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Molecular phylogeny of *Aphelidium arduennense* sp. nov. – new representative of Aphelida (Opisthosporidia)

Victoria S. Tcvetkova[1], Natalia A. Zorina[1], Maria A. Mamkaeva[1] and Sergey A. Karpov[1,2]

[1] *St. Petersburg State University, St. Petersburg 199034, Russia*
[2] *Zoological Institute, Russian Academy of Sciences, St. Petersburg 199034, Russia*

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Summary

Aphelids (Aphelida) are poorly known parasitoids of algae that have raised considerable interest because of their phylogenetic position as phagotrophic protists sister to Fungi. Together with Rozellida and Microsporidia they have been classified in the Opisthosporidia but seem to be more closely related to the Fungi rather than to the Cryptomycota and Microsporidia, the other members of the Opisthosporidia. Molecular environmental studies have revealed high genetic diversity within the aphelids, but only four genera have been described: *Aphelidium, Amoeboaphelidium, Paraphelidium* and *Pseudaphelidium*. Here, we describe the life cycle of a new species of *Aphelidium, Aph. arduennense*. Molecular phylogenetic analysis of its 18S rRNA indicates that *Aph. arduennense* is sister to *Aph. tribonematis,* and together with *Aph. melosirae* they form a monophyletic cluster. Within the aphelids, this cluster is distantly related to *Paraphelidium* and *Amoeboaphelidium*.

Key words: aphelids, Holomycota, Opisthosporidia, Rozellosporidia, taxonomy

Introduction

Aphelids are a divergent group of intracellular parasitoids of green, yellow-green and diatom algae (Gromov, 2000; Karpov et al., 2014a). The four known genera have different ecological preferences: *Aphelidium, Amoeboaphelidium* and *Paraphelidium* occur in freshwater and *Pseudaphelidium* is found in marine environments. Although with only these four described genera, the group is highly diverse, including many environmental sequences from diverse ecosystems (Karpov et al., 2013; 2014a). The phyla Aphelida, Microsporidia and Rozellosporidia (Cryptomycota) formed the superphylum Opisthosporidia, the deepest branch of the Holomycota lineage, separated from the Fungi (Karpov et al., 2014a; Letcher et al., 2015; 2017; Torruella et al., 2015). Several biological peculiarities of the aphelids do not conform the classical definition of the Fungi. The most fundamental of these is that, unlike osmotrophic fungi, the trophonts of Aphelida and Rozellosporidia engulf the host cytoplasm by phagocytosis, like amoebae (Powell, 1984; Gromov, 2000; Karpov et al., 2014a). According to a multigene phylogeny of the Aphelida based on the transcriptomic analysis of

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Paraphelidium tribonematis the aphelids are a sister group to Fungi thus having a common ancestor with the latter (Torruella et al., 2018).

Because of great interest on the aphelids, more studies have been published in recent years including a recent taxonomic revision of the aphelids (Letcher and Powell, 2019). At present, several species have been studied by modern molecular methods: two species of *Amoeboaphelidium*: *Am. protococcarum* (Karpov et al., 2013; Letcher et al., 2013; 2015), and *Am. occidentale* (Letcher et al., 2015), three species of *Aphelidium: Aph. melosirae* (Karpov et al., 2014b), *Aph. tribonematis* (Karpov et al., 2016) and *Aph. desmodesmi* (Letcher et al., 2017), and two species of *Paraphelidium: P. tribonematis* (Karpov et al., 2017a) and *P. letcheri* (Karpov et al., 2017b). Zoospore structure of *Aphelidium* and *Paraphelidium* with evolutionary explications for the aphelid ancestral stages have been recently presented (Karpov et al., 2019).

Here, we report the morphological and molecular phylogenetic study of the strain B-0, which forms a sister branch to the *Aphelidium tribonematis* and corresponds in general to it morphologically. This isolate represents a new species *Aph. arduennense* differing from *Aph. tribonematis* by zoospore morphology and 18S sequence.

Material and methods

Isolation and cultivation of *Aphelidium arduennense* sp. nov.

