***Esquamula lacrimiformis* n. gen., n. sp., a New Member of Thaumatomonads that Lacks Siliceous Scales**

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ABSTRACT. We report a new naked cercozoan flagellate, *Esquamula lacrimiformis* n. gen., n. sp., collected from a sandy beach in Japan. Its cells were 4.5--11.3 μm in length and 3.9--8.8 μm in width and possess two unequal flagella. Cells move in a smooth gliding motion and have a trailing long posterior flagellum. Phylogenetic analyses with small and large subunit ribosomal RNA genes revealed that *E. lacrimiformis* forms a novel lineage within the Thaumatomonadida, the members of which are flagellates with siliceous scales. However, our light and electron microscopic observations indicated that *E. lacrimiformis* cells do not possess any siliceous structures. Furthermore, other morphological characteristics, such as the shape of the extrusomes and the structural arrangement of the microbody, were clearly different from those of previously described thaumatomonads. On the basis of a combination of these morphological observations and our phylogenetic analyses, we conclude that *E. lacrimiformis* should be treated as a new species of a new genus and placed into a new family, Esquamulidae n. fam., under Thaumatomonadida.

Key Words. Cercozoa, extrusome, microbody, phylogeny, taxonomy, thaumatomonad, ultrastructure.

CERCOZOA is a rhizarian phylum established by Cavalier-Smith (1998) on the basis of the result of a molecular phylogenetic analysis with the small subunit ribosomal RNA (SSU rRNA) gene. Cercozoa is divided into two subphyla, Endomyxa and Filosa. Endomyxa comprise heterotrophic filose or reticulose amoebae (e.g. *Gromia*, *Filoreta*) and parasites of plants and crustaceans (e.g. plasmodiophorids, ascetosporeans); Filosa consists of photosynthetic amoebae (i.e. chlorarachniophytes) and several heterotrophic amoeboid and flagellate groups (e.g. cercomonads, euglyphids, and thaumatomonads) (Adl et al. 2005; Cavalier-Smith and Chao 2003). Cercozoans are common in marine, freshwater, and soil environments; however, their morphological and genetic diversity are still unclear, and recent studies with environmental sequences have shown that many member species are still undescribed (Bass and Cavalier-Smith 2004; Bass et al. 2009). Therefore, further taxonomical study on Cercozoa is required to reveal its hidden diversity.

Thaumatomonadida is an amoeboflagellate cercozoan group comprising species that live in freshwater and marine environments (Shirkina 1987). The cell surfaces of thaumatomonads are decorated with many small siliceous scales, which are synthesized in silica deposition vesicles (SDVs) that are associated with the mitochondria (e.g. Beech and Moestrup 1986; Karpov 1987; Ota, Eikrem, and Edvardsen 2012; Preisig 1994; Swale and Belcher 1974, 1975; Thomsen et al. 1993). All thaumatomonads harbor two-tiered siliceous scales on their cell surface, according to previous electron microscopic investigations (e.g. Howe et al. 2011; Ota, Eikrem, and Edvardsen 2012; Swale and Belcher 1974, 1975; Thomsen et al. 1993; Wylezich et al. 2007; Zolotarev, Mylnikova, and Myl'nikov 2011). The shape, structure, and size of the scale show a wide range of diversity, and those features are used to describe the group's main taxonomic characteristics (e.g. Beech and Moestrup 1986; Thomsen et al. 1993; Thomsen and Ikävalko 1997; Swale and Belcher 1974, 1975; Wylezich et al. 2007). Howe et al. (2011) rearranged the classification system of thaumatomonads on the basis of cell and scale morphology and molecular phylogeny. Under the revised system, thaumatomonads were divided into two families, Thaumatomonadidae and Peregriniidae. The Thaumatomonadidae

contains gliding flagellates with unequal flagella and currently consists of the five genera *Thaumatomonas, Thaumatomastix, Reckertia, Allas,* and *Hyaloselene*, which are classified mainly on the basis of the structural differences of their siliceous scales. The Peregriniidae is currently composed of only two genera: *Peregrinia* and *Gyromitus*.

Herein, we report a novel heterotrophic flagellate, strain YPF708, isolated from a sandy beach in Japan. Phylogenetic analyses showed that this flagellate is clearly distinguishable from other known thaumatomonad lineages, with its closest relative being the lineage of Peregriniidae. Morphological observations under light and electron microscopes revealed that this flagellate has several novel characteristics and is separable from all of the previously described thaumatomonads. Especially, the absence of siliceous scales is noteworthy, and this is the first report of such characteristic among thaumatomonads.

MATERIALS AND METHODS

Isolation and cultivation. A beach sand sample was collected in Shizugawa, Motoyoshi, Miyagi, Japan (latitude = 38.6707, longitude = 141.4620) on February 2, 2007. For the initial cultivation, the sample was placed into a flask filled with ESM medium (Kasai et al. 2004) and kept at 20 °C under a 14-h light/10-h dark cycle. A culture of strain YPF708 was established using a single-cell isolation technique from the initial cultivation. The culture, which also contained bacteria as prey was maintained in the same condition as the initial cultivation.

Light microscopy. The cells of strain YPF708 growing on glass-bottomed dishes were observed using an Olympus IX71 inverted microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP71 CCD camera (Olympus).

Electron microscopy. Preparation for transmission electron microscopy (TEM) was performed as described in Eikren and Moestrup (1998). Ultrathin sections were prepared on a Reichert Ultracut S ultramicrotome (Leica, Vienna, Austria), double stained with 2% (w/v) uranyl acetate and lead citrate (Reynolds 1963), and observed using a Hitachi H-7650 electron microscope (Hitachi High-Technologies Corp., Tokyo, Japan) equipped with a Veleta TEM

CCD camera (Olympus Soft Imaging System, Munster, Germany). For the observation of whole-mount cells under TEM, specimens were prepared as described in Yabuki and Ishida (2011) and observed under the same TEM system.

Fluorescence microscopy. For Fluorescence microscopy to confirm if there is not any siliceous structures in/on the cells, 2-(4-pyridyl)-5-{[4-(2-dimethylaminoethylaminocarbamoyl)methoxy]phenyl}oxazole (PDMPO) (LysoSensor™ Yellow/Blue DND-160; Molecular Probes, Eugene, OR, USA) was added to cultures in the growth phase, and the cultures were cultivated for 1 day. The cultivated cells were fixed with 5% (v/v) glutaraldehyde and observed using a Leica DMRD microscope (Leica, Wetzlar, Germany) equipped with an Olympus DP71 CCD camera (Olympus).

DNA extraction, polymerase chain reaction amplification, and sequencing. Total DNA was extracted from cells harvested from ~ 300 ml of a 2-wk-old culture of strain YPF708 using DNeasy Plant mini kit (Qiagen Science, Valencia, CA), according to the manufacturer's instructions. The SSU rRNA and large subunit ribosomal RNA (LSU rRNA) genes of strain YPF708 were amplified by polymerase chain reaction (PCR) with the following primer sets: 25F-1256R (Bass and Cavalier-Smith 2004) and EugF1-UNIR2 (Van Borm and Boomsma 2002) for SSU rDNA and LR1-LR12 (Yabuki, Inagaki, and Ishida 2010) for LSU rDNA. Amplifications consisted of 30 cycles of denaturation at 94 °C for 30 s, annealing at 55—50 °C for 30 min, and extension at 72 °C for 1--4 min, depending on the expected size of PCR fragments. An additional extension for 7 min at 72 °C was performed at the end of the reaction. Amplified DNA fragments were purified after gel electrophoreses with a QIAquick Gel Extraction Kit (Qiagen Science), and then cloned into the p-GEM® T-easy vector (Promega, Tokyo, Japan). The insert DNA fragments were completely sequenced on both strands by 3130 Genetic Analyzer (Applied Biosystems, Monza, Italy). The SSU and LSU rDNA sequences of strain YPF708 were deposited as AB714270 and AB714271, respectively, in GenBank.

