate (Sato 1968) for 5

min. The sections were viewed with the Hitachi H-7650 TEM.

2.3 Molecular phylogenetic analyses

To amplify the 18S rDNA of the strains obtained, single cells of each strain were isolated with micropipettes using a single-cell isolation technique and were transferred into polymerase chain reaction (PCR) tubes with autoclaved distilled water. The tubes were first stored overnight at room temperature to digest the engulfed feed and then placed in a freezer at −20◦C overnight to break the cell membranes. 18S rDNA was amplified by PCR with the primer pairs described by Nakayama et al. (1998). In the first round of PCR, primersSR1 and SR12 were used. The obtained PCR products were amplified again using the following primer pairs: SR1 andSR5; SR4 and SR9; and SR8 and SR12. Nonspecific PCR products were detected electrophoretically, and specific PCR products were purified using the QIAquick® Gel Extraction Kit (Qiagen, Venlo, Limburg, Netherlands). The purified PCR products were sequenced with a BigDye Terminator v. 1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using a 3130 Genetic Analyzer (Applied Biosystems). The other sequences used were obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/, retrieved April 1, 2016) and automatically aligned with CLUSTALX, version 1.81, using default parameters (Thompson et al.1997; ftp://ftp.ebi.ac.uk/pub/software/clustalw2/, retrieved April 1, 2016). For phylogenetic analyses, ambiguously aligned regions were manually adjusted or deleted using the BioEdit Sequence Alignment Editor, version 7.0.9.0 (Hall1999), and finally, 1274 base pairs (bp) of 18S rDNA were used for the analyses. Phylogenetic trees were constructed based on a 1274-bp alignment using both

maximum-likelihood (ML) and Bayesian approaches, with three sequences of Alveolata used as the outgroup. We used PHYLIP version 3.69 (Felsenstein2005) for the ML method and MrBayes 3.2.1 (Ronquist et al. 2012) for the Bayesian analysis. For the ML analysis, the Jones–Taylor–Thornton (JTT) + G model with global rearrangement was used. For the Bayesian analysis, the GTR + I + G models were selected using Mr Modeltest 2.3(Nylander 2004; https://github.com/nylander/MrModeltest2,retrieved April 1, 2016). The stability of the relationships was assessed using bootstrap analyses based on 100 resamplings. The Bayesian analysis was run for 1,000,000 generations, with a sampling frequency of every 100th generation. All other settings were retained at their default values.

CHAPTER 3: RESULTS

3.1 Taxonomic treatments

Based on the morphological characteristics and the result of molecular phylogenetic analyses using SSU rDNA sequences, I describe a new species of the genus *Diplophrys*, *D. mutabilis* sp. nov. and a new genus of the family Amphifilidae, *Fibrophrys* gen. nov., and a new species, *F. columna* sp. nov.

3.2 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 m in diameter) displaying an incrassate rim. The cells divided 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-3361Type habitat/locality: Nojiri Lake, Nagano Prefecture, Japan (36.830585N, 138.20848E).

Etymology: Specific epithet "mutabilis" means changeable cell shapes.

Gene sequence: AB856527

Fibrophrys gen. nov.

The cell shape is nearly orbicular or broadly elliptical, asymmetric to the axis connecting the two antipolar ends. Fine filamentous, branching ectoplasmic elements emanate from both polar ends of the cell and are spread evenly, similar to a fibrous root system of a plant. An internal membrane system exists in the filamentous ectoplasmic elements. In the body of the organism, generally one (but up to five) orange, amber, or colorless lipid bodies are immersed. In colonies, cells maintain an equal distance using the ectoplasmic elements and rarely make direct contact with each other. Gliding motility is not observed, and cells move over a limited distance.

Taxonomic summary

Chromalveolata, Stramenopiles, Labyrinthulomycetes (Labyrinthulea), Amphifiloidea, Amphifilidae.

Type species: *Fibrophrys columna*.

Fibrophrys columna sp. nov. The cells measure 5.0–8.3 × 5.6–10.3 μm. Sometimes, instead of the ectoplasmic elements, hernia-like prongs emanate from the cells. An axis-like electron-dense body exists in the mitochondria.

Type material: Holotype: EM block.TNS-AL-58929 in TNS (Department of Botany, National Museum of Nature and Science), Japan.

Type habitat/locality: Hiuchigaike Pond, Ibaraki Prefecture, Japan (36.202516◦N, 140.087326◦E).

Etymology: the specific epithet “columna” means pillar, referring to the electron-dense body-like pillar evident in mitochondria on examination by TEM.

Gene sequence: AB856528 as Amphifilidae sp. H-1 gene for 18S ribosomal RNA, partial sequence (Takahashi et al.2014).

3.3 General Morphology

Diplophrys mutabilis

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.

Fibrophrys

The morphology of the members of the genus *Fibrophrys* was orbicular or broadly elliptical in shape. The cells contained refractive bodies, a single nucleus, a contractile vacuole, and ectoplasmic elements emanating from the poles (Figs 8, 9). Generally, one (but up to five) refractive body was observed in each cell. The cells measured 5.0–8.3 × 5.6–10.3 μm for *F. columna* and 2.4–6.1 × 3.4–7.2 m for *Fibrophrys* sp. E-1. Gliding motility was not observed in either species. Instead, *Fibrophrys* moved like a moored body within a loose colony. Although individual cells sometimes gathered around water fleas, single cells moving out of colonies were not observed. This implies that *Fibrophrys* exhibits some chemotactic properties in order to feed and can move separately. The

ectoplasmic elements were branching but not anastomosing and evenly spread (Fig. 10A). Although it was difficult to recognize the ectoplasmic elements by optical microscopy, *Fibrophrys* cells maintained an equal distance from each other in colonies using their ectoplasmic elements and rarely made close contact with each other (Figs 8A, 9A). Sometimes, globular protrusions of the ectoplasmic elements were evident (Fig. 8A, upper left).

3.4 Ultrastructural Observations

Diplophrys mutabilis

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 Figure 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.

Fibrophrys

By examination of thin sections using TEM, we observed the nucleus, mitochondria, lipid bodies, Golgi bodies, and a complex membrane system, which we termed “the unidentified membrane system” (Figs 8B, C, 9B–D). The ectoplasmic elements contained ribosome-free cytoplasm and tubular internal membrane system elements in both species (Fig. 10B).Many layers of ectoplasmic elements surrounded the surface of water fleas (Fig. 10C). Bothrosomes and bothrosome-like bodies were not observed. Both *Fibrophrys* species possessed mitochondria containing distinctive cristae with short, stubby branches (Figs 8B, 9D). In the mitochondria of *F. columna*, an axis-like electron-dense body was observed, which was not evident in *Fibrophrys* sp. E-1. In the latter strain, a ladder-like pattern was observed between the cytoplasmic membrane

and mitochondrial outer membrane, close to mitochondria. In addition, to some extent, the distribution of certain organelles in *Fibrophrys* cells was fixed. The Golgi body was situated close to the nucleus, and many small vesicles, which seemed to be a cis-Golgi network, were observed between these organelles in both species. The unidentified cytoplasmic membranes were connected to the nuclear membrane, lipid membrane, and mitochondrial outer membrane in *Fibrophrys* sp. E-1 (Fig. 9B–D), but not in *F. columna*. The unidentified cytoplasmic membranes and the neighboring endoplasmic reticulum were ribosome-free in both species.

3.5 Molecular Phylogenetic Analyses

Diplophrys mutabilis

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 analyses 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. (2011). All identified members in this clade display a bipolar cell shape and are unicellular, solitary organ-isms that do not form developed ectoplasmic networks. They also share the characteristic of having solid cell coverings; however, *Amphitrema* and *Archerella* have a monolithic testa, whereas *Diplophrys* have layers of discrete scales.

Fibrophrys

Phylogenetic analyses based on 18S rDNA gene sequences revealed that *F. columna* and the other *Fibrophrys* species were new members of the class Labyrinthulomycetes and belonged to the same clade, Amphifiloidea (Fig. 11). The whole topology of the phylogenetic tree was similar to those reported previously (Anderson and Cavalier-Smith 2012; Leander and Porter 2001; Takahashi et al. 2014). Our analysis revealed a close phylogenetic relationship among the sequences of *F. columna*, *Fibrophrys* sp. E-1 (LC096096), *Af. marina*, and many environmental sequences in the family Amphifilidae. It also revealed significant independence of the organisms from two representative clades of the Labyrinthulomycetes, Labyrinthulida and Amphitremida. Although the relationship between Amphifiloidea, to which the genus *Fibrophrys* belongs, and Thraustochytrida is still unclear (Anderson and Cavalier-Smith 2012; Gomaa et al. 2013; Takahashi et al. 2014), Amphifiloidea may be a separate group of Thraustochytrida.

CHAPTER 4: DISCUSSION

4.1 *D.mutabilis*

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

A swelling in the basal part of the ectoplasmic elements was observed in *D. mutabilis* (Fig. 2A, B). Similar swellings have been observed in *Af. marina* and *S. stercorea*. However, their swellings occurred in the middle part of the ectoplasmic elements (Porter 1984), not in the basal part as observed in *D. mutabilis*. Although pseudopodial features are important morphological characteristics of amoeboid organisms in general, it is unclear whether this difference reflects phylogenetic relationships in this group.

