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Microscopical Studies on *Ministeria vibrans* Tong, 1997 (Filasterea) Highlight the Cytoskeletal Structure of the Common Ancestor of Filasterea, Metazoa and Choanoflagellata

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Ministeria vibrans **(Filasterea) is a tiny amoeboid species described by Tong in 1997. It has been sporadically found in different habitats, and cultured strains were established. *M. vibrans* is well characterised by molecular phylogeny but until now was not ultrastructurally investigated in detail. Here, we provide the ultrastructure for this species based on a new strain isolated from oxygen-depleted water of the Baltic Sea. A thin vibrating flagellum could be observed but no vibrating movement of the cell body and no stalk. Our first ultrastructural study of a filasterean taxon revealed radial microvilli supported by bundles of microfilaments. Two centrioles located in the nuclear pit can migrate to the cell periphery and transform into the kinetid: the centriole orthogonal to the kinetosome with a fibrillar root and a basal foot that initiates microtubules. Microvilli in *Ministeria* suggest their presence in the common ancestor of Filasterea and Choanoflagellata. The kinetid structure of *Ministeria* is similar to that of the choanocytes of the most deep-branching sponges, differing essentially from the kinetid of choanoflagellates. Thus, kinetid and microvilli of *Ministeria* illustrate features of the common ancestor of three holozoan groups: Filasterea, Metazoa and Choanoflagellata.**

Key words: Electron microscopy; microvilli; kinetid; vibrating flagellum; Filasterea; coastal picoplankton

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Introduction

Many protist species have been described based on light microscopical findings only (for example in Larsen and Patterson 1990). Ultrastructural or molecular characterisations are often missing for species' descriptions done during the light microscopic period, leaving their taxonomic assignments uncertain (compare list of genera with uncertain affiliation in Adl et al. 2012). This is especially true for species of small size and infrequent occurrence. Meanwhile, the fraction of very small protists is found to exhibit the highest taxonomic diversity at least in the euphotic parts of the oceans (de Vargas et al. 2015). This indicates a huge backlog in documenting the taxonomy, morphology, physiology and ecological role of eukaryotic picoplankton, which is protists within a size range of 0.8 to 3 μm. Picoeukaryotes are important contributors to pivotal ecosystem functions like primary production and bacterivory and enter into symbiotic relationships with other eukaryotes, including parasitism. Many picoeukaryote taxa, for example marine stramenopiles (MAST) and marine alveolates (MALV), are globally distributed. In comparison, a large amount of picoeukaryotes likely belongs to the rare biosphere (Massana 2011).

One taxon that seems to have a rather infrequent occurrence in marine habitats is the genus *Ministeria*. Specimens of the genus were first found in a study investigating detritus-associated protists from the deep sea (Patterson et al. 1993). The type species *Ministeria marisola* was described as a small protist with equally long, stiff, non-tapering radiating cytoplasmic arms that did not show any internal skeletal elements. A second species, *Ministeria vibrans* (Tong 1997), was described and separated from *M. marisola* based on the presence of a stalk for attachment, resulting in a vibrating movement of the cell, as well as a greater number of arms (Tong 1997; Tong et al. 1998). This stalk was inferred to be a flagellum or a flagellum-derived structure (Cavalier-Smith and Chao 2003; Lee et al. 2003).

Early observations on *Ministeria* reported on the type species *M. marisola* (Patterson et al. 1993; Tong et al. 1997, 1998; Vørs 1992) whereas more recent observations were only of the second described species, *M. vibrans* (Cavalier-Smith and Chao 2003; Kiss et al. 2009; Lee et al. 2003; Tong 1997; this study). Unfortunately, *M. marisola* has not been brought into pure culture, in contrast to *M. vibrans,* which has been isolated and maintained in pure culture on two occasions (Cavalier-Smith and Chao, 2003; Tong, 1997).

Based on comparisons of ribosomal gene sequences, *Ministeria* was found to be a member of the Opisthokonta, a clade containing fungi, animals and other protist lineages (Cavalier-Smith and Chao 2003). The class Filasterea Cavalier-Smith, 2008 was erected to unify *Ministeria* and the amoeboid protist *Capsaspora owczarzaki* into one group (Shalchian-Tabrizi et al. 2008). Recently, this group was extended by the inclusion of the newly established flagellate genus *Pigoraptor* (Hehenberger et al. 2017).

We were able to obtain and to cultivate a new strain of *Ministeria vibrans* isolated from oxygen-depleted waters of the Baltic Sea (Weber et al. 2017). Since details on the ultrastructure of this crucial holozoan taxon are missing, we here provide a light and electron microscopical study to fill this knowledge gap.

Results

Sampling Location and Taxonomic Classification

Ministeria vibrans was detected in one pre-culture (L27) taken in the Landsort Deep, Baltic Sea (58°350′N,18°140′E, Table 1) and was then cultured in culture flasks as described in Weber et al. (2017). The sample was isolated from a depth of 105 m representing sulfidic water (9.8 μM sulphide, temperature 5.5 °C, salinity 10). The culture L27 containing *Ministeria vibrans* and *Paraphysomonas* sp. is maintained at the IOW-Institute for Baltic Sea Research (Rostock-Warnemünde, Germany). A re-isolated *Ministeria* culture free of *Paraphysomonas* is maintained in the "Live culture collection of free-living amoebae, heterotrophic flagellates and heliozoans" at the Institute for the Biology of Inland Waters, Russian Academy of Science (Borok, Russia). The culture was additionally deposited in the DSMZ culture collection (Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) in July 2017.

