Protist, Vol. 164, 195–205, March 2013
http://www.elsevier.de/protis
Published online date 9 October 2012

Protist

ORIGINAL PAPER

Obligately Phagotrophic Aphelids Turned out to Branch with the Earliest-diverging Fungi

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Submitted April 17, 2012; Accepted August 9, 2012
Monitoring Editor: Hervé Philippe

Reconstructing the early evolution of fungi and metazoans, two of the kingdoms of multicellular eukaryotes thriving on earth, is a challenging task for biologists. Among extant organisms having characters intermediate between fungi and hypothetical protistan ancestors, from which both fungi and metazoans are believed to have evolved, aphelids are unfairly neglected. The phylogenetic position of these microalgal endoparasites remained uncertain, since no nucleotide sequence data have been reported to date. Aphelids resemble some primitive zoosporic fungi in life cycle, but, unlike fungi, they live by phagotrophy. Here we present a phylogeny, in which a cultured aphelid species, *Amoeboaphelidium protococcarum*, forms a monophyletic group with *Rozella* and microsporidia as a sister group to Fungi. We also report a non-canonical nuclear genetic code in *A. protococcarum*.

Key words: Aphelids; fungi; nuclear genetic code; phylogeny.

Introduction

Transitions from unicellularity to multicellularity are believed to have occurred several times in the history of life. According to current concepts on the origins of multicellularity, two kingdoms of multicellular organisms, the Fungi and Metazoa, share a common ancestry (Baldauf and Palmer 1993; Burki et al. 2007; Carr and Baldauf 2011; Medina et al. 2003; Steenkamp et al. 2006). On the tree of life, the Fungi and Metazoa form a monophyletic clade, named the Opisthokonta (Adl et al. 2005; Cavalier-Smith 1987, 1998), which includes also a variety of lineages consisting of unicellular organisms. Some of them, such as choanoflagellates and ichthyosporeans, are closely related to metazoans, whereas the others, e.g., nucleariids, were shown to be a sister group to the Fungi (Liu et al. 2009; Medina et al. 2003; Steenkamp et al. 2006). Among unicellular lineages that are expected to be close extant relatives of fungi or metazoans,

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http://dx.doi.org/10.1016/j.protis.2012.08.001

