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Protistology

Reviews

Rozellids and aphelids: cryptic diversity and distribution in the environment

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Summary

Rozellids and aphelids represent the basal holomycotan lineages that attract attention as an intermediate link between protists and fungi and can shed light on their early evolution and phylogeny. They appear to be both abundant and diverse in many aquatic and terrestrial habitats all over the world, and in some ecotopes their abundance is comparable or even exceeds that of other holomycots. Despite this, these groups remain poorly studied. Few species belonging to these groups were isolated and analyzed at the organismal level; the diversity is known mainly from the metagenomic data. The sequences of rozellids and aphelids were found in the samples collected from a huge range of ecotopes and geographically distant habitats on all continents. The majority of sequences were isolated from freshwater as well as from activated sludge and wastewater. They were also found in soil, groundwater, perennial ice, invertebrate guts, marine and anoxic sediments. These organisms are able to survive extreme conditions, such as extremely low pH, low temperatures, and considerable oxygen depletion. They appear to have an important ecological role as parasites of zooplankton, zoosporic fungi and algae. The information on ecological properties of rozellids and aphelids is still scarce. Further metagenomic surveys of aquatic and terrestrial ecosystems are expected to enlarge our knowledge of their diversity and functional ecology.

Key words: rozellids, aphelids, metagenomics, metabarcoding, environmental sequences, cryptic diversity

Introduction

In the modern research of the diversity of eukaryotic microorganisms, application of high-throughput sequencing approaches, such as metabarcoding of universal marker genes, is playing an increasingly important role. This group of methods, also known as "environmental sequencing" or "metagenomics", is based on isolation of total DNA or RNA from samples collected in natural habitats, amplification of marker gene fragments, constructing of metagenomic libraries, sequencing using NGS (e.g., Illumina MiSeq platform) and subsequent phylogenetic identification (Bass et al., 2015; Taberlet et al., 2018; Burki et al., 2021). Metagenomic surveys of recent years have shown

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that the world of living organisms is significantly larger and more diverse than we could have imagined until now, studying the morphological diversity of life using classical methods (Eisen, 2007). A very small percentage of sequences obtained in the metagenomic projects correspond to known species, the rest of them are new species and often new phylogenetic lineages. Thus, so far, we have seen and known only the very tip of the iceberg, having a large diversity of pico- and nano-organisms missed (Šlapeta et al., 2005).

Metagenomic approaches have given new insights into the diversity of basal groups of Holomycota over the last 15 years. The early-branching holomycotan lineages, in particularly rozellids and aphelids, demonstrated enormous cryptic diversity (Richards et al., 2015; Tedersoo et al., 2017). Rozellida (Lara et al., 2010), also known as Cryptomycota (Jones et al., 2011a, 2011b; Karpov et al., 2014a), Rozellomycota (James and Berbee, 2012; Doweld, 2013; Corsaro et al., 2014b), and Rozellosporidia (Karpov et al., 2017b) is a highly diverse and widely distributed clade of the parasitic Holomycota. Their diversity is believed to be enormous; however, it is mainly cryptic since there are a lot of identified environmental sequences, from metagenomic studies, but only a few described representatives (Lara et al., 2010; Jones et al., 2011a; James and Berbee, 2012; Corsaro et al., 2014b; Karpov et al., 2017b).

What are rozellids and aphelids? Host range and life cycles for the known representatives

In the framework of this review, we use the term 'rozellids' *sensu lato*, although we are aware that this group is, in fact, heterogeneous. It includes the representatives of the genus *Rozella* and related clades (which can be considered as rozellids *sensu stricto*), and a large group of microsporidia-like organisms, which altogether were termed 'short-branch' microsporidia (Bass et al., 2018). Alternatively, these groups are recognized as different orders within the phylum Rozellomycota (Corsaro, 2022). It is a very diverse group, which includes the endoparasites of a broad range of eukaryotic organisms. Currently, the group includes five genera characterized at the organismal level. All genera except *Rozella* possess microsporidia-like morphological traits (Hoffmann et al., 1998; Michel et al., 2000, 2009a, 2009b, 2012; Corsaro et al., 2014a, 2014b, 2016, 2020).

• *Rozella* Cornu 1872 – includes 27 species infecting lower fungi (Chytridiomycota, Blastocladiomycota, Monoblepharomycota), oomycotes and green algae. Recent revision of the genus was provided by Letcher and Powell (2019).

• *Paramicrosporidium* Corsaro, Walochnik, Venditti, Steinmann, Müller et Michel 2014 – contains two recently described species (there is also a mentioning of one new species, not yet described – Corsaro, 2022). All of them are intranuclear parasites of Amoebozoa (Fig. 1, A, B).

• *Nucleophaga* Dangeard 1895, emend. Corsaro, Walochnik, Venditti, Müller, Hauröder et Michel 2014 – contains three valid species with modern descriptions. There are many reports of *Nucleophaga*-like parasites in early literature, summarized in Gordetskaya et al. (2019) and in Blackwell et al. (2019). All of them are intranuclear parasites of Amoebozoa (Fig. 1, C, D).

• *Morellospora* Corsaro, Walochnik, Venditti, Hauröder et Michel 2020 – the single recently described species is a cytoplasmic parasite of amoebae (Fig. 1, E, F).