Based on 18S rDNA sequence comparison, *Ministeria vibrans* strain L27 is closely related to the type strain of this species (ATCC 50519) and the strain 'South Africa' (99.8% and 99.7% sequence similarity, respectively). In addition, the three cultured *M. vibrans* strains are also closely related to a short environmental clonal 18S rDNA sequence from a cultivation-independent study (Piquet et al. 2014) (Fig. 1).

Table 1. Overview of records of the genus *Ministeria* for *M. marisola* and *M. vibrans*, including detection method, location and references. Abbreviations: EM, electron microscopy, LM light microscopy.

Species	Method	Location	Reference
M. marisola[1]	LM (?)	Coastal seawater, Baltic Sea	Vørs 1992
M. marisola	EM, LM, culture[2]	Deep marine water, mid North Atlantic	Patterson et al. 1993
M. marisola	LM, culture[3]	Coastal marine water, Prydz Bay, Antarctic Ocean	Tong et al. 1997
M. marisola	LM, culture[3]	Coastal marine surface water, Port Jackson, Sydney	Tong et al. 1998
M. vibrans	LM, EM, culture[3,4,*]	Coastal marine water, Southampton, North Atlantic Ocean	Tong 1997
M. vibrans	LM	Water column of coastal marine water, Darwin Harbor, Pacific Ocean	Lee et al. 2003
M. vibrans	LM, EM, culture[4]	Coastal marine water, South Africa, South Atlantic Ocean	Cavalier-Smith & Chao 2003
M. vibrans	LM	Freshwater, River Danube	Kiss et al. 2009
M. vibrans	LM, EM, culture[4]	Oxygen-depleted water, Baltic Sea at Landsort Deep	This study

[*]Type strain culture for *M. vibrans*.
[1] *M. marisola* is named mistakenly as *M. maricola* in Vørs 1992.
[2]Cultured at the pressure of sample site, taken from 1515 m depth.
[3]Enrichment culture.
[4]Pure culture.

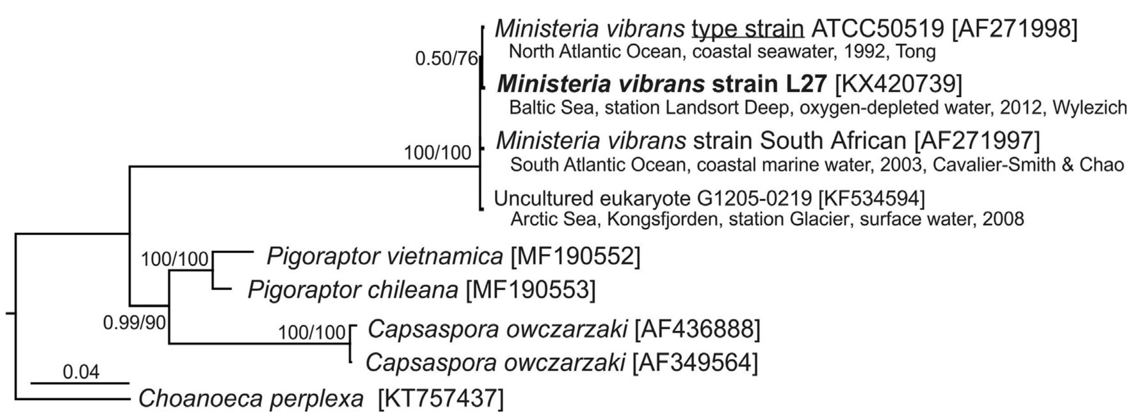

Figure 1. Phylogenetic tree of the class Filasterea using the 18S rDNA (phyML tree with corresponding MrBayes support values). The phylogenetic tree was constructed from an alignment of 1,748 positions using the choanoflagellate *C. perplexa* (KT757437) as an outgroup. Support values of the Maximum Likelihood (bootstrap) and the Bayesian (Posterior Probabilities) analyses are shown (BPP/ML) if values were above 50/0.50.

Microscopical Investigations

In live material viewed by light microscopy, the cell of *Ministeria vibrans* is more-or-less spherical and typically 2.1–3.6 μm (n = 50) in diameter (Fig. 2A, B). About 14 stiff arms emerge from the cell surface (including the upper cell side) and are symmetrically distributed (Fig. 2J, K). Some of the arms are forked at the ends (Fig. 2C, M, arrow). The arms vary in length but are typically 2–3 times longer than the cell's body (8–10 μm) and about 0.17 μm wide in live cells. Elongated oval cells with several thicker straight projections were also observed (Fig. 2D). Those are straight and more rigid compared with pseudopodia (see below and Fig. 2G). In culture conditions the organism feeds on small (~1.5 μm) *Pseudomonas fluorescens* bacteria that stick to arms or the cell body (Fig. 2E, F). One cell with long pseudopodial protrusions was observed (Fig. 2G). Also, one flagellated cell was identified among several hundred examined cells. The single flagellum (~3.6 μm) is slightly longer than the cell body, very