The strain B-0 of *Aphelidium* was isolated by M.A. Mamkaeva in 2019 from sample collected in September of 2018 from a roadside ditch in the Ardennes forest, commune of Jedinne, Walloon municipality, province of Namur, Belgium (49°57' 03.6"N 4°50'44.0"E). The strain was maintained in culture on *Tribonema gayanum* (strain 20 CALU) as the host. The culture of the host was grown on mineral medium (KNO_3, 2 g L^{-1}; KH_2PO_4, 0.3 g L^{-1}; $MgSO_4$, 0.15 g L^{-1}; EDTA, 10 mg L^{-1}; $FeSO_4$, 5 mg L^{-1}; $NaBO_3$, 1.4 mg L^{-1}; $(NH_4)_6Mo_7O_2$, 4.1 mg L^{-1}; $CaCl_2$, 0.6 mg L^{-1}; $ZnSO_4$, 0.1 mg L^{-1}; $CuSO_4$, 50 mg L^{-1}, $Co(NO_3)_2$, 20 mg L^{-1}) at room temperature in the presence of white light. After inoculation with the parasite, the cultures were incubated for 1–2 weeks to reach the maximum infection of host cells. Light and DIC microscopy observations of living cultures were carried out on a Zeiss Axioplan microscope equipped with a color MRm Axiocam camera.

Molecular analyses

We collected zoospores from the B-0 culture with a micromanipulator and stored each of them in 1 µl of mineral media in PCR-tubes at -21 °C. We added PCR mix (Encyclo Plus PCR kit, Evrogen) directly to the tube. The aphelid 18S rRNA gene was amplified with the fungal primers UF1 (5'-CGAATCGCATGGCCTTG) and AU4 (5'-RTCTCACTAAGCCATTC) (Kappe et al., 1996). PCR reactions consisted of 5 min denaturation at 94 °C; 39 cycles of a denaturation step at 94 °C for 15 s, a 30 s annealing step at 50°C and an extension step at 72 °C for 2 min; and a final elongation step of 7 min at 72 °C. Negative controls without template DNA were used at all amplification steps. Fragments of the expected size (~1,400 bp) were purified with Clean Up Standard kit (Evrogen) and then used for direct sequencing.

Molecular phylogenetic analyses

We aligned the *Aphelidium arduennense* sp. nov. 18S rDNA sequence with sequences previously used in Karpov et al. (2017, 2019) using MUSCLE (Edgar, 2004) and manually trimmed the multiple alignment to eliminate spuriously aligned sites. A total of 1,570 unambiguously aligned sites were retained to reconstruct a phylogenetic tree applying Bayesian Inference (BI) and Maximum Likelihood (ML) methods. BI analyses were carried out with MrBayes (Ronquist et al., 2012) applying the GTR+G+I model with four chains and 10,000,000 generations per run. ML analyses were done with RAxML 8 (Stamatakis, 2014). The best tree was obtained out of 1000 best tree searches applying a GTR+G+I model of nucleotide substitution, taking into account a proportion of invariable sites, and a Gamma-shaped distribution of substitution rates with four rate categories. Bootstrap values were calculated using 1000 non-parametric replicates with the same substitution model. *Aphelidium arduennense* sp. nov. 18S rDNA sequence has been deposited in GenBank with accession number MN 733418.

Results

Molecular phylogeny

(CCPP ZIN RAS) The near-full 18S rRNA gene sequence from strain B-0 of *Aphelidium arduennense*