An internal membrane system in ectoplasmic elements is widely observed in Labyrinthulomycetes species (Perkins 1972), and the system of *D. mutabilis* is apparently more developed than those in other organisms. The system has been observed in *S. stercorea* (Dykstra 1976a), but not in *Af. marina* and *D. parva*. The

ectoplasmic element of *Af. marina* appears to consist of fine fibrous structures rather than a bundle of membranous tubes (Dykstra and Porter 1984). Labyrinthulomycetes species have mitochondria with tubular cristae, which are also observed in Stramenopiles, but *D. mutabilis* has mitochondria containing distinctive cristae with short, stubby branches (Fig. 4C). This characteristic has also been recognized in *S. stercorea* (Dykstra 1976a,b), *D. parva* (Anderson and Cavalier-Smith 2012), and *Af. marina* (Porter 1984), but these mitochondrial features have not been observed in other members of Labyrinthulomycetes. This characteristic could be a synapomorphic or apomorphic characteristic of the genus *Diplophrys* and related lineages. Nevertheless, it remains a matter of debate because *Archerella flavum*, closely related to genus *Diplophrys*, has mitochondria with tubular cristae (Bonnet et al. 1981).

In TEM observations, unidentified concentric and helical cytoplasmic membranes were observed (Fig. 5). Similar cytoplasmic membranes have been observed in *S. stercorea* (Dykstra et al.1975) and *D. parva* (Anderson and Cavalier-Smith2012), but they have not been reported in other Labyrinthulomycetes species including *Af. marina*. The function of these 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. This would means that this organelle could be an unusual type of smooth endoplasmic reticulum. Although further investigations are needed to answer the question, this organelle may be a key structure in the development of prominent 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 their relationships.

D. mutabilis resembles *D. archeri* in several ways. Based on the original description of *D.archeri* (Barker 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* has1–10 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, B, 6A). These differences distinctly separate *D. mutabilis* from *D. archeri* .

D. parva appears to be the closest relative of *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 (Andersenand 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 internal membrane system of ectoplasmic elements (Dykstra and Porter 1984); they are contrastingly well developed in *D. mutabilis* (Table 1). Furthermore, 18S rRNA analysis (Fig. 7) demonstrated that they are distantly related within Labyrinthulomycetes.

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

Elaeorhanis cincta, a filopodial amoeba with debris on its cell surface, has been

suggested tobe closely related to *Diplophrys* species (Patterson1996). They share filopodia, an oil-like refractivebody of an orange or amber color, and some other features. Although *Diplophrys* and *Elaeorhanis* are easily distinguished from one another by the presence or absence of the debris layer, it is still possible that they may be closely related species or sim-ply different ecotypes of the same organism. No *Elaeorhanis* strain or its rRNA gene sequences are available at present even though the genus is com-mon in freshwater habitats. A detailed comparison between these two organisms is required to settle this question.

From the phylogenetic tree, *D. mutabilis* clearly belonged to Labyrinthulomycetes, Amphitremida, Diplophryidae, being positioned near *At. wrightianum* and *Ar. flavum*. The two genera are very different from *Diplophrys* concerning cell size and the presence of a monolithic brown or hyaline testa and endosymbiotic algae. Endosymbionts, or preyed bacteria have never been reported in *Diplophrys* despite the presence of bacteria attached to the scale surface and ectoplasmic elements (Fig. 2A, C). Thus, according to our results, *Diplophrys* does not appear to display phagocytosis. On the contrary, the ultrastructure of their ectoplasmic elements and roots is similar to that of *D. mutabilis*, including the absence of bothrosomes and the presence of an internal membrane system (Table 1). *Diplophrys* is phylogenetically similar to these two genera, but it presumably diverged before its species could obtain endosymbiotic algae.

4.2 Fibrophrys

The light microscopic appearance of the *Fibrophrys* species resembles that of *Diplophrys archeri*, *D. parva*, *D. mutabilis*, *Af. marina*, and of the vegetative cells of

Sorodiplophrys stercorea (Anderson and Cavalier-Smith2012; Dykstra and Olive 1975; Dykstra and Porter 1984).These organisms are nearly orbicular or broadly elliptical in shape, and the cells feature refractive bodies, a contractile vacuole, and ectoplasmic elements emanating from the poles. In the mitochondria of *F. columna*, an axis-like electron-dense body was observed (Fig. 8B). This electron-dense body was not visible in *Fibrophrys* sp. E-1 and has not been reported in any other species of the Labyrinthulomycetes. In *Fibrophrys* sp. E-1, a ladder-like pattern consisting of unidentified cytoplasmic membranes connected to the mitochondria was evident. This ladder-like pattern resembles that of the rumposome of chytrids, which is connected to the lipid body and associated with the flagellar apparatus and plasmamembrane in some chytrid species (Dorword and Powell1982). Because the ladder-like pattern seen in *Fibrophrys* sp. E-1 exists between the mitochondria and unidentified cytoplasmic membranes, no robust topological similarity was recognized with the rumposome.

Fibrophrys resembles *Af. marina*, the type species of the Amphifilidae family. The most apparent difference between the genera *Fibrophrys* and *Amphifila* is their habitat. The habitat of *Af. marina* is, as its name suggests, a marine environment. *Af. marina* is a heterotrophic protist associated with marine vascular plants such as *Spartina alterniflora* and *Zostera marina* (Porter 1972). On the other hand, the *Fibrophrys* species were isolated from freshwater habitats, an inland pond, and a lake. The second difference is the motility of the cells. *Af. marina* exhibits gliding motility on substrates, while *F. columna* and *Fibrophrys* sp. E-1 did not show this type of motility. *F. columna* usually floated and moved like a moored ship. The third difference is the presence or absence of the unidentified membrane system and internal membranous tubes within ectoplasmic elements. Both of these peculiar structures are evident in *F. columna* and

Fibrophrys sp. E-1 (Figs 8C 9B–D, 10B, C), but neither is present in *Af. marina* (Dykstra and Porter 1984). Based on these differences, we conclude that the organisms belong to separate genera.

In light microscopy images, the vegetative cells of *S. stercorea* resemble *Fibrophrys* cells in terms of their morphology and structure of organelles, such as the unidentified cytoplasmic membranes and internal membrane systems (Dykstra and Olive 1975). However, the aggregative behavior, terrestrial habitat, and complex life cycle (which includes a sorocarp) of *Sorodiplophrys* are sufficient to distinguish it from *F. columna* (Table 1). The independence of the genus *Sorodiplophrys* should be confirmed based on molecular data when the DNA sequence of *Sorodiplophrys* becomes available. In a recent study, a new strain of *Sorodiplophrys* has been established, and its 18S rDNA sequence has been determined (Tice et al.2016). In the phylogenetic trees, *S. stercorea* is closely related to *Af. marina*. The above study is currently under review/inpress and will provide insight not only into *Sorodiplophrys* but also into the Labyrinthulomycetes in their entirety.

F. columna also resembles *D. archeri*, *D. parva*, and *D. mutabilis*. We compared the *Fibrophrys* and *Diplophrys* species as shown in Table 1. The average size of *D. archeri*, the type species of the genus *Diplophrys*, was reported to be 1/2000 inch, i.e., 12.7 μm (Anderson and Cavalier-Smith2012; Baker 1868). This cell size is larger than that of *F.columna*. In TEM images, *D. parva* can be seen to possess a bothrosome-like electron-dense body in the base of its ectoplasmic elements (Anderson and Cavalier-Smith 2012). In the ectoplasmic elements of *D. mutabilis*, internal membrane systems are more developed than in those of *Fibrophrys* (Fig. 10B; Takahashi et al. 2014). Moreover, *D. mutabilis* shows gliding motility, whereas *Fibrophrys* does not.

Based on these morphological differences, *Fibrophrys* and *Diplophrys* are distinguishable and should be considered two independent genera.

The definitions of the families Diplophryidae (Takahashi et al. 2014) and Amphifilidae (Anderson and Cavalier-Smith2012) remain unclear. As shown in Table 1, there is no definitive behavior or ultrastructure that would allow us to distinguish between Diplophryidae and Amphifilidae. At present, they can only be separated by phylogenetic analysis. Furthermore, the morphological differentiation between *Diplophrys* and freshwater Amphifilidae performed in some past studies (e.g., Anderson and Cavalier-Smith 2012) is insufficient because no cultured strain of freshwater Amphifilidae was available to the public. For example, Baker (1868) stated that "in the body of the organism (*D. archeri*) is immersed an oil-like refractive globule of an orange or amber color;" however, there are up to 10 lipid bodies present in *D.parva* and *D. mutabilis*. On the contrary, generally only one lipid body is present in *Fibrophrys*. In addition, as shown in Table 1, *D. archeri*, *D. parva*, and *F. columna* do not show gliding motility, but *D. mutabilis* does. *F. columna* matches the description of *D. archeri*, except for the cell size. To definitively distinguish these species, the election of a neo-type of *D. archeri* and the establishment of a cultured strain that satisfies all definitions are necessary.

Furthermore, *Elaeorhanis cincta*, a filopodial amoeba with debris on its cell surface, has been suggested to be closely related to *Diplophrys*-like species (Patterson 1996). The organisms share filopodia, an oil-like refractive body of an orange or amber color, and some other morphological features. Although *Elaeorhanis* and *Diplophrys*-like species are easily distinguished by the presence or absence of a debris layer, they may be closely related species or simply different ecotypes of the same species of the genus *Diplophrys*.