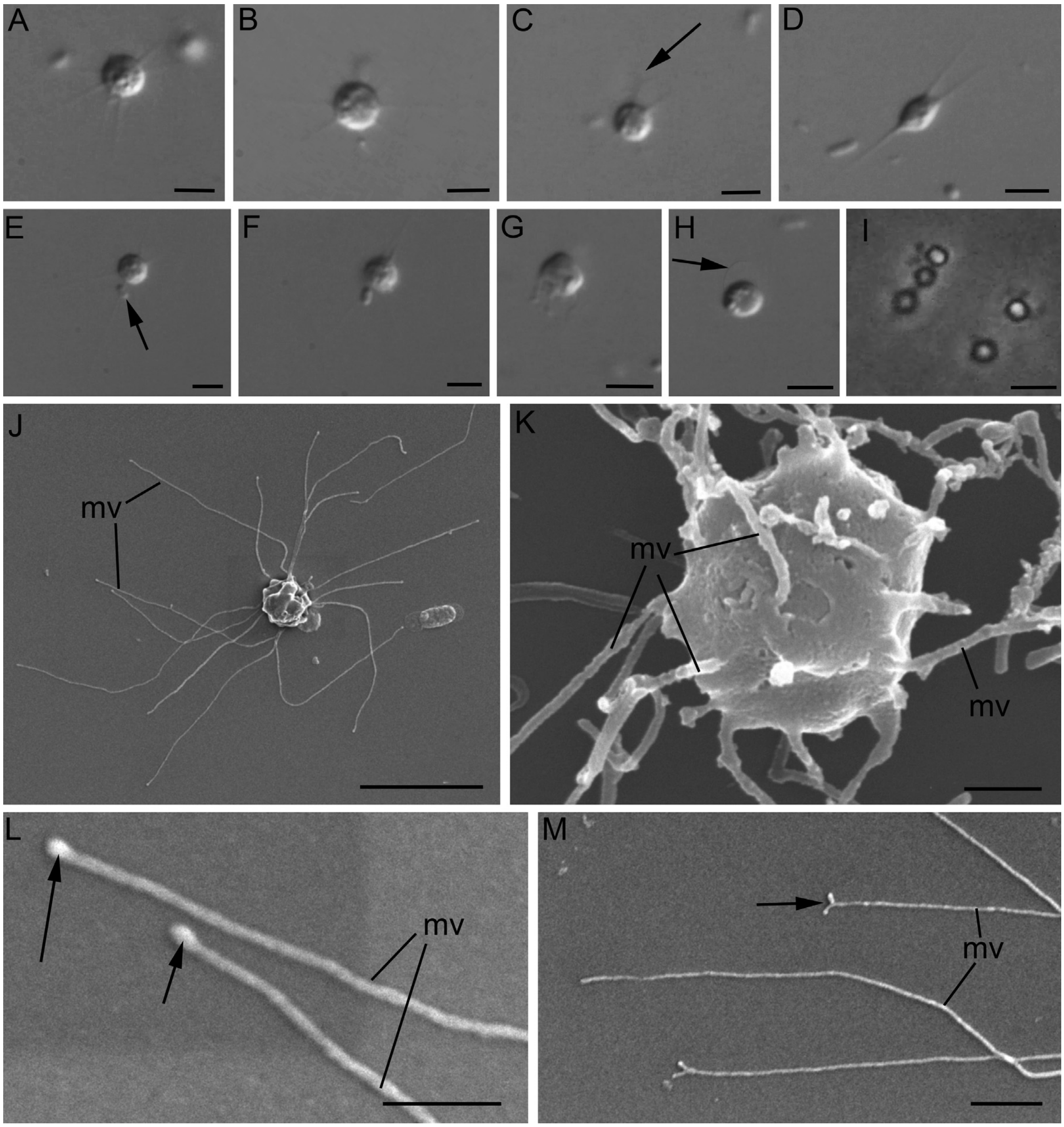

Figure 2. Light microscopic (**A–I**) and SEM (**J–M**) images of *Ministeria vibrans* strain L27. The emergence of microvilli (mv) is seen (**A–C, J, K**). The microvilli (mv) end by thickenings or bifurcations (arrows) (**C, L, M**). Presence of thicker straight projections and pseudopodial protrusions (**D, G**). Feeding on bacteria (**E, F**). Flagellated cell (**H**) and floating cell cluster (**I**). Measure bars indicate 0.5 μm (K, L), 1 μm (M), 3 μm (A–H), 4 μm (J), 6 μm (**I**).

thin and poorly visible, with vibrating movements (Fig. 2H, arrow, Supplementary Material Video S1). The flagellated cell lacks any cytoplasmic protrusions and is attached to the substrate. Swimming using the flagellum was not observed. Cells can slightly shift their position on the surface with the aid of the lightly bending arms. Previous records of this species report a cytoplasmic stalk, attachment of the cells to the substrate using a stalk and a vibrating cell movement, however, none of these features was observed in culture L27. Floating cell clusters, composed of 2–7 cells attached to each other by arms, have been seen in culture (Fig. 2I, Supplementary Material Video S2).

