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Molecular phylogeny of *Aphelidium tribonemae* reveals its sister relationship with *A.* aff. *melosirae* (Aphelida, Opisthosporidia)

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Summary

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

Key words: aphelids, Opisthosporidia, molecular phylogeny, Holomycota

Introduction

The aphelids are a divergent group of intracellular parasitoids of green, yellow-green and diatom algae (Gleason et al., 2014; 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) form the superphylum Opisthosporidia, the deepest branch of the Holomycota lineage, separated from the fungi (Karpov et al., 2014a; Letcher et al., 2015; Torruella et al., 2015). Not only does their phylogenetic position place them as sister to true fungi, but also several of their biological peculiarities do not conform with the classical definition of fungi. The most remarkable of those is that, unlike

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osmotrophic fungi, the trophonts of Aphelida and Rozellosporidia (but not Microsporidia, which are extremely specialized derived parasites) engulf the host cytoplasm by phagocytosis, like amoebae (Gromov, 2000; Karpov et al., 2014a).

Because of great interest in the aphelids, more and more studies have been published in recent years. At present, several strains have been studied by modern molecular methods: three strains of *Amoeboaphelidium*: *A. protococcarum*: X-5 CALU (Karpov et al., 2013), FD95 (Letcher et al., 2015), and *A. occidentale* FD01 (Letcher et al., 2013), 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., 2016). Here, we report the morphological and molecular phylogenetic study of the strain X-102, which forms a sister branch to *Aphelidium* aff. *melosirae* and corresponds morphologically to *Aphelidium tribonemae* Scherffel 1925.

Material and methods

Isolation and cultivation of *Aphelidium tribonemae*

The strain X-102 of *Aphelidium tribonemae* was isolated by M.A. Mamkaeva in 2012 from sample P-2 collected in the vicinity of the Kuty village, Kingisepp District, Leningrad Province, Russia. 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; KH_2PO_4, 0.3 g/L; $MgSO_4$, 0.15 g/L; EDTA, 10 mg/L; $FeSO_4$, 5 mg/L; $NaBO_3$, 1.4 mg/L; $(NH_4)_6Mo_7O_2$, 4.1 mg/L; $CaCl_2$, 0.6 mg/L; $ZnSO_4$, 0.1 mg/L; $CuSO_4$, 50 mg/L, $Co(NO_3)_2$, 20 mg/L) 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. Cells were then harvested and used directly for DNA extraction. Light and DIC microscopy observations of living cultures were carried out using a Zeiss Axioplan microscope equipped with black and white MRm Axiocam camera.

Molecular analyses

We centrifuged 2 ml of infected *Tribonema* culture and extracted DNA from pelleted cells with the DNA purification kit PowerSoil (MoBio) following the manufacturer's instructions. To avoid amplifying an excess of host genes, the aphelid 18S rRNA gene was amplified by polymerase chain reaction (PCR) with the fungi-like specific primers UF1 (5'-CGAATCGCATGGCCTTG) and AU4 (5'-RTCTCACTAAGCCATTC) (Kappe et al., 1996). PCR was carried out in 25 µl of reaction buffer, containing 1 µl of DNA, 1.5 mM $MgCl_2$, dNTPs (10 nmol each), 20 pmol of each primer, and 0.2 U of TaqPlatinum DNA polymerase (Invitrogen). PCR reactions consisted of 2 min denaturation at 94 °C; 35 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. We cloned the amplified 18S rRNA gene fragments using the Topo TA Cloning System (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Clone inserts were PCR-amplified using flanking vector primers and inserts of the expected size (1,400 bp) were sequenced bidirectionally with vector primers (Beckman Coulter Genomics, Takeley, UK).

Molecular phylogenetic analyses

The *Aphelidium tribonemae* 18S rDNA sequence was aligned together with the sequences previously used in Karpov et al. (2014b) and Letcher et al. (2015) using Mafft (Katoh et al., 2002). The multiple alignment was then manually trimmed to eliminate spuriously aligned sites. A total of 1,391 unambiguously aligned sites were retained to reconstruct a phylogenetic tree applying Maximum Likelihood (ML) methods with RAxML 8 (Stamatakis, 2014). The best tree was obtained out of 500 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 with 500 non-parametric replicates with the same substitution model. The *Aphelidium tribonemae* 18S rRNA sequence has been deposited in GenBank with accession number KY129663.