• *Mitosporidium* Haag, James, Pombert, Larsson, Schaer, Refardt et Ebert 2014 – the single described species is a parasite of the water flea *Daphnia magna*.

There is a discussion (Mikhailov et al., 2022b; Kamyshatskaya and Nassonova, 2022) about whether the genus *Chytridiopsis* Schneider 1884 containing no less then eight species (recent revision of the genus was provided by Larsson, 2014) including the parasites of insects (Coleoptera, Trichoptera) and Myriapoda, should be classified among primitive microsporidia or among rozellids. There are several more genera close to *Chytridiopsis* from a morphological point of view (*Nolleria, Intexta, Burkea, Hessea, Buxtehudea*) that have not been studied using molecular methods. Their status, therefore, also remains uncertain.

Two fundamentally different variants of the life cycle have been described in rozellids. The first is typical for representatives of the genus *Rozella*. The dispersion stage is the flagellated zoospore. It finds the host and adheres to its surface. It then encysts on the host surface and forms a penetration tube that grows into the host cell. Through the tube, the parasite enters the host, where its trophont engulfs the cytoplasm by phagocytosis, grows, and eventually turns into a multinucleated plasmodium. The latter divides into flagellated zoospores that are

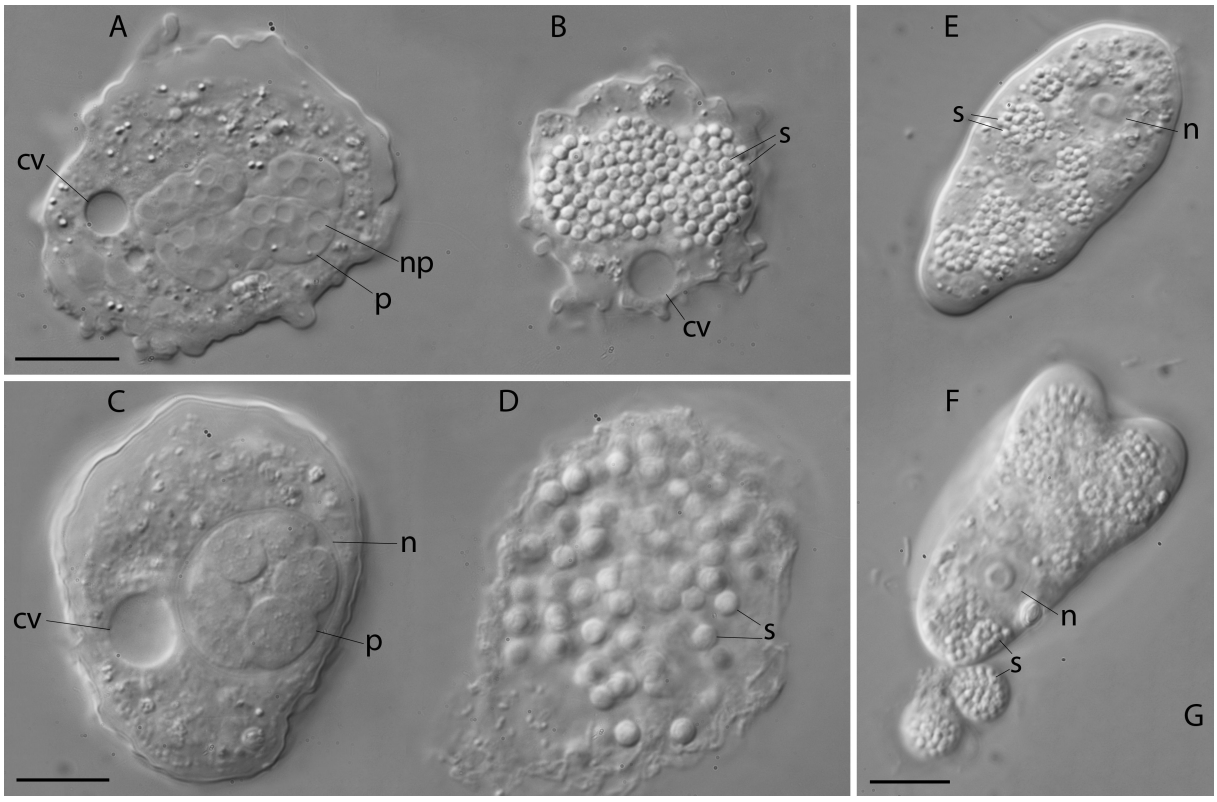

Fig. 1. Diversity of microsporidia-like parasites of amoeba, DIC. A–B – *Paramicrosporidium vannellae* KAUN, an intranuclear parasite of *Vannella* sp. (A – multinucleate plasmodia developing within amoeba nucleus; B – mature spores); C–D – *Nucleophaga amoebae* KTq-2, an intranuclear parasite of *Thecamoeba quadrilineata* (C – multinucleate plasmodia developing within the nucleus of amoebae; D – young spores in the dead amoebae cell); E–F – *Morellospora* sp. KSL8, intracytoplasmic parasite of *Saccamoebae* sp. *Abbreviations*: cv – contractile vacuole, n – nucleus of amoeba, np – nucleus of parasite, p – plasmodium, s – spore. Scale bars: 10 μm. Micrographs courtesy of Yelisei S. Mezentsev, St. Petersburg University. The cultures of parasites and amoebae were kindly provided by Rolf Michel (Germany).

released into the environment (Held, 1981; Letcher et al., 2017a, 2017b; Letcher and Powell, 2019; Karpov and Paskerova, 2020).