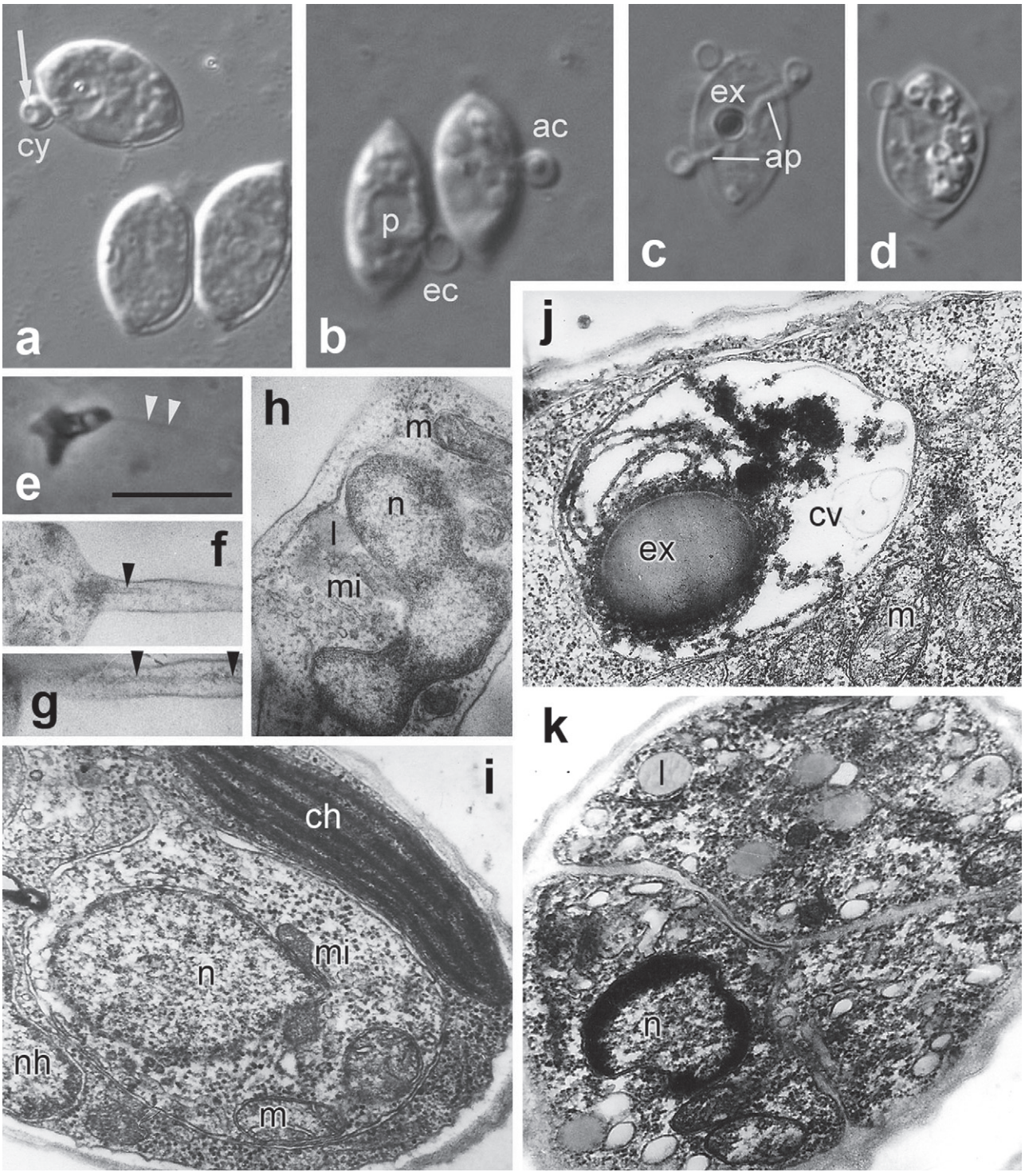

Figure 1. Light (a–e) and electron (f–k) micrographs of *A. protococcarum* (strain x-5). (**a**) Cyst with the enlarged posterior vacuole (arrow). (**b**) Recently attached zoospore and empty cyst after injection of the parasite into the host. (**c**) Two empty cysts with germ tubes and excretory body of the developed parasite. (**d**) Mature zoospores inside an empty host cell. (**e**) Amoeboid zoospore with posterior pseudocilium

the Aphelidea, a group of intracellular parasites that feed on microalgae are currently referred to as incertae sedis opisthokont protists (Adl et al. 2005; Carr and Baldauf 2011; Cavalier-Smith 1998). Molecular phylogenetic analyses have not been performed for this group. Known since the 19th century (Cienkowski 1865) and described substantially decades ago (Gromov and Mamkaeva 1968, 1970a,b, 1975; Gromov 2000; Schweikert and Schnepf 1996, 1997), aphelids were placed within phycomycetes, rhizopods, or ichthyosporeans by different authors (Adl et al. 2005; Carr and Baldauf 2011; Cavalier-Smith 1998; Gromov 2000). The aphelids share similarities with chytridiomycetes in life cycle; however, they use phagotrophy for the uptake of nutrients like nucleariids, whereas the overwhelming majority of fungi live by absorption of nutrients across a cell wall. The transition from phagotrophy to osmotrophy is among the evolutionary changes that have promoted the dramatic success of fungi on land and seems to have occurred very early in the fungal radiation, though several fungal lineages may retain the ability for phagocytosis at some phases of their life cycle.

In this paper, we present a multigene phylogeny of an aphelid species, *Amoeboaphelidium protococcarum* and reinvestigate its morphology and life cycle. In the molecular phylogeny, inferred from rDNA (18S, 5.8S, and 28S) and two RNA polymerase II subunit gene sequences (*RPB1* and *RPB2*), *A. protococcarum* branched as sister group of the Fungi. We also report a non-canonical nuclear genetic code in this aphelid species. We discuss the results within a framework of current concepts of early opisthokont evolution. Our results support the idea that the limits defining the fungal kingdom may be extended beyond those generally accepted, which is in accord with the current opinion of researchers exploring the early evolution of fungi (James and Berbee 2011; Jones et al. 2011a; Lara et al. 2010).

Results

Morphology and life cycle of *A. protococcarum*: A posteriorly uniflagellate zoospore having a specific ultrastructure is the main characteristic feature of opisthokonts, since such a complex structure is believed to have evolved only once. To provide more evidence for the opisthokont nature of aphelids, we reinvestigated the ultrastructure of the amoeboid zoospore of *A. protococcarum* and found a pseudocilium, which was not described earlier (Gromov and Mamkaeva 1970b) (Fig. 1e, Supplementary Fig. S1, Movie S1). This permanent immotile posterior projection contains microtubules (Fig. 1f, g), so it may be considered as a reduced posterior flagellum. The ultrastructure of *A. protococcarum* zoospores and intracellular stages is shown in Figures 1h to 1k and in Supplementary Figure S1. A zoospore contains a membrane-bound granular microbody that is normally associated with the nucleus, a mitochondrion with flat cristae, several lipid globules, and ribosomes spread over the cytoplasm. This ultrastructure is similar to that reported earlier in other aphelids, *Aphelidium* (Gromov and Mamkaeva 1975) and *Pseudaphelidium* (Schweikert and Schnepf 1996, 1997), which have a typical opisthokont zoospore. Thus, the dispersal stage of the life cycle of all known aphelids is a true opisthokont zoospore.