Results

Molecular phylogeny

We amplified and sequenced a near-full 18S rRNA gene from the strain X-102 of *Aphelidium*

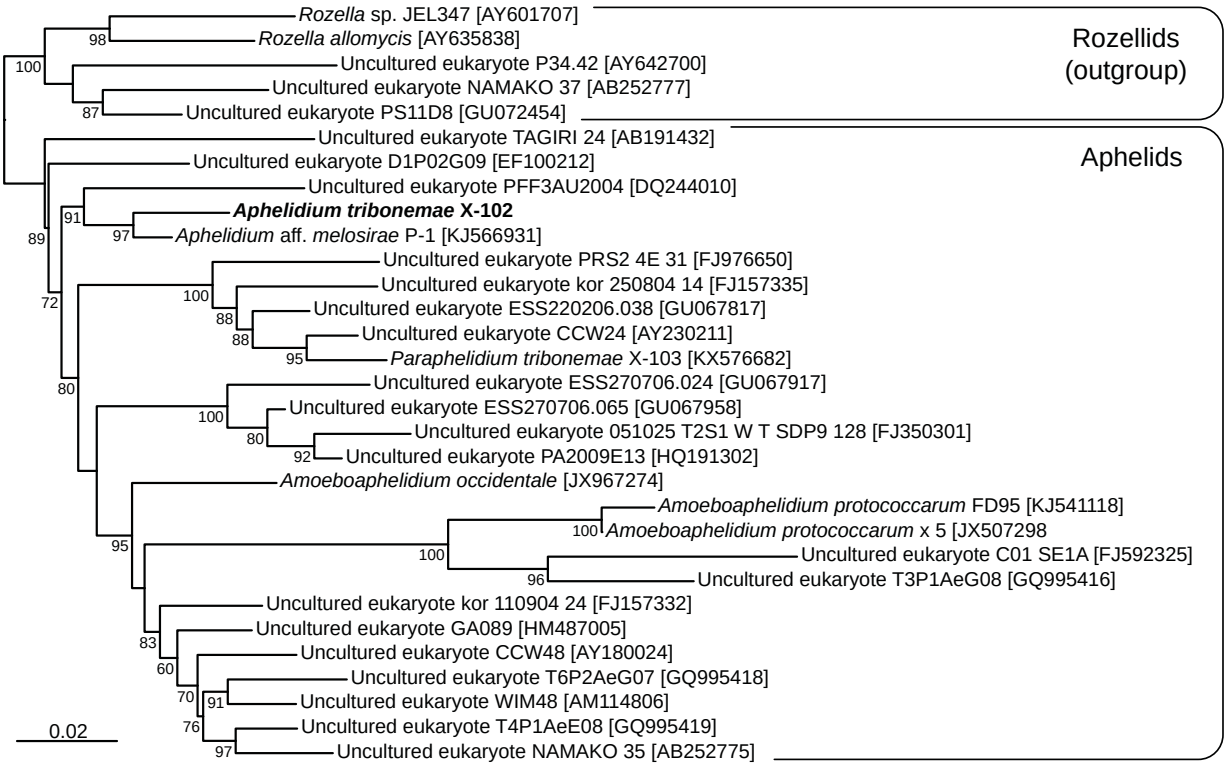

Fig. 1. Molecular phylogeny of *Aphelidium tribonemae*. Maximum likelihood tree based of aphelid 18S rRNA gene sequences rooted on rozellid representatives. The tree is based on an alignment of 1391 conserved positions. Sequence accession numbers are provided within brackets. Numbers at branches are bootstrap proportions >50 %.

tribonemae (CCPP ZIN RAS) maintained in culture on the xanthophyte alga *Tribonema gayanum* (strain 20 CALU). This sequence was only 86% identical to that of the other described *Aphelidium* species, *Aphelidium* aff. *melosirae* (Karpov et al., 2014b), clearly indicating that they are two distantly related *Aphelidium* 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, *A. tribonemae* formed a clade with *A.* aff. *melosirae* with strong statistical support (bootstrap value of 97). This clade branches very distant from the other two sequenced aphelid genera, *Paraphelidium* and *Amoeboaphelidium*, supporting the distinction between them.