Using electron microscopy, microfilaments were found inside the arms (Figs. 3A, G, 4 A–F; Sup-

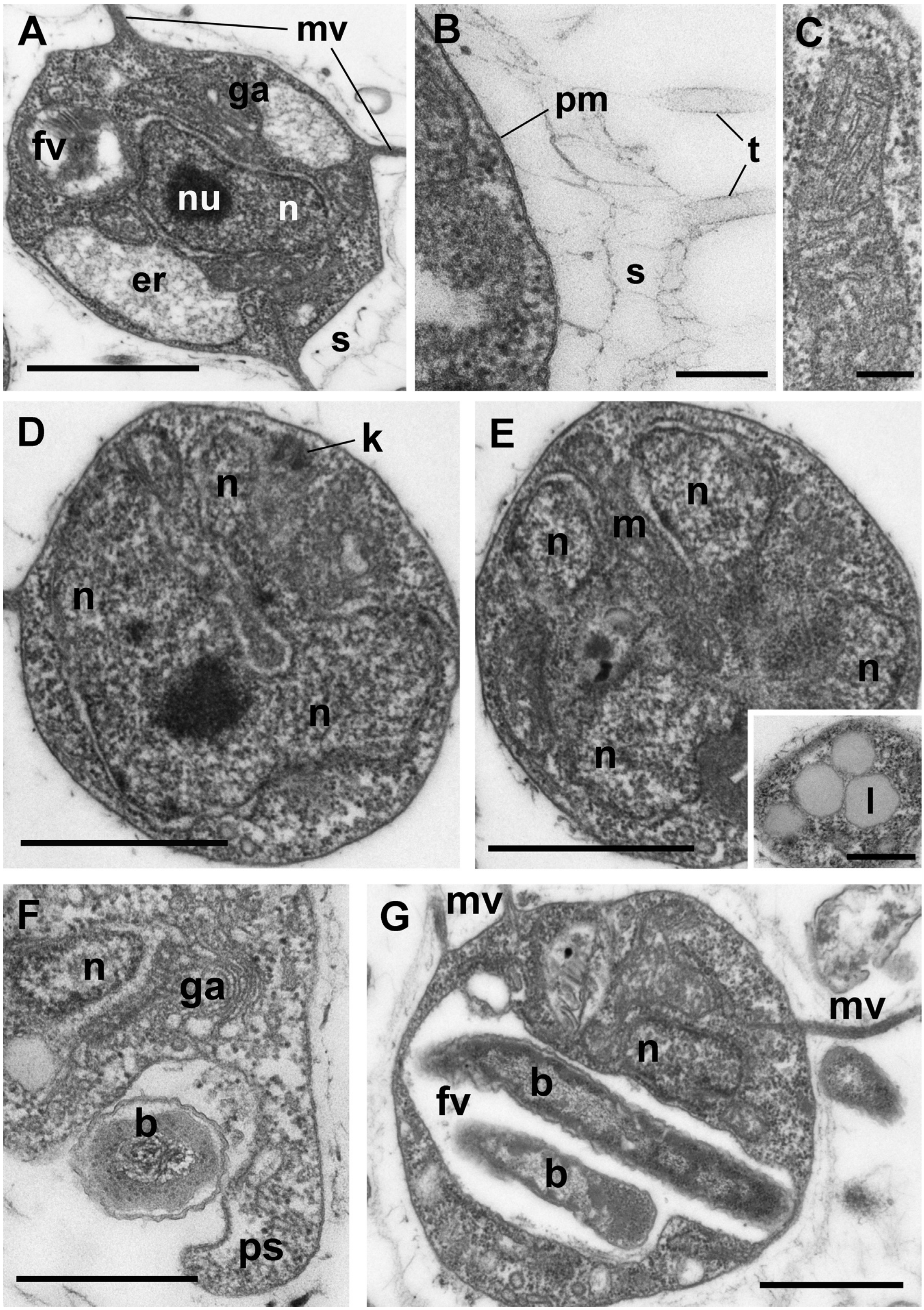

Figure 3. Ultrastructural features of *Ministeria vibrans* strain L27. Sections of the whole cell show microvilli (mv), shell (s), central position of the nucleus (n) with two dilations of endoplasmic reticulum (er), nucleolus

plementary Material Fig. S1), which are therefore called microvilli below. Some microvilli end with tiny nodules or a short bifurcation (Fig. 2L, M). A delicate shell (Fig. 3 A–G) covers the complete cell body and the proximal part of the microvilli. This covering is 0.1–0.5 μm thick and consists of two layers connected by reticulate structures (Fig. 3B). Thin-walled tube-like appendices emerge occasionally from the shell (see Fig. 3B for detail).

The cell has a peculiar predominantly fibrillar cytoskeleton. Bundles of microfilaments support the microvilli and continue in the cytoplasm for rather long distances (Fig. 4 A, C–F) but have no obvious connection with cell organelles or other structures. At the same time, cells with retracted microvilli have a huge cluster of microfilaments, tentatively called here a ‘microfilament organising centre’ (MFOC). This cluster has a dense central region and radiating bundles of microfilaments, and is located near the centre of the cell, in the nuclear pit (Fig. 4B; Supplementary Material Fig. S2). The MFOC seems to be a pool of F-actin formed in the process of microvilli contraction; if so, it may differ from microtubular organising centres (MTOCs) by not acting as a site where new units are nucleated. Kinetosomes were rarely detected, presumably because of their unstable location (Figs. 3D, 4 G–J).