Other life cycle stages are similar to those of other aphelids (Gromov 2000; Gromov and Mamkaeva 1970a,b; Schweikert and Schnepf 1996, 1997). After attachment to the host cell, a zoospore loses its flagellum, produces a cyst wall, and penetrates the host cell wall by means of a germ tube (Fig. 1a, c). Once the tube is inside the host cell, the posterior vacuole of the parasite enlarges and seems to push the contents of the cyst into the host cell (Fig. 1b, d), resembling sporoplasm injection in microsporidia (Franzen 2004). At the intracellular stage, the parasites engulf the host cell contents by pseudopodia, like true amoebae phagocytising their food, and transporting food vacuoles to the large central vacuole for digestion (Fig. 1j). The residual (excretory) body formed by non-digested material is stored inside the central vacuole (Fig. 1c, j). The parasite grows and gradually replaces the host cell contents. Then it forms a multinuclear plasmodium by nuclear divisions, which undergoes multiple fissions, producing uninuclear zoospores (Fig. 1d) to be released from the host cell through the holes made by cyst stalks.

(arrowheads). (**f,g**) Serial longitudinal sections of pseudocilium with microtubules inside (arrowheads). (**h**) Zoospore ultrastructure: nucleus, mitochondrion with flat cristae, microbody with granular contents, and lipid globules. (**i**) A young parasite in the host. (**j**) Excretory body in the central digestive vacuole. (**k**) A divided parasite forming zoospores inside the host cell wall. ac, attached cyst; ap, apparatus for penetration; ch, chloroplast; cv, central vacuole; cy, cyst; ec, empty cyst; ex, excretory body; l, lipid globule; m, mitochondrion; mi, microbody; n, nucleus; nh, nucleus of the host cell; p, parasite. Scale bars: 5 μm (**a–e**); 500 nm (**f,g**), and 1 μm (**h–k**).

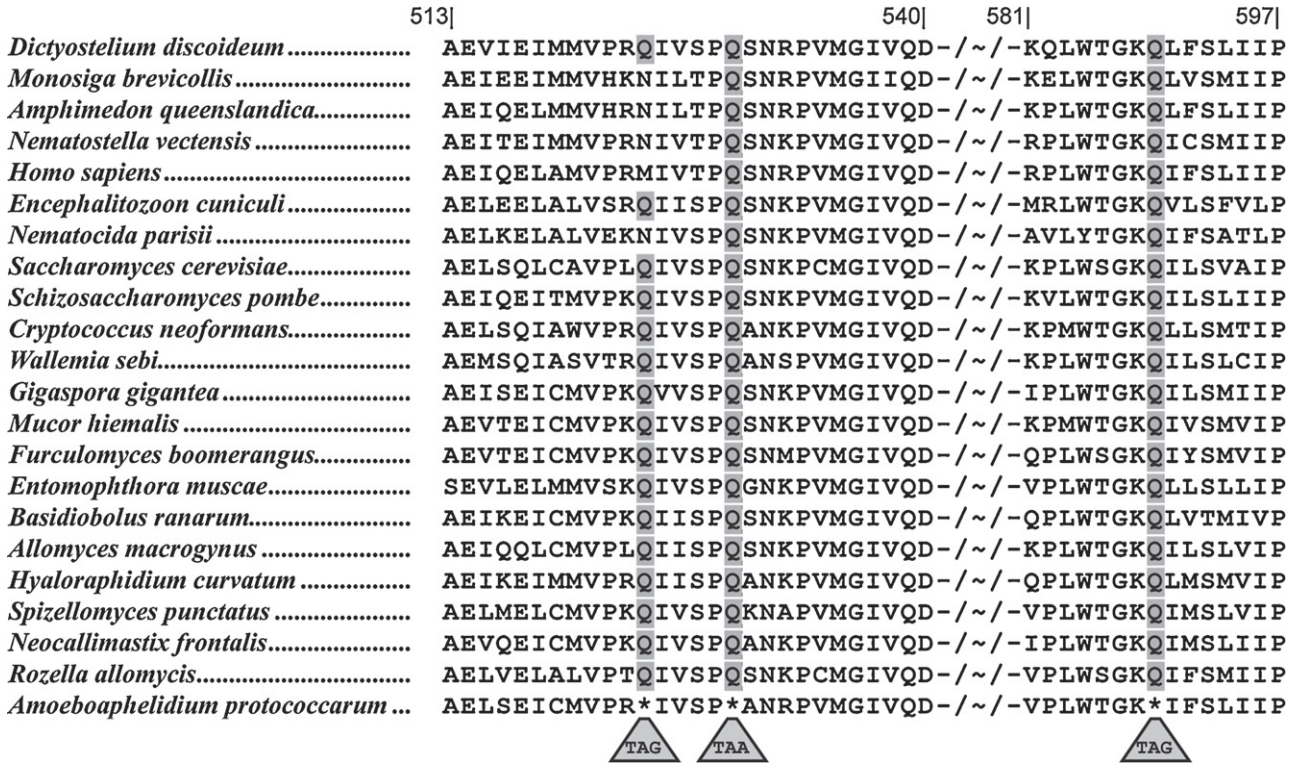

Figure 2. Non-canonical genetic code in *A. protococcarum*. Two fragments of the *RPB1* alignment are shown. Codons are numbered according to the human *RPB1* amino acid sequence (P24928). Conserved glutamines encoded by TAA or TAG codons are shown in grey.