Life cycle

The life cycle of strain X-102 corresponds to that of *Aphelidium tribonemae* and the other *Aphelidium* species as well (Gromov, 2000). It encompasses several phases as follows (Fig. 2). The opisthokont zoospore attaches to the host alga, and encysts (Figs 2, I; 3). The cyst germinates and penetrates the host cell wall with an infection or penetration tube (Fig. 2, I, J). A conspicuous enlarging vacuole pushes the contents of the cyst towards the interior of the host cell through the penetration tube (Fig. 2, I). The parasitoid becomes a trophont, which engulfs the host cytoplasm forming food vacuoles (Fig. 2, L). The parasitoid grows and forms a plasmodium with residual bodies while it totally consumes the cytoplasm of the host cell (Fig. 2, M–O). A multinucleate plasmodium has a large central vacuole with a residual excretion body. The latter is composed either of several (Fig. 2, M), or more often of one large globule (Fig. 2, N, O).The mature plasmodium (Fig. 2, O) then divides into a number of uninucleated cells, which become zoospores releasing from the host cell and infecting other host algal cells (Fig. 2, P).

Zoospores

The most informative feature for aphelid taxonomy is considered to be the structure of zoospores (Gromov, 2000; Karpov et al., 2014a). Zoospores

of strain X-102 are able to swim with a posterior flagellum, but also to crawl on the substrate like amoebae, producing short pseudopodia. Swimming X-102 cells are spherical, 3–3.5 μm in diameter, with an acronematic flagellum of 10–12 μm including an acroneme of 2.5–3.3 μm (Fig. 2, G). In the vicinity of the host algal filament zoospores move slower and demonstrate amoeboid appearance (Fig. 2, B-H): they produce short (up to 0.5 μm) posterior or lateral filopodia and a broad anterior hyaline lamellipodium up to 1 μm long without subfilopodia. The zoospore body elongates up to 4 μm and sometimes has irregular outlines, producing lateral and anterior lamellipodia at the same time (Fig. 2, H).

Encystment

After attachment to the algal filament, the zoospore retracts its flagellum. We were lucky to document all stages of this quick process for one zoospore (Fig. 3). The attached zoospore spent about one minute slowly waving with flagellum and then suddenly rotated quickly clockwise at about 120° along the lateral axis while the flagellum twisted like a belt around the cell (Fig. 3, A, B). Then it took about 1-2 minutes to retract the main part of flagellum into the cytoplasm, and only the distal end of the flagellum remained outside the zoospore body (Fig. 3, C-H). The speed of flagellar retraction increased at the last steps (Fig. 3, I-N) when its distal end disappeared in the cell; it is possible to see the groove (arrows in Fig. 3, J, L, M) where the flagellum entered the zoospore body.

After flagellar retraction, 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, I, K). 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, I). Empty cysts remain attached to host cells by their penetration tubes for a long time (Fig. 2, J).

Discussion

Its life cycle and type of zoospore, which is able to produce filopodia, but not subfilopodia, firmly places strain X-102 in the genus *Aphelidium* (Gromov, 1972, 2000; Gromov and Mamkaeva, 1975; Karpov et al., 2014b, 2016). Further, our 18S rRNA gene phylogeny strongly supports this generic placement because strain X-102 branches close to *A.* aff. *melosirae* (Fig. 1). Among four species and one forma known for the genus *Aphelidium*, strain X-102 appears to be most similar to *A. tribonemae* Scherffel 1925. According to the original description of *A. tribonemae* (Scherffel, 1925), this parasitoid of the yellow-green alga *Tribonema* has a cyst diameter of about 2 μm, a zoospore body of 4 μm in length and a flagellum of 11 μm length (measured from Figure 107a, Taf. 3 in: Scherffel, 1925), measurements that are in accordance with our data for X-102. The acroneme was not obviously present in Scherffel's pictures, but it could have been easily overlooked at that time. Scherffel (1925) noticed that *A. tribonemae* zoospores become amoeboid when encountering an obstacle, but he did not describe the shape of pseudopodia. Later, Gromov (1972) described a similar aphelid growing on *Tribonema gayanum* Pasch. and *Botridiopsis intercedens* Visch. et Pasch, which he referred to *A. tribonemae*; it had zoospores of 2–3 μm in diameter and a flagellum of about 12 μm including a 5 μm acroneme. Zoospores produced filopodia and moved like amoebae with an immotile flagellum. These data also correspond, in general, to the X-102 strain in having zoospores that never exceed 4 μm, but can be less than 3 μm. One more *Aphelidium* species, *A. chlorococcarum* forma *majus* Gromov et Mamkaeva 1970, also has zoospores of 2–3 μm in diameter, but it has a longer flagellum (14 μm) and parasitizes green algae *Chlorococcus* sp. (Gromov and Mamkaeva, 1970), therefore being clearly different from strain X-102. Zoospores of all these *Aphelidium* species are able to produce filopodia from different sides of the cell body, but never form a lamellipodium with subfilopodia like the *Paraphelidium* spp. do (Karpov et al., 2016).