Sequence alignments and phylogenetic analysis. We newly created the SSU rDNA and concatenated the SSU+LSU rDNA alignment sets of Cercozoa. The SSU and LSU rDNA

sequences of strain YPF708 were added to these two alignment sets, respectively. The sequences of both alignment sets were automatically aligned with MAFFT (Katoh and Toh 2008), and then edited manually with SeaView (Galtier, Gouy, and Gautier 1996). For phylogenetic analyses, ambiguously aligned regions were manually deleted from each alignment. Finally, we prepared two alignment datasets: one comprising only 1,514 positions of SSU rDNA and the other comprising 4,062 positions of the combined SSU and LSU rDNA. The alignment files that were used in the analyses are available on request. Both datasets were analyzed under the same conditions. The maximum likelihood (ML) tree was heuristically searched using RAxML v.7.2.6 (Stamatakis 2006) under the GTR+ Γ model. Tree searches started with 20 randomized-taxonaddition maximum-parsimony trees, and the highest log likelihood (lnL) was selected as the ML tree. A ML bootstrap analysis of 1,000 replicates was conducted under the GTR+ Γ model with rapid bootstrap option.

A Bayesian analysis was run using MRBAYES v. 3.1.2 (Ronquist and Huelsenbeck 2003) with the GTR +Γ model for each dataset. One cold and three heated Markov chain Monte Carlo with default chain temperatures were run for 5×10^6 generations, sampling lnL values and trees at 100-generation intervals. The first 1.0×10^6 generations of each analysis were discarded as "burn-in." Bayesian posterior probability (BPP) and branch lengths were calculated from the remaining trees.

RESULTS

Light microscopy. Cells of *Esquamula lacrimiformis* n. gen., n. sp. were ovoid (tear-drop in shape), with a rigid surface, and were 7.8 μm (4.5--11.3 μm) long and 6.5 μm (3.9--8.8 μm) wide (n = 52) (Fig. 1--5). Many tiny particles that were probably tips of extrusomes were visible in the cell (Fig. 1). Each cell possessed two flagella of unequal length (Fig. 2). The short anterior flagellum was 4.1 μm long (1.4--6.5 μm, n = 31) and the long posterior flagellum was 14.9 μm long (12.5--17.4 μm, n = 29). Both flagella emerged from the same flagellar pit located at the subapical ventral side of the cell (Fig. 2, 3). The flagellar pit was elliptical in shape (Fig. 2) and was continuous with a shallow ventral groove (Fig. 2, 3).

The groove extended to nearly the posterior end of the cell (Fig. 2, 3). Cells showed a smooth gliding movement. The distal half of the posterior flagellum was trailing and the basal half lay within the groove. The anterior flagellum laterally curved from the basal insertion (Fig. 2) and usually did not show active motion. On occasion, a nongranular thin filopodium was extended from the ventral groove (Fig. 5). The filopodia were sometimes branched and reached about 1--2 times of the length of the cell. On occasion, a lobose pseudopodium also extended from the ventral groove (Fig. 4) or the posterior end of the cell. While the pseudopodia were emerging, the cells were immobile and tightly attached to the culture dish. Such cells with pseudopodia were easily recognizable in the culture at the early stages of inoculation; however, they were hard to find in an older culture in which many non-amoeboid cells were recognizable. Cells showing binary fission were observed on several occasions. During the binary fission, cells were still moving actively. Plasmodial stage and cyst formation without the flagella were not observed in our culture conditions.

Transmission electron microscopy. Cells were covered only by plasma membrane, and any additional structures, such as scales, spines, and sheath structures, were not observed on the cell surface (Fig. 6). A food vacuole containing prey cells was occasionally observed at the posterior half of the cell (Fig. 6). A nucleus with a conspicuous nucleolus was located at the anterior or middle of the cell (Fig. 6, 10). The shape of the nucleus was generally roundish and in many sections the nucleus showed a small hollow at the ventral anterior side near the basal bodies (Fig. 7). However, amorphously shaped nuclei invaginated by either cytoplasm or microbody material, or both, were also occasionally observed (Fig. 8, 9). The cells had a few microbody profiles, which are probably lobes of a single microbody (Fig. 6--10). The microbody was always closely associated with the nucleus (Fig. 6--10). The microbody itself was also sometimes invaginated by the cytoplasm and/or the nuclear membrane (Fig. 9). Several roundish mitochondria with tubular cristae were scattered throughout the cell (Fig. 6). A Golgi apparatus was observed near the anterior end of the cell (Fig. 6, 10), and a single large vesicle was frequently observed near the Golgi apparatus (Fig. 11, 12). This large vesicle was

0.4--0.8 μm in diameter. The vesicle was generally filled with slightly dense materials. Occasionally, membranous structures were also observed in the vesicles (Fig. 11).

Two flagella were seen emerging from a single shallow pit at the anterior part and near the nucleus (Fig. 10). Basal bodies were connected to each other by several fibrous structures; the left side of the basal bodies were connected by a highly condensed thin fiber (arrowhead in Fig. 13) and the right side was connected by amorphous and thick fibrous substances (arrow in Fig. 13). The basal bodies were at a 45--70° degree angle (Fig. 10, 12). A rhizoplast with very clear bundle patterns was seen emerging from the base of the posterior basal body and running toward the nucleus (Fig. 12). The posterior end of the flagellar pit was connected to the ventral groove (Fig. 14). Several microtubules that originated near the posterior basal body ran toward the ventral groove (Fig. 15) and underlined the groove from the inside (Fig. 16).

Several slender extrusomes, 1.2--2.0 μm long, consisted of a shaft with a horizontal-stripe pattern and a cap structure with a high density (Fig. 17, 18). They were located beneath the cell membrane and surrounded by a single membrane (Fig. 17). Some interspaces were found between the membrane and the contents of extrusome at the anterior end of the extrusome (Fig. 17), which may have been caused by artifacts of fixation. The cap structure was likely to have collapsed in the discharged extrusome (Fig. 18).

In the whole-mount cell observation under the transmission electron microscope, neither spines nor scales were observed on the cell surface (Fig. 19). The posterior flagellum bears numerous tiny and simple flagellar hairs emerging from both sides of the flagellum (Fig. 19, 20). Those hairs were not detected on the anterior flagellum (Fig. 19). In a fixed and mounted cell, several discharged extrusomes were also observed (Fig. 19). From our observations, a maximum of 15 discharged extrusomes were observed around one cell. A large globular structure was attached on the tip of the discharged extrusomes (Fig. 21).

Fluorescence microscopy. To confirm if *E. lacrimiformis* n. gen., n. sp. really do not produce any siliceous structure, fluorescence microscopic observations with PDMPO as the fluorescent marker were conducted cells (Fig. 22) and two other heterotrophic cercozoan strains,

Thaumatomastix sp. (strain SRT005; Fig. 24), which possesses siliceous scales on its cell surface, and *Cercomonas* sp. (strain YPF926; Fig. 23), which does not produce any siliceous structures. Silica-induced PDMPO fluorescence was observed on the cell surface of *Thaumatomastix* sp. (Fig. 24B). Neither *E. lacrimiformis* nor *Cercomonas* sp. cells showed any confirmable fluorescence (Fig. 22B, 23B).

