Published in final edited form as:

J Eukaryot Microbiol. 2017 September ; 64(5): 573–578. doi:10.1111/jeu.12389.

Molecular Phylogeny of *Paraphelidiumletcheri* sp. nov. (Aphelida, Opisthosporidia)

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Abstract

Aphelids remain poorly known parasitoids of algae and have recently raised considerable interest due to their phylogenetic position at the base of Holomycota. Together with Cryptomycota (Rozellosporidia) and Microsporidia, they have been recently re-classified as the Opisthosporidia, which constitutes the sister group to the fungi within the Holomycota. Molecular environmental studies have revealed a huge diversity of aphelids, but only four genera have been described: *Aphelidium*, *Amoebaphelidium*, *Paraphelidium*, and *Pseudaphelidium*. Here, we describe the life cycle of a new representative of Aphelida, *Paraphelidiumletcheri* sp. nov., and provide the 18S rRNA gene sequence for this species. Molecular phylogenetic analysis indicates that *P.letcheri* is sister to *Paraphelidiumtribonemae* and together they form a monophyletic cluster which is distantly related to both, *Aphelidium*, with flagellated zoospores, and *Amoebaphelidium*, with amoeboid zoospores

Keywords

Aphelids; Holomycota; Rozellosporidia; systematics; taxonomy

The aphelids are intracellular parasitoids of green, yellowgreen and diatom algae (Gleason et al. 2014; Karpov et al. 2014a). In 2013, they were suggested to form the deepest branch of the Holomycota lineage, appearing as sister to cryptomycotes (Rozellosporidia) and Microsporidia (Karpov et al. 2013). Later, because of their high genetic diversity, including environmental sequences, the aphelids were proposed to constitute the phylum, Aphelida, which together with the Microsporidia and Cryptomycota (Rozellosporidia nom. nov.¹) form the superphylum Opisthosporidia, separated from the fungi (Karpov et al. 2014a; Letcher et

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¹Phylum Rozellosporidia nom. nov. Basionym: Cryptomycota (Jones et Richards) Karpov et al. 2014a. We propose here to call this group Rozellosporidia instead of Cryptomycota or Rozellomycota: “mycota” makes reference to classical fungi, and phagotrophic rozellids as the other opisthosporidians are not; in addition, there is an incompatibility issue with the fungal nomenclature, as “Crypto” appears to allude to Cryptomyces or Cryptococcus, which are true fungal genera.

al. 2015; Torruella et al. 2015). Recent molecular phylogenetic and morphological studies have led to the description of a new genus of aphelids, *Paraphelidium*, in addition to three known genera: two from freshwater *Aphelidium* and *Amoeboaphelidium*, and one marine genus *Pseudaphelidium* (Karpov et al. 2016a). Although only four genera have been officially described, the group is highly diverse, including many environmental sequences from diverse ecosystems (Karpov et al. 2013, 2014a). Several biological peculiarities of the Opisthosporidia do not conform to the classical definition of fungi. Unlike osmotrophic fungi, the trophonts of Aphelida, and Rozellosporidia engulf the host cytoplasm by phagocytosis, like amoebae (Gromov 2000; Karpov et al. 2014a; Powell 1984).

Because of the phylogenetic and ecological interest on aphelids, an increasing number of studies about members of this group have been published in recent years. At present, several strains have been studied by molecular methods: three strains of *Amoeboaphelidium*: *A. protococcarum*: X-5 CALU (Karpov et al. 2013), FD95 (Letcher et al. 2015), and *Amoeboaphelidium occidentale* FD01 (Letcher et al. 2013, 2015), a strain of *Aphelidium* *aff. melosirae* P-1 CALU (Karpov et al. 2014b) and three strains of the recently described genus and species *Paraphelidium tribonemae* (Karpov et al. 2016a). Recently, one species, *Aphelidium tribonemae*, has found its place in molecular phylogenetic analyses as sister to *A. aff. melosirae* (Karpov et al. 2016b). This is important to connect the classical morphological taxonomy to the new molecular phylogenetic systematics of the aphelids and thus integrate classical morphological, biological (e.g. life cycle) and ecological knowledge within an evolutionary framework. Here, we report on the morphology and molecular phylogeny of strain X-129, which forms a sister branch to *P. tribonemae*, and which we describe as the new species *Paraphelidiumletcheri*.

Material and Methods

Isolation and cultivation of *Paraphelidiumletcheri* sp. nov.