Non-canonical nuclear genetic code in *A. protococcarum*: The gene sample that we chose to infer the phylogeny of *A. protococcarum* included the gene sequences for RNA polymerase II subunits (*RPB1* and *RPB2*), proven markers for fungal phylogenetic studies (James et al. 2006a; Liu et al. 2006; Tanabe et al. 2004). Analysing the nucleus-encoded *RPB1* and *RPB2* gene sequences of *A. protococcarum*, we unexpectedly found a non-canonical genetic code: UAG and UAA apparently encode glutamine rather than termination. The analysed partial *RPB1* and *RPB2* sequences of *A. protococcarum* contain 54 standard CAR glutamine codons and 48 UAR codons: many of the latter were found in positions, where glutamine is expected based on the alignment (Fig. 2).

Multigene phylogeny of *A. protococcarum*: The multigene phylogeny of *A. protococcarum* was inferred based on *RPB1*, *RPB2*, and rRNA (18S, 5.8S, and 28S) gene sequences (Fig. 3). In the multigene phylogenetic tree, *A. protococcarum* forms a well-supported monophyletic group with microsporidia, intracellular parasites of animals, and *Rozella allomycis*, an endoparasite of the blastocladiomycete *Allomyces* (Karling 1942). The *Amoeboaphelidium* + *Rozella* + Microsporidia (ARM) group branched sister to the Fungi. Microsporidian rDNA sequences were excluded from analysis for their extremely accelerated rate of evolution (Corradi and Keeling 2009).

When the protein-coding gene sequences, *RPB1* and *RPB2*, were analysed alone (Supplementary Fig. S2), the tree topology remained similar to that in Figure 3, and the monophyly of the ARM group was also well supported. When reconstructing trees with PhyloBayes, we found that the monophyly of the ARM group is independent of the selected evolutionary model for a large range of models

divided into five partitions by genes. The tree topology was obtained with MrBayes under GTR + Γ_{12}; key nodes are marked by support values (Bayesian posterior probability/bootstrap support by ML method - RAxML). Filled circles indicate that support values are 1.0/100. The scale bar indicates substitutions per site.