Phylogenetic analysis. We determined the SSU and LSU rDNA sequences of *E. lacrimiformis* n. gen., n. sp. to be 2,920 and 4,042 bp, respectively. Both SSU and LSU rDNAs contained a single putative group 1 intron. The intron in the SSU rDNA was 1,160 bp long and located between positions 543 and 1704, and its inferred size after exclusion of the intron was 1760 bp. The intron in the LSU rDNA was 575 bp long and located between positions 1382 and 1958, and its inferred size after exclusion of the intron was 3499 bp. In the phylogenetic tree inferred from the SSU rDNAs of various cercozoans, including the environmental sequences, *E. lacrimiformis* branched with *Peregrinia clavideferens* (Peregriniidae, Thaumatomonadida) and an environmental sequence (EF024794) with a bootstrap probability (BP) value of 100% and a BPP of 1.00. This clade was sister to the Thaumatomonadidae, which also included several environmental sequences, but the monophyly of *E. lacrimiformis*, *P. clavideferens*, and Thaumatomonadidae did not show high statistical support values (i.e. 45% BP and 1.00 BPP) (Fig. 25).

In the phylogenetic tree inferred from the combined SSU and LSU rDNAs, the tree topology was generally similar to our SSU rDNA tree and combined SSU and LSU rDNA tree in Chantangsi, Hoppenrath, and Leander (2010). The sister relationship between *E. lacrimiformis* and *P. clavideferens* was recovered with a BP of 100% and a BPP of 1.00 also in this tree. The monophyly of thaumatomonads including *E. lacrimiformis* increased to 96% for BP and to 1.00 for BPP (Fig. 26).

DISCUSSION

***Esquamula lacrimiformis* n. gen., n. sp. is a novel member of thaumatomonads.** Light microscopic observations did not show the conclusive taxonomic/phylogenetic position of

E. lacrimiformis n. gen., n. sp. since the major morphological characteristics in light microscopy (e.g., the existence of the shorter anterior flagellum, posterior long flagellum, and nonpermanent pseudopodia, and the teardrop-shaped cell with many tiny granules) are shared by several "core" cercozoans, such as cercomonads, glissomonads, cryomonads, and thaumatomonads. The precise identification of those members is difficult under light microscopic observation alone. In particular, the scales of thaumatomonads except *Thaumatomastix* cannot be recognized well under a light microscope.

While the identity of *E. lacrimiformis* was unclear on light microscopic observations, its phylogenetic position was clearly specified by our molecular phylogenetic analyses. In the SSU rDNA tree, *P. clavideferens*, a thaumatomonad flagellate, appeared as the closest relative of *E. lacrimiformis* with a high statistical support. Although SSU rDNA analysis did not strongly support the monophyly of the Thaumatomonadida, including *E. lacrimiformis* and *P. clavideferens*, the combined SSU and LSU rDNA analysis strongly supported the inclusion of *E. lacrimiformis* in the Thaumatomonadida. The ultrastructural observations, however, demonstrated that *E. lacrimiformis* possesses several unique characteristics that have not been reported in other thaumatomonads.

Absence of siliceous scales. While siliceous scales on the cell surface is regarded as a synapomorphic characteristic of thaumatomonads (Ota, Eikrem, and Edvardsen 2012), we could not detect any putative related structures in *E. lacrimiformis* even under the electron microscope. The SDV and its putative related structure were also not detected in the cell. Since *E. lacrimiformis* was kept in ESM medium, a natural seawater-based medium, this medium is expected to contain sufficient dissolved silica for scale formation. In fact, by using ESM medium, we successfully established a culture of a siliceous scale-bearing thaumatomonad, *Thaumatomastix* sp., and have kept it in culture for more than four years (deposited to the National Institute for the Environmental Sciences (NIES) as NIES-2378). The presence of siliceous scales on the cell surface of this strain has also been confirmed (data not shown). We consider that the absence of siliceous scales in *E. lacrimiformis* is not caused by cultivation

artifacts. Moreover, we confirmed that *E. lacrimiformis* cells grown in a medium with PDMPO showed no siliceous fluorescence. Hence, all of our data indicate that *E. lacrimiformis* does not possess any siliceous structure. Since *E. lacrimiformis* is not a uniquely deep-branching lineage in the thaumatomonads, it is reasonable to consider that the loss of the silica synthesis abilities has occurred in this species alone. Interestingly, while this is the first case to show the secondary loss of the siliceous scales in thaumatomonads, another example of the secondary loss of the ability to synthesize siliceous structures was also recently reported in euglyphids (Howe et al. 2011). These findings indicate that the secondary loss of the siliceous scales may have been occurred in Cercozoa more frequently previously thought.

Extrusomes with novel characteristics. The presence of extrusomes was reported in at least five thaumatomonad genera: *Thaumatomonas, Reckertia, Thaumatomastix, Peregrinia,* and *Gyromitus* (e.g. Karpov 1993; Karpov and Zhukov 1987; Ota, Eikrem, and Edvardsen 2012; Swale and Belcher 1974, 1975; Zolotarev, Mylnikova, and Myl'nikov 2011). The detailed structure of extrusomes differs among these genera. With the exception of *Thaumatomastix*, the extrusomes of the other four genera share several common features: 1) an ovoid or globular shape, 2) a < 500 nm length, and 3) a heterogeneous axial element structure (Mikrjukov 1995). The extrusomes of *Thaumatomastix* are cylindrical, with lengths > 1 μm (Ota, Eikrem, and Edvardsen 2012; Fig. 4D, E), have neither a heterogeneous axial element nor a cap structure, and contain only fibrous and amorphous materials (Ota, Eikrem, and Edvardsen 2012). The extrusome of *E. lacrimiformis* is similar to that of *Thaumatomastix* in terms of size and the absence of a heterogeneous axial element. However, they are clearly distinguishable from those of *Thaumatomastix* by the presence of a cap structure. The extrusomes of *E. lacrimiformis* also resemble those of thecofilosean flagellates (i.e. *Protaspa* (=*Protaspis*)*, Cryothecomonas,* and *Mataza*) in terms of their slender shape and the presence of a cap structure (Hoppenrath and Leander 2006; Schnepf and Kühn 2000; Yabuki and Ishida 2011). However, the horizontal-stripe pattern found in the shaft of *E. lacrimiformis* extrusomes has never been reported, not only in thecofiloseans but also in thaumatomonads. Thus, the extrusome of *E.*

lacrimiformis is unique and easily distinguishable from that of other thaumatomonads.

Amorphous microbody. The microbody is a ubiquitous organelle in protists and has been reported in many cercozoans, such as the cercomonads Sainouroidea, *Massisteria*, *Metromonas*, *Clautriavia*, and *Thaumatomonas* (Cavalier-Smith et al. 2008, 2009; Chantangsi and Leander 2010; Karpov 2011; Mylnikov and Karpov 2004; Mylnikova and Mylnikov 2011; Patterson 1990). The microbody of *E. lacrimiformis* is always associated with the nucleus, similar to other cercozoan microbodies. The amorphous shape is another shared characteristic between them. Cercozoans with those microbodies are not so closely related with each other within the phylum Cercozoa; thus, the features of the microbody (i.e. the amorphous shape and the association with the nucleus) are probably ancestral. However, the invagination of the microbody into the nucleus is a rare characteristic in eukaryotes. A similar microbody arrangement was recognized in *Clautriavia biflagellata* (Chantangsi and Leander 2010; Fig. 3A), but has not been reported in any other cercozoans. Since *C. biflagellata* is not phylogenetically related to thaumatomonads, the similar invagination may have evolved independently in the Cercozoa. We further found that the cytosol, instead of the microbody, invaginated into the nucleus in some cells. The nucleus of *E. lacrimiformis* might be capable of being invaginated by other closely associated cellular compartments.

