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# **New member of Gromochytriales (Chytridiomycetes) – *Apiochytrium granulosporum* nov. gen. et sp.**

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## **Abstract**

Molecular phylogenetic analysis of 18S rRNA gene sequences of nearly any species of Chytridiomycota has typically challenged traditional classification and triggered taxonomic revision. This has often led to the establishment of new taxa which, normally, appear well supported by zoospore ultrastructure as diagnostic character. To construct a meaningful and comprehensive classification of Chytridiomycota, the combination of molecular phylogeny and morphological studies of traditionally defined chytrid species is needed. In this work, we have studied morphological and ultrastructural features based on light and transmission electron microscopy as well as molecular phylogenetic analysis of a parasite of the yellow-green alga *Tribonema gayanum* (strain X-124 CCPP ZIN RAS) morphologically similar to *Rhizophydium granulosporum*. Phylogenetic analysis of the 18S rRNA gene sequence of this strain supports that it represents a new genus and species affiliated to the recently established order *Gromochytriales*. The ultrastructure of X-124 confirms its phylogenetic position sister to *Gromochytrium* and serves as the basis for the description of the new genus and species *Apiochytrium granulosporum*.

**Key words:** chytrids, molecular phylogeny, ultrastructure

## INTRODUCTION

The systematics of Chytridiomycota has been under continuous revision after a global molecular phylogenetic tree of Fungi based on six gene markers was published some years ago (James et al. 2006a). From that time, the phylogenetic investigation of 18S rRNA gene sequences of nearly any species or strain has triggered the revision of neighboring taxa, often leading to the establishment of new higher rank taxa, e.g. family, order and class, divisions normally supported by zoospore ultrastructure. In the past few years, chytrid taxonomy has experienced considerable changes. For instance, James et al. (2006b) described the new phylum Blastocladiomycota; Letcher et al. (2006) redescribed the *Rhizophyidium* clade (James et al. 2000, 2006a) as the order *Rhizophydiales*; Mozley-Standridge et al. (2009) established the order *Cladochytriales* for the *Cladochytrium* clade (James et al. 2006a) and Simmons et al. (2009) redescribed the clade formerly represented in phylogenetic trees by *Chytriomyces angularis* (James et al. 2006a) as the order *Lobulomycetales*. New orders have also been proposed: *Polychytriales* (Longcore & Simmons 2012), *Gromochytriales* and *Mesochytriales* (Karpov et al. 2014) and *Synchytriales* (Longcore et al. 2016). Moreover, some genera with typical chytrid morphology and hence thought to branch early in the fungal tree, such as *Olpidium* (Sekimoto et al. 2011), *Amoeboradix* and *Sanchytrium* (Karpov et al. 2017b), appeared to branch among more apical fungal lineages, out from the Chytridiomycota clade. It is clear that we need molecular data for each traditionally described species of chytrids to construct a meaningful and comprehensive classification of Chytridiomycota (see also Powell 2016). Transmission electron microscopy (TEM) sometimes reveals peculiarities that can be used as new taxonomic characters or, on the contrary, may show the unimportance of some commonly accepted ultrastructural features. However, zoospore ultrastructure has so far provided useful discriminatory characters for taxonomy in agreement with the molecular phylogeny of fungi. Some chytrids have been found to contain insertions in ribosomal DNA, which may or may not be present in close relatives, but that are rather common in Dikarya and green algae (Karpov et al. 2017b). Here we present light microscopy observations, ultrastructure and molecular phylogenetic analysis of strain X-124 (CCPP ZIN RAS), a parasite of algae isolated from freshwater samples using *Tribonema gayanum* as a host. Phylogenetic analysis of 18S rRNA gene sequences of this strain supports that it represents a new genus and species affiliated to the recently established order *Gromochytriales*. The ultrastructure of X-124 confirms its phylogenetic position sister to *Gromochytrium* and serves as the basis for the description of this new genus and species.

## MATERIALS AND METHODS

### Collection and isolation

Strain X-124 was isolated by M.A. Mamkaeva in 2012 from the freshwater sample x-22 collected in a small pond in Kutuy village, Kingisepp District, Leningrad Province, Russia. A strain was maintained in culture on *Tribonema gayanum* (strain 20 CALU) as a host as described in Karpov et al. (2016).

### Light and transmission electron microscopy

Light and DIC microscopy observations of living cultures were carried out on a Zeiss Axioplan microscope (Leica Microsystems, St. Petersburg, Russia) equipped with black and white MRm Axiocam (Leica Microsystems, St. Petersburg, Russia).

### Electron microscopy

Infected algal filaments were fixed with 0.3% OsO<sub>4</sub> in 0.1M cacodylate buffer during two minutes, then, glutaraldehyde was added to 2% final concentration for 30 min in the dark. Then the fixative was removed and 2% glutaraldehyde in 0.1M cacodylate buffer was added for 1 hour, rinsed in 0.1M cacodylate buffer and postfixed in 1% OsO<sub>4</sub> in the same buffer for one hour. All fixation stages were carried out on ice. After rinsing in 0.1M cacodylate buffer for 5 min, the filaments were dehydrated in alcohol series and in propylene oxide, and finally embedded in Spurr resin (Sigma Aldrich, St Louis, USA). Ultrathin sections were prepared using a Leica Ultracut ultratome with a diamond knife. After double staining, the sections were observed using a JEM 1400 microscope (Jeol, Japan) equipped with the digital camera Olympus Veleta (Japan).