No strains or sequence data are currently available for *Elaeorhanis*, although the genus is common in freshwater habitats. A detailed comparison between *Elaeorhanis* and *Diplophrys*-like organisms is required to resolve this issue.

As mentioned earlier, *Sorodiplophrys* is closely related to the Amphifilidae family. This is consistent with the findings of Anderson and Cavalier-Smith (2012) who demonstrated a relationship between Sorodiplophryidae and Amphifilidae and determined that they comprise the Amphifiloidea super-family. One of the two distinct, deep-branching soil lineages in the Amphifilidae clade, namely, Soil, US, not Eimeriidae (Fig. 11; Lesaulnier et al. 2008), is related to the dung-dwelling *Sorodiplophrys* (Tice et al. 2016). Because the organisms belonging to Amphifilidae were isolated from soil samples moistened with distilled water obtained from the campus of University of Tsukuba, Tsukuba, Japan (data not shown), some of the registered environmental sequences obtained from soil may belong to the genus *Sorodiplophrys* or neighboring genera. This implies that some freshwater members of the Amphifilidae family exhibit potentially high resistance to desiccation and exist in soil. This may be one reason why environmental sequences derived from soil are polyphyletic (Fig. 11).

Based on our phylogenetic tree, the monophyletic group including *F. columna* is composed of sequences obtained from freshwater habitats. This clade also includes sequences from samples collected from a suboxic pond in Paris (Slapetaet al. 2005). Our culture of *F. columna* is maintained in a medium with many bacteria, which includes water fleas as an organic substance and smells a little rotten, implying that it is probably suboxic. Accordingly, this species exhibits tolerance to low-oxygen conditions. Based on these findings and morphological features, we propose that this monophyletic group be named *Fibrophrys*. Although the type species of the family Amphifilidae is a

marine species, the preference for marine habitats seems rare among members of the Amphifiloidea superfamily because no environmental sequences of Amphifiloidea, derived from marine habitats, are currently available. As shown in the phylogenetic tree, there are many clades without cultured strains associated with diverse environments, such as the Rio Tinto, which has a pH of 2 and contains much higher concentrations of heavy metals than typically found in fresh waters (Zettler et al.2002). To fully appreciate the diversity of members of the Amphifiloidea superfamily, more cultured strains are necessary.

4.3 General discussion

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* tends 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;

Quick1974), 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*, *F. columna* and *S. stercorea* lack bothrosomes, a shared characteristic of Labyrinthulomycetes species, but *D. parva* was reported to have bothrosome-like structures.

More studies, both morphological and molecular phylogenetic are required to establish a robust phylogenetic relationship among Labyrinthulomycetes species.

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Genus/species (sources)	Internl membrane system	unidentified cytoplasmic membranes	Cell size (μ m)	gliding motility	sagenogenetosome (= bothrosome, sagenogen)	habitat	aggregation (**)	endosymbiotic algae	zoospores
Diplophrys mutabilis (This study)	++	++	3.1 – 8.3 × 3.4 – 10.3	+	−	freshwater	−	−	−
Fibrophrys columna (This study)	+	+	5.0 – 8.3 × 5.6 – 10.3	−	−	freshwater	−	−	-
Diplophrys archeri Barker, 1868 (Anderson and Cavalier-smith 2012, Barker 1868, Patterson 1996)	No data	No data	12.7	−	No data	freshwater	−	−	−
Diplophrys parva Anderson et Cavarier-smith, 2012 (Anderson and Cavarier-smith 2012)	−	++	6.5 ± 0.08 × 5.5 ± 0.06; mean ± SE	−	+?	freshwater	−	−	−
Amphifila marina Dykstra et Porter, 1984 (Dykstra And Porter 1984)	−	−	3.7 – 5.9 × 5.1 – 8.5	+	−	marine	−	−	−
Sorodiplophrys stercorea (Cienkowski) Olive et Dykstra, 1975 (Dykstra And Olive 1975)	+	+	2.4 – 4.8 × 4.8 – 9.6	+	−	terrestrial	+	−	−
Elaeorhanis cincta Greeff, 1873 (Lee 2000, Patterson 1996)	No data	No data	10 – 20 in diameter	No data	No data	freshwater	No data	−	No data
Amphitrema wrightianum Archer, 1869 (Edmondson1959, Gomma 2013)	No data	No data	61 – 95 in diameter	+	No data	freshwater	−	+	−
Archerella flavum Loeclich et Tappan, 1961 (Bonnet et al. 1981, Edmondson1959, Gomma 2013)	+?	−	45 – 77 in diameter	+	−	freshwater	−	+	−
Labyrinthula zosterae Muehlstein et Porter, 1991 (Muehlstein and Porter 1991)	+* (Perkins 1972)	−	15.5 – 19.5 × 3.5 – 5.0	+	+	marine	+	−	+(Perkins and Amon 1969)
Aplanochytrium stocchinoi Morro et al. 2003 (Morro et al. 2003)	No data	−	4 – 8 in diameter	+	+* (Watanabe 2012)	marine	−	−	aplanospore
Schizochytrium aggregatum Goldstein et Belsky, 1964 (Goldstein and Belsky 1964)	+* (Perkins 1972)	−	6 – 12 in diameter	−	+	marine	−	−	+

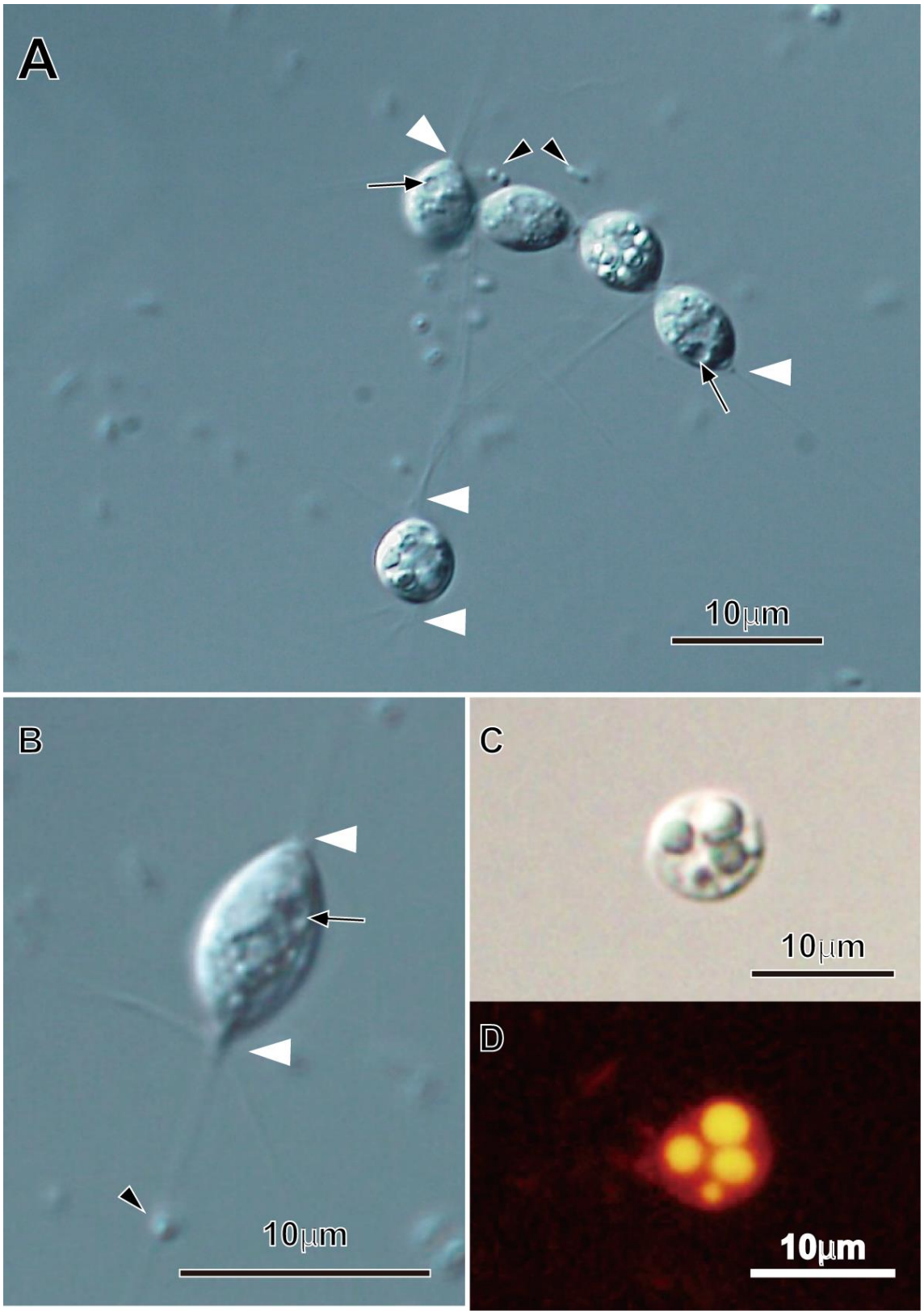

Figure 1. Light micrographs of *Diplophrys mutabilis*.

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.