Other characteristics. Parallel basal bodies are considered a shared character among thaumatomonads and spongomonads, which is considered the sister lineage of thaumatomonads (Howe et al. 2011). However, the arrangement of basal bodies in *Peregrinia limax* (=*Gyromitus limax*) appears to be non-parallel (Swale and Belcher 1975; Fig. 19). The basal bodies of *E. lacrimiformis* are also not arranged in parallel. Considering the phylogenetic position of *E. lacrimiformis*, non-parallel basal bodies may be a synapomorphic feature of *Esquamula* and *Peregrinia*. This interesting subject should be carefully and continuously examined in further studies, as the sister lineage of thaumatomonads has not been robustly specified by our phylogenetic analyses.

Flagellar hairs are widely distributed in various cercozoan lineages (e.g. *Aurigamonas*

solis, *Protaspa longipes*, and *Auranticordis quadriverberis*) (Chantangsi, Esson, and Leander 2008; Schnepf and Kühn 2000; Vickerman et al. 2005), but those hair-harboring members are just a minority in Cercozoa as a whole. The flagellar hairs of cercozoans are simple and resemble each other, although they are found on organisms not phylogenetically closely related to each other. In thaumatomonads, flagellar hairs have been reported only on the anterior flagellum of *Thaumatomonas coloniensis* and on the posterior flagellum of *Thaumatomonas lauterborni* (Karpov 1987; Wylezich et al. 2007). However, *E. lacrimiformis* possesses flagellar hairs like those of *Thaumatomonas* despite their distant phylogenetic positions among the thaumatomonads.

Taxonomic conclusion. Since our phylogenetic analyses clearly showed that *E. lacrimiformis* branches within the thaumatomonads, it is undoubtedly a member of the Thaumatomonadida clade. However, *E. lacrimiformis* can be distinguished from other thaumatomonads on the basis of the above-mentioned morphological characteristics, as well as phylogenetically. Therefore, we conclude here that *E. lacrimiformis* should be treated as a new species in a new genus, *Esquamula* n. gen., of the thaumatomonads. The absence of siliceous scales is a particularly notable morphological difference between *E. lacrimiformis* and other thaumatomonads. Since the members of both Peregriniidae and Thaumatomonadidae produce siliceous scales, we propose a new family, Esquamulidae n. fam., in the order Thaumatomonadida, which will require emendation of the description, in order to accommodate the new genus.

***Esquamula lacrimiformis* n. gen., n. sp. and further hidden diversity within the thaumatomonads.** We established cultures of *E. lacrimiformis* n. gen., n. sp. from the samples of a rather ordinary sandy beach in Japan. However, neither *E. lacrimiformis* itself nor closely related environmental sequences have been reported previously, which indicate that this species may not be sufficiently abundant in the natural habitat to be detected by environmental survey. To understand the precise diversity of unicellular eukaryotes, continuous and careful observations of the various environmental samples, including usual samples, such as beach sand,

must be further performed. Our detection of *E. lacrimiformis* strongly implies that we have underestimated the diversity of thaumatomonads: some undetected organisms may offer hints to understanding their morphological evolution of this group.

TAXONOMIC TREATMENT

Rhizaria

Cercozoa

Filosa

Imbricatea

Thaumatomonadida Shirkina 1987, emend. Karpov 1990. (ICZN)

Emended description. Heterotrophic biflagellates. Cells with many siliceous scales formed in vesicles attached to mitochondria or without any scales. Cells with a rigid surface or amoeboid cells. Presence of filose, lobose, or finger-like pseudopodia. Movement by gliding, swimming, or amoeboid creeping. Mitochondria with tubular cristae. Presence of Golgi apparatus and extrusomes.

Esquamulidae n. fam. (ICZN)

Description. Unicellular heterotrophic flagellates with a short anterior flagellum and a long posterior flagellum. Cells gliding with a posterior flagellum. Both flagella emerge from the same flagellar pit. Cells with a rigid surface and without thecae or scales. Filose or lobose pseudopodia sometimes emerging. Slender extrusomes consist of shaft with a horizontal-stripe pattern and cap structure on the tip.

Type genus. *Esquamula*.

Esquamula n. gen. (ICZN)

Description. Gliding biflagellates with subapical and ventral flagellar pit. Posterior end of the flagellar pit continuous with the ventral groove. Basal bodies not parallel. Amorphous microbody attached to the nucleus.

Type species. *Esquamula lacrimiformis* n. sp.

Etymology. The genus name *Esquamula* derived from Latin refers to “cells without scales” (*e* is a negator prefix and *squamula* means a small scale). *Esquamula* is considered to be of female gender.

Esquamula lacrimiformis n. sp. (ICZN)

Description. Cells ovoid to teardrop, 7.8 μm (4.5--11.3 μm) long by 6.5 μm (3.9--8.8 μm) wide. Ventral groove nearly reaching the posterior end of the cell. Movement by smooth gliding. Cells with many tiny intracellular particles that probably the tips of the extrusomes. Anterior flagellum, 4.1 μm (1.4--6.5 μm) long, curved, with subtle movement. Posterior flagellum, 14.9 μm (12.5--17.4 μm) long, trailing, with simple and tiny hairs. Microbody sometimes invaginates into the nucleus.

Hapantotype. One microscope slide (TNS-AL-56983a), deposited in the herbarium of the National Museum of Nature and Science (TNS), Tokyo.

Isotype. One EM block (TNS-AL56983b) in TNS. These cells are derived from the same sample as the holotype.

Iconotype. Figure 1

DNA sequence. Small subunit ribosomal DNA, AB714270.

Type locality.: Sandy beach in Shizugawa Bay, Japan (latitude = 38.6707, longitude = 141.4620).

Collection date. February 2, 2007

Authentic culture. The strain DA172 used for describing this species is deposited in and maintained in the National Institute for the Environmental Sciences, Tokyo, as NIES-xxxx.

Etymology. .

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Fig. **1**--**5**. Differential interference contrast (DIC) micrographs of living cells of *Esquamula lacrimiformis* n. gen., n. sp. Fp, flagellar pit. Arrows indicate the ventral groove. Arrowheads indicate the flagella. Double arrowheads indicate the pseudopodium. Scale bar = 10 μm. **1**--**3**. Typical gliding cells. **4**. Non-gliding cell with lobose pseudopodium emerging from the ventral groove. **5**. Non-gliding cell with filose pseudopodium (double arrowhead).

Fig. **6**--**9**. Transmission electron micrographs of *Esquamula lacrimiformis* n. gen., n. sp. Fv, food vacuole; G, Golgi body; Mb, microbody; Mt, mitochondrion; N, nucleus; n, nucleolus;. **6**. Longitudinal section of whole cell. Scale bar = 1 μm. **7**. The nucleus with a small hollow near the basal body. Scale bar = 1 μm. **8**. Amorphous-shaped nucleus invaginated by the cytoplasm and microbody. Scale bar = 1 μm. **9**. Nucleus deeply invaginated by the microbody. Double arrowhead indicates the membrane structure invaginating into the microbody. Scale bar = 1 μm.