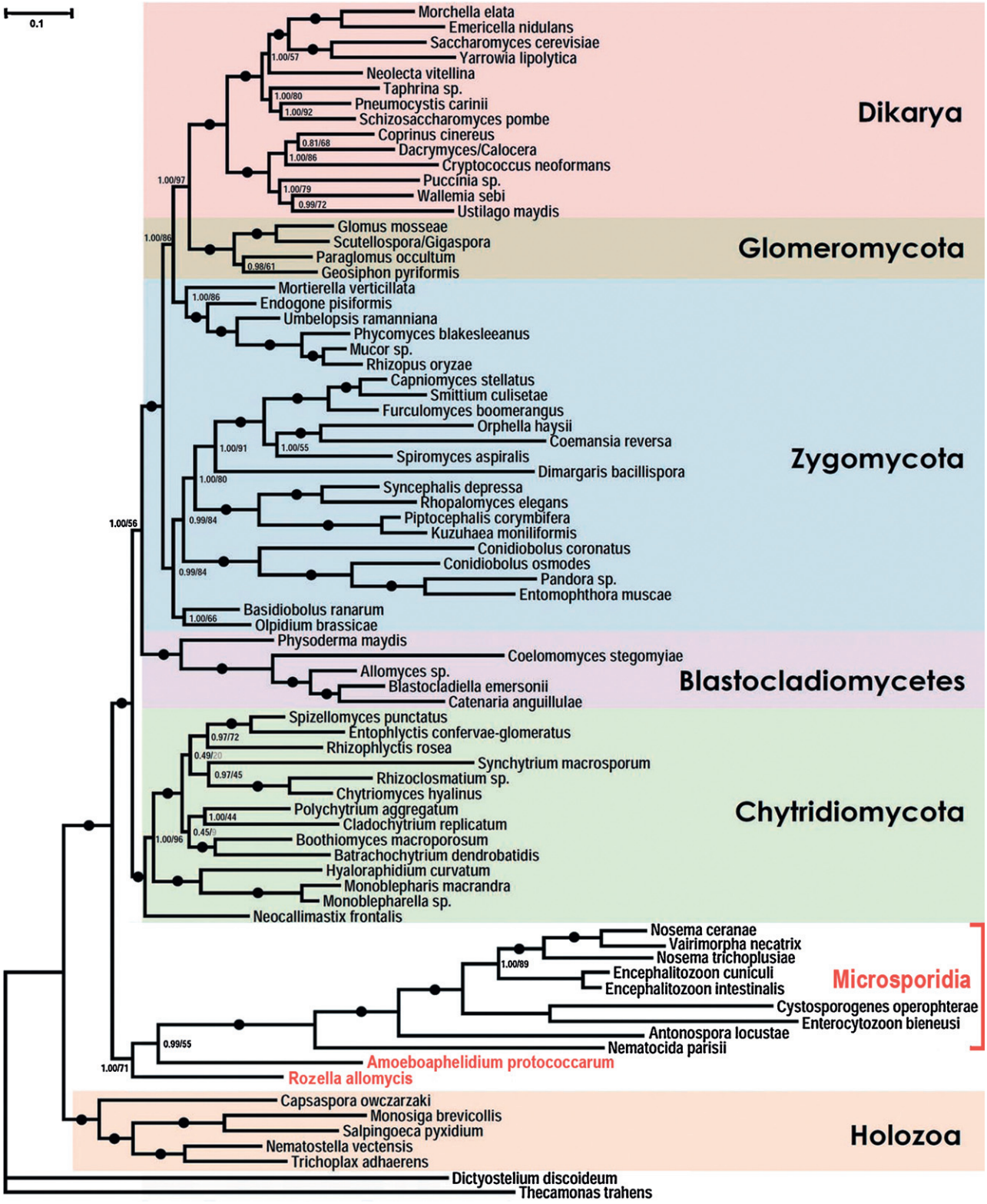

Figure 3. Multigene phylogenetic tree with the *Amoeboaphelidium* + *Rozella* + Microsporidia (ARM) clade at the base of the Fungi. The total length of concatenated rRNA (18S, 5.8S, and 28S) and protein-coding (*RPB1* and *RPB2*) gene sequences after the removal of ambiguous alignment regions was 6,273 positions

(Supplementary Table S1). To eliminate the possibility of long branch attraction (LBA) effects, we used several different approaches. The removal of either fast-evolving (Microsporidia) or divergent (Holozoa) sequences from the analysis did not significantly affect either tree topology or posterior probability of the ARM clade (see Alignments B and C in Supplementary Table S2); the same applies to the removal of sequences not conforming to the LG substitution model (see Alignment D in Supplementary Table S2). Another simple method to minimize the LBA artifact is the "fast site removal" approach (Dacks et al. 2002), which eliminates the most common source of homoplasies – rapidly evolving sites. The site-wise substitution rates were estimated with TREE-PUZZLE using the topology in Figure 3 as a constraint. After the stepwise removal of fast-evolving sites from the analysis, the ARM group remained monophyletic, and the clade was characterized by moderate to high posterior probabilities (see Alignments G–I in Supplementary Table S3). The approximately unbiased (AU) and Kishino–Hasegawa tests reject any tree topology other than the monophyletic ARM group, except for a more basal placement of Microsporidia. When fast-evolving sites were removed from the analysis, the monophyletic ARM group remained the only accepted topology (Supplementary Fig. S3). However, we can not exclude the possibility that a different method of site-wise rate estimation would give a different picture as it was shown that the results of a site removal test are influenced by the topology used for parameter estimation (Rodríguez-Ezpeleta et al. 2007).

We also analysed rDNA alone, using an enlarged taxon sample of 144 operational taxonomic units (OTUs), which included aphelid sequences, a specifically selected set of opisthokont sequences, and a broad selection of environmental sequences available from GenBank. In the rDNA tree presented in Supplementary Figure S4, aphelids are clustered with sequences from various habitats (freshwater and marine sediments, soil, and invertebrate gut), branching from the stem of the Fungi after the divergence of nucleariids suggested earlier as sister group to the Fungi (Liu et al. 2009; Medina et al. 2003; Steenkamp et al. 2006). Alternative topology tests rejected the placement of aphelids in any of the major fungal groups; however, some topologies with either an earlier divergence of rozellids or joining rozellids and aphelids in a monophyletic cluster are not significantly worse and therefore should not be neglected (Supplementary Fig. S5).

Discussion

Our results suggest a close relationship of aphelids with rozellids and microsporidia. Earlier, in the first large-scale molecular phylogeny of the Fungi by James et al. (2006a), *Rozella allomycis* was suggested as the earliest diverging branch of the fungal kingdom, and its relationship to microsporidia was also predicted in that study, though some alternative positions for both *Rozella* and microsporidia could not be rejected (James et al. 2006a, b). Recent rDNA studies by Lara et al. (2010) and Jones et al. (2011a) revealed that *Rozella* fell within a fungal clade comprising the uncultured fungi obtained from environmental samples from various habitats and earlier referred to as LKM11 group (Van Hannen et al. 1999). This putative basal-most fungal group was named the Rozellomycota (James and Berbee 2011) or Cryptomycota (provided with a diagnosis) (Jones et al. 2011b) and was given the rank of phylum. The latter name hints at the cryptic nature and diversity of the group, as well as the probable non-conformity of its members to a typical fungal body plan (Jones et al. 2011a); the former refers to *Rozella* as the type genus, which has hitherto been the only culturable and morphologically identified member of the group. Estimated by branch length, the diversity of the LKM11-affiliated group is thought to rival that of the rest of the fungi (Jones et al. 2011a), which may imply that the group of cryptomycota consists of more than one phylum.