Strain X-129 was isolated by M.A. Mamkaeva in May 2016 from sample 22 collected in the dried stream in the village Opolye, Kingisepp region, Leningrad Province, Russia. The strain was maintained in culture on *Tribonema geyanum* (strain 20 CALU) as described in Karpov et al. (2016a). Light and DIC microscopy observations of living cultures were carried out on a Leica Axioplan microscope (Leica Microsystems, St. Petersburg, Russia) equipped with color Axiocam camera (Leica Microsystems). We used the same culture methods to test the ability of the strain X-129 to infect strains of the green algae *Cosmarium* sp. (strains VN11 and VN81) and *Closterium* (strain Mn139) provided by the Algae Culture Collection BOROK (WDCM 602), and *Scenedesmus obliquus* (strain CALU 13).

Molecular analyses

After inoculation with the parasite, cultures were incubated for 1–2 wk to reach the maximum infection of host cells. Cells were then harvested and used directly for DNA extraction. A total of 40 µl of infected *Tribonema* culture were centrifuged and DNA was extracted from pelleted cells with the Arcturus PicoPure DNA Extraction Kit (ThermoFisher, Waltham, MA) following the manufacturer's instructions. The 18S rRNA gene was first PCR-amplified using the general eukaryotic primers EK-82F (50-

GAAACTGCGAA TGGCTC) and EK-1520R (50-CYGCAGGTTACCTAC), followed by a nested amplification step using the fungi-like specific primer UF1 (50-CGAATCGCATGGCCTTG) (Kappe et al. 1996) and the general eukaryotic primer 18S-1498R (50-CACCTACGGAAACCTTGTTA). PCR reactions were carried out in 25 µl of reaction buffer, containing 1 µl of the resulting DNA suspension, 1.5 mM MgCl₂, dNTPs (10 nmol each), 20 pmol of each primer, and 0.2 U of TaqPlatinum DNA polymerase (Invitrogen, Carlsbad, CA). PCR reactions comprised the following incubation cycles: denaturation at 94 °C for 2 min; 35 cycles consisting of a denaturation step at 94 °C for 15 s, an annealing step at 50 °C for 30 s and an extension step at 72 °C for 2 min; followed by a final elongation step of 7 min at 72 °C. Negative controls without template DNA were used at all amplification steps. The final PCR product was Sanger-sequenced bidirectionally with UF1 and 18S-1498R primers (Genewiz, Takeley, U.K.).

Molecular phylogenetic analyses

The *P.letcheri* sp. nov. 18S rDNA sequence was aligned with a collection of sequences of available aphelids and other opisthokonts as previously used in Karpov et al. (2014b) and Letcher et al. (2015) using Mafft (Katoh et al. 2002) with default parameters. The multiple alignment was then manually trimmed to eliminate gaps and ambiguously aligned sites. A total of 1,391 unambiguously aligned sites were retained to reconstruct a phylogenetic tree with Maximum Likelihood (ML) using RAxML 8 (Stamatakis 2014) as similarly done in our previous work (Karpov et al. 2016a). The *P.letcheri* 18S rRNA sequence has been deposited in GenBank with accession number KY412789.

Results

Molecular phylogeny

We amplified and sequenced a near-full 18S rRNA gene from the strain X-129 of *P.letcheri* sp. nov. (CCPP ZIN RAS) maintained in culture on the xanthophyte alga *T.gayanum* (strain 20 CALU). This sequence was 91% identical to that of the other described *Paraphelidium* species, *P.tribonemae* (Karpov et al. 2016b), suggesting that they are two closely related *Paraphelidium* species. We reconstructed a maximum likelihood (ML) phylogenetic tree including the new 18S rDNA sequence and a selection of aphelid sequences together with several rozellid sequences as outgroup (Fig. 1). In our tree, *P.letcheri* sp. nov. formed a clade with *P.tribonemae* with strong statistical support (bootstrap value of 83). This clade branches distantly from the other two sequenced aphelid genera, *Aphelidium* and *Amoeboaphelidium*, supporting the distinction between them.

Life cycle

The life cycle of strain X-129 corresponds to that of the type species of *Paraphelidium*. It encompasses several phases as follows (Fig. 2). The opisthokont zoospore attaches to the host alga, and encysts (Fig. 2A–H). The cyst germinates and penetrates the host cell wall with an infection or penetration tube and the parasitoid becomes a trophont which engulfs the host cytoplasm forming food vacuoles (Fig. 2F–H). The parasitoid grows and forms a plasmodium that totally replaces the host cytoplasm (Fig. 2I–L). This multinucleate plasmodium has a large central vacuole with a residual excretion body which is unusually

big for the aphelids, reaching 5–6 μm in diameter and totally occupying a central vacuole. The residual body is always composed of several loosely packed granules of different size in late trophic stage (Fig. 2G), but the granules then become more compact and are associated with big lipid globules in the plasmodium (Fig. 2I–L). The mature plasmodium then divides into a number of uninucleated cells (Fig. 2M), which become zoospores. Zoospores develop flagella inside the sporangium and are released from the empty host cell probably one by one through the gap between the halves of the algal wall, or several cells can come out simultaneously when the host cell is broken (Fig. 2N). Interestingly, in the latter case they do not swim away from the broken host cell, but passively fall out and stay without movement for some time (Fig. 2N). The resting spore, or sporocyst, is spherical and covered with a smooth thick wall; a residual body is normally attached to the outer surface of the sporocyst wall (Fig. 2O).