The second variant is characteristic of representatives of the genera *Paramicrosporidium, Nucleophaga*, and *Morellospora*. The infective stage of these parasites is an immobile spore with internal structures that can be interpreted as the organelles of invasion apparatus characteristic of microsporidian spores. Spores are phagocytized by amoeba. *Morellospora* develops in the cytoplasm of the host cell, while the proliferative stages of *Paramicrosporidium* and *Nucleophaga* by not yet clear way appear inside the cell nucleus. Inside the host, parasites actively grow and form a multinucleate plasmodium. Subsequently, it fragments into numerous mononucleate sporoblasts, from which spores are formed. After the spores occupy the entire volume of the nucleus or cytoplasm, the host cell is destroyed, and the spores end up in the environment (Corsaro et al., 2014a, 2014b, 2016, 2020; Blackwell et al., 2019; Gordetskaya et al., 2019). The nature of the life cycle and the structure of spores in these organisms show a certain similarity to microsporidia.

Aphelida Karpov, Aleoshin et Mikhailov 2014 (syn. Aphelidiomycota Tedersoo et al. 2018) are intracellular parasites and parasitoids of green algae, xanthophytes and diatoms with a moving dispersion and infective stage (zoospores or amoebae) and a phagotrophic intracellular stage (Karpov et al. 2013, 2014a, 2014b, 2016, 2017a, 2017b, 2020; Letcher et al., 2013; Seto et al., 2020). Four genera of aphelids, in total containing about 20 species were described:

- *Aphelidium* Zopf 1885, emend. Gromov 2000
- *Amoeboaphelidium* Scherffel 1925 (Fig. 2)
- *Pseudaphelidium* Schweikert et Schnepf 1996

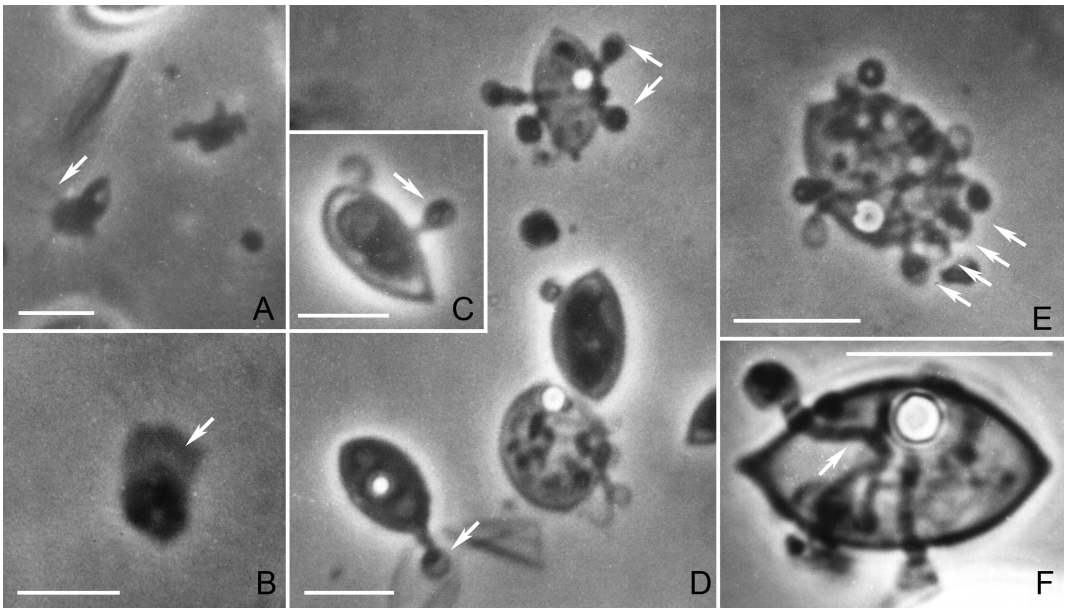

Fig. 2. Propagation of *Amoeboaphelidium protococcarum* X-1 in the culture of *Scenedesmus obliquus* CALU-13, Phaco. A – Amoeboid stage; two specimens, one showing flattened locomotive form with filopodia (*arrowed*) and another one demonstrating eruptive motility; B – amoeboid stage with clearly visible frontal zone of the hyaloplasm (*arrowed*); C – infective cyst (*arrowed*) on the surface of algae cell; D – various cases of infection: single, double and multiple (infective cysts are *arrowe*d); E – a case of very intensive infection; not less than seven parasites attack an algae cell (four closely located cysts of infection are *arrowed*); F – penetration tubes (*arrowed*) inside the completely digested algae cell. Scale bars: 5 μm.

• *Paraphelidium* Karpov, Tcvetkova, Mamkaeva, Torruella, Timpano, Moreira, Mamanazarova et López-García 2017a.