Separate stages of flagellar retraction before zoospore encystment have been described in *A.* aff. *melosirae* (Karpov et al., 2014b), but the complete documentation of this process is presented here for aphelids for the first time. Thus, this form of flagellar retraction might be a feature of the genus *Aphelidium* only, or characterize the aphelids in general, but we do not have data on *Paraphelidium* and *Pseudaphelidium*, the two other genera with flagellated zoospores. Flagellar retraction is similar to the process in some blastocladialeans like *Catenaria* spp. (Deacon and Saxena, 1997), but differs from others such as the rhizophydialean *Dinomyces arenysensis* (Lepelletier et al., 2014), where zoospores retract the flagellum by folding and simultane-

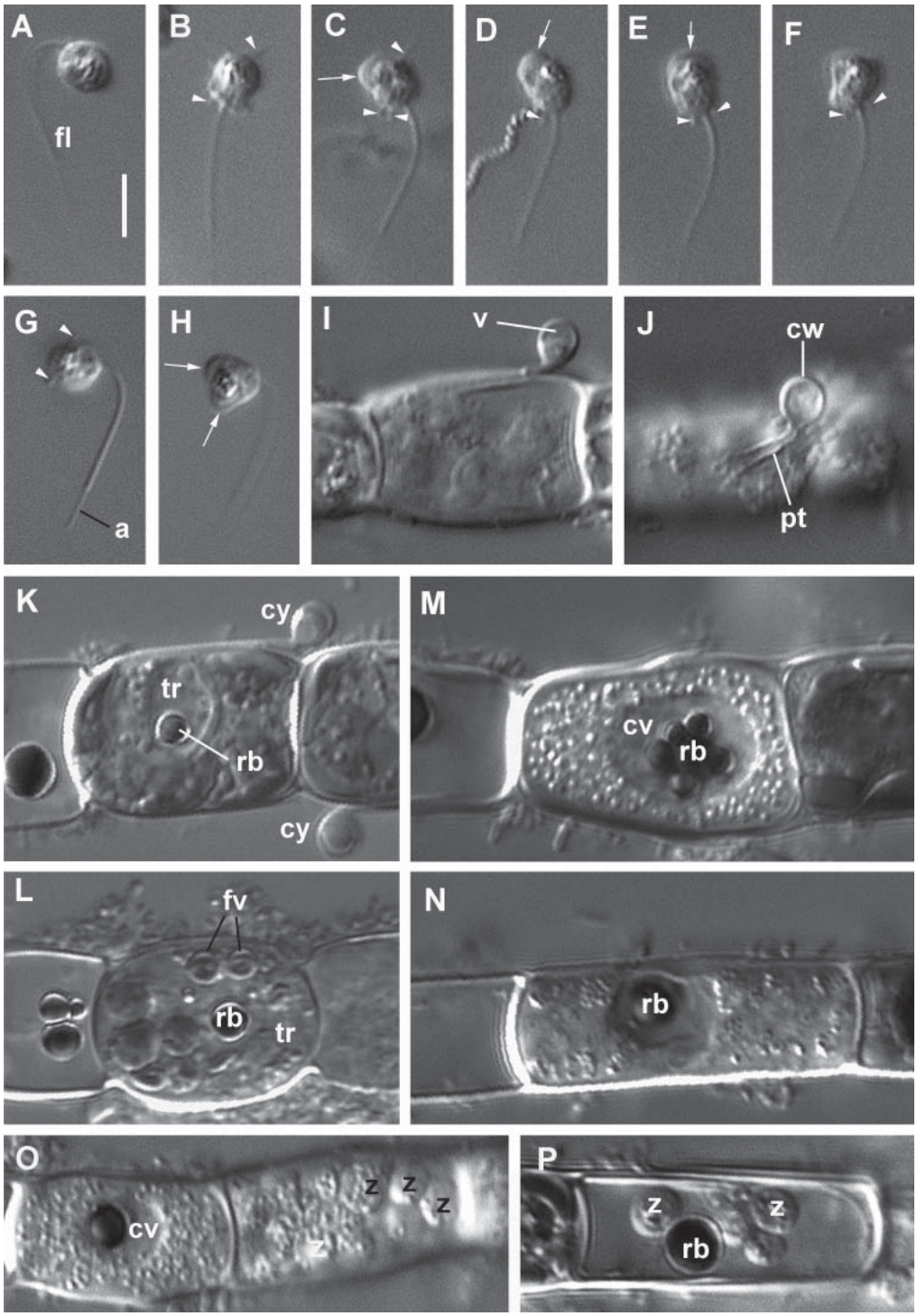

Fig. 2. Stages of the life cycle of *Aphelidium tribonemae* strain X-102 observed in living material by differential interference contrast (DIC) microscopy. A-F – One zoospore producing anterior lamellipodia (*arrow*) and short filopodia (*arrowheads*); G-H – another zoospore with filopodia (*arrowheads*) and lamellipodia (*arrows*), a – acronema; I – cyst on the tribonema filament with enlarging vacuole (v); J – empty cyst wall (cw) with penetration tube (pt); K – young