### **18S rRNA gene sequencing and molecular phylogeny**

Cells from infected cultures were pelleted by centrifugation and their DNA was purified using the PowerSoil DNA purification kit from MoBio (Carlsbad, USA). 18S rRNA gene fragments were then amplified by PCR using the fungal specific primers Fun\_UF1 (5'-CGAATCGCATGGCCTTG) (Kappe et al. 1996) and Fun\_AU4 (5'-RTCTCACTAAGCCATTC) (Vandenkoornhuyse et al. 2002). PCR reactions were carried out in a final volume of 25 µl. The reaction mix contained 1 µl of the eluted DNA, 1.5 mM MgCl<sub>2</sub>, dNTPs (10 nmol each), 20 pmol of each primer, and 0.2 U TaqPlatinum DNA polymerase (Invitrogen). After a first denaturation step at 94 °C for 2 min, the PCR reaction consisted of 35 cycles of the following iterative cycle: denaturation at 94 °C for 15s, annealing at 50°C for 30 sec, and polymerization at 72 °C for 2 min. A final elongation at 72 °C for 7 min was carried out. We cloned the resulting 18S rRNA amplicons to identify individual fungal sequences using the Topo TA Cloning System (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Clone inserts were then amplified by PCR using flanking vector primers and the inserts of expected sizes were sequenced bidirectionally with vector primers (Beckman Coulter Genomics, Takeley, UK). Two closely related chytrid sequences were identified and compared with sequences in the GenBank nr database using BLAST (Altschul et al. 1990). The new sequences and those retrieved by BLAST were aligned using MAFFT with the accurate -linsi option (Katoh et al. 2002). The multiple alignment was trimmed with TRIMAL (Capella-Gutierrez et al. 2009) and a Bayesian phylogenetic tree was reconstructed using MrBayes (Ronquist et al. 2012) applying the GTR + G + I model with four chains and 10,000,000 generations. After checking for convergence, the first 15,000 trees were eliminated as burn-in, and a consensus tree was constructed by sampling every 100 trees. The new *Apiochytrium granulosporum* strain X-124 18S rRNA sequences have been deposited in GenBank under accession numbers XXXXX.

### **Intron S943 phylogeny**

Intron localization was defined by comparing assembled contig with other species from NCBI nr database using BLAST. Insertion positions were identified using the 16S gene reference of *Escherichia coli* (Cannone et al. 2002; [http://www.rna.icmb.utexas.edu/SAE/2C/rRNA\\_Introns](http://www.rna.icmb.utexas.edu/SAE/2C/rRNA_Introns)). Additional SSU sequences containing intron S943 were downloaded from GenBank. Sequences were aligned with MUSCLE (Edgar 2004) and manually adjusted in BioEdit (Hall 1999). Bayesian inference was calculated with MrBayes applying the GTR + G + I model with four chains and 10,000,000 generations. After checking for convergence, the first 50% trees were eliminated as burn-in, and a consensus tree was constructed. The resulting Bayesian tree was visualized using the iTOL web tool (Letunic & Bork 2016).



## RESULTS

### Morphology

#### Zoospores

This fungus produces slightly elongated or spherical zoospores 2.3-2.7  $\mu\text{m}$  in diameter with a posterior single lipid globule and a comparatively short flagellum about 8-9  $\mu\text{m}$  in length having a short acroneme of approximately 1  $\mu\text{m}$  (Fig. 1A, B). In released zoospores, the single prominent lipid globule locates at a posterior position, near the point of flagellum emergence (Fig. 2A-D). The lipid globule is associated with a flat microbody and covered with fenestrated cisternae facing the plasma membrane (Fig. 2D). The elongated nucleus is slightly curved and oriented in posterior-anterior direction. Its posterior end is located near the microbody-lipid complex (MLC) (Fig. 2D), the lateral cavity of the nucleus is filled with small vesicles and rare cisternae of the Golgi apparatus (not shown). Mitochondrial profiles with flat cristae are scattered in the cytoplasm, and the ribosomes do not aggregate (Fig. 2A, D). Mature intrasporangial zoospores are predominantly of amoeboid appearance, and in general exhibit the same location of MLC, nuclear cavity, Golgi apparatus, dispersed ribosomes and mitochondria (Fig. 4B-D). A non-functional kinetosome (centriole) is located at sharp angle (ca. 60°) with the flagellar kinetosome and connected to it with a broad fibrillar bridge (Fig. 4C, D). The flagellar transition zone contains a spiral fiber, or cylinder (Fig. 2B, C). A short posterior root of two microtubules passes, between kinetosome and centriole, to the plasma membrane (Fig. 4D). Traces of one more root (probably anterior) of 2-3 microtubules can be found between the plasma membrane and the fenestrated cisternae (Fig. 2A), but its origin could not be fully tracked.