Our data indicate that some of the published rDNA sequences representing the diversity of cryptomycota may be affiliated with aphelids, which are clustered separately from rozellids in our rDNA tree (Supplementary Fig. S4).

The life cycle stages in aphelids and rozellids are similar to each other and correspond in general to those of chytridiomycetes with exogenous development and an endobiotic sporangium (Barr 2001). There might be some structural differentiation in sporangia formation and the details of zoospore discharge (through the apical papillae of the host in *Rozella*, or through the holes in the host wall left after germ tube penetration in aphelids), but these characters do not mark high level taxa.

A posteriorly uniflagellate zoospore and a wall-less form feeding on host cytoplasm are characters shared by *Rozella* species (Held 1975; Powell 1984) and aphelids, as well as the capability of phagocytosis (Gromov 2000; Powell 1984).

Zoospores of *R. allomycis*, *Aphelidium* and *Pseudaphelidium* are similar to each other in general ultrastructure: ribosomes do not aggregate,

a microbody-lipid complex (MLC) is composed of a microbody with several lipid globules (Gromov and Mamkaeva 1975; Held 1975; Schweikert and Schnepf 1997). At the same time, the flagellar kinetosome has a prominent rhizoplast connected to the mitochondrion in *Rozella* (Held 1975), but the rhizoplast was not found in aphelids (Gromov 2000; Gromov and Mamkaeva 1975). Further investigations of flagellar apparatus structure in aphelids are necessary to clarify its peculiarities, which may have important taxonomic implications.

At the intracellular stage the aphelids phagocytise the host cell contents by pseudopodia like true amoebae (Gromov 2000; Gromov and Mamkaeva 1975; Karling 1942). Unlike *Rozella polyphagi* that seems to digest food in separate vacuoles (Powell 1984), the aphelids transport the food vacuoles to the large central vacuole for digestion (Gromov 2000).

Thus, the morphological characters are still not complete and do not show a strong separation of rozellids from aphelids at this stage of investigation.

The main peculiarity of both the cryptomycota and the aphelids is their ability of phagocytosis at the intracellular stage, which is absent in chytrids and other fungi. This common feature is reflected by the phylogenetic trees (Fig. 3, Supplementary Fig. S4) separating the *Amoeboaphelidium* and *Rozella* clades from the fungal lineage.

The well-supported ARM clade looks reasonable, given the endoparasitic nature of its members. However, they differ fundamentally in host range. As pointed out earlier, the lineage leading to *Rozella* diverged before that leading to its host, as did the lineage leading to Microsporidia (Stajich et al. 2009), which may indicate that the sister position of the ARM group relative to Fungi is an artifact. Be it as it may, the penetrative apparatus of aphelids (a germ or infection tube for penetration and transport into the host cell, a posterior vacuole, and a rigid cyst wall protecting the cell from the pressure generated by the enlargement of the posterior vacuole) shares many morphological similarities with that of *Rozella* and microsporidia, which argues in favour of ARM grouping. To support the placement of the ARM clade and to resolve the branching order within the clade, an increased gene sample should be analysed.

The placement of cryptomycota and aphelids at the primary nodes of fungal radiation suggests that characteristics of the fungi + ARM common ancestor should include phagotrophy and absence of cell walls. We can propose an evolutionary scenario in these two branches involving two alternative paths: 1) absorptive nutrition with a cell wall rich in chitin appeared in the fungal lineage, or 2) a secondary loss of typical fungal characters could have occurred as an adaptation to intracellular parasitism (James and Berbee 2011). Considering that microsporidia also have these fungal characters, the common ancestor seems to have had an ability to synthesize chitin as opposed to acquiring this ability in two branches independently.

Thus, our study places the morphologically described organisms, class Aphelidea Gromov, 2000, in a sister position to the fungi with the ARM group also including cryptomycota and microsporidia. The aphelids are capable of growing in culture and are therefore promising candidates for further phylogenomic studies, along with *Rozella*. Another important finding of this study is a non-canonical nuclear genetic code in *A. protococcarum.* Deviations from standard nuclear code were described in a number of organisms (Cocquyt et al. 2010; De Koning et al. 2008; Lozupone et al. 2001). As for opisthokonts, a non-canonical nuclear genetic code was only described in *Candida* yeasts: the leucine CUG codon is reassigned to serine (Sugita and Nakase 1999). The question whether the deviation revealed in this study is characteristic of all aphelids or is only restricted to *A. protococcarum* and its close relatives remains open, but this feature of *A. protococcarum* genome should be considered in further genomic studies.