Fig. **10**--**16**. Transmission electron micrographs of *Esquamula lacrimiformis* n. gen., n. sp. AB, anterior basal body; AF, anterior flagellum; Fv, food vacuole; G, Golgi body; Mb, microbody; mt, microtubules; N, nucleus; n, nucleolus; PB, posterior basal body; PF, posterior flagellum; r, rhizoplast; VG, ventral groove. Asterisk indicates an electron-dense vesicle. **10**. Longitudinal section of whole cell. Scale bar = 1 μm. **11**. High magnification view of dense vesicle located near the Golgi body. Scale bar = 1 μm. **12**. Longitudinal section of 2 basal bodies. Rhizoplast connects to the posterior basal body and the nucleus. Scale bar = 1 μm. **13**. Transverse section of 2 basal bodies. Arrow indicates a dense region. Arrowhead indicates the connecting fiber. Scale bar = 500 nm. **14**. Transverse section of 2 flagella in flagellar pit. Posterior end of the flagellar pit connects to the ventral groove. Scale bar = 1 μm. **15**. Section showing the orientation of basal bodies, ventral groove, and connecting microtubules. Several microtubules ran towards ventral groove from the base of the PB. Scale bar = 1 μm. **16**. High magnification view of the ventral groove and its supporting microtubules. Scale bar = 500 nm.

Fig. **17**, **18**. Transmission electron micrographs of extrusomes of *Esquamula lacrimiformis* n. gen., n. sp. Arrowheads indicate the shaft with horizontal-stripe pattern.

Asterisk indicates dense material in a cap structure. Scale bar = 500 nm. **17**. Longitudinal section of an undischarged extrusome. **18**. Longitudinal section of a discharged extrusome.

Fig. **19--21**. Whole-mount transmission electron micrographs of *Esquamula lacrimiformis* n. gen., n. sp. **19**. Whole-cell image of *E. lacrimiformis* cell with two flagella. Arrowheads indicate discharged extrusomes. Scale bar = 2 μm. **20**. High-magnification image of the posterior flagellum with fine flagellar hairs. Scale bar = 500 nm. **21**. High-magnification image of discharged extrusome. A large globular structure attached on the tip of the discharged extrusome. Scale bar = 1 μm.

Fig. **22--24**. Fluorescence microphotographs of 3 cercozoan cultures grown with a fluorescent dye that detects silicon (see Materials and Methods). **22A, 23A, 24A**. DIC images. **22B, 23B, 24B.** Fluorescence images. Scale bars = 10 μm**. 22**. *Esquamula lacrimiformis* n. gen., n. sp. **23**. *Cercomonas* sp. **24**. *Thaumatomastix* sp.

Fig. **25**. Maximum-likelihood tree of 102 cercozoans and 4 radiolarians, using 1,514 positions of the small subunit (SSU) rDNA. Environmental sequences were labeled with accession numbers. Only bootstrap support ≥ 50% is shown. Nodes supported by Bayesian posterior probabilities ≥ 0.95 are highlighted by bold lines.

Fig. **26**. Maximum-likelihood tree of 29 cercozoans, using 15,45 bp of the small subunit (SSU) and 2,517 bp of the large subunit (LSU) rDNA. Only bootstrap support ≥ 50% is shown. Nodes supported by Bayesian posterior probabilities ≥ 0.95 are highlighted by bold lines.