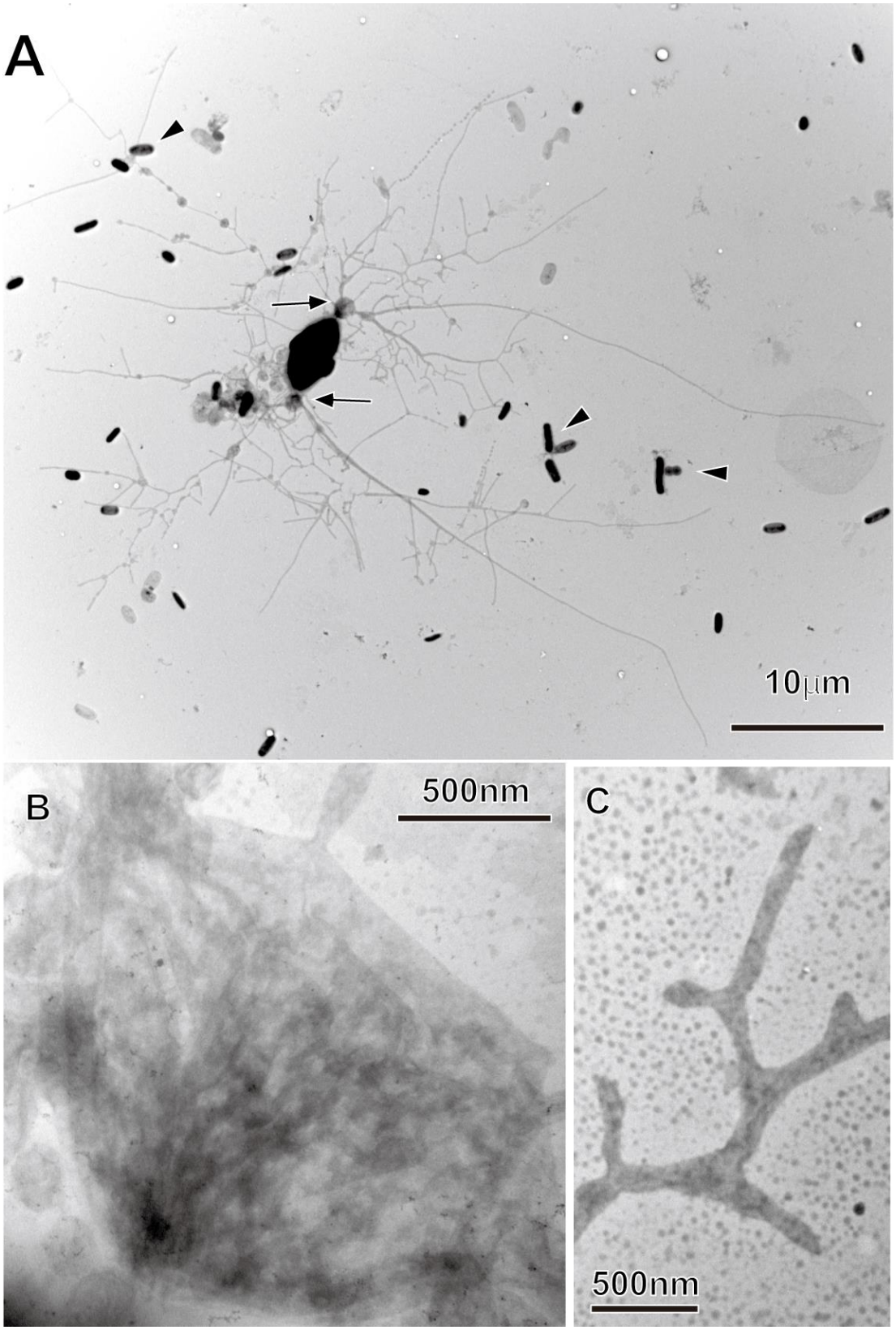

Figure 2. Whole-mounted cells of *Diplophrys mutabilis*.

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 in homogeneous texture.

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

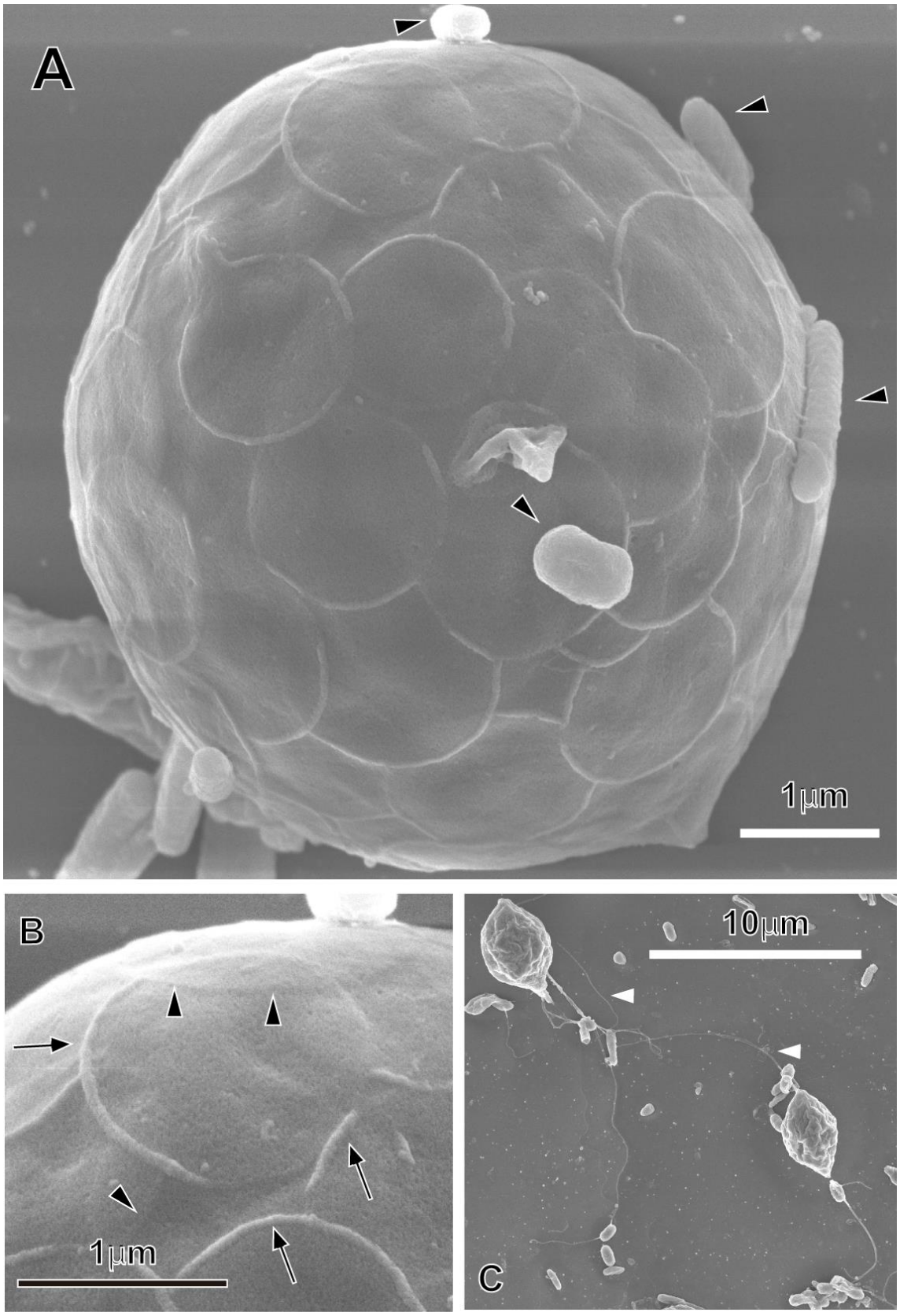

Figure 3. SEM images of *Diplophrys mutabilis*.

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).