Our results are in line with the current concept of defining the Fungi, which was adequately formulated by James and Berbee (2011) in their words: “the lines dividing fungus from the protozoan soup from which they evolved may be fuzzier than appreciated.” While it is generally accepted that fungi share a common ancestry with metazoans, the details on how the divergence between the two kingdoms of multicellular organisms have occurred during evolution need further exploration. Therefore, the organisms residing in the early diverging branches of both kingdoms or in the borderland between them take on special significance for future phylogenetic and phylogenomic studies aimed at understanding the origins of multicellularity in opisthokonts.

Methods

Strains and cultivation: The strains mentioned here are part of the Culture Collection of St. Petersburg State University collection of microorganisms (CALU) (Pinevich et al. 2004). The strain CALU x-5 of *Amoeboaphelidium protococcarum* Gromov and Mamkaeva 1968 was originally isolated from the springs of Kamchatka region in August 1966.

The cultures of the hosts *Scenedesmus obliquus* and *Chlorococcum minutum* were grown on Petri dishes in a standard solid (2% Bacto-Agar) mineral medium (Pinevich et al. 1997) (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}; $(NH_4)_6Mo_7O_{24}$, 1 mg L^{-1}; $CaCl_2$, 0.6 mg L^{-1}; $ZnSO_4$, 0.1 mg L^{-1}; $CuSO_4$, 50 μg L^{-1}, $Co(NO_3)_2$ 20 μg L^{-1}) supplemented with meat pancreatic peptone (1.0 g L^{-1}) at room temperature in the presence of white light. After inoculation with strains of aphelids, the cultures were incubated for 1-2 weeks in order to reach the maximum infection of host cells. The cells were then harvested and used directly for DNA extraction.

DNA extraction and sequencing: The aphelid DNA was extracted with Diatom DNA Prep (IsoGen Lab, Moscow). The *RPB1*, *RPB2*, and rRNA gene sequences were amplified using Encyclo PCR kit (Evrogen) and a set of primers (Medlin et al. 1988; Van der Auwera et al. 1994) and sequenced either directly or after cloning into the pTZ57R vector (Fermentas). The aphelid rRNA and *RPB* sequences were deposited in GenBank under no. JX507298 – JX507301.

Light and electron microscopy: Leica and Zeiss microscopes were used for light microscopic observations. The microscopes were equipped with digital video cameras; DIC and phase contrast objectives (100x) were used. For electron microscopy, pellets of parasites were fixed with a fresh mixture of 0.6% osmium tetroxide and 1.4% glutaraldehyde in an 0.05 M phosphate buffer, pH 7.3 (final concentrations) at 0 °C in the dark for 30 to 40 minutes, dehydrated, and embedded in Araldite. Ultrathin sections were treated with uranyl acetate and lead citrate and studied using a JEM 100CX electron microscope operating at 80 kV.

Data set construction: The alignments used for phylogenetic analyses were obtained from the Assembling the Fungal Tree of Life project, AFTOL (http://aftol1.biology.duke.edu/pub/alignments/download_alignments) and updated with sequences from GenBank (http://www.ncbi.nlm.nih.gov), Whole Genome Shotgun Sequences (ftp://ftp.ncbi.nih.gov/genbank/wgs), DOE JGI (http://genome.jgi-psf.org), and the Broad Institute Origins of Multicellularity Sequencing Project (http://www.broadinstitute.org/annotation/genome/multicellularity_project/MultiHome.html). Accession numbers are listed in Table S4. The alignments were added with new aphelid sequences with the help of MUSCLE (Edgar 2004) and then manually adjusted using BioEdit (Hall 1999). In the amino acid data, the UAR codons of *RPB1* and *RPB2* genes from *A. protococcarum* were interpreted as glutamine-encoding. Ambiguous regions were manually removed from further analysis. For multigene phylogenetic analysis, single gene alignments were concatenated into a supermatrix with SCaFoS (Roure et al. 2007). To minimize the amount of missing data, we set chimeric OTUs of closely related sequences (*Allomyces arbusculus* + *A. macrogynus*, *Emericella nidulans* + *Aspergillus oryzae*, *Dacrymyces chrysospermus* + *D. stillatus* + *Calocera cornea*, *Neocallimastix* sp. + *N. frontalis*, *Mucor hiemalis* + *M. racemosus*, *Pandora neoaphidis* + *P. dipterigena*, *Podochytrium dentatum* JEL30 + *Chytriomyces* sp. JEL378, *Taphrina wiesneri* + *T. deformans*, *Gigaspora gigantea* + *Scutellospora heterogama*, *Coprinopsis cinerea* + *C. calospora*, *Morchella elatagi* + *Tuber melanosporum*, *Orbilia auricolor* + *O. vinosa*, and *Schizangiella* sp. ARSEF 2237 + *S. serpentis*).