#### Cyst

Zoospores encyst on the surface of the host alga and produce a rhizoid penetrating the host cell wall (Figs 1A; 2E, F). In addition to the chitin wall, the cyst is covered with a rather thick layer of electron dense amorphous material (Fig. 2E). Each cyst contains a nucleus with a large central nucleolus and a lipid globule that can be visible in the thin sections (Fig. 2E). The rhizoids were not visible on the living material, but the ultrastructural observation revealed a very thin rhizoidal stem penetrating the algal cell wall and its thickened part just under the wall of the host (Fig. 2E, F). Such a narrow hole (less than 200 nm as measured in serial sections) in the algal cell wall for thin rhizoids has been also found in the mature sporangium (not shown). The rhizoid distal part branches inside the cell, sometimes enveloping the host nucleus, which looks deformed (Fig. 3B).

#### Sporangium

Monocentric epibiotic sessile slightly broaden pear-shaped sporangia up to 10-12  $\mu\text{m}$  in height with a broad apical papilla for zoospore releasing locate on the *Tribonema* filament (Fig. 1C-E). The immature sporangia have characteristic granulated contents (Fig. 1 E) with several nuclei and one big lipid globule (Fig. 3A). At a later stage of sporangium maturation (Fig. 4A), this globule disappears, likely as a consequence of its use as an energy source for zoospore formation. Interestingly, in spite of the fact that the plasmodium division just has started and the flagella are not yet formed, the papilla is already open (Fig. 4A). In the mature sporangium, each newly formed zoospore contains a single big globule immediately prior to zoospore release. Thus, each intrasporangial zoospore already contains a posterior lipid globule near the flagellar base (Fig. 4B-D).

### 18S rRNA phylogeny

The two 18S rRNA gene sequences of the strain X-124 were very similar (99.4% identical) and formed a clade sister to the cluster containing *Gromochytrium mamkaevae* and some environmental sequences with high support (Fig. 5). We ignore if the two X-124 sequences correspond to two closely related chytrid strains present in our culture or to two slightly divergent 18S rRNA genes present in a single species. According to our tree, this strain represents a new deep-branching lineage within the order Gromochytriales, clearly distinct from *Gromochytrium*. In addition, the strain X-124 sequences contains a large (1476 bp) insertion absent in the other Gromochytriales sequences. The presence of this large insertion may have impacted the whole 18S rRNA gene structure of X-124, which may explain the relatively long branch of this strain compared with the other Gromochytriales. Taking into account its phylogenetic distance, we consider that strain X-124 represents a new genus and species within the order Gromochytriales. Our tree also supports strongly the sister relationship between the Gromochytriales and the Mesochytriales, and the position of the recently described Polyphagales as sister of the Gromochytriales+Mesochytriales cluster (Fig. 5), in agreement with previous analyses (Karpov et al. 2016).

### **S943 intron phylogeny**

The 18S rRNA gene of the strain X-124 contains a group I intron with an insertion position at S943 according to *E. coli* reference (Cannone et al. 2002). The intron locates on the antisense strand of P8 and carries a homing endonuclease gene coding His-Cys box protein 244 amino acid residues long. Homing endonuclease genes are rare but widely distributed among S943 introns (Haugen et al. 2004). The predicted homing endonuclease protein of the strain X-124 does not have at its C-terminus with the 2nd zinc-binding motif and dimerization tail which are common for His-Cys box family proteins (Chevalier and Stoddard, 2001). However, zinc-binding conservative motif can be detected if multiple frame shifts are introduced into the nucleotide sequence. Previously, pseudogenes/remnants of homing endonuclease genes were found in S943 introns (Haugen et al. 2004). Within chytridiomycetes homing endonuclease genes in 18S rRNA genes were also found in *Gonapodya* sp. (intron insertion position S943) and *Phlyctochytrium planicorne* (intron insertion position S508). According to tblastn search against *nr/nt* database of NCBI, the endonuclease from *Gonapodya* sp. is the most similar toward endonuclease from the strain X-124. S943 is one of the most common positions among all group I intron insertion sites in 18S rRNA genes (Haugen et al. 2004; Karpov et al. 2017b). S943 introns are widely distributed in different fungi, amoeba, algae, cercozoans and ciliates. Most fungi group together in the Bayesian phylogenetic tree of S943 introns, forming a monophyletic Dikarya group (Supplementary Figure S1). With a few exceptions, ascomycetes form a large cluster, which include one basidiomycete and one zygomycete species. Basidiomycetes form three paraphyletic groups and one group inside the ascomycetes cluster. Viridiplantae form a monophyletic group which include two amoeba species. Strain X-124 intron branches off from the tree right before the dikaryan radiation. There are other species branch around X-124 with unclear branching order – two uncultured chytrid clones, five monoblepharids, the aphelid *Amoeboaphelidium occidentale*, and some oomycetes and cercozoans.