Fig. 1--5

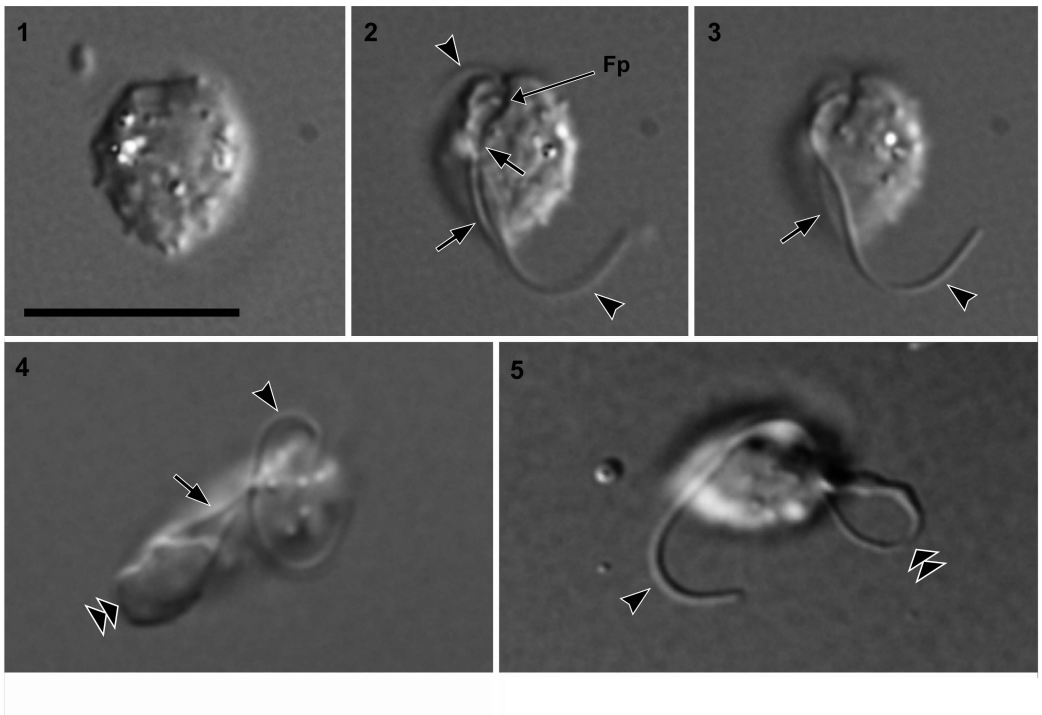

Fig. 6--9

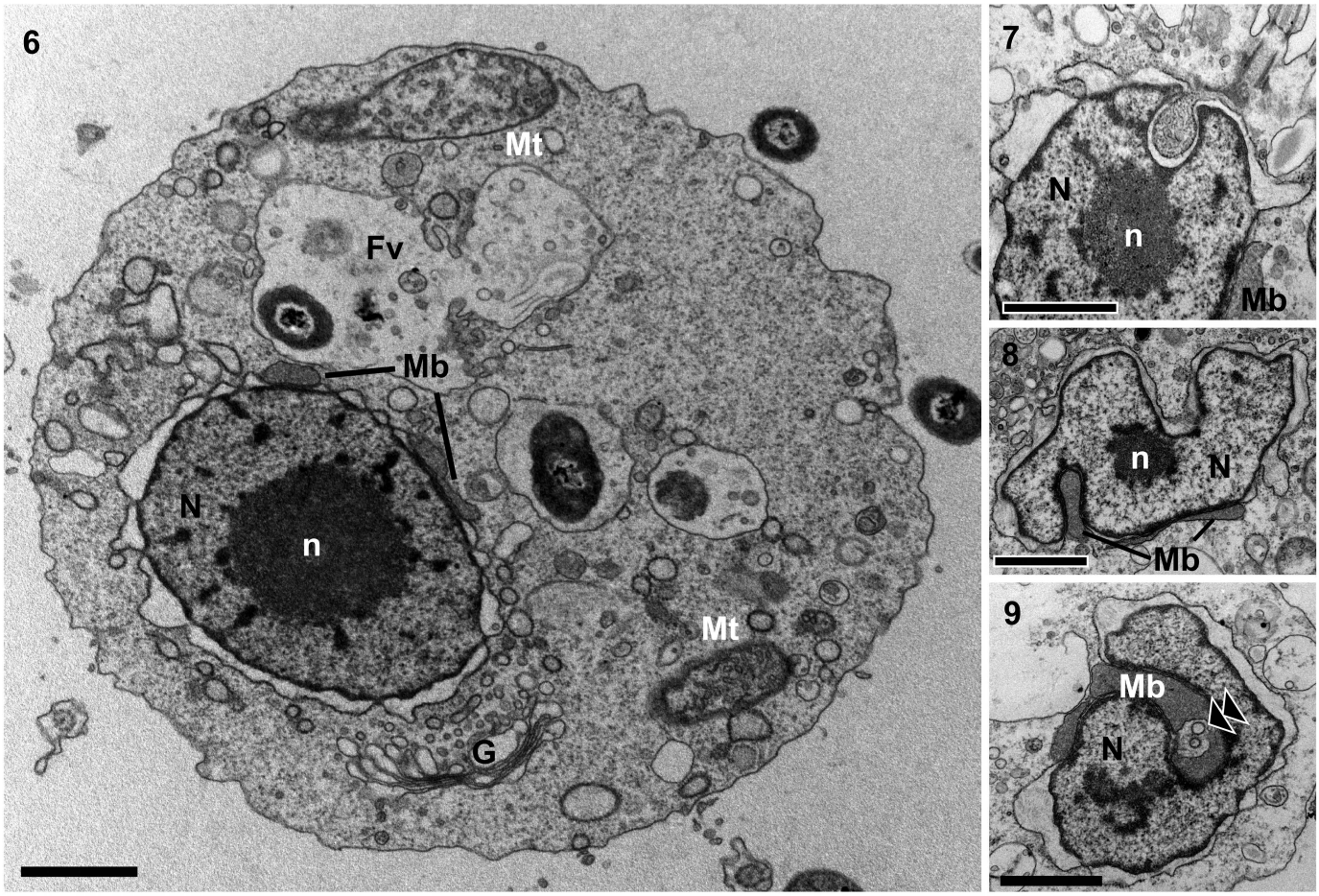

Fig. 10--16

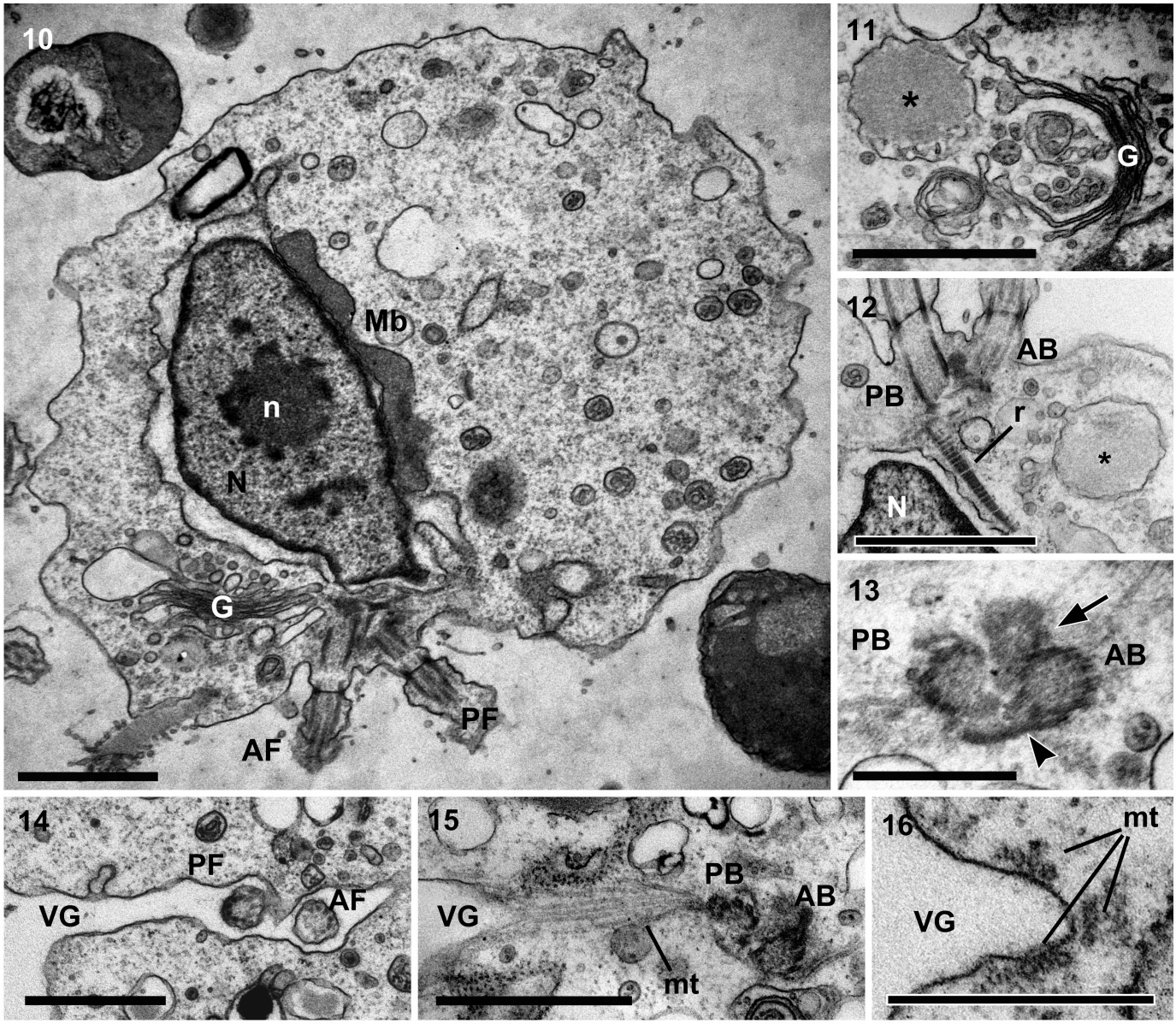
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Fig. 17, 18