trophont (tr) in the host cell with residual body (rb), two cysts (cy) on the host surface; L – developed trophont (tr) with food vacuoles (fv) and residual body (rb); M, N – plasmodium with central vacuole (cv) and different appearance of residual bodies (rb); O – plasmodium (left cell) and sporangium (right cell) with mature zoospores (z); P – sporangium with few zoospores (z) and residual body (rb). Scale bar: 4 μm.

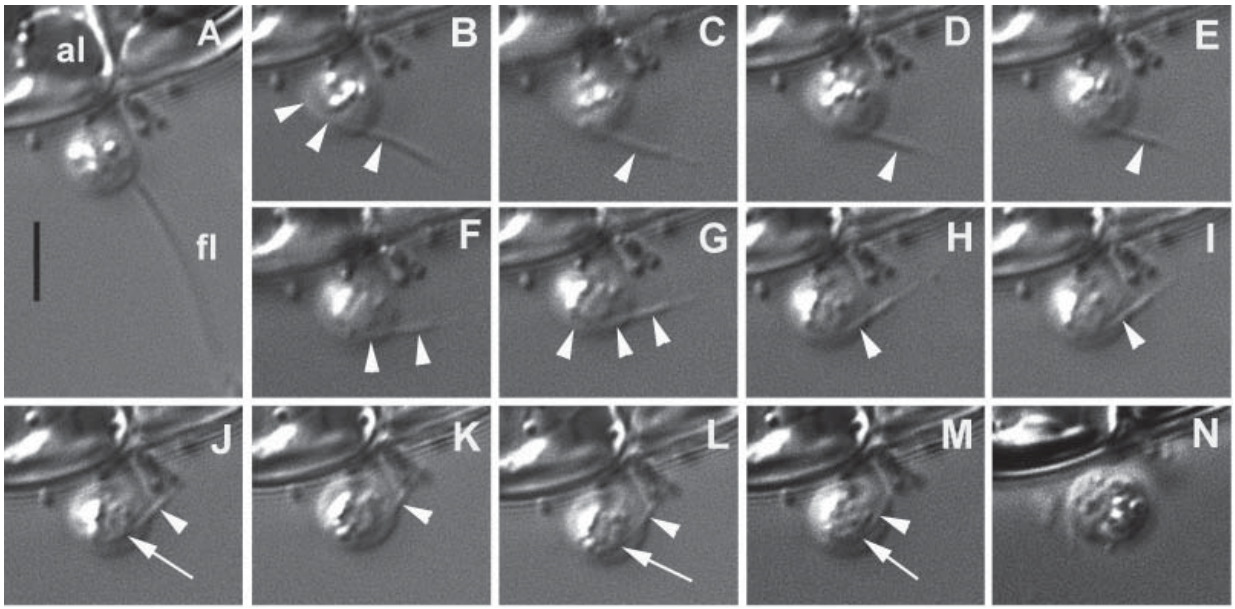

Fig. 3. Zoospore of *Aphelidium tribonemae* strain X-102 involves a flagellum before encystment. A-N – Consecutive stages of the flagellar retraction in one cell. *Abbreviations*: al – algae, fl – flagellum. *Arrowheads* trace a flagellum, *arrows* show a shallow furrow where the flagellum submerges. Scale bar: 4 μm.

ously retracting the flagellum at the posterior end of zoospore. Intracellular stages of the parasitoid life cycle are similar to each other not only within genera, but seem not to differ 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.

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References

Deacon J.W. and Saxena G. 1997. Orientated zoospore attachment and cyst germination in *Catenaria anguillulae*, a facultative endoparasite of nematodes. Mycological Research. 101, 513–522.