## **DISCUSSION**

### **Phylogeny and ultrastructure**

The 18S rRNA gene sequences of the X-124 strain form a monophyletic cluster with some environmental sequences (Fig. 5) that corresponds to the recently described order Gromochytriales (Karpov et al. 2014). This order includes one cultured chytrid,

*Gromochytrium mamkaevae*, having zoospores with posterior ribosome aggregates unbounded by endoplasmic reticulum and one MLC with anterior location with a rather thick microbody. By contrast, X-124 shows dispersed ribosomes and a posterior MLC with a thin microbody. Both species exhibit a single lipid globule with fenestrated cisternae facing towards the exterior, posterior and probably anterior microtubular roots, a cylinder or spiral filament in the flagellar transition zone and several dispersed mitochondria. *G. mamkaevae* has a centriole at an angle of approx. 30° with respect to the kinetosome vs. 60° in X-124. We did not observe a centriole in released zoospores; nonetheless, we can propose that the angle of centriole location in the X-124 intrasporangial zoospore may probably be changed during complete maturation in the released zoospore. Although a centriole-kinetosome bridge was certainly present, our material did not allow studying it in detail. Some zoospore characters of X-124, i.e. dispersed ribosomes and posterior MLC, are similar to those of *Mesochytrium penetrans* (Karpov et al. 2010), which belongs to the closely related order Mesochytriales. However, other zoospore characters of *M. penetrans* essentially differ from those of X-124: the ER bounding MLC associated with a single big mitochondrion, fenestrated cisterna facing the flagellar base, and the presence of a vacuole and several fibrils associated with the centriole and kinetosome (Karpov et al. 2010). The flagellar apparatus of the *M. penetrans* zoospore contains a spiral fiber in the transition zone, but does not have any microtubular roots (Karpov et al. 2010). In summary, the zoospore morphology and, in particular, the flagellar apparatus of X-124 are more similar to those of *G. mamkaevae* than to those of *M. penetrans*, confirming the affiliation to the Gromochytriales. The partial zoospore similarity of X-124 to *M. penetrans* is in agreement with a somewhat intermediate phylogenetic position of X-124 between *Gromochytrium* and *Mesochytrium*. In fact, the X-124 18S rRNA sequences branch deep in the Gromochytriales (Fig. 5), which might explain that X-124 has retained some features of the last common ancestor of Gromochytriales and Mesochytriales, sharing characters with both orders. From a taxonomy perspective, our data provide additional material for revision the key diagnostic characters defining the order Gromochytriales. However, at this stage of investigation, such a revision is obviously immature. More molecular and ultrastructural data from other representatives of the order will be needed to attempt that task.

### **S943 intron phylogeny**

In general, the results of the phylogenetic analysis of S943 intron sequences are similar to those of the 18S rDNA phylogeny of these groups (Supplementary Figure S1), as previously seen in Haugen et al. (2004). High level taxa such as Viridiplantae, Dikarya, Ascomycota and Basidiomycota appear monophyletic, which supports that vertical gene transfer is the main mechanism of S943 intron heredity among these taxa. However, the incongruent localization of a few branches might suggest some cases of horizontal transfer, as also seen in Haugen et al. (2004). For example, a transfer between ascomycetes and basidiomycetes might have happened in the case of *Ballistosporomyces sasicola*. The majority of taxa include nonintronized and intronized species, although the former situation predominates across the phylogenetic tree. The observed mosaic distribution of introns might argue against the hypothesis of their vertical heredity. Whether vertical inheritance followed by losses or horizontal gene transfer from preferred sources are the cause of this pattern will require further study.

### **Taxonomy**

According to light microscopic observations, strain X-124 is similar to *Rhizophydium granulosporum* Scherff., a parasite of *Tribonema* with a broadly pyriform sporangium of

close dimensions (7–14 µm), delicate rhizoids arising from a short main axis and ovoid zoospores of nearly the same dimensions (3 µm long and 2 µm diameter) with a large colorless globule (Sparrow 1960; Letcher & Powell 2012). However, the globule in *Rh. granuloporum* zoospore is slightly eccentric, not posterior as in X-124, and the flagellum of *Rh. granuloporum* seems to be longer than in X-124, although the flagellar length was not shown in the diagnosis of *Rh. granuloporum*. The resting spore was not found in X-124. Thus, zoospores of X-124 strain differ essentially from those of *Rh. granuloporum* and belong to a new genus.

*Apiochytrium* Karpov et Moreira gen. nov.

*MycoBank* MB 827963

Zoospores contain posterior MLC with single lipid globule and flat microbody near the flagellar base, dispersed ribosomes and several scattered mitochondria. Kinetid structure as for the order.

Etymology: Apio (Greek) – pear, referring to the pyriform sporangium.

Type species *A. granuloporum* Karpov et Moreira sp. nov.

*Apiochytrium granuloporum* Karpov et Moreira sp. nov (Fig. 1).

*MycoBank* MB 827964

Monocentric epibiotic sessile pyriform sporangia up to 12 µm in height and 9 µm broad with 1 broad apical papilla for zoospore releasing. Branching rhizoid is invisible on the living material. Elongated to spherical zoospores 2.3–2.7 µm in diameter or up to 3 µm in length with posterior single lipid globule and flagellum 8–9 µm in length. A non-functional kinetosome (centriole) is located at sharp angle to the flagellar kinetosome.

Parasite of yellow-green alga *Tribonema gayanum*.

**Etymology:** after the name *Rh. granuloporum* – most similar to the strain X-124.

GenBank Accession Number XXXXXXXX.

**Type:** Fig. 1. Karpov et al. this publication. RUSSIA, Leningrad Province, Kingisepp District, small pond in Kutuy village (59°48'62" N, 28°94'66" E). Sample collected by Maria Mamkaeva in April 2012. Ex type culture deposited in Culture Collection of Parasitic Protists of Zoological Institute of Russian Academy of Science (CCPP ZIN RAS) (Malysheva et al. 2016) under No: X-124.