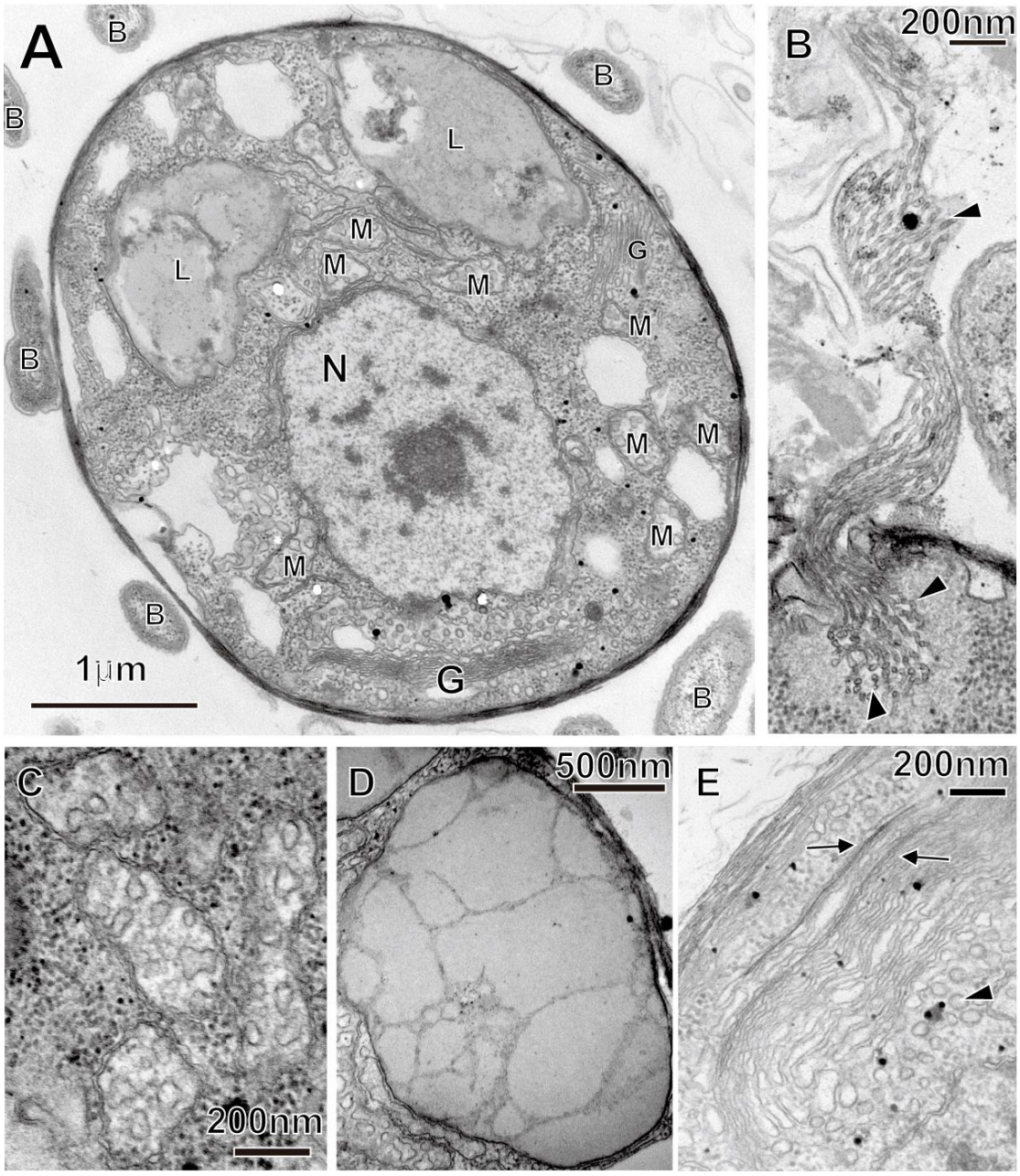

Figure 3. SEM images of *Diplophrys mutabilis*.

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).

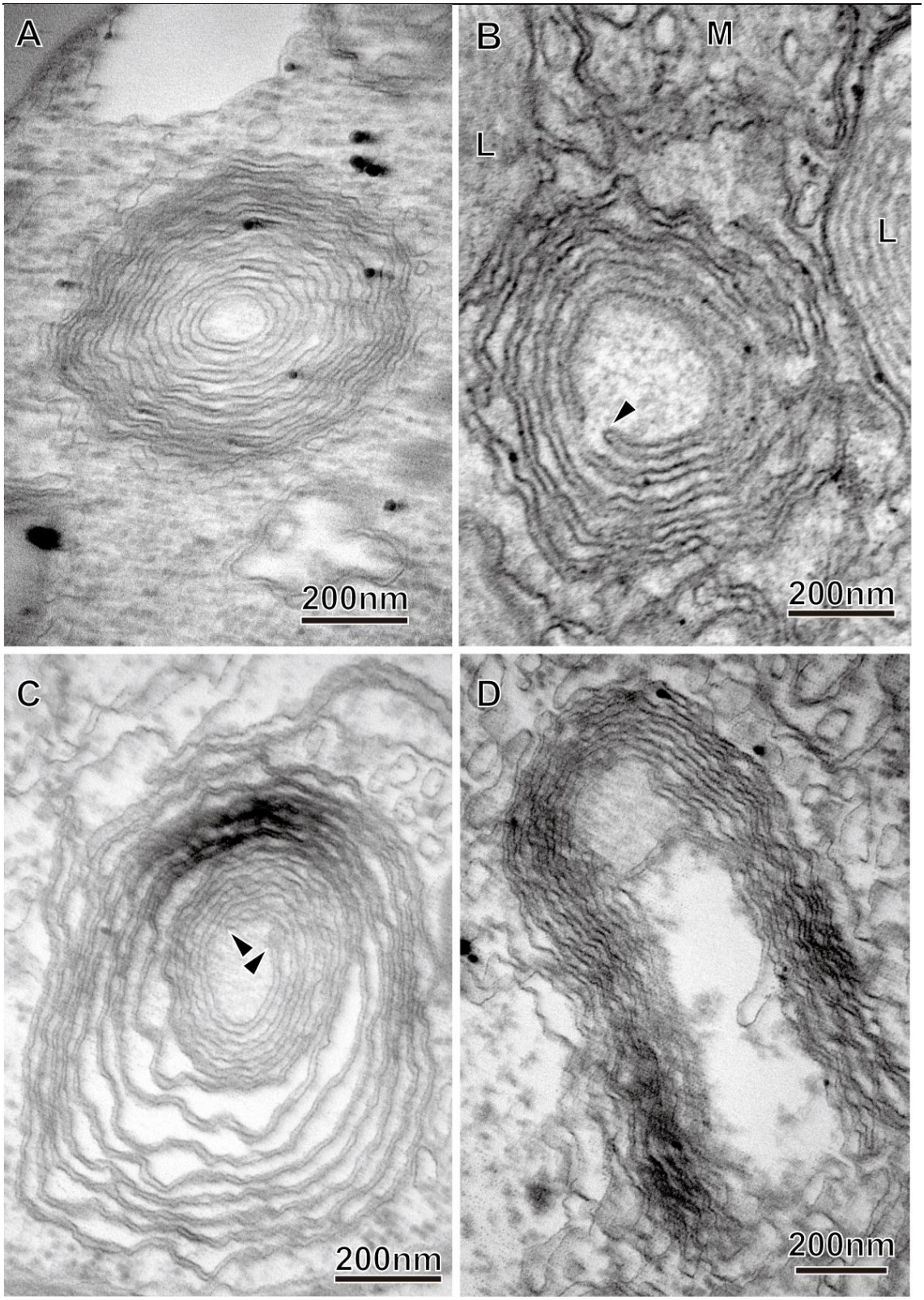

Figure 5. TEM images of unidentified cytoplasmic membranes revealing different

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.

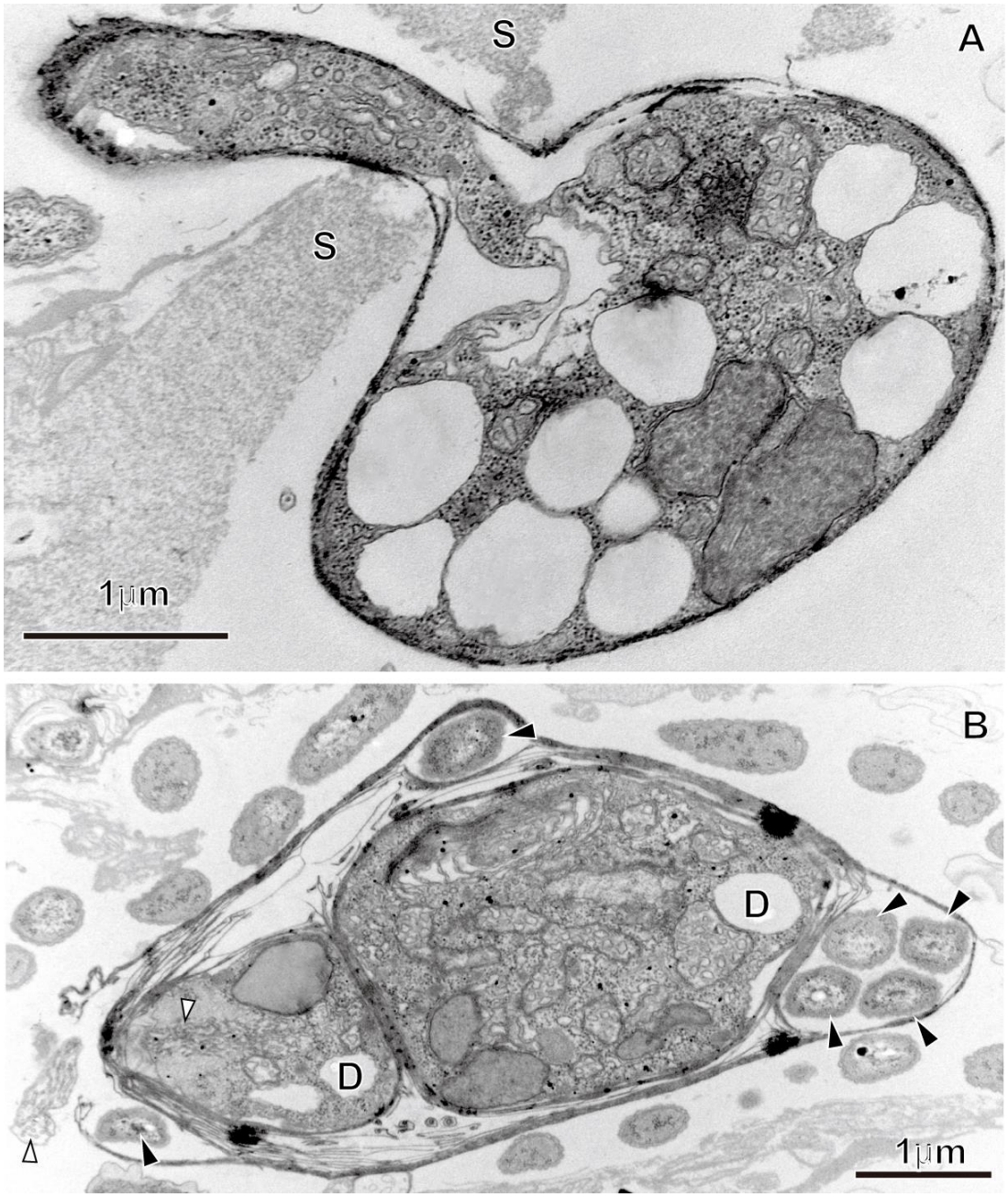

Figure 6. TEM images of *Diplophrys mutabilis*.