In some cells, the centrosome system was observed. This usually consisted either of two short centrioles located in the nuclear pit (Fig. 4G), or of one centriole and one kinetosome, arranged orthogonally, with the kinetosome located near the cell surface (Figs. 3D, 4 H). In some sections, these kinetid structures have an intermediate position: a centriole is still associated with the nucleus but also connected to the kinetosome facing towards the plasma membrane (Fig. 4I, J). The centrosome is most likely composed of two centrioles. These lie in the nuclear pit and can probably migrate to the cell periphery to become a base for the flagellum. The kinetosome has a basal foot that initiates microtubules at its distal end (Fig. 4J) and an opposite fibrillar cross-striated root (Fig. 4H–J).

A flagellum was not found in all sections. Single microtubules are visible, sometimes parallel to the plasma membrane or possibly forming a bundle in the deep cytoplasm (Fig. 4A, bracket). The microvilli are supported by parallel actin microfilaments, as demonstrated by transverse sections of the microvilli (Fig. 4C, arrows).

A vesicular nucleus with central nucleolus is located in the cell centre. It has an erythrocyte-like shape (Figs. 3D, E, 4 G) containing the mitochondrion, Golgi apparatus and centrosome in the nuclear pit and, in some cases, the MFOC (see above). This gives the nucleus a lobed appearance in some sections (Fig. 3E). Mitochondria have typical lamellar cristae (Fig. 3C). Bacterial food particles are engulfed by a pseudopodium (Fig. 3F). The food vacuole can contain two bacteria (Fig. 3G).

Discussion

The taxon Filasterea currently includes the genera *Capsaspora*, *Ministeria* and *Pigoraptor*, and branches off as a sister group to animals plus choanoflagellates (e.g., Hehenberger et al. 2017). Electron microscopic investigations for the members of this group are still rare. Here we provide new light and electron microscopic observations of the filasterean taxon *Ministeria* based on a strain closely related to previously isolated strains of *Ministeria vibrans* (Fig. 1), the only molecularly investigated species.

General Morphology of *Ministeria* Strain L27

The two *Ministeria* species already described, *M. marisola* and *M. vibrans*, have the same size range but are separated from each other by the possession of a stalk and a resulting vibrating movement in *M. vibrans,* as observed by several investigators (Cavalier-Smith and Chao 2003; Lee et al. 2003; Tong 1997). In the present study, a cytoplasmic stalk and attachment of cells to the substrate using a stalk was not observed with either light or electron microscopy. As noted, the stalk formation and active vibration can be rare in clonal culture (Tong 1997), a possible reason for the missing observation in the present study. According to Lee

(nu) and food vacuole (fv) (**A**). The network shell (s) covers the plasma membrane (pm), and forms thin-wall tubes (t) (**B**). Structure of mitochondrion (**C**). Two consecutive sections show nuclear (n) pit at one side with mitochondrion (m) and kinetosome (k) (**D, E**), and lipid globules (l) at the cell periphery (**E**, insert). The cell captures bacterium (b) by pseudopodium (ps); Golgi apparatus (ga) is near the nucleus (n) (**F**). Two just engulfed bacteria (b) in food vacuole (fv), nucleus (n) and microvilli (mv) are present (**G**). Scale bars: **A, D, E, G** – 1 μm; **B**, **C** – 0.2 μm; **E** insert – 0.5 μm; **F** – 0.4 μm.