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## Legends to the figures

**Fig. 1.** DIC images of living zoospores (A, B) and sporangia (C-H) of *Apiochytrium granuloporum* sp. nov. (X-124 CCPP ZIN RAS). A – released zoospores, cyst (cy) and empty sporangium (sp), B – zoospores at high magnification. Arrows on B show posterior lipid globule. C, D – young sporangia, E – premature sporangium. Scale bars: A – 5  $\mu$ m, B – 3  $\mu$ m, C-E – 5  $\mu$ m.

**Fig. 2.** Transmission electron micrographs of *Apiochytrium granuloporum* sp. nov. (X-124 CCPP ZIN RAS). A-D – sections of free-swimming zoospores, B, C - consecutive sections of flagellar transition zone with spiral filament (arrowheads), E – encysted zoospore penetrating the algal cell wall (aw) with a rhizoid (rh), F – rhizoid (rh) inside the alga. Abbreviations: aw-algal cell wall, chl-chloroplast, fc-fenestrated cisterna, fl-flagellum, hc-host cytoplasm, l-lipid globule, mm-mitochondrion, mi-microbody, n-nucleus. Arrowheads on A show microtubules of the anterior root. Scale bars: A – 500 nm, B, C – 200 nm, D – 300 nm, E – 800 nm, F – 400 nm.

**Fig. 3.** Transmission electron micrographs of *Apiochytrium granuloporum* sp. nov. (X-124 CCPP ZIN RAS). A – young sporangium with several nuclei and a big lipid globule, B – host nucleus (hn) surrounded by rhizoids (rh). Abbreviations: aw-algal cell wall, hn-host nucleus, l-lipid globule, n-nucleus, nu-nucleolus, sw-sporangial wall. Scale bars: A – 2  $\mu$ m, B – 1  $\mu$ m.

**Fig. 4.** Transmission electron micrographs of *Apiochytrium granuloporum* sp. nov. (X-124 CCPP ZIN RAS). A – immature sporangium with open papilla, several nuclei and without lipid globule. Arrowheads show developing cell borders, B – sporangium with mature and releasing zoospores, C – intrasporangial zoospore. Arrowhead shows a fibrillar bridge between centriole (c) and kinetosome (k). D – longitudinal section of centriole adjacent to kinetosome which is obliquely sectioned here. Arrowheads show a posterior root of two microtubules. Abbreviations: aw-algal cell wall, fl-flagellum, l-lipid globule, m-mitochondrion, n-nucleus. Scale bars: A – 2.5  $\mu$ m, B – 2  $\mu$ m, C – 200 nm, D – 200 nm.

**Fig. 5.** 18S rRNA gene Bayesian phylogenetic tree based on 1456 conserved sites. The tree is rooted using sequences of Lobulomycetales and Polychytriales. Numbers at branches are Bayesian posterior probabilities (only values higher than 0,7 are shown).