Gleason F.H., Karpov S.A., Lilje O., Macarthur D.J., van Otgen F.F. and Sime-Ngando T. 2014. Chapter 13. Zoosporic parasites of phytoplankton. In: Freshwater Fungi and Fungal-like organisms. (Eds Jones E.B.G., Hyde K.D. and Pang K.L.). De Gruyter, Berlin, Germany, pp.273–298.

Gromov B.V. 1972. *Aphelidium tribonemae* Scherffel parasitizing yellow green algae. Mikol. Fitopatol. 6, 443–445 (in Russian).

Gromov B.V. 2000. Algal parasites of the genera *Aphelidium, Amoeboaphelidium* and *Pseudoaphelidium* from the Cienkovski's "Monadea" group as representatives of new class. Zool. Zhurn. 79, 517–525 (in Russian).

Gromov B.V. and Mamkaeva K.A. 1970. The culture of *Aphelidium chlorococcarum* f. *majus* f. *nova*. Acta Protozool. 7, 263–267.

Gromov B.V. and Mamkaeva K.A. 1975. Zoospore ultrastructure of *Aphelidium chlorococcarum*. Fott. Mikol. Fitopatol. 9, 190–193 (in Russian).

Kappe R., Fauser C., Okeke C.N. and Maiwald M. 1996. Universal fungus-specific primer systems and group-specific hybridization oligonucleotides for 18S rDNA. Mycoses. 39, 25–30.

Karpov S.A., Mikhailov K.V., Mirzaeva G.S., Mirabdullaev I.M., Mamkaeva K.A., Titova N.N. and Aleoshin V.V. 2013. Obligately phagotrophic aphelids turned out to branch with the earliest-diverging fungi. Protist. 164, 195–205.

Karpov S.A., Mamkaeva M.A., Aleoshin V.V., Nassonova E.S., Lilje O. and Gleason F.H. 2014a. Morphology, phylogeny and ecology of the aphelids (Aphelidea, Opisthokonta) with proposal of new superphylum Opisthosporidia. Front. Microbiol. 5, 112. doi: 10.3389/fmicb.2014.00112.

Karpov S.A., Mamkaeva M.A., Benzerara K., Moreira D. and López-García P. 2014b. Molecular phylogeny and ultrastructure of *Aphelidium* aff. *melosirae* (Aphelida, Opisthosporidia). Protist. 165, 512–526.

Karpov S.A., Tcvetkova V.S., Mamkaeva M.A., Torruella G., Timpano H., Moreira D., Mamanazarova K.S. and López-García P. 2016. Morphological and genetic diversity of Opisthosporidia: new aphelid *Paraphelidium tribonemae* gen. et sp. nov. J. Eukaryot. Microbiol. Published online 23 AUG. 2016. doi:10.1111/jeu.12352.

Katoh K., Misawa K., Kuma K. and Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30, 3059–3066.

Lepelletier F., Karpov S.A., Alacid E., Le Panse S., Bigeard E., Garcès E., Jeanthon C. and Guillou L. 2014. *Dinomyces arenysensis* gen. sp. nov. (Rhizophydiales), the first chytrid infecting marine dinoflagellates. Protist. 165, 230–244.

Letcher P.M., Lopez S., Schmieder R., Lee P.A., Behnke C., Powell M.J. and McBride R.C. 2013. Characterization of *Amoeboaphelidium protococcarum*, an algal parasite new to the cryptomycota isolated from an outdoor algal pond used for the production of biofuel. PLoS One. 8, 2. doi: 10.1371/journal.pone.0056232.

Letcher P.M., Powell M.J., Lopez S., Lee P.A. and McBride R.C. 2015. A new isolate of *Amoeboaphelidium protococcarum*, and *Amoeboaphelidium occidentale*, a new species in phylum Aphelida (Opisthosporidia). Mycologia. 107, 522–531. doi:10.3852/14-064.

Scherffel A. 1925. Endophytische Phycomyceten-Parasiten der bacilleriaceen und einige neue Monadinen. Arch. Protistenkd. 52, 1–141.

Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 30,1312–1313. doi: 10.1093/bioinformatics/btu033.

Torruella G., de Mendoza A., Grau-Bove X., Anto M., Chaplin M. A., del Campo J., Eme L., Perez-Cordon G., Whipps C. M., Nichols K.M., Paley R., Roger A. J., Sitja-Bobadilla A., Donachie S. and Ruiz-Trillo I. 2015. Phylogenomics reveals convergent evolution of lifestyles in close relatives of animals and fungi. Curr. Biol. 25, 2404–2410.

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