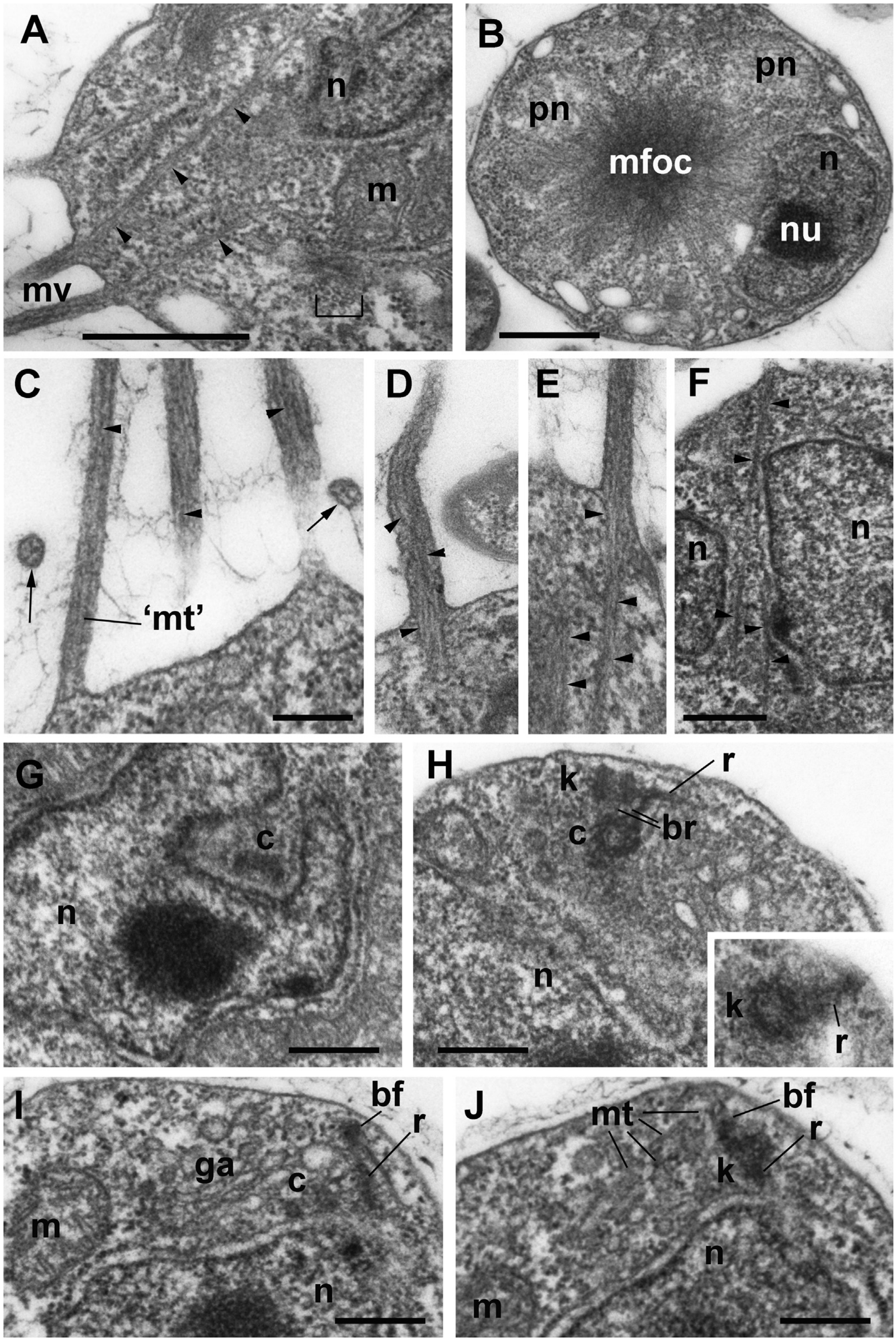

Figure 4. Cytoskeletal structures of *Ministeria vibrans* strain L27. Microvilli (mv) with long bundles of microfilaments in the cytoplasm (arrowheads) nearby nucleus (n) and mitochondrion (m), and a group of cytoplasmic microtubules (bracket) (**A**). Microfilament organising centre (mfoc) in the nuclear pit (n): note perinuclear space (pn) in tangential section; nucleolus (nu) (**B**). Microfilaments (arrowheads) at longitudinal and transversal

et al. (2003) the cell vibration was found only occasionally and can be therefore easily overlooked. In addition, most records of *Ministeria* came from coastal marine locations (Cavalier-Smith and Chao 2003; Lee et al. 2003; Tong 1997) where a higher amount of substrate is available due to coastal disturbances. In contrast, our strain was isolated from the water column of the central Baltic Sea where lower amounts of substrate are available, possibly indicating a reduced need for a stalk for attachment. Interestingly, for culture L27, we could observe a vibrating movement of the flagellum on an attached cell instead of a vibrating cell movement during attachment.

It is also possible our strain is able to connect to the substrate both by microvilli and by the flagellum, comparable to the bicosoecid *Cafeteria* (stramenopiles), which can attach by the posterior flagellum to detrital particles or other surfaces (Larsen and Patterson 1990). A structure assumed the stalk was made visible in *M. vibrans* via tubulin immunolabeling, indicating it is a flagellar structure rather than an actin-containing pseudopodial structure (Torruella et al. 2015). This would differ from the stalk-formation in other holozoans as shown for *Salpingoeca rosetta*, which attach to substrates using microvilli-like structures as revealed by immunostaining (Sebé-Pedrós et al. 2013). Indeed, the typical stalk of *Ministeria* as originally mentioned by Tong is much more likely to be a filipodial structure because it is thicker than the arms (microvilli) at the proximal end and frequently extended by a fine cytoplasmic thread which seems identical to the arms except that it can greatly exceed them in length (2–16.5 μm) (Tong 1997). According to Lee et al. (2003), the stalk consists of a thickened proximal and a thinner distal region resembling the arms (microvilli) of *Ministeria*.

Ministeria is generally capable of forming a flagellum (Patterson et al. 1993) as already demonstrated via electron microscopy (Cavalier-Smith and Chao 2003; Torruella et al. 2015) and transcriptomic findings reveal that *Ministeria vibrans* strain ATCC 50519 has a reduced flagellar toolkit (Torruella et al. 2015). In the present study, the flagellum was observed only once and in a very thin state, indicating it to have a reduced axoneme. But this assumption cannot be documented by electron microscopy for the strain under study. The flagellum might be formed only rarely. Earlier, Torruella et al. (2015) demonstrated a flagellum with normal axoneme (9 + 2) for *M. vibrans* strain ATCC 50519. It should also be noted that the flagellum could easily be overlooked because of its diminutive size. For the purpose of movement, *Ministeria* does not use the flagellum but instead the microvilli by bending them. With the aid of the microvilli, *Ministeria* cells can also build attachments to each other enabling them to float.

Ultrastructure of *Ministeria* Highlights Cytoskeleton of Ancestor of Filasterea, Choanoflagellata and Metazoa

Ministeria is a small cell with equally long radiating microvilli supported by axial bundles of microfilaments, as in choanoflagellates (Karpov and Leadbeater, 1998). At first, we viewed these arms as filopodia, but, unlike filopodia, they have an isodiametric appearance and a well-developed bundle of filaments at their base. In addition, the cell may contain a special MFOC with retracted bundles of F-actin. The microvilli of choanoflagellates are also isodiametric and have stable bundles of microfilaments at their bases; however, they can elongate and retract, as occurs with the arms of filasterids. Thus, Filasterea is the second group of protists, besides choanoflagellates, with true microvilli – permanent projections supported by axial bundles of microfilaments. By contrast, the haptopodia of the cercozoan *Aurigamonas solis* are also permanent projections, but are supported by a cylindrical core of microfilaments (Vickerman et al. 2005).