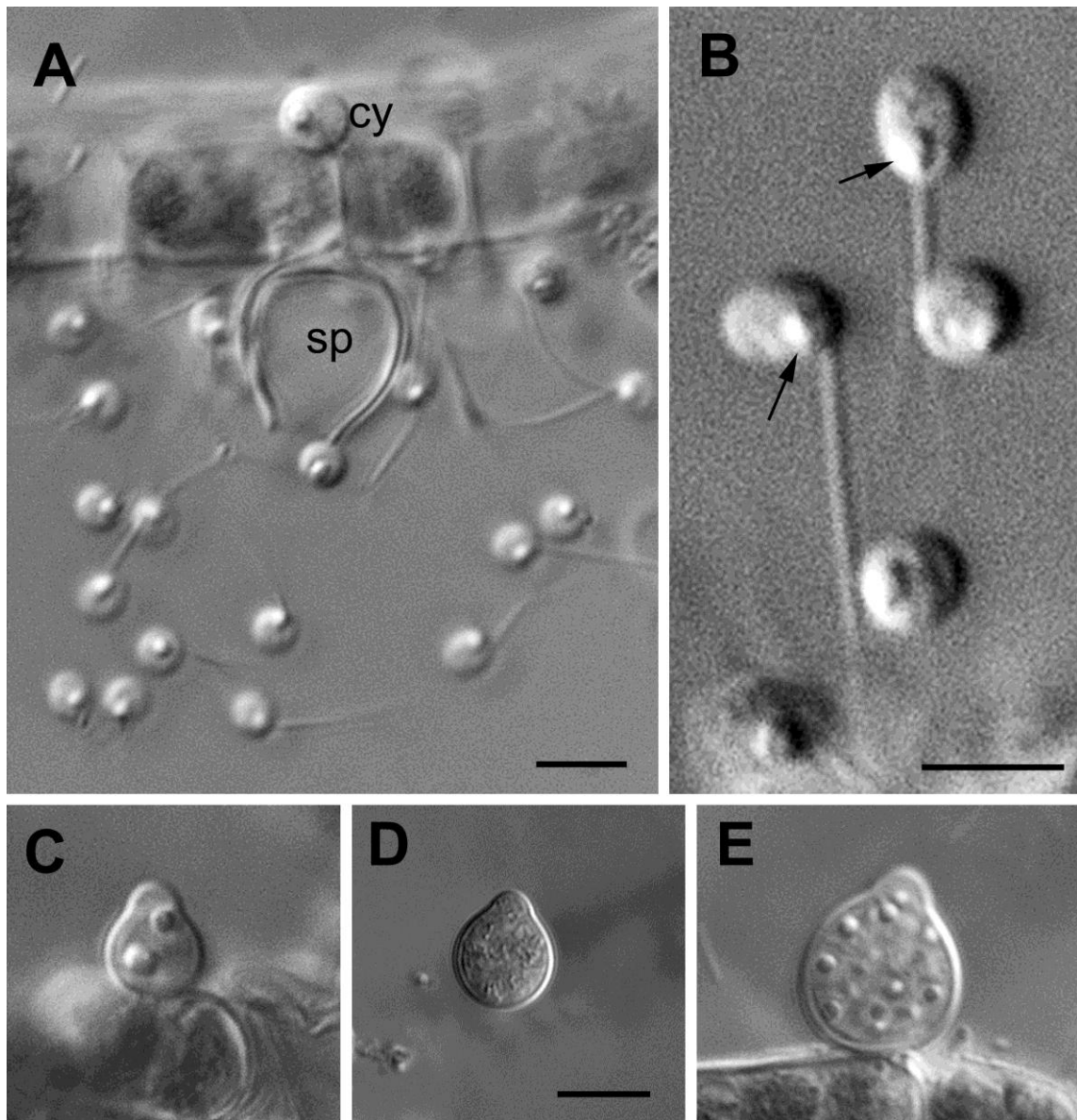


Figure 1.

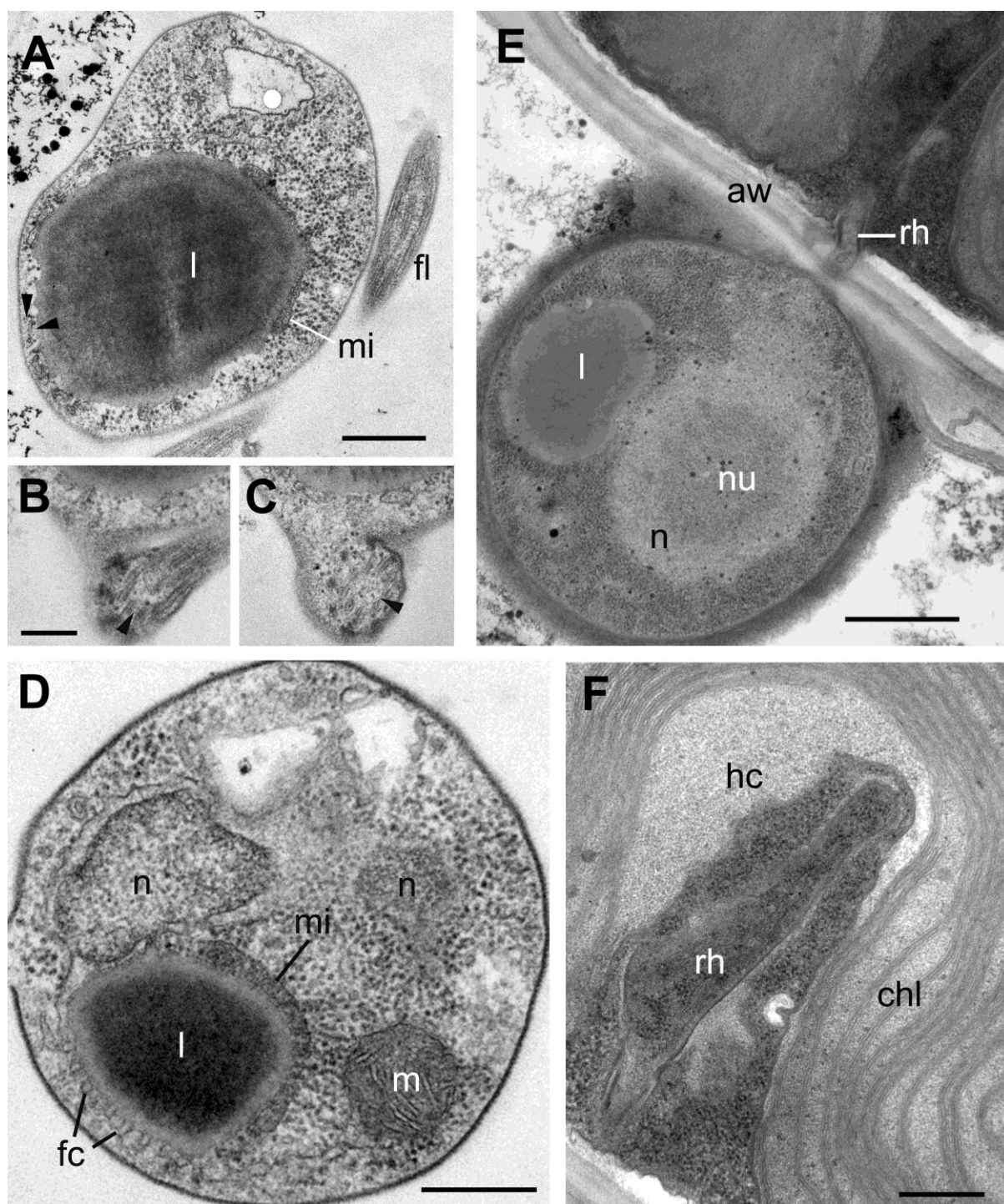


Figure 2.