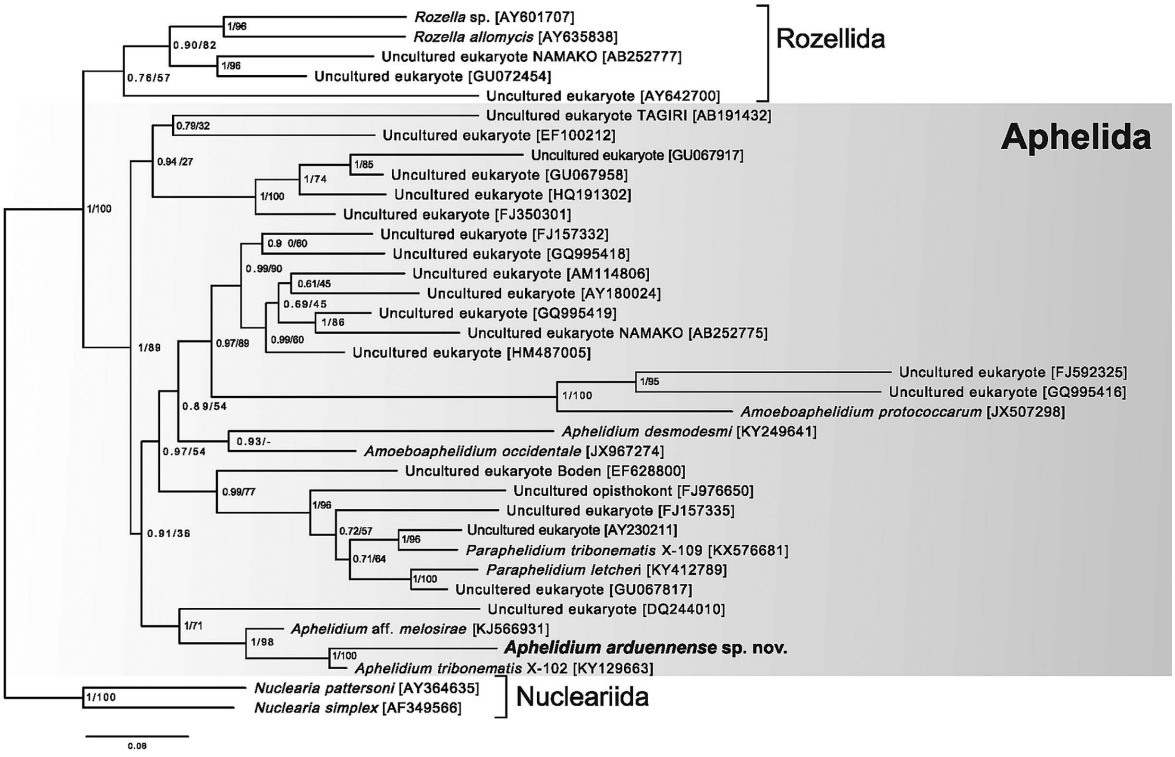

Fig. 1. Position of *Aphelidium arduennense* sp. nov. on the tree inferred from 18S rDNA analyses by Bayesian and ML methods. The tree contains a sampling of sequences from Rozellida and Aphelida, rooted on Nucleariida. The tree was constructed using 1570 nucleotide characters. Node support values are given as follows: Bayesian posterior probabilities (MrBayes) followed by bootstrap values (RAxML).

sp. nov. was 94% identical to that of *Aph. tribonematis* (Karpov et al., 2016). We reconstructed a phylogenetic tree including the new 18S rDNA sequence and a selection of Aphelida and Rozellida sequences together with two Nucleariida sequences as outgroup (Fig. 1). In our tree, *Aph. arduennense* sp. nov. formed a clade with *Aph. tribonematis* with strong statistical support (bootstrap value of 100), and this clade grouped with *Aph. melosirae* forming a highly supported branch of the aphelid tree. This *Aphelidium* branch is sister to the remaining Aphelida representatives, including the genera *Paraphelidium* and *Amoeboaphelidium*, the species *Aphelidium desmodesmi*, and numerous uncultured representatives.

Life cycle

The life cycle of strain *Aphelidium arduennense* sp. nov. corresponds to that of *Aph. tribonematis* and the other *Aphelidium* species as well (Gromov, 2000). When near the algal host, zoospores are amoeboid with many filopodia (Fig. 2 A-E). After attachment to the algal filament the cell produces a cyst wall and penetrates the alga via a germ tube, which extends into the gap between the inner and outer halves of the host cell wall (Fig. 2 F). The penetration tube provides a way for injecting the cyst contents into the host and the growing cyst vacuole pushes out the contents of the cyst (Fig. 2 G, H). Empty cysts remain attached to host cells by their penetration tubes for a long time. The growing parasitoid engulfs the host cytoplasm forming food vacuoles (Fig. 2 H). As the parasitoid grows and forms a plasmodium with residual body, it totally consumes the cytoplasm of the host cell (Fig. 2 H, I). The multinucleate plasmodium has a large central vacuole with a residual excretion body, which is composed of one red and 2–3 colorless lipid globules (Fig. 2 H, I). The mature plasmodium then divides into a number of uninucleated cells (Fig. 2 J), which become zoospores (Fig. 2 K), are released from the host cell, and infect other host algal cells.

Zoospores

The most informative feature for aphelid taxonomy is considered to be the structure of