Zoospores

Zoospores of *P.letcheri* sp. nov. strain X-129 swim with a posterior flagellum, but also crawl on the substrate like amoebae, producing short pseudopodia. Swimming cells are spherical, 2–2.5 μm in diameter, with a flagellum of 8–10 μm including an acroneme of 4 μm . In the vicinity of the host algal filament zoospores move slower and demonstrate amoeboid appearance (Fig. 2A–C). The zoospore can become flat with the body elongated up to 4 μm and produce a broad anterior or lateral hyaline lamellipodia (flattened pseudopodia) up to 1 μm long. The anterior lamellipodium often forms several rather long filopodia (up to 1.8 μm) named here as subfilopodia (Fig. 2D,E). Zoospores can also produce lateral or posterior filopodia independently of the lamellipodium (not shown).

Taxonomy

Paraphelidiumletcheri Karpov et Torruella sp. nov.

Etymology—After the family name of the renowned mycologist Peter M. Letcher.

Crawling flagellated zoospores have a body up to 4 μm long and able to produce a lamellipodium with subfilopodia up to 1.8 μm in length; swimming zoospores with spherical body 2–2.5 μm in diameter, and a flagellum 8–10 μm including an acroneme of 4 μm . Large residual body associated with one or two lipid globules totally occupies a central vacuole of plasmodium. Sporocyst spherical 6–8 μm in diameter with smooth wall.

Type—Figure 2 Karpov et al. this publication. RUSSIA, Kingisepp region, dried stream in the village Opolye (59°25'059"N; 28°49'019"W). Sample collected by Maria Mamkaeva in May 2016. Ex type culture deposited in ZIN collection (CCPP ZIN RAS) under No: X-129.

Notes—*P.letcheri* can be clearly distinguished from type species *P.tribonemae* by much larger residual body associated with big colorless lipid globules in plasmodium. None of the green algae, *Cosmarium* sp. (2 strains), *Closterium* sp. (1 strain) and *S.obliquus* (1 strain), became infected when inoculated with *P.letcheri*.

Discussion

The aphelid strain X-129 can be attributed with certainty to the genus *Paraphelidium* based on its life cycle and type of flagellated zoospore, which is able to produce a lamellipodium with subfilopodia (Karpov et al. 2016a,b). The 18S rRNA gene phylogeny strongly supports this affiliation as strain X-129 branches close to *P. tribonemae* (Fig. 1). *Paraphelidium letcheri* can be easily distinguished from other aphelids by the large residual body totally occupying a central vacuole in old trophonts and plasmodia.

This residual body usually associates with one or two big lipid globules. In trophonts (Fig. 2H) and in plasmodia (Fig. 2K), two residual bodies sometimes occur in a single host cell; this seems to point to the presence of two parasites in a single algal cell as a result of double infection. Morphological variability at the light microscopic level is very low among the aphelids. All known aphelids have the same life cycle stages: zoospores, cysts, trophonts, multinuclear plasmodia that produce multiples zoospores, and resting spores, or sporocysts (Gromov 2000; Karpov et al. 2014a). Consequently, zoospore morphology is used to distinguish aphelid genera from each other: *Amoeboaphelidium* has amoeboid zoospores with a highly reduced flagellum, a pseudocilium, thus, they cannot swim; *Aphelidium* and *Paraphelidium* have flagellated zoospores able to produce filopodia and lamellipodia; and the marine *Pseudaphelidium* produces amoeboid zoospores that encyst immediately after being released and subsequently release flagellated zoospores from these cysts (Gromov 2000; Karpov et al. 2013, 2014a, 2016a; Letcher et al. 2013, 2015; Schweikert and Schnepf 1996). *Paraphelidium* zoospores differ from those of *Aphelidium* by their ability to produce subfilopodia from lamellipodia. This trait was not observed in *Aphelidium* species, which seem to produce filopodia straight from the cell body and never from lamellipodia (Karpov et al. 2016a,b). The amoeboid appearance of zoospores has been described in all aphelids, so we can distinguish the genera by the morphology of this stage.