The life cycle of aphelids includes dispersal and trophic stages. A dispersal stage is a motile cell, which is either flagellated (*Aphelidium, Pseudaphelidium*), amoeboid (*Amoebaphelidium*, with a posterior immotile pseudocilia) or is an amoeboflagellate (*Paraphelidium*). The dispersal stage encysts upon contact with the host cell. From the cyst, an amoeboid sporoplasm invades the algae cell, where it begins to feed by phagocytosis. The intracellular amoeboid trophic stage phagocytizes the contents of the algal cell, occupying the entire volume of the host cell and turning into a multinucleated plasmodium. Having engulfed the cytoplasm of the host, the plasmodium divides into mononuclear zoospores (or into amoeboid cells) that are released through the penetration site (Karpov et al., 2016, 2017a, 2017b).

In addition to the limited number of morphologically described species, much higher environmental diversity of rozellids and aphellids is assumed, since a large number of environmental sequences of ribosomal RNA genes branching together with the known representatives of these groups were discovered during metagenomic studies (Jones et al., 2011a; Karpov et al., 2014a; Lazarus and James, 2015; Richards et al., 2015; Tedersoo et al., 2017; Bass et al., 2018).

The history of study and discovery of cryptic diversity of rozellids and aphelids

The first sequences of uncultivated rozellids were discovered from the experimental study of a eukaryotic community developed on algal detritus in continuous-flow systems filled with water from a freshwater lake. Three of the sequences designated as LKM11, LKM15, and LKM46 formed a unique 'terminal cluster' with the fungi as the closest relatives (van Hannen et al., 1999). This work provoked a surge of surveys on uncultivated fungi and their close relatives by environmental sequencing, i.e., by the analysis of 18S ribosomal RNA gene sequences amplified from environmental DNA. These studies demonstrated that some environmental sequences formed an important novel clade located at the basal position within Holomycota (Lefèvre et al., 2007). The study using tyramide signal amplification–fluorescent *in situ* hybridization (TSA-

FISH) showed that the cells targeted by LKM11 probes represented the second in abundance group among heterotrophs of a freshwater lake (Lepère et al., 2010). Lara et al. (2010) reported that the monophyletic and strongly supported clade LKM11, in addition to numerous environmental sequences, included only two sequences of known organisms – two sequenced species from the genus *Rozella*. They designated this group as Rozellida (but did not provide any formal description or taxonomic status) and formulated the hypothesis that this group of mainly unknown organisms might be composed largely (if not entirely) of parasites.

Jones et al. (2011a) proposed an informal name 'cryptomycota' (crypto, hidden; mycota, of the kingdom Fungi) for this group. Using TSA-FISH, they showed that the cryptomycotan cells that they detected in the probes were small eukaryotes of 3–5 μm in size, capable of forming a microtubule-based flagellum. They identified at least three morphotypes of these microorganisms: uniflagellate zoospores, variably shaped cells without flagella attached to other eukaryotic microscopic organisms (e.g., diatom hosts), and cysts with no flagella. Co-staining with calcofluor white and lectin wheat germ agglutinin demonstrated that representatives of the clade did not produce a chitin-rich cell wall. The authors suggested that the molecular diversity of this group might be as high as the rest of the known Fungi according to the rRNA gene markers. Later that year, Jones et al. (2011b) established the phylum Cryptomycota Jones et Richards 2011 and published its Latin diagnosis. The scientific name underlined the cryptic nature of this group and that they were initially revealed by molecular methods rather than using morphological approach.

James et al. (2013) published the results of sequencing and analysis of the genome of *Rozella allomycis* (11.9 Mb) and showed that Cryptomycota share many unique traits with microsporidia. In the phylogenomic trees, Cryptomycota occur to be the closest relatives of Microsporidia.

The phylogenetic reconstructions based on the rRNA genes and two protein-encoding genes (rpb1, rpb2) demonstrated that Microsporidia and Cryptomycota grouped together with another lineage of endoparasites, the aphelids (Karpov et al., 2013, 2014a). These three groups formed a distinct monophyletic clade ARM (Aphelida – *Rozella* – Microsporidia), which was a sister to Fungi. The new clade received a superphylum rank and was named Opisthosporidia Karpov, Aleoshin et Mikhailov, 2014.

Karpov et al. (2017b) proposed to call the group Rozellosporidia instead of Cryptomycota or Rozellomycota justifying this by the fact that "mycota" refers to classical fungi,while phago-trophic rozellids and the other opisthosporidia are evidently not similar to them. They also noted that in the name Cryptomycota, there is an incompatibility issue with the fungal nomenclature, as "Crypto" appears to allude to *Cryptomyces* or *Cryptococcus*, that are true fungal genera.

Hoffmann et al. (1998) and Michel et al. (2000, 2009b) discovered spore-forming intranuclear parasites of amoebae belonging to the genera *Vannella* and *Saccamoeba*. These organisms, named *Paramicrosporidium* spp., demonstrated some morphological similarity to microsporidia but branched within Cryptomycota in the rDNA-based phylogenetic tree (Corsaro et al., 2014b). This finding disclosed one more missing link to the evolutionary puzzle, filling the morphological gap between *Rozella* and microsporidia. Corsaro et al. (2014b) provided a broadened taxonomic characterization of the phylum Cryptomycota and proposed its renaming. They suggested an old name with a new meaning: Rozellomycota (James et Berbee 2012; Doweld 2013) emend. Corsaro et Michel 2014.