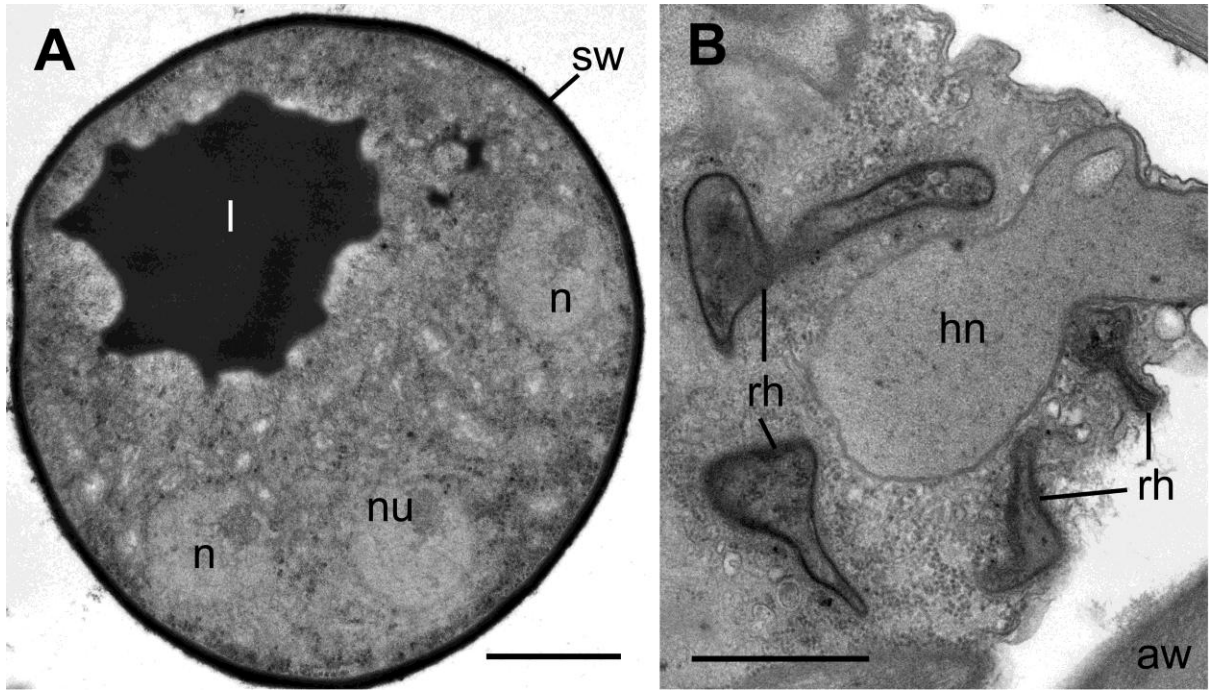


Figure 3.