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.

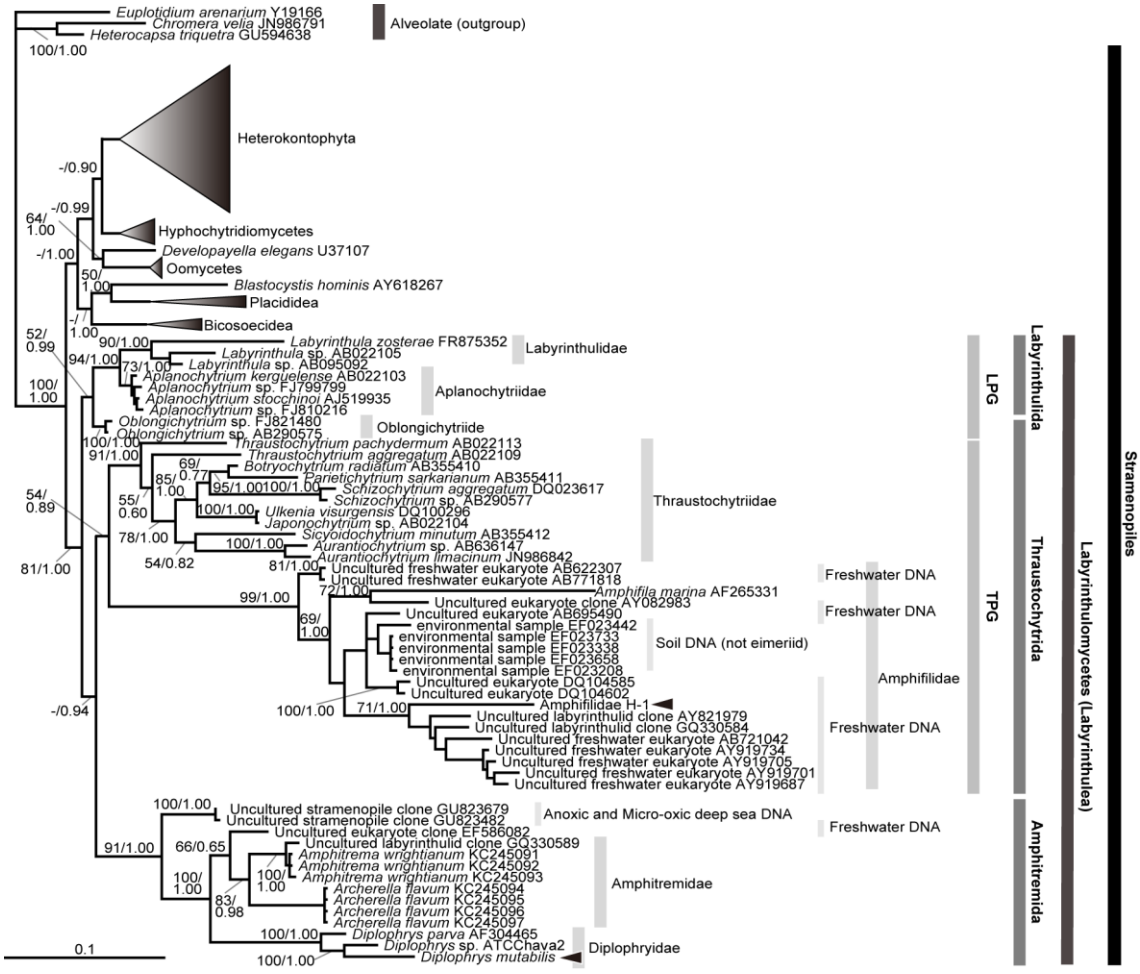

Figure 7. Phylogenetic tree based on the 18S rDNA sequences and constructed using the maximum likelihood method based on a 1230-bp alignment. Bayesian approach also estimated the same topology of the tree (not shown). Support values at each node are presented for ML/Bayes. Bootstrap values larger than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

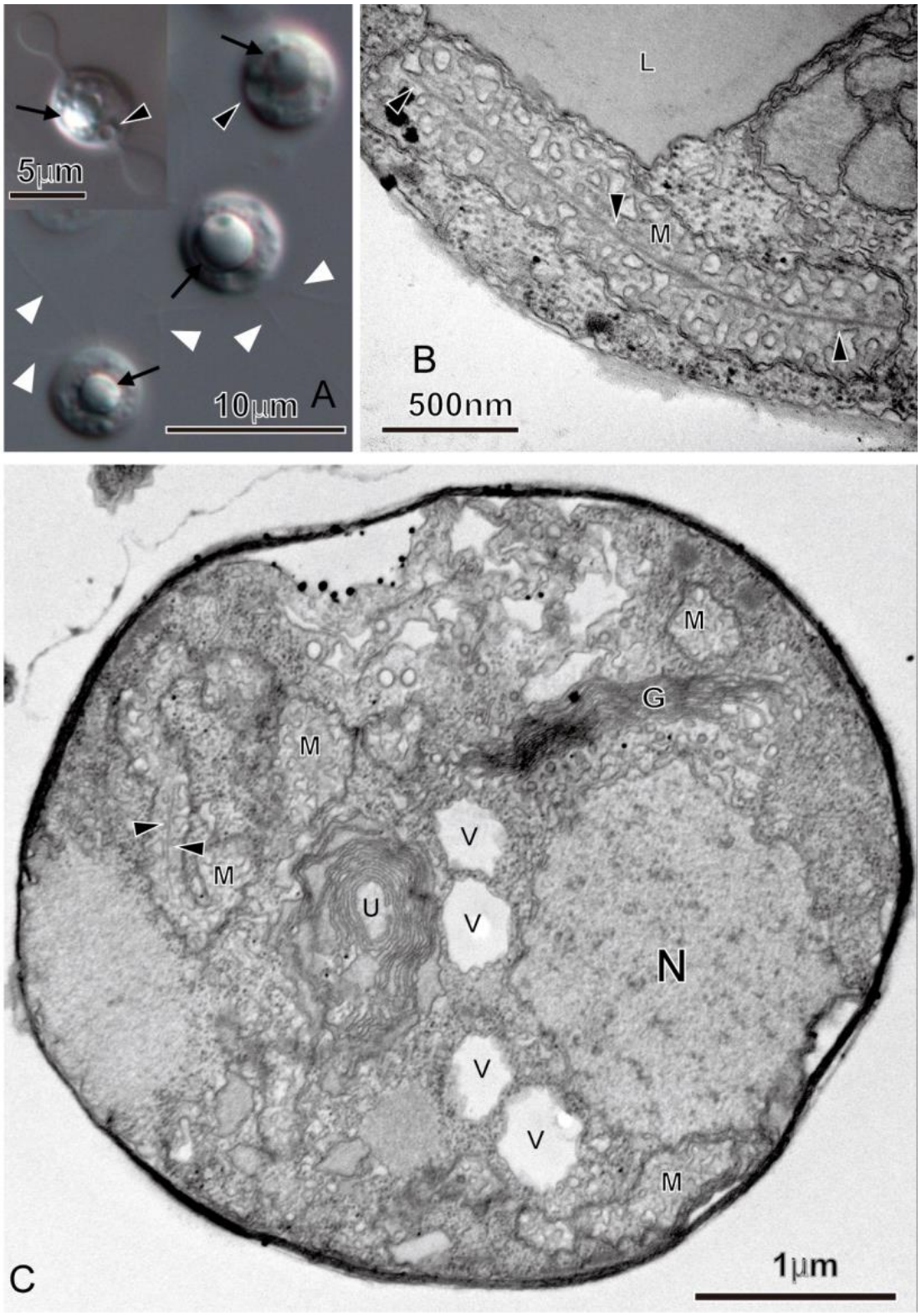

Fig. 8. Light micrograph and transmission electron microscopy images of *Fibrophrys columna*.

A. Colonial cells connected through ectoplasmic elements (white arrowheads). Cells contain refractive lipid bodies (arrows) and contractile vacuoles (arrowheads). Sometimes, globular protrusions of the ectoplasmic elements are visible (upper left window).

B. Section near the cell surface. An electron-dense body-like pillar is evident in the mitochondria (arrowheads) in the transmission electron microscopy images. L: lipid body, M: mitochondria.

C. Diametric section of a spherical cell. An electron-dense structure resembling a pillar is observed in the mitochondria (arrowheads). G, Golgibody; L, lipid body; M, mitochondria; N, nucleus; U, unidentified cytoplasmic membranes; V, vacuole.