In the same year, *Mitosporidium daphniae*, a spore-forming parasite of *Daphnia magna*, was described. Its microsporidia-like spores possess the structures interpreted as the components of an extrusion apparatus (a short and thick polar filament, a polar sac and a rudimentary polaroplast). The phylogenomic analysis, which included six species of microsporidia and the only rozellid with the sequenced genome, *Rozella allomycis*, showed that *M. daphniae* branches at the base of the clade corresponding to Microsporidia (Haag et al., 2014). Moreover, this parasite retained the mitochondrial genome, which contains genes involved in the process of oxidative phosphorylation (however it lacks the complex I of the oxidative phosphorylation pathway), and its nuclear genome (5.6 Mb) is less reduced than the genomes of typical microsporidia. In the phylogenetic tree based on the analysis of the SSU rRNA gene, *M. daphniae* branches together with unknown endoparasite of plasmodial slime molds *Lamproderma* sp. described by Yajima et al. (2013) and with the environmental sequences from the clade LKM15 (Corsaro et al., 2016, 2018; Bass et al., 2018).

A relatively abundant and diverse uncultured lineage named Basal Clone Group I (BCGI) (Nagahama et al., 2011) or Novel Chytrid-Like-Clade

1 (NCLC1) (Richards et al., 2015) discovered in the marine water column and sediments have been shown to be intracellular parasites of diatom algae (Chambouvet et al., 2019). Using lineage-specific rRNA-targeted TSA-FISH, it was shown that NCLC1 cells form intracellular infection of diatom species from the genera *Chaetoceros, Skeletonema*, and *Pseudonitzschia*. In the phylogenetic reconstructions they are always found as the most basal branch of the rozellids, that is why Corsaro et al. (2020) suggested to call them Basal Marine Group A.

In the following years several isolates of microsporidia-like parasites infecting the amoebae were characterized and sequenced: *Nucleophaga amoebae* KTq-2, *N. terricolae* KTt-1 and *N. striatae* KTsa – intranuclear parasites of Thecamoebae (Michel et al., 2009a, 2009b, 2012, 2021; Corsaro et al., 2014a, 2016) and *Morellospora saccamoebae* KSL6 – intracytoplasmic parasite of Saccamoebae (Corsaro et al., 2020). It is interesting to note that microsporidia-like organisms are distributed throughout rRNA tree and do not form a single clade (Corsaro et al., 2014b, 2020). *Paramicrosporidium saccamoebae* KSL3 and *P. vannellae* KAUN formed an independent branch within the rozellid clade III *sensu* Lazarus and James (2015). *Morellospora saccamoebae* branched within the rozellid clade LKM15 (the clade X *sensu* Lazarus and James, 2015), which also includes *Mitosporidium daphniae* and a parasite of *Lamproderma* sp. The highly derived sequences of *Nucleophaga* spp. formed a long branch that grouped closely to microsporidia, hence with low support (Corsaro et al., 2014a, 2016). The backbone of the tree in the intermediate zone between microsporidia and rozellids is usually not resolved, which might have been caused by the rapid evolutionary rate and highly derived character of these sequences. Most of the sequences in this part of the tree form very long branches, while the distances at the basal part of the tree are much shorter, so the tree has low stemminess, which complicates recovery of correct topology (Smith, 1994).

The genome sequencing project on *Paramicrosporidium saccamoebae* (Quandt et al., 2017) demonstrated that this parasite has a rather canonical mitochondrial genome, similar to the mitochondrial genomes of fungi, which encodes the genes necessary for oxidative phosphorylation, including cytochrome oxidase genes. The nuclear genome of *P. saccamoebae* (7.6 Mb) shows many typically "fungal" features. It seems the evolution of the genomes within rozellids was accomplished with repeated and independent gene gains and losses, possibly because of changing parasitic strategies.

The relationship between the early-branching groups of Holomycota

The most intriguing question concerning the evolution of early holomycotan lineages is where the border between rozellids and microsporidia is. In the phylogenetic reconstructions based on the analysis of the available sequences of the SSU rRNA gene from the described rozellid representatives (*Mitosporidium, Paramicrosporidium, Nucleophaga, Rozella*), aphelids (*Amoeboaphelidium, Paraphelidium*) and a small sample of canonical microsporidia, as well as a representative set of related environmental sequences (SSU rDNA, V4 region), Bass et al. (2018) revealed a monophyletic clade with a fairly high level of support (96% ML bootstrap, 0.98 Bayesian posterior probability), which included most of rozellids (with the exception of sequences grouping with *Rozella* spp.), as well as metchnikovellids and canonical microsporidia. Based on this analysis, they concluded that the phylogenetic volume of Microsporidia is greatly underestimated, since most of the diversity previously considered to belong to rozellids actually belongs to microsporidia. It suggests a new understanding of the evolution of this group of highly specialized parasites. The authors suggested that these new microsporidia should be considered as 'short-branch' ones, since they are characterized by typically eukaryotic sequences in contrast to canonical Microsporidia (that are proposed to be called 'long-branch' microsporidia) with their highly derived, shortened sequences forming long tree branches. However, these conclusions were made from the analysis of alignment, which included a large number of short environmental sequences. To confirm the topology observed by Bass et al. (2018), it is desirable to include longer sequences in the analysis to get more genomic data from target groups to perform a phylogenomic analysis.