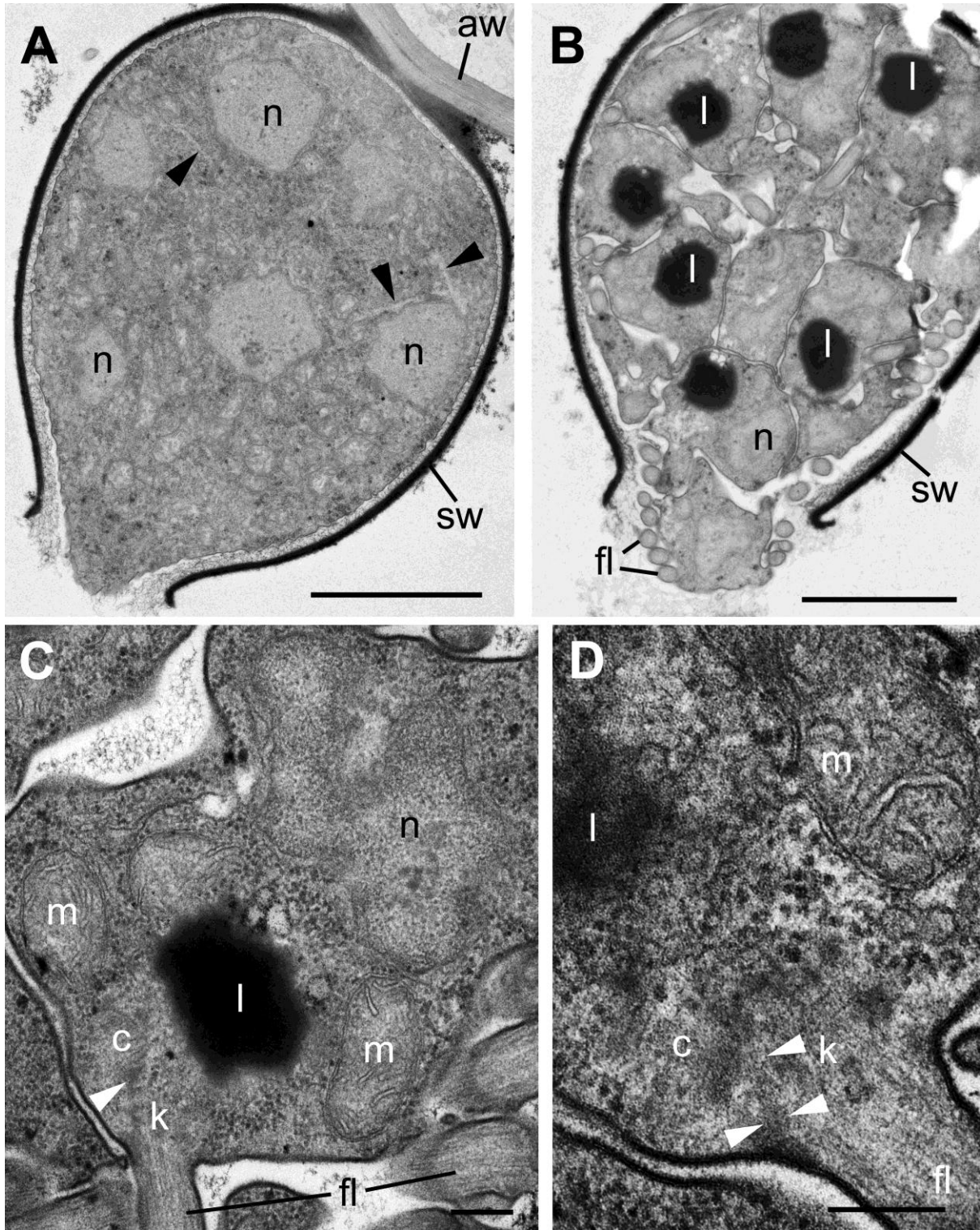


Figure 4.

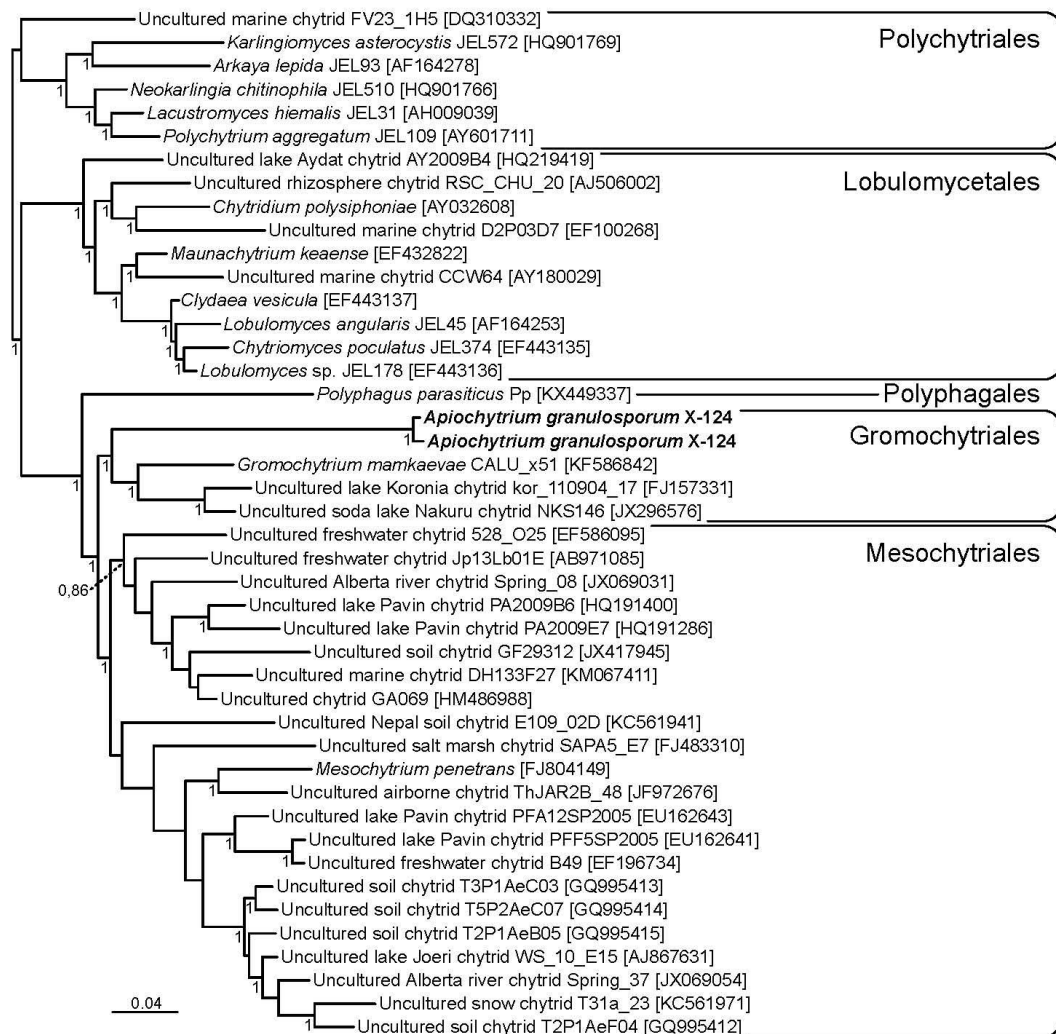


Figure 5.