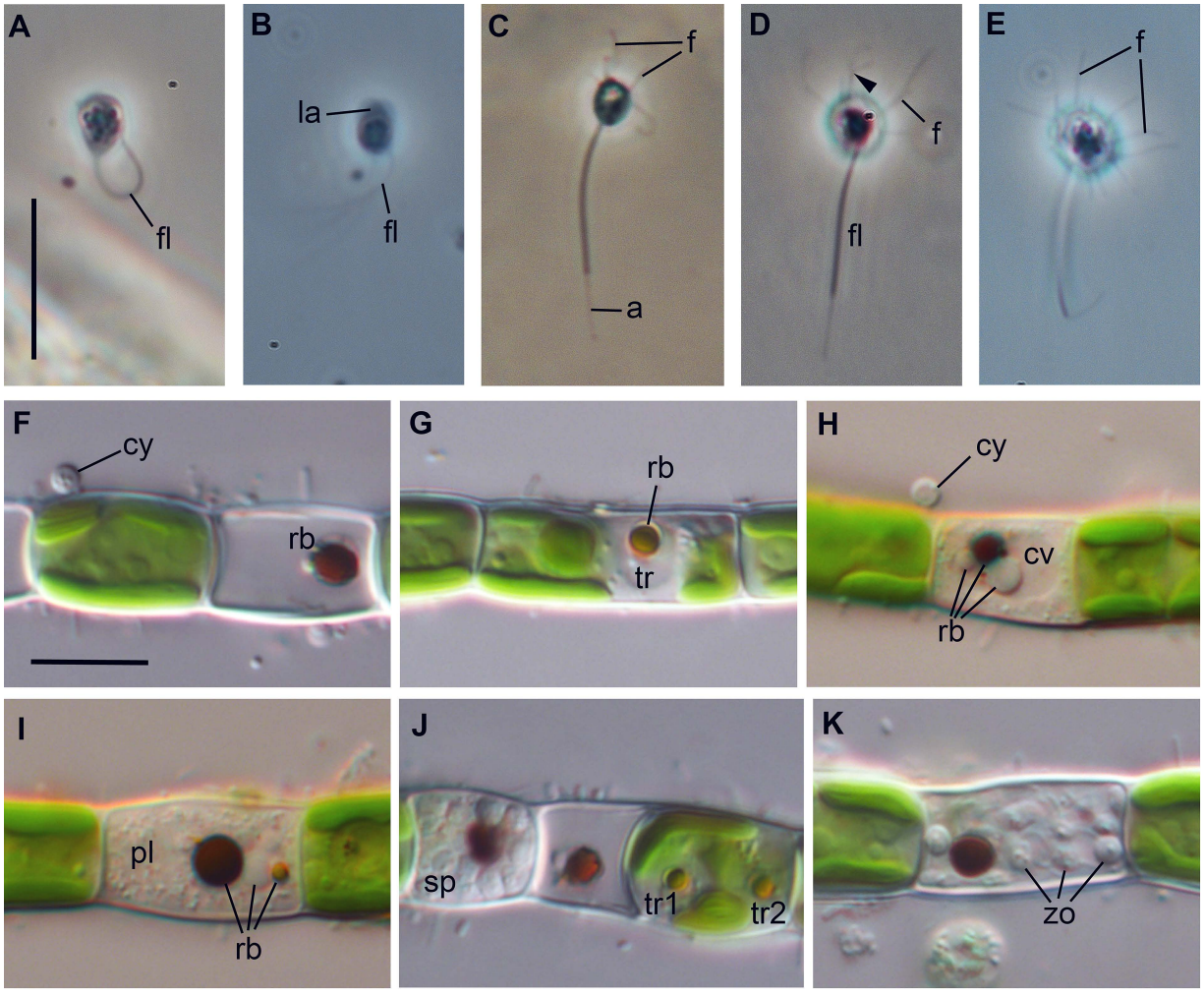

Fig. 2. Stages of the life cycle of *Aphelidium arduennense* sp. nov. observed in living material by phase (Ph) and differential interference contrast (DIC) microscopy. A–F – Diversity of zoospores (Ph): free-swimming (A) and crawling with anterior lamellipodium (B), few (C) and many filopodia (D, E). *Arrowhead* points branching filopodium. F-K (DIC): F – Cyst on the healthy host cell and empty neighbor cell with red residual body; G – trophont with residual body; H – plasmodium with central vacuole containing red and colorless lipid globules of residual body; empty cyst wall on the algal surface; I – plasmodium with residual body; J – sporangium with recently divided plasmodium into uninucleate cells (to the left); two trophonts with residual bodies in one host cell (to the right); K – mature zoospores with residual body inside sporangium. *Abbreviations:* a – acronema, cv – central vacuole, cy – cysts, f – filopodia, fl – flagellum, la – lamellipodium, pl – plasmodium, rb – residual body, sp – sporangium, tr – young trophont, tr1, tr2 – two different trophonts in a host cell, zo – mature zoospores. Scale bars: A-E – 10 μm, F-K – 10 μm.

zoospores (Gromov, 2000; Karpov et al., 2014a; 2016). Zoospores of Aph. arduennense sp. nov. are able to swim with a posterior flagellum (Fig. 2 A), but also to crawl on the substrate like amoebae, producing long often branching filopodia (Fig. 2 B-E). Swimming B-0 cells are spherical, 3–3.5 μm in diameter, with an acronematic flagellum of 9-10 μm including an acroneme of 3 μm. In the vicinity of the host algal thread zoospores move slower and become amoeboid (Fig. 2 C-E): they produce long (up to 3.5 μm) filopodia radiating from any part of the body cell, or produce a broad anterior hyaline lamellipodium up to 1 μm long without subfilopodia (Fig. 2 B).