Traditionally, the basal groups of Microsporidia include chytridiopsids and metchnikovellids (Larsson, 2014). Phylogenomic studies of metchnikovellids confirmed their sister's relationship with core microsporidia (Mikhailov et al., 2017; Galindo et al., 2018; Nassonova et al., 2021), while genomic data on chytridiopsids remain missing. An analysis of the rRNA gene sequence of *Chytridiopsis typographi*, a

parasite of the bark beetle *Ips typographus*, placed it in the phylogenetic tree as a sister group to canonical Microsporidia + Metchnikovellida (Corsaro et al., 2018). The overall structure of *Ch. typographi* rRNA is more similar to typical eukaryotic rRNA than to a microsporidian one. Many regions in *Ch. typographi* rRNA are not that shortened as in canonical microsporidia. Its ITS2 region is also close to typical eukaryotic one. It suggests the processing of rRNA and the presence of separate 5,8S and 28S rRNAs (in typical microsporidia these regions are fused, while ITS2 is absent). The numerous autapomorphic deletions noted in a number of helices of *Ch. typographi* must be verified by an independent study of other isolates (Mikhailov et al., 2022a). Because of these differences and numerous nucleotide substitutions a branch of *Ch. typographi* in the microsporidian SSU rRNA tree is very long. It is the next in length after the branches of typical microsporidia. Long branch attraction artefacts may contribute to the positioning of this organism in the tree. In rRNA trees, the placement of *Ch. typographi* with typical microsporidia is not highly supported. In a number of trees, *Ch. typographi* occurs to be in the rozellid clade LKM15 (the clade X *sensu* Lazarus and James, 2015), which also includes *Mitosporidium daphniae*, *Morellospora saccamoebae* and a parasite of *Lamproderma* sp. (Frolova et al., 2021; Mikhailov et al., 2022a). The structure of the invasion apparatus in the spores of *Morellospora saccamoebae* and *Ch. typographi* is similar and this fact also supports its affiliation with the rozellids (Kamyshatskaya and Nassonova, 2022). To determine the true position of chytridiopsids, it is necessary to obtain genomic data for this organism and perform phylogenomic analysis.

Torruella et al. (2018) sequenced and analyzed the transcriptome of *Paraphelidium tribonemae*. Phylogenomic analysis of different sets of protein-coding genes suggested that the aphelids are the closest relatives of Fungi, excluding rozellids and microsporidia. Therefore, Opisthosporidia turns out to be a paraphyletic clade. The genomes of two species of *Amoeboaphelidium* (Mikhailov et al., 2022b) and the transcriptomes of two species of the genus *Aphelidium* (Galindo et al., 2022) are now available. Phylogenetic analysis including species from the three genera of aphelids convincingly rejected the Opisthosporidia hypothesis. In addition, comparative analysis of genomes also confirmed the hypothesis that the aphelids are a sister group to Fungi (Aphelida + Fungi). Molecular synapomorphies of this clade include 19 orthogroups, unique to these two lineages. Galindo et al. (2022) proposed to divide the former Opisthosporidia into two groups: Phytophagea (Aphelida + Fungi) and Opisthophagea (Microsporidia + Rozellida). The former is ancestrally more specialized in infecting and/or degrading photosynthetic eukaryotes, while the latter mostly feed on other opisthokonts.

The early-branching lineages of Holomycota are the subject of endless discussions between protistologists and mycologists for the right to classify them and to include in their sphere of interest. While some researchers believe that they are branching within the fungal radiation (and suggest the subkindoms Rozellomyceta and Aphelidiomyceta – Tedersoo et al., 2018a; Naranjo-Ortiz and Gabaldón, 2019; James et al., 2020), others consider them as protistan relatives of Fungi (Galindo et al., 2022; Mikhailov et al., 2022b). These groups should probably be considered as biregnal.

At present, the robust reconstruction of the relationship between the early-branching groups of Holomycota is rather problematic because genomic data is available only for a small fraction of species. The number of isolated and described species in these groups remains limited. However, as mentioned above, the metagenomic screenings demonstrated that the "cryptic" diversity of these groups is very high and the genetic distances between sequences are large (Jones et al., 2011a; Karpov et al., 2014a; Lazarus and James, 2015). Further development of modern morphological, molecular, ecological, phylogenomic and comparative genomic studies, involving a wider range of studied organisms from these groups, are required in order to trace the evolutionary history of early Holomycota. This probably will help us understand key morphological and genomic changes accompanying the evolution of these parasites, to demarcate the border between rozellids and microsporidia and, on a global scale, between fungi and protists.