Two data sets were used for phylogenetic analysis of rDNA: an alignment of 144 OTUs enriched with SSU rDNA sequences from environmental samples (see the rDNA tree in Supplementary Fig. S4), and an alignment of 78 OTUs selected to represent all major fungal taxa evenly and to minimize the missing data for the five-gene analyses (see the multigene tree in Fig. 3). For phylogenetic analysis of *RPB1* and *RPB2* gene sequences (a total of 1889 amino acid sites) we used nine alignment files: the initial alignment of 78 OTUs (alignment A) and eight additional test alignments. Five of them were constructed by removing one of the following groups: (1) microsporidia (alignment B, 69 OTUs), (2) outgroup outside the Holomycota (alignment C, 71 OTUs), (3) taxa failed the χ^2 test for compliance with the LG model at a 5% significance level (alignment D, 71 OTUs), (4) aphelids, so as to make the taxon sample comparable to the one studied earlier (James et al. 2006) (alignment E, 77 OTUs), and (5) rosellids (aligment F, 77 OTUs). The remaining three additional alignments (G, H, and I) were made by stepwise removal of fast-evolving sites for the "fast site removal" test (Dacks et al. 2002). The rate categories for sites were calculated with TREE-PUZZLE under the LG + I + Γ_8 model using the topology in Figure 3 as a constraint. The alignments were generated by removing sites in the 8th (G), 8th and 7th (H), 8th, 7th, and 6th (I) categories (up to 1737, 1512, and 1276 amino acid sites, respectively). All alignments are available on request.

Phylogenetic analyses: Phylogenetic analyses for individual genes and concatenated alignments were performed using the Bayesian (MrBayes version 3.1.2 [Ronquist and Huelsenbeck 2003] and PhyloBayes version 3.2 [Lartillot et al. 2009]) and maximum likelihood (ML) methods (TREE-PUZZLE version 5.2 [Schmidt et al. 2002] and RAxML version 7.2.6 [Stamatakis 2006]) (http://wwwkramer.in.tum.de/exelixis/software.html). Partition by genes was used for the concatenated gene sample with all parameters unlinked, except the topology and branch lengths for MrBayes tree construction. For nucleotide data, the GTR (Lanave et al. 1984), GTR + Γ_{12}, and GTR + CAT + DP (Lartillot and Philippe 2004) models were used in RAxML, MrBayes, and PhyloBayes tree reconstruction software, respectively. Optimization of site-specific evolutionary rates in RAxML was carried out using categories (GTRCAT option). For amino acid data, a mixed model was preliminarily used to select the best-fit model. We added the LG model with an improved general amino acid replacement matrix (Le and Gascuel 2008) to the list of models implemented in MrBayes and TREE-PUZZLE and applied it to amino acid data with the across-site rate heterogeneity modelled using Γ_8. The LG substitution model was selected as the best-fit model for amino acid data, therefore the LG + I + Γ_8 model was used for MrBayes and TREE-PUZZLE tree reconstructions. PhyloBayes analyses for amino acid data were performed under the CAT + GTR/CAT + LG models. The LG model fit for a selected OTU was evaluated using the χ^2 test implemented in TREE-PUZZLE. Bootstrap support was evaluated on the basis of ML analyses by 100 bootstrap replicates. The alternative topologies were tested using the AU (Shimodaira 2002) and Kishino–Hasegawa (Kishino and Hasegawa 1989; Shimodaira 2002) tests (CONSEL [Kishino and Hasegawa 1989; Shimodaira 2002; Shimodaira and Hasegawa 2001]). The topologies were visualized using TREEVIEW (Page 1996), and site-wise log likelihood values were computed with the help of TREE-PUZZLE under the LG + I + Γ_8 model (Schmidt 2009). For MrBayes Markov Chain Monte Carlo (MCMC) computation, eight independent runs were conducted (each with eight chains) for 3,000,000 to 5,000,000 generations, sampling every 1,000 generations. The degree of convergence was assessed by PSRF (potential scale reduction factor [Gelman and Rubin 1992]) and average standard deviation of split frequencies. The trees generated before attaining to convergence were discarded. For PhyloBayes MCMC computation, four independent runs were performed with

30,000 cycles. The first 20,000 points were discarded. The alignment was deposited in the TreeBase under ID 13099.

Acknowledgements

We thank A. Kobseva and A. Smirnov for help with photos and movies, and H. Talanova for help with English. We are grateful to the staff of the Chebyshev and Lomonosov Supercomputer Center of the Moscow State University (http://parallel.ru/cluster) and the Bioportal of the University of Oslo (www.bioportal.uio.no), whose resources we used for computation. This work was supported by grants from the Russian Foundation for Basic Research (grants No 12-04-00154, 12-04-01486, and 12-04-90903), the Ministry of Education and Science of the Russian Federation (the program "Scientific and scientific-pedagogical personnel of innovative Russia"), a grant of the Deutsche Forschungsgemeinschaft GRK 1563 (RECESS), and by the program "Problems of life origin and biosphere development," launched by the Presidium of the Russian Academy of Sciences.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.protis.2012.08.001.

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