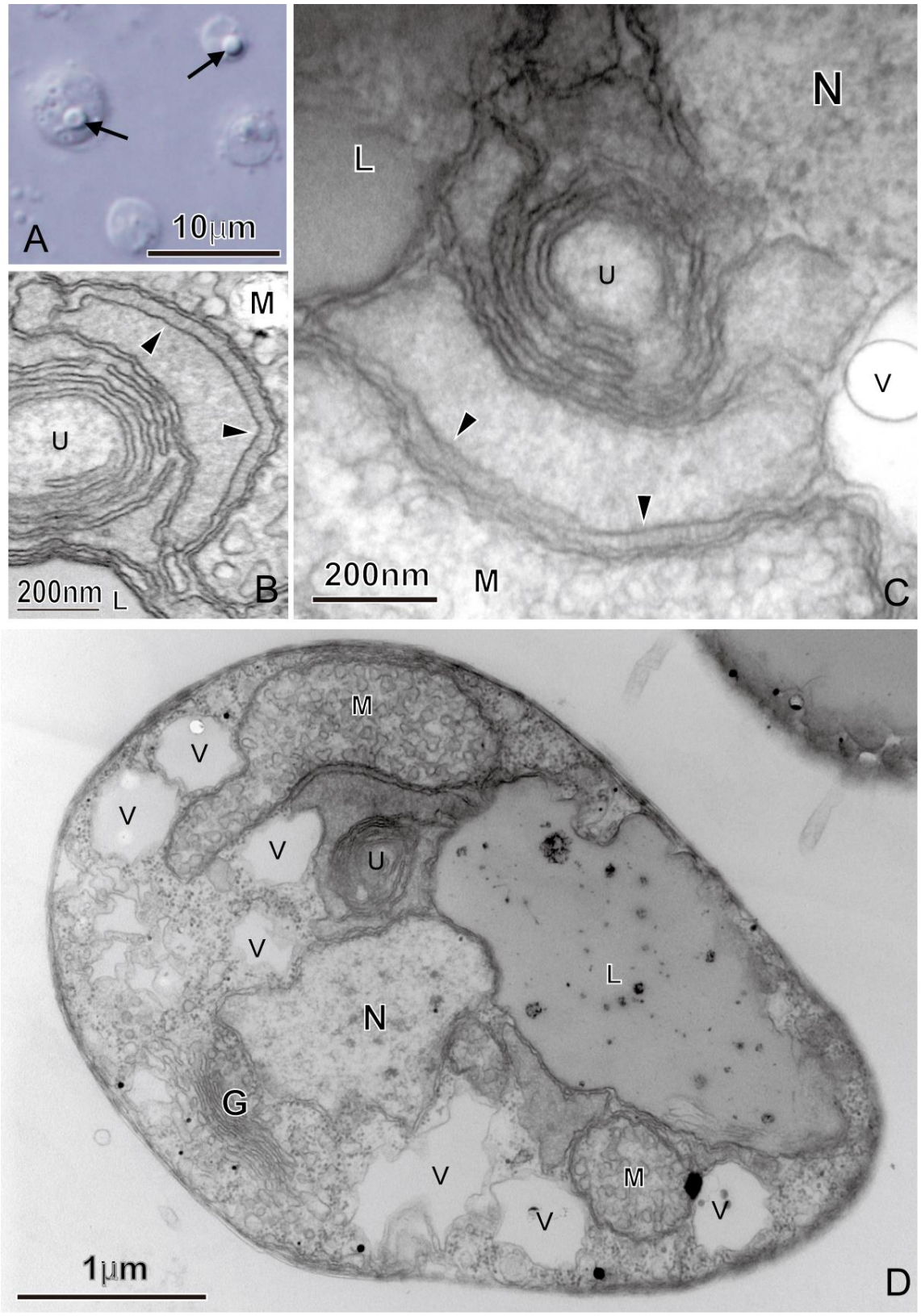

Fig. 9. (A)–(D) Light micrograph and transmission electron microscopy images of

Fibrophrys sp. E-1.

A. Colonial cells. The cells contain refractive bodies (arrow). Some bacteria and dust are also visible.

B. Unidentified cytoplasmic membranes and mitochondria. A ladder-like pattern is visible between the cytoplasmic membrane and mitochondrial outer membrane (arrowheads). L, lipid body; M, mitochondria; U, Unidentified cytoplasmic membranes.

C. Lipid body, mitochondria, nucleus and unidentified cytoplasmic membranes connected by the endoplasmic reticulum. A ladder-like pattern is evident, bordered by mitochondria (arrowheads).

D. Diametric section of a spherical cell. G,Golgi body; L, lipid body; M, mitochondria; N, nucleus; U, unidentified cytoplasmic membranes; V, vacuole.

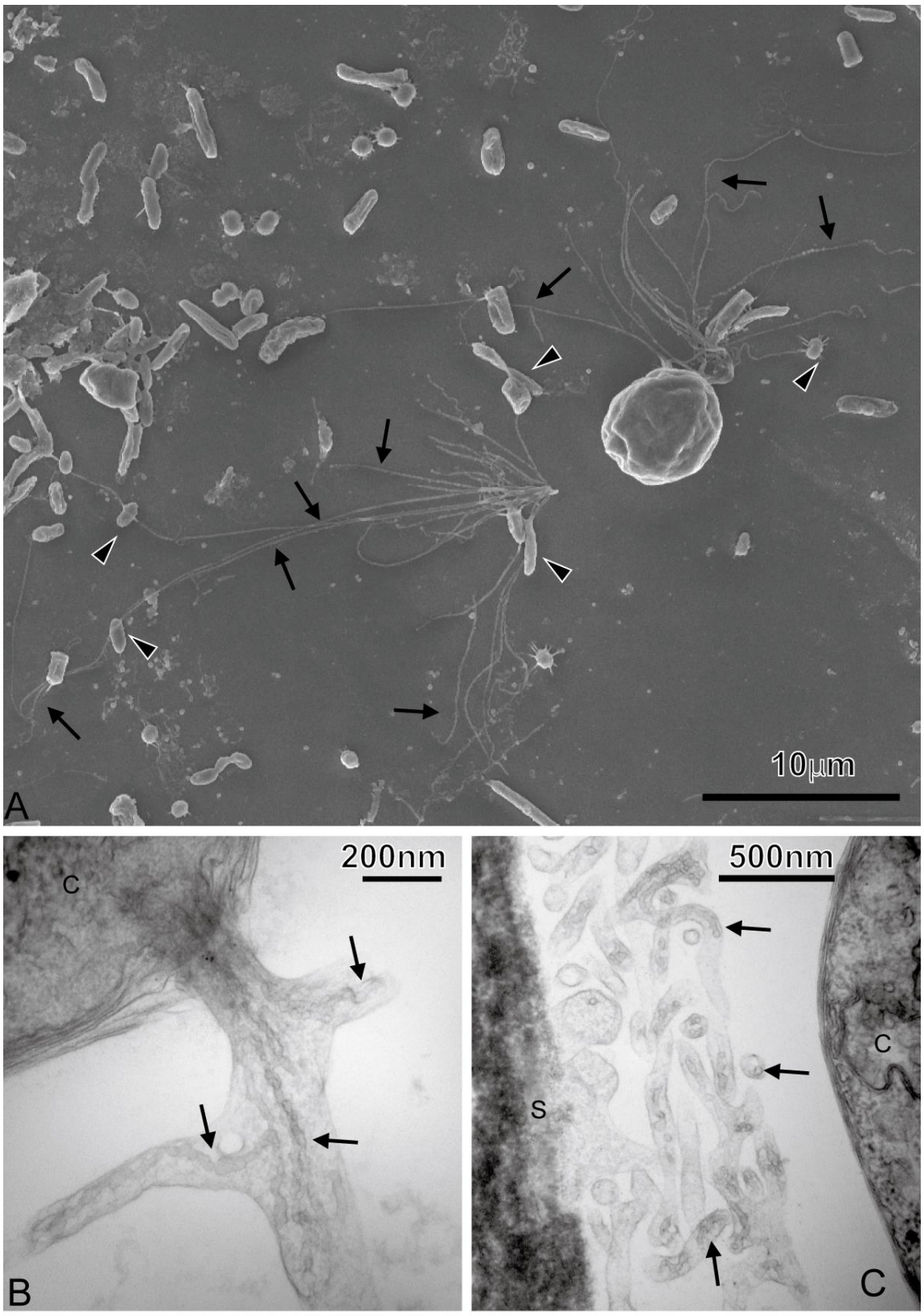

Fig. 10. Scanning electron microscopy image of *Fibrophrys columna*. Some bacteria

(arrowheads) are attached to the ectoplasmic elements (arrows). Section of the basal part of an ectoplasmic element of *Fibrophrys* sp. E-1. Internal membrane systems are running in the ectoplasmic element (arrows). Section of distal parts of the ectoplasmic elements surrounding the surface of the substrate. Left side: water flea substrate. Right side: cell body of *Fibrophrys* sp. E-1. Internal membrane systems are running in the ectoplasmic element (arrows). C, cell; S, substrate.