Biogeographic distribution and inhabited ecotopes

The sequences of rozellids and aphelids were recovered from diverse habitats and geographical locations on all six continents (Figs 3 and 4). They appeared from freshwater environment, such as lakes (Lefranc et al., 2005; Šlapeta et al., 2005; Lepère et al., 2006, 2007, 2008; Mangot et al., 2009;

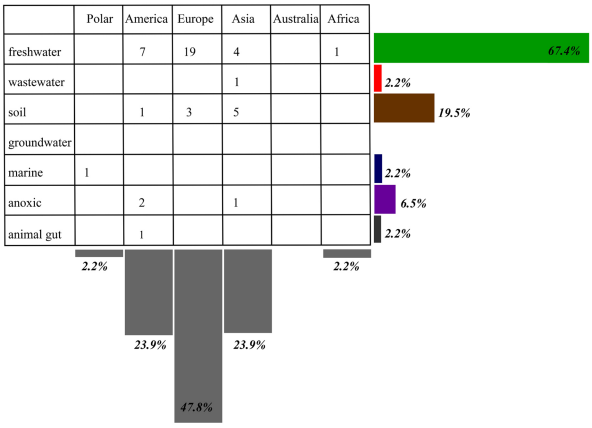

Fig. 3. Distribution of the sequences of aphelids within ecotopes of origin and regions of sampling.

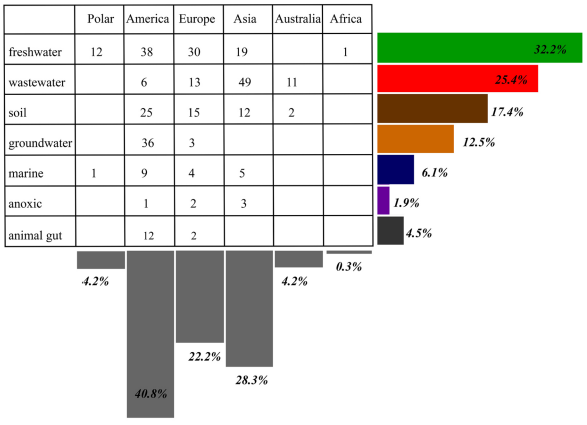

Fig. 4. Distribution of the sequences of rozellids within ecotopes of origin and regions of sampling.

Takishita et al., 2007a; Rojas-Jimenez et al., 2017), water springs (Luo et al., 2005), artificial water systems (van Hannen et al., 1999), wastewaters (Matsunaga et al., 2014). These organisms were also found in brackish-water and marine habitats (Savin et al., 2004; Massana et al., 2004; Takishita et al., 2005, 2007b; Livermore and Mattes, 2013; Richards et al., 2015; Wang et al., 2018; Chambouvet et al., 2019) including anoxic coastal sediments (Dawson and Pace, 2002) and deep-sea sediments (Nagano et al., 2010). Wide distribution of rozellids and aphelids in freshwater habitats makes it necessary to evaluate the functional role of these parasites in freshwater environment. Some authors suggested that, together with zoosporic fungi, they play a significant role in the microbial loop of freshwater ecosystems (Lefèvre et al. 2008; Gleason et al. 2014, 2017). In several studies, it was shown that the rozellids could act as parasites of freshwater phytoplankton (Jones et al., 2011a; Ishida et al., 2015; Lepère et al., 2019; Chambouvet et al., 2019).

Soil habitats (Freeman et al, 2009; Bass et al., 2018; Lepère et al, 2019) including the root rhizosphere (Lesaulnier et al. 2008) and oxygen-depleted environments (van Hannen et al., 1999; Dawson and Pace, 2002; Stoeck and Epstein, 2003; Takishita et al., 2005, 2007a; Matsubayashi et al., 2017) were also found to harbor rich diversity of rozellids.

Some rozellid sequences originate from extreme environments – e.g., river Rio Tinto (Spain), extremely acidic habitat with high levels of iron and heavy metals (Amaral-Zettler et al., 2002); anoxic hydrocarbon rich Zodletone Spring in the USA (Luo et al., 2005), in deep sea cold-seeps (Nagahama et al., 2011). Extremophiles would be expected among rozellids because some of their hosts, zoosporic fungi, were also found in extreme environments (Gleason et al., 2012). The rozellid and aphelid sequences were also documented from polar sampling sites (Nakai et al., 2012, Antonya et al., 2016, Rojas-Jimenez et al 2017) and perennial ice in the caves (Brad et al 2018).

Global soil fungal metagenomic survey by Tedersoo et al. (2014) showed very little differentiation of rozellids by ecotopes. No strictly habitat-specific clades were found for freshwater, soil or marine ecosystems (Livermore and Mattes, 2013). Most clades contain sequences obtained from geographically distant habitats and ecotopes of various types. Grossart et al. (2015) suggested that this finding points to a non-specific mechanism of dispersal without significant dispersal limitations. Currently it is reasonable to suggest that both rozellids and aphelids have ubiquitous distribution in the environment, while the geographic distribution and host range of individual species requires further studies. Biogeography of parasitic protists is generally described as associated with biogeography of their hosts. Since common hosts of rozellids and aphelids are different groups of protists, biogeorgaphic issues of free-living protist species may be applied to both of them as well.