Superficially microtubule-like profiles can be found in some longitudinal and oblique sections of *Ministeria* microvilli, but cross sections of these structures refute the presence of microtubules. Immunolabeling of *M. vibrans* strain ATCC 50519 also did not reveal microtubules in the arms, but instead microfilaments (Torruella et al. 2015). In some cases, retraction of microvilli does not seem to disintegrate all the microfilament bundles, most

(arrows) sections of microvilli and in cytoplasm; ‘mt’ shows microtubular-like profile absent at cross sections (arrows) (**C–E**). Long bundles of microfilaments (arrowheads) in the nuclear pit (n) (**F**). Centriole (c) in the nuclear (n) pit (**G**). Kinetosome (k) connected to centriole (c) by a bridge (br), with cross-striated fibrillar root (r) apart from nucleus (n) (**H**, insert). Two consecutive sections show centriole (c) and kinetosome (k) with basal foot (bf) producing microtubules (mt) and fibrillar root (r) still connected to nucleus (n) (**I, J**). Scale bars: **A, B** – 0.5 μm; **C–E** – 0.2 μm; **F–J** – 0.4 μm.

of which are stored in the MFOC near the nucleus. Morphologically this centre is similar to the axoplast (the MTOC that organises the microtubular axonemes of axopodia) known for heliozoans and radiolarians (Anderson 1983), however, the MFOC is composed of actin and seems to serve as a source of F-actin for microvilli. The MFOC could only be found in a few rounded cells of the *Ministeria* strain L27, which were devoid of microvilli, and F-actin labelling did not reveal the MFOC in *M. vibrans* (Torruella et al. 2015). Thus, we suggest that this centre is probably a temporary F-actin cluster completely used for microvilli rebuilding. Based on the current molecular phylogeny of Holozoa (Hehenberger et al. 2017; Sebé-Pedrós et al. 2013; Torruella et al. 2015), we infer that microvilli were present in the common ancestor of Filasterea and Choanoflagellata + Metazoa.

Interestingly, an "internal skeleton of microfilaments" as shown here for *Ministeria* was already mentioned in the description of the class Filasterea (Shalchian-Tabrizi et al. 2008) referring to the species description of *Capsaspora owczarzaki* (Hertel et al. 2002), though this observation was not made for *Capsaspora* (Hertel et al. 2002; Owczarzak et al 1980; Stibbs et al. 1979). A cell covering as detected here was not found in *Capsaspora* (Owczarzak et al 1980; Stibbs et al. 1979). For the genus *Pigoraptor*, no ultrastructural investigations are available for comparison.

While still incomplete, our study means that *Ministeria* gives us the best picture available to date of the kinetid structure of filastereans. For the first time, we received a mostly complete picture of the kinetid structure of a filasterean. It has a normal basal apparatus composed of a kinetosome with a basal foot and a short cross-striated fibrillar root, and an orthogonal centriole. In *M. vibrans* it can produce a flagellum with a typical axoneme, as documented in a single section by Torruella et al. (2015; their fig. 3C). In some sections of our strain (Fig. 4I, J), a fibrillar root connects the kinetosome to the nucleus. Comparisons of the kinetids of sponges (probably the first metazoans) and choanoflagellates has shown their cardinal differences (Pozdnyakov et al. 2017). However, the kinetid of *Ministeria* generally has the same structure as the most ancient kinetid type in sponges (Pozdnyakov et al. 2017): both have an orthogonal centriole, a basal foot with microtubules and a nuclear-kinetosome fibrillar connection. This type of kinetid most likely represents the ancestral state of the structure for the clade consisting of Filasterea and Metazoa plus Choanoflagellata. The kinetid of choanoflagellates seems to have undergone a complex evolution from the ancestral type with microtubular bands radiating from the flagellar kinetosome (Hibberd 1975; Karpov 2016). In general, the ultrastructure of *Ministeria* illuminates two main cytoskeletal features probably characteristic for the common ancestor of Filasterea, Metazoa and Choanoflagellata: the microvilli and the kinetid structure.

The formation of colonies or cell clusters is widespread in different genera of choanoflagellates (Leadbeater 2015), but also in the filasterean genus *Pigoraptor* (Hehenberger et al. 2017). *Ministeria* cells seem to use microvilli to build attachments to each other, enabling them to float. Thus, the ability for cell-to-cell interaction may have appeared in the Holozoa before the origin of choanoflagellates, in a common ancestor with Filasterea, as supported by genomic data (Torruella et al. 2015) and the light microscopic observations of the present study (Fig. 2I, Supplementary Material Video S2).