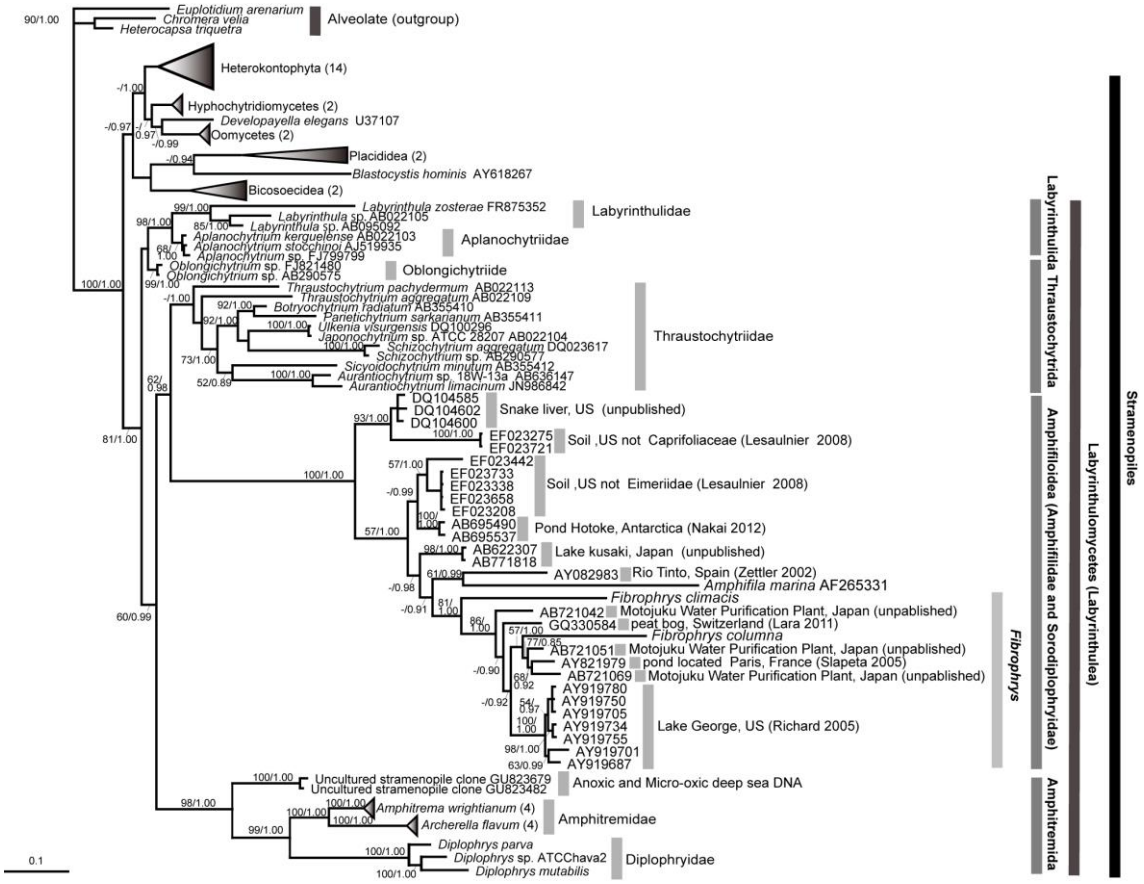

Fig. 11. Phylogenetic tree based on alignment of 1274 base pairs of 18S ribosomal DNA sequences, constructed using the maximum-likelihood method. The Bayesian approach resulted in the same topology (Supplementary File). Support values at each node are presented for the maximum-likelihood/Bayesian approaches. Bootstrap values larger than 50% and posterior probabilities larger than 0.80 are shown. Smaller values are represented by "–".

APPENDIX

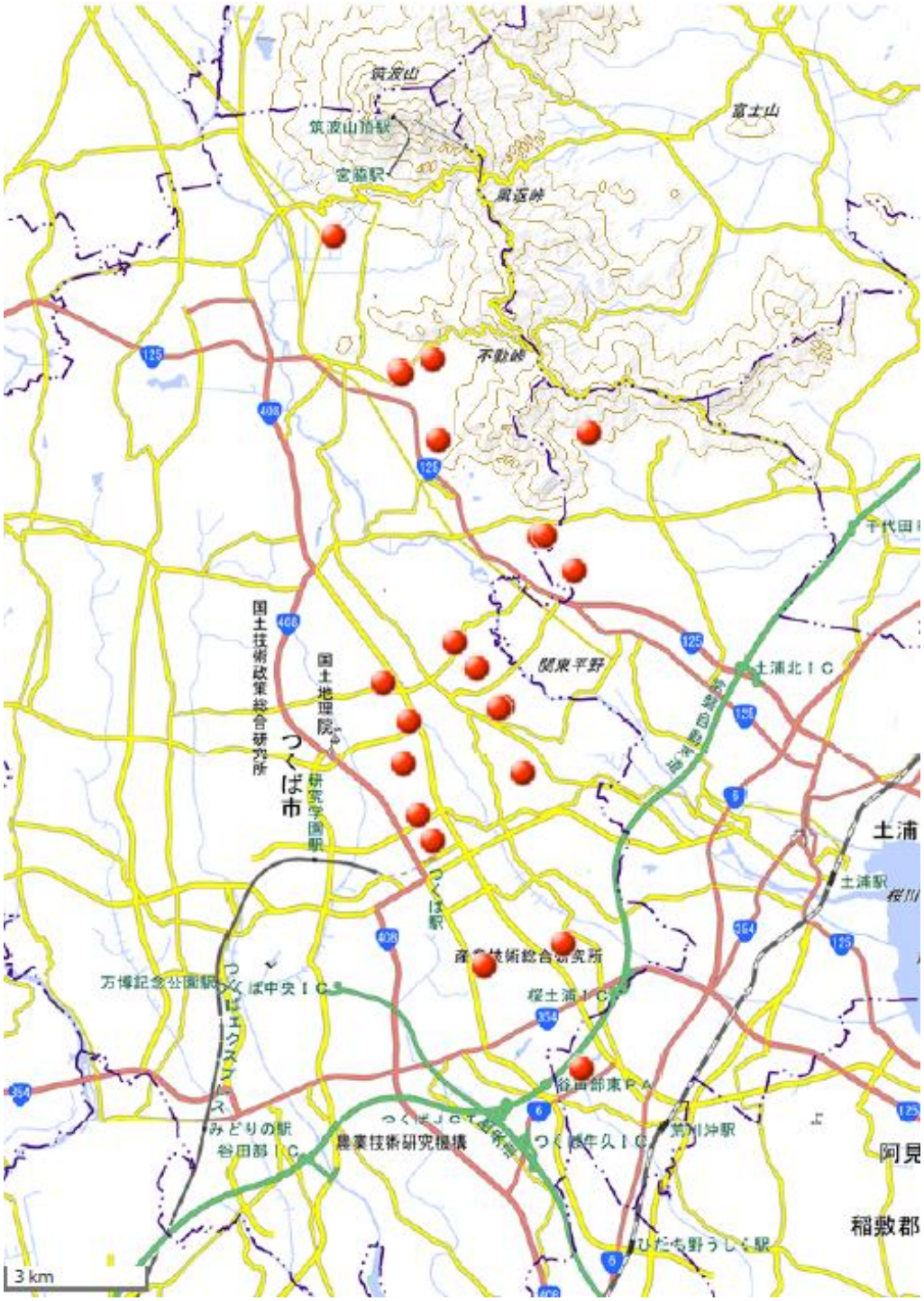

Fig. s1. The map of sampling sites around Tsukuba.

This map is a reproduction of the Digital Map 25000(Map Image) published by Geospatial Information Authority of Japan

Fig. S2. Detection sites of Diplophrys-like organisms in other literature and Environmental Diplophrys-like organisms sequences.

Environmental Diplophrys-like organisms sequences from freshwater habitat
Reporting Diplophrys-like organisms Sightings from freshwater habitat
Diplophrys-like organisms sequences from marine habitat
Reporting Diplophrys-like organisms Sightings from marine habitat
Environmental Diplophrys-like organisms sequences from marine habitat
Reporting Diplophrys-like organisms Sightings from marine habitat

発見地	県	採集日	株化
五色沼	福島県	2011/5/30	×
ボトリオコッカス ドーム培養	茨城県	2011/6/8	×
北条大池(1)	茨城県	2011/7/11	×
燧ヶ池	茨城県	2011/7/27	○
野尻湖	長野県	2011/12/2	○
西表島池	沖縄県	2012/2/8	×
北条大池(2)	茨城県	2012/4/18	○
筑波大学下水	茨城県	2012/4/19	×
下横場下水	茨城県	2012/5/22	×
越後沼	北海道	2012/8/10	○
熱海	静岡県	2012/8/10	×
宍道湖	島根県	2012/12/25	×
山中湖	山梨県	2013/1/18	○
チョウザメ飼育プール(1)	茨城県	2013/2/25	×
チョウザメ飼育プール(2)	茨城県	2013/2/25	×
二次処理水	宮城県	2013/3/12	×
活性汚泥	宮城県	2013/3/12	×
荒地水たまり	宮城県	2013/4/12	×
山口池	茨城県	2013/4/12	×
北条大池（3）	茨城県	2013/4/13	×
金田池	茨城県	2013/4/19	×
兵太郎池（1）	茨城県	2013/5/18	×
草木湖	群馬県	2013/6/12	○
兵太郎池（2）	茨城県	2013/6/24	×
赤沼	宮城県	2013/8/5	×
加瀬沼	宮城県	2013/8/5	×
芦ノ湖	神奈川県	2013/8/22	○
松川浦洞窟	福島県	2013/11/5	×
蛭沢溜池	福島県	2013/11/5	×
東が丘公園1	福島県	2013/11/5	×
東が丘公園2	福島県	2013/11/5	×
大堤溜池	福島県	2013/11/5	×
新横峰溜池	福島県	2013/11/5	○
猪子坂溜池	福島県	2013/11/5	×
藤金沢溜池	福島県	2013/11/5	×

Table S1. Detection sites of Diplophrys-like organisms in this study.