Ecological and practical importance

Aphelids and rozellids are beleived to play a key role in regulating the size, composition and dynamics of populations of zoospore fungi, oomycetes

(heterotrophic stramenopiles) and phytoplankton. These organisms can be very abundant. In some cases, they may achieve absolute dominance in sequencing libraries over other protists (Taib et al., 2013; Debroas et al., 2015). In the study of DNA samples from the Rhode Island Estuary (USA, Atlantic Coast), up to 25% of all the "fungal" phylotypes appeared to be the sequences of rozellids and aphelids (Mohamed and Martiny, 2011). These widespread parasites (including highly virulent organisms) are considered among the key factors determining the complexity and stability of aquatic and terrestrial ecosystems. Fine-tuning of the structure and dynamics of food webs by fungi-like parasites may have a huge impact on the maintenance of various microbial communities (Gleason et al., 2012, 2014, 2017). Like other parasitic microorganisms, they influence the structure and dynamics of food networks, affecting both primary and secondary consumers. Preferred groups of hosts determine the ecological roles of these parasites as primary consumers (for parasites of producers) or secondary consumers (for parasites of heterotrophic protists and hyperparasites). It is suggested that they affect significantly the population of both primary and secondary consumers. Grazing zooplankton, including cladocerans, large ciliates and some filter-feeding metazoans, is known to feed on zoospores produced by zoosporic fungi and fungi-like organisms in freshwater ecosystems (Gleason et al., 2012, 2014). The zoospores of these organisms are smaller than 5 μm and would be expected to be proper food sources for many metazoans because of the high content of stored fatty acids and sterols ("mycoloop concept" by Kagami et al., 2014). In this way zoospores facilitate the transfer of carbon and energy from producers to tertiary consumers (Kagami et al., 2014; Gleason et al., 2017). However quantitative data on population dynamics of rozellids and aphelids are still lacking (Lefèvre et al., 2008).

Hyperparasitic organisms, numerous among rozellids, provide fine regulation of multicomponent host-parasite systems, ensuring their stability and polyvalence (Parratt and Laine, 2016). Recent studies have clearly shown that among microorganisms parasitic and symbiotic taxa prevail, and their diversity far exceeds any previous estimates. It is generally believed that every free-living species has parasites, if it is studied well enough. However, the parasites themselves can be the hosts of hyperparasites. There are often at least four parasitic species for any given host, and the better the host is explored, the longer becomes the list of parasites that can infect it (Windsor, 1998). The results of recent metagenomic studies confirm that the diversity of parasitic and symbiotic organisms far exceeds the number of free-living species. At the same time, the significant influence of parasitic and symbiotic organisms on the dynamics of food chains, biogeochemical cycles, ecosystem functioning and evolution of their hosts becomes more and more obvious (Grami et al., 2011, Gozlan et al., 2014, Kagami et al., 2014, Geisen et al., 2015). The life cycle of zoosporic hyperparasites is usually shorter than the life cycle of their hosts, so hyperparasites may accelerate the turnaround times of nutrients within the ecosystem. Hyperparasites may increase the complexity of food webs and play significant roles in regulating population size and population dynamics of their hosts. Sufficient role of (hyper)parasites is shown in suppression of animal, plant and algae pathogens, that is directly related to such applied problems as increasing the efficiency of agriculture, animal husbandry (in particular, commercial fisheries and aquaculture) and biotechnology production (Gleason et al., 2014, 2017).

Future perspectives

There is still a huge knowledge gap and the future research is essential to understand the phylogeny and ecology of the rozellids and aphelids. Poor knowledge of early lineages seriously complicates the reconstruction of a robust evolutionary scenario for the holomycotan branch of Opisthokonta. Further studies of rozellids and aphelids will unveil their cryptic environmental diversity, ecological role and significance in the evolution and origin of Fungi.

Small number of species, isolated and studied at the organismal level seriously limits phylogenomic research of these groups. One of the most actual and important tasks is to increase this number. 'Reverse metagenomics', e.g., using of TSA-FISH as well as application of fluorescence-activated cell sorting (FACS) and single-cell genomics (single amplified genomes for unculturable microorganisms – SAGs) could be a solution (Roy et al., 2014; Seeleuthner et al., 2018). Metagenome assembled genomes (MAGs) can be helpful in the more distant future (Massana and López-Escardy, 2022). TSA-FISH techniques could also provide further insights into the interactions between novel uncultivated parasites and their hosts in the environment. Classical methods, sometimes sadly and undeservedly

considered old-fashioned, can also be useful – e.g., extensive sampling and cultivation from the sites where the novel interesting clades were identified.

To infer co-occurrences between environmental lineages of parasites and their potential hosts, the network analyses for metabarcoding data can be applied (Doliwa et al., 2021). It is the first step to identify potential hosts of novel environmental lineages of parasites, which can be targeted in future both in molecular and microscopic studies.

The main disadvantage of metabarcoding by second-generation high-throughput technologies (e.g., Illumina MiSeq) is that it produces short sequences (e.g., short reads of the V4 region of the SSU rRNA gene below 500 bp), that provide limited phylogenetic information for species identification and taxonomic assignment. The third-generation Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT) instruments provide longer amplicons for metabarcoding – e.g., full-length internal transcribed spacer (ITS) barcodes and longer rRNA gene amplicons – up to 3000 bp (Tedersoo et al., 2018b, 2022; Jamy et al., 2020). However, the sequence quality in the case of PacBio barcodes remains slightly inferior to Illumina sequencing. Further development of technological and bioinformatic pipelines, which would allow us to overcome these shortcomings, will probably make PacBio amplicon sequencing a promising tool for studying novel organisms and hidden diversity and greatly improve taxonomic identification at the species and phylum levels.

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