We expect that the ultrastructure of zoospores will provide more morphological characters for taxonomy and phylogeny of this group and also for the Holomycota as a whole, but up to now ultrastructural information is rather poor. It is available for a few amoeboid zoospores of *Amoeboaphelidium chlorellavorum* (Gromov and Mamkaeva 1970a), *Am. protococcarum* (Gromov and Mamkaeva 1970b; Karpov et al. 2013; Letcher et al. 2015) and *Am. occidentale* (Letcher et al. 2013), and free swimming flagellated zoospores have been investigated only in *Aphelidium chlorococcarum* (Gromov and Mamkaeva 1975), and *Aph. aff. melosirae* in sporangia with immature flagella (Karpov et al. 2014b). The most informative structure for zoospore comparison is the flagellar apparatus, which has not yet been studied in detail. In summary, ribosomes are scattered throughout the cell; the microbody-lipid complex is present: there are several lipid globules associated with small microbodies; the rumposome, or fenestrated cisternum, is absent; mitochondrial cristae are flat or rhomboid, and the flagellar basal body with short nonflagellar centrioles does not connect to the nucleus (Gromov and Mamkaeva 1975; Karpov et al. 2014b, 2016a).

The structure of the sporocyst seems to vary from species to species. Regarding the taxonomy we have to take into account that the sporocyst variability has been noticed even in the culture of one strain, perhaps due to different stages of sporocyst maturation (Karpov

et al. 2016a). Morphological features provide only limited discriminant power; therefore, molecular phylogenetic studies are of utmost importance to assess taxonomic classifications in Aphelida. Available 18S rRNA sequences for aphelids are genetically highly divergent, yet they underlie only slightly different morphological features. For instance, the genetic divergence between 18S rRNA genes of the two morphospecies *P.letcheri* and *P.tribonemae* is 9%, that between *Aph.aff.melosirae* and *Aph.tribonemae* is 14% (Karpov et al. 2016b) and that between *Am.protococcarum* and *Am.occidentale* is 14% (Letcher et al. 2013, 2015). This high degree of divergence within individual genera might be possibly related to the fact that parasite genes tend to evolve faster than those of free-living species. Assigning new taxonomic descriptions to aphelids along with molecular sequence information will help match environmental sequence information with identifiable organisms. In the future, generating transcriptome/ genome sequence data should help unravel molecular features associated with the seemingly general parasitic strategy of the aphelid clade.

Acknowledgments

The isolation, cultivation, morphological study of strain X-129 and the manuscript writing have been supported by RSF Grant No. 16-14-10302, and the molecular analysis by ERC Advanced Grant No. 322669 'ProtistWorld'. SK also thanks for support the RFBR Grant No. 15-29-02734 and the scholarship program "Jean d'Alembert" of Paris-Saclay University; GT is funded by the MSCA-IF fellowship 704566 AlgDates. The investigated strain has been cultivated at the Center for Culturing Collection of Microorganisms of Research park of St. Petersburg State University.

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Figure 1.

Molecular phylogeny of *Paraphelidium letcheri*. Maximum likelihood tree based on aphelid 18S rRNA gene sequences rooted on rozellosporidian representatives. Tree is based on alignment of 1,391 conserved positions. Sequence accession numbers are provided within brackets. Numbers at branches are bootstrap proportions > 50%.

Figure 2.

Stages of the life cycle of *Paraphelidiumletcheri* strain X-129 observed in living material by differential interference contrast (DIC) microscopy. (A–C) one zoospore producing lamellipodia (arrow); (D, E) another zoospore with subfilopodia (arrowheads) appearing from the lamellipodia (arrows). fl – flagellum with acroneme (a); (F, G) cyst wall (cw) on the *Tribonema* filament after recent injection of cyst contents into the host; note young trophonts (tr); plasmodium with residual body (rb) (G); (H) two cysts (cy) probably initiated development of two trophonts (tr) in a host cell; chloroplast in the food vacuole (fv) is visible in another trophont (tr); (I–L) plasmodia with large residual bodies (rb) associated with lipid globules (l) inside a central vacuole (cv); (K) two residual bodies (rb) probably belong to different plasmodia inside the same host cell; (M) zoospore (zo) formation in sporangia; (N) zoospores (zo) released from broken *Tribonema* cell, fl-flagellum of zoospore

in the host cell; (**O**) sporocyst (resting spore) covered with a smooth wall (sw), and residual body (rb) outside the sporocyst; both within the empty host cell wall. Scale bar: 5 μ m.