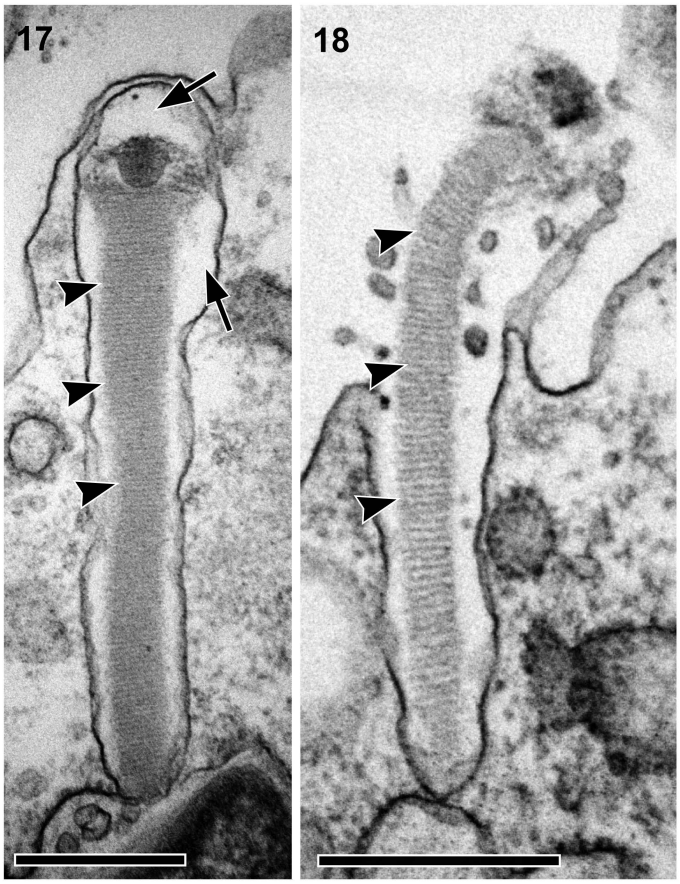


Fig. 19--21

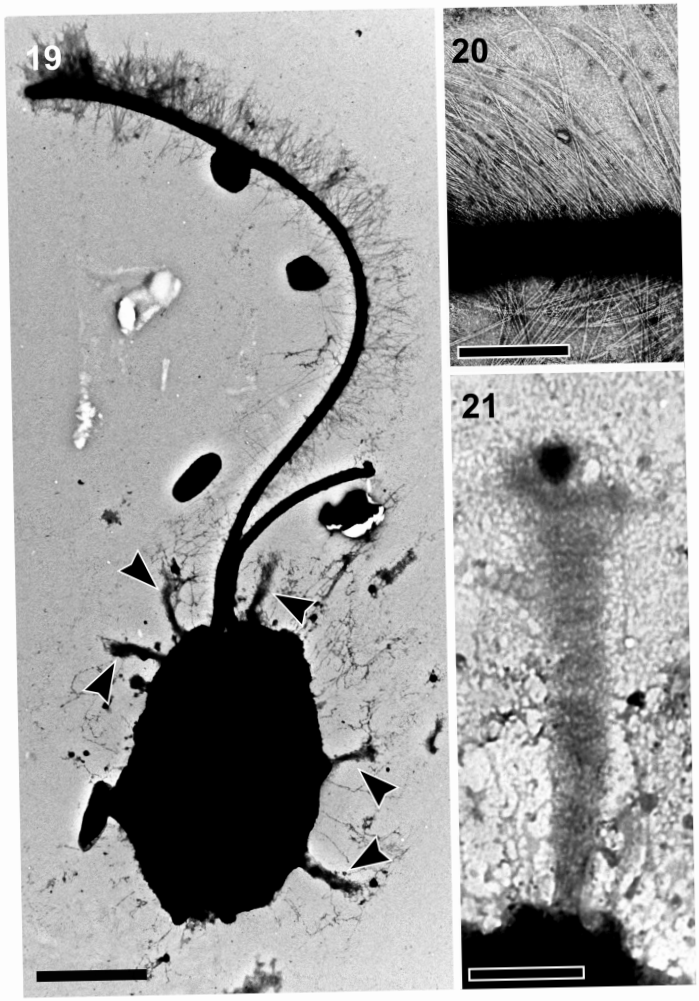


Fig. 22–24

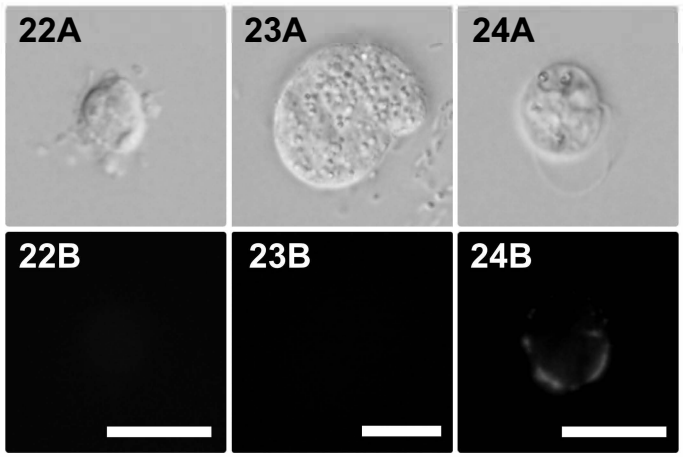
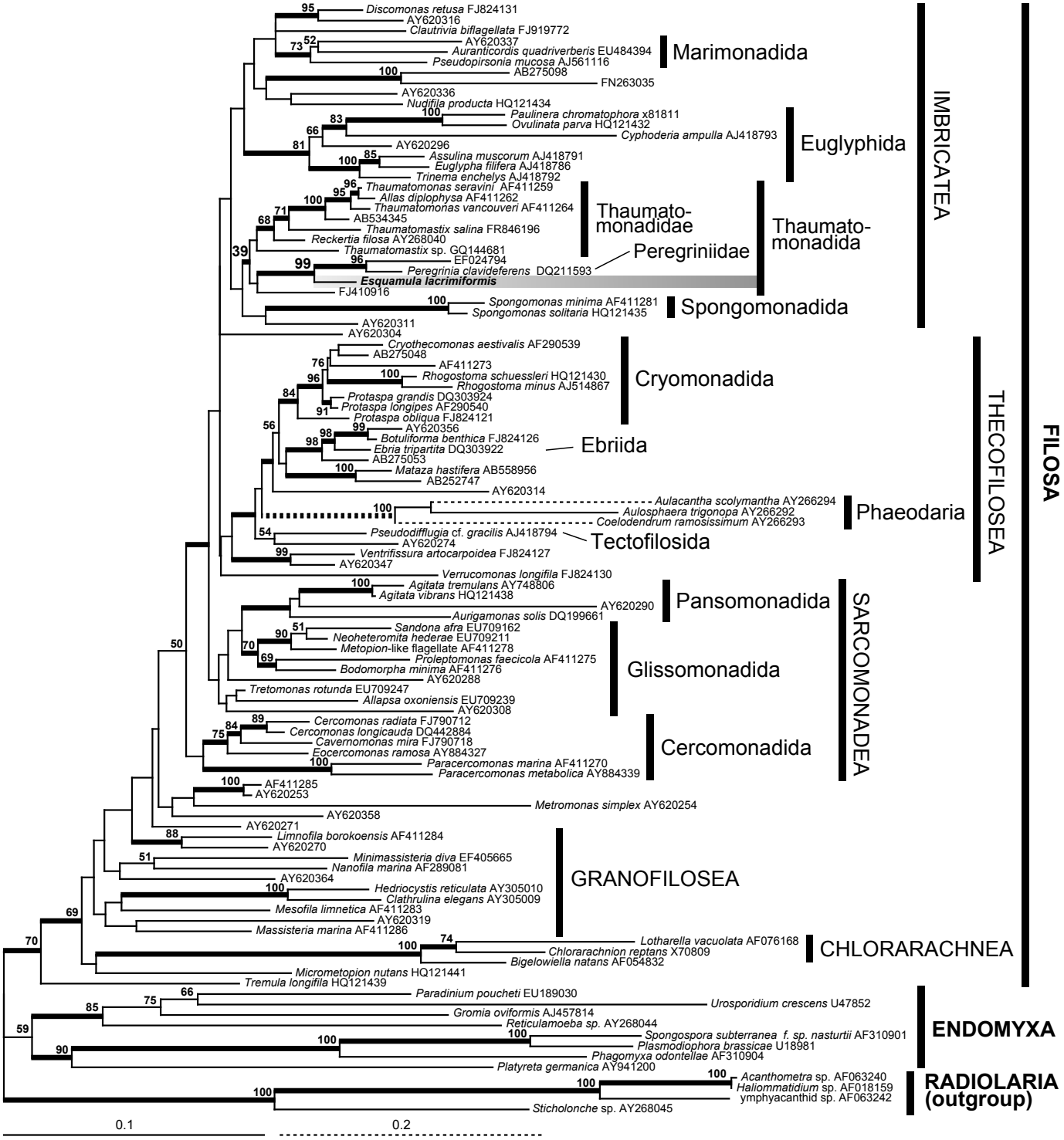