Discussion

The 18S gene sequence analysis unambiguously places isolate B-0 close to *Aph. tribonematis* with high bootstrap support (Fig. 1). Its life cycle and type of zoospore, which is able to produce filopodia, but not subfilopodia, firmly place strain B-0 in the genus *Aphelidium* (Gromov, 1972, 2000; Gromov and Mamkaeva, 1975; Karpov et al., 2014b, 2016).

Among six species known for the genus *Aphelidium* (*Aph. chaetophorae* Scherff., 1925, *Aph. chlorococcorum* Fott, 1957 (with two forms: f. *chlorococcorum* Letcher and Powell, 2019 and f. *majus* Gromov and Mamkaeva, 1970), *Aph. deformans* Zopf, 1885, *Aph. desmodesmi* Letcher, 2017, *Aph. melosirae* Scherff., 1925, *Aph. tribonematis* Scherff., 1925), zoospores of B-0 appear to be most similar to those of *Aph tribonematis* (Gromov, 1972; Karpov et al., 2016; Letcher and Powell, 2019). Their dimensions mostly correspond to those of the strain studied by Scherffel (1925), but differ essentially in having very long and numerous filopodia, which were not described in the diagnosis of that species. The only sequenced strain of *Aph. tribonematis* (X-102; Karpov et al., 2016) also has zoospores with filopodia, but they are much shorter than those of strain B-0 (0.5 vs. 3,5 μm). The nearly full length 18S sequence of B-0 differs from that of *Aph. tribonematis* by about 6%, which corresponds to the average degree of difference between *Aphelidium* species. Based on these morphological and molecular characteristics we describe the strain B-0 as a new species of *Aphelidium*.

Aphelidium arduennense Tcvetkova, Zorina, Mamkaeva et Karpov sp. nov. (Fig. 2).

Crawling flagellated zoospores with body up to 4 μm long; able to produce lamellipodium or numerous radiating often branching filopodia up to 3,5 μm in length; swimming zoospores spherical (3 μm dia), and flagellum 10–11 μm including an acroneme of 3 μm. Round residual body associated with one or two colorless lipid globules.

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Type: this publication fig. 2. Belgium, province of Namur, Walloon municipality, commune of Jedinne, Ardennes forest, 49°57'03.6"N, 4°50' 44.0"E. Sample collected by Maria Mamkaeva in September of 2018 from a roadside ditch. Ex type culture deposited in ZIN collection (CCPP ZIN RAS) under No: X-132.

Etymology: after the name of the type locality, Ardennes forest.

This is the 7th species of *Aphelidium* and has zoospores typical for this genus; an anterior lamellipodium or filopodia appeared from different sides of the cell body, but it never has a lamellipodium with subfilopodia like the *Paraphelidium* spp. (Karpov et al., 2017). Intracellular stages of the parasitoid life cycle are similar to each other not only within genera, but even among genera. Differences in morphology and measurements appear to be totally dependent on host cell size and shape in aphelid genera. Although zoospore morphology and flagellar length vary among genera and species, molecular sequences are needed both to place aphelids with certainty into genera and to populate the databases so that environmental sequences can be identified. Long numerous filopodia of similar type and length have been described in amoeboid zoospores of *Amoeboaphelidium radiatum* (Gromov and Mamkaeva, 1969), but its zoospores have no flagellum and are much smaller (2 μm in diameter).

According to our molecular phylogeny *Aph. desmodesmi* is not in the *Aphelidium* clade (Fig. 1), but, instead is on a long branch sister to *Am. occidentale*, i.e. inside the *Amoeboaphelidium* clade but with low ML support (Fig. 1). Similar results have been published earlier (Karpov et al. 2019) where the 18S sequence of *Aph. desmodesmi* clustered with *Amoeboaphelidium* clade also with low bootstrap support. The morphology of zoospores of studied *Aphelidium* species (Gromov, 2000; Karpov et al. 2014a, 2019; Letcher et al., 2017; this paper), supports monophyly rather than paraphyly for studied *Aphelidium* spp. The problem of the *Aphelidium* monophyly/paraphyly can be solved only with further molecular phylogenetic study of the aphelids.

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Collection of Microorganisms of Research park of St. Petersburg State University and sequenced at the Research Resource Center for Molecular and Cell Technologies (RRC MCT) at St. Petersburg State University (SPbSU).

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Address for correspondence: Sergey Karpov. Zoological Institute, Universitetskaya nab. 1, 199034 St. Petersburg, Russia; e-mail: *sakarpov4@gmail.com.*