Ecology of Ministeriidae

In contrast to other protist groups like chrysophytes, bicosoecides or choanoflagellates (e.g., del Campo and Massana 2011; Wylezich et al. 2012), there are only few sequences originating from environmental studies that branch with filastereans (del Campo et al. 2015; Heger et al. 2018). For example, environmental sequences branching in the *Capsaspora* clade (Heger et al. 2018) are not closely related to the described species and to each other. The same is true for *Ministeria*, for which only one closely related environmental sequence is available in GenBank (from the study of Piquet et al. 2014). The overall scarcity of environmental sequences of filasterean taxa nearly identical to described species might indicate rather infrequent occurrence, though primer bias discriminating against filasterean sequences cannot be excluded (del Campo et al. 2015). Nonetheless, the genus *Ministeria* seems to be able to thrive in different habitats like coastal (Tong et al. 1997, 1998; Vørs 1992) and off-shore (Patterson et al. 1993) marine waters (Table 1). The new isolate L27 was obtained from a sulfidic sample of the Baltic Sea and then cultured under oxic conditions. With this record, the genus is detected for the first time in an oxygen-depleted environment, hinting at a broad ecological capability. Because of the presence of typical mitochondria (Fig. 3C), we assume that well oxygenated environments are its typical habitat. The freshwater record of *Ministeria* (Kiss et al. 2009) is of great interest and might be a candidate for genome comparisons within this genus.

The cells feed on bacteria, as seen in the culture L27, and as already described by Tong et al. (1998). Bacteria stick to the microvilli or the cell body (Fig. 2E, F) and are ingested by protruding pseudopodia (Tong et al. 1998). *Ministeria* cells were found in association with detritus particles (Patterson et al. 1993) and may feed on bacteria colonising such particles.

Methods

Sampling: The strain L27 was isolated from the central Baltic Sea. Samples were taken from anoxic and sulfidic waters in the Landsort Deep (IOW station 284, 58°350′N, 18°140′E) during June 2012. Pre-cultures of a 500 μl sample were established in 24-well microtiter plates filled with 500 μl autoclaved F2 medium (Guillard and Ryther 1962) and a quinoa grain as carbon source for ambient bacteria (see Weber et al. 2017). Pure cultures deposited in the IOW culture collection were routinely cultured using F2 medium with a salinity of 8–16, a mixture of bacteria from the sampling site as a food source, and a wheat grain at 10 °C (Weber et al. 2017).

Gene sequence analysis: For taxonomic classification, DNA was extracted as described by Weber et al. (2017). The 18S rDNA was PCR-amplified using 25f (Bass and Cavalier-Smith 2004) and the newly designed primer MinRev (5′-TTTAGATCCTCTAAATGACC-3′). The PCR mixture, containing 0.5 μM of each primer, 200 μM of dNTPs, 1× PCR buffer, and 0.5 μl of Herculase II Fusion DNA polymerase (Agilent Technologies) was heated to 94 °C for 2 min, after which the 18S rDNA was amplified in 35 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 80 s. The PCR products were purified using the Nucleospin II kit (Machery Nagel) and then sequenced commercially (LGC Genomics, QIAGEN) using PCR primers and internal primers (Weber et al. 2017).

The 18S rDNA sequence of the new strain (deposited in GenBank under accession number KX420739) was compared to other filasterean sequences available in GenBank using MAFFT v7.388 (Katoh and Standley 2013) using the slow, iterative refinement method (1,000 iterations). The resulting alignment was trimmed to the length of the sequence of *Ministeria* strain L27 in Geneious version 10.2.3 (Biomatters, Auckland, New Zealand) resulting in a final sequence alignment of 1,748 positions. Phylogenetic analyses were performed for both data sets using MrBAYES 3.2.6 (Ronquist and Huelsenbeck 2003) and PhyML 3.0 (Guindon et al. 2010) as implemented in Geneious version 10.2.3 (Biomatters, Auckland, New Zealand). The GTR model of substitution (Lanave et al. 1984) and a gamma-shaped distribution of substitution rates among sites with eight rate categories and a proportion of invariable sites were used. The Bayesian analysis was performed for 1,000,000 generations and sampled every 1,000 generations with two independent runs, each with four simultaneous chains, discarding the first 20% of generations as burn-in. Convergence in topology was confirmed via MrBayes (Average standard deviation of split frequencies between runs was low, <0.03; potential scale reduction factor was $\tilde{1}$). For the likelihood analysis, all model parameters were estimated from the data set. To estimate branch support, 1,000 bootstrap replicates were performed.

Microscopy investigations: Living cells were observed by light microscopy with an AxioScope A1 microscope using phase contrast and difference interface contrast (DIC) optics (Carl Zeiss, Jena, Germany). A 63× water immersion objective and an AVT HORN MC-1009/S video camera were used. Video clips were digitised using the Behold TV 409 FM tuner. For scanning electron microscopy (SEM), cells placed on the surface of cover slips were fixed with glutaraldehyde (2% final concentration) for 5–15 min at 22 °C. After dehydration in a series of alcohols (from 30% to 96%) and anhydrous acetone, the specimens were dried using a critical point device (HCP-2, Hitachi, Japan). Afterwards, the dried cover slips were mounted on aluminum stubs, which were coated with gold-palladium and then studied with a JSM-6510LV microscope.

For transmission electron microscopy (TEM) of sectioned cells, the flagellates were concentrated by centrifugation and the resulting pellets were fixed as described by Mylnikov et al. (2015). After dehydration in a series of alcohols and anhydrous acetone, specimens were embedded in an araldite-epon mixture, cut using a diamond knife (LKB Ultramicrotome, Sweden) and examined using a JEM-1011 microscope (JEOL, Tokyo, Japan).

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Appendix A. Supplementary Data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.protis.2019.07.001.

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