Fig. 25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AB275098
FN263035
AY620336
Nudifila producta HQ121434
Paulinera chromatophora x81811
Ovulinata parva HQ121432
Cyphoderia ampulla AJ418793
AY620296
Assulina muscorum AJ418791
Euglypha filifera AJ418786
Trinema enchelys AJ418792
Thaumatomonas seravini AF411259
Allas diplophysa AF411262
Thaumatomonas vancouveri AF411264
AB534345
Thaumatomastix salina FR846196
Reckertia filosa AY268040
Thaumatomastix sp. GQ144681
EF024794
Peregrinia clavideferens DQ211593
Esquamula lacrimiformis
FJ410916
Spongomonas minima AF411281
Spongomonas solitaria HQ121435
AY620311
AY620304
Cryothecomonas aestivalis AF290539
AB275048
AF411273
Rhogostoma schuessleri HQ121430
Rhogostoma minus AJ514867
Protaspa grandis DQ303924
Protaspa longipes AF290540
Protaspa obliqua FJ824121
AY620356
Botuliforma benthica FJ824126
Ebria tripartita DQ303922
AB275053
Mataza hastifera AB558956
AB252747
AY620314
Aulacantha scolymantha AY266294
Aulosphaera trigonopa AY266292
Coelodendrum ramosissimum AY266293
Pseudodifflugia cf. gracilis AJ418794
AY620274
Ventrifissura artocarpoidea FJ824127
AY620347
Verrucomonas longifila FJ824130
Agitata tremulans AY748806
Agitata vibrans HQ121438
AY620290
Aurigamonas solis DQ199661
Sandona afra EU709162
Neoheteromita hederae EU709211
Metopion-like flagellate AF411278
Proleptomonas faecicola AF411275
Bodomorpha minima AF411276
AY620288
Tretomonas rotunda EU709247
Allapsa oxoniensis EU709239
AY620308
Cercomonas radiata FJ790712
Cercomonas longicauda DQ442884
Cavernomonas mira FJ790718
Eocercomonas ramosa AY884327
Paracercomonas marina AF411270
Paracercomonas metabolica AY884339
AF411285
AY620253
Metromonas simplex AY620254
AY620358
AY620271
Limnofila borokoensis AF411284
AY620270
Minimassisteria diva EF405665
Nanofila marina AF289081
AY620364
Hedriocystis reticulata AY305010
Clathrulina elegans AY305009
Mesofila limnetica AF411283
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Massisteria marina AF411286
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Chlorarachnion reptans X70809
Bigelowiella natans AF054832
Micrometopion nutans HQ121441
Tremula longifila HQ121439
Paradinium poucheti EU189030
Urosporidium crescens U47852
Gromia oviformis AJ457814
Reticulamoeba sp. AY268044
Spongospora subterranea f. sp. nasturtii AF310901
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Platyreta germanica AY941200
Acanthometra sp. AF063240
Haliommatidium sp. AF018159
ymphyacanthid sp. AF063242
Sticholonche sp. AY268045
0.1
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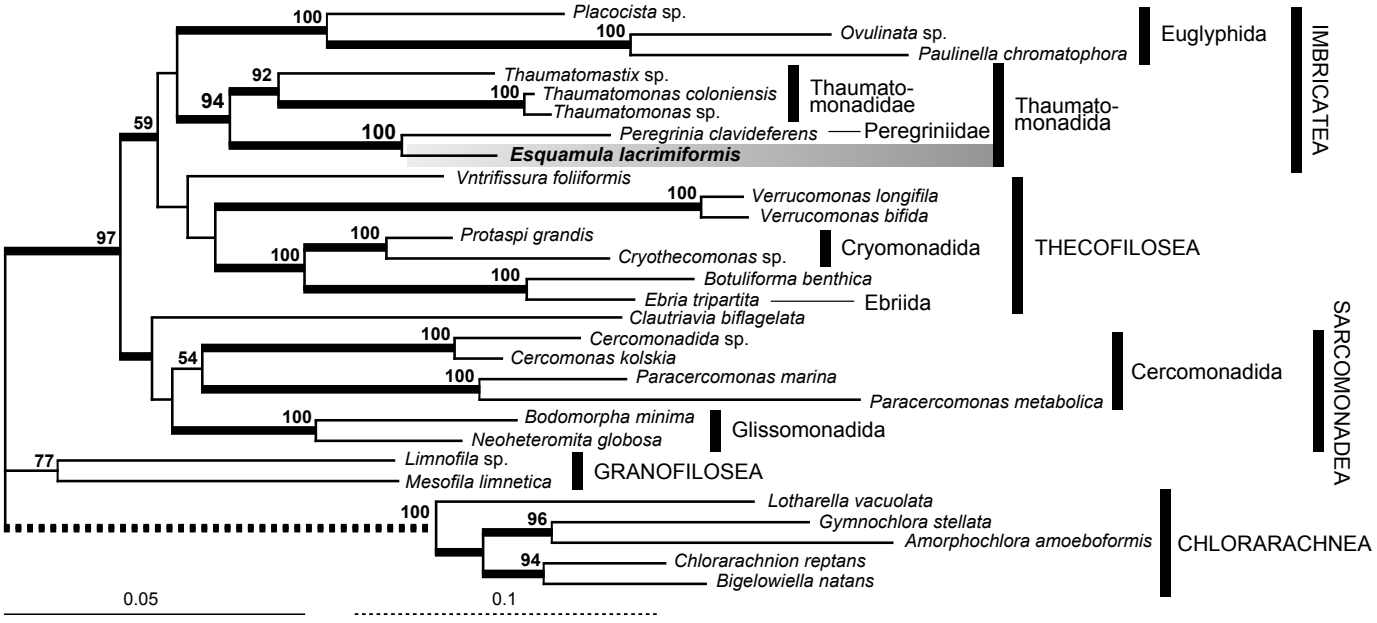
Marimonadida
Euglyphida
Thaumato-monadidae
Peregriniidae
Thaumato-monadida
Spongomonadida
IMBRICATEA
Cryomonadida
Ebriida
Phaeodaria
Tectofilosida
THECOFILOSEA
Pansomonadida
Glissomonadida
Cercomonadida
SARCOMONADEA
GRANOFILOSEA
CHLORARACHNEA
ENDOMYXA
RADIOLARIA (outgroup)
FILOSA

Fig. 26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Thaumato-monadidae
Peregrinia clavideferens
Peregriniidae
Thaumato-monadida
Esquamula lacrimiformis
Vntrifissura foliiformis
Verrucomonas longifila
Verrucomonas bifida
Protaspi grandis
Cryothecomonas sp.
Cryomonadida
THECOFILOSEA
Botuliforma benthica
Ebria tripartita
Ebriida
Clautriavia biflagelata
Cercomonadida sp.
Cercomonas kolskia
Paracercomonas marina
Paracercomonas metabolica
Cercomonadida
SARCOMONADEA
Bodomorpha minima
Neoheteromita globosa
Glissomonadida
Limnofila sp.
Mesofila limnetica
GRANOFILOSEA
Lotharella vacuolata
Gymnochlora stellata
Amorphochlora amoeboformis
Chlorarachnion reptans
Bigelowiella natans
CHLORARACHNEA
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94
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