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Discovery of dark matter fungi in aquatic ecosystems demands a reappraisal of the phylogeny and ecology of zoosporic fungi

Hans-Peter GROSSART[a,b,*], *Christian WURZBACHER*[a], *Timothy Y. JAMES*[c], *Maiko KAGAMI*[d]

[a]*Department of Experimental Limnology, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany*
[b]*Institute for Biochemistry and Biology, Potsdam University, Potsdam, Germany*
[c]*Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI 48109, USA*
[d]*Department of Environmental Science, Faculty of Science, Toho University, Funabashi, Chiba, Japan*

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ABSTRACT

Our knowledge of zoosporic fungal phylogeny, physiology, and ecological functions, in particular their role in aquatic food web dynamics and biogeochemistry, is limited. The recent discovery of numerous dark matter fungi (DMF), i.e., uncultured and poorly known taxa belonging to early diverging branches of the fungal tree (namely the Rozellomycota and Chytridiomycota) calls for reconsideration of the phylogeny and ecology of zoosporic fungi. In this opinion paper, we summarize the exploration of new, recently discovered lineages of DMF and their implications for the ecology, evolution, and biogeography of the rapidly growing fungal tree. We also discuss possible ecological roles of zoosporic fungi in relation to recent methodological developments including single cell genomics and cultivation efforts. Finally, we suggest linking explorative with experimental research to gain deeper insights into the physiology and ecological functioning of zoosporic fungi DMF in aquatic habitats.

Introduction

The existence of dark matter in the universe can only be inferred indirectly. With the advent of environmental DNA sequencing, microbiologists discovered an analogous phenomenon through the discovery of cryptic organisms known only from genetic material, which has been referred to as biological dark matter (Filée et al., 2005; Marcy et al., 2007). Molecular analyses of environmental DNA samples have also revealed an unexpectedly large diversity of undescribed fungi, the "dark matter fungi" (DMF). DMF are ubiquitous and abundant in the environment, but have not been cultured and are missing from taxonomies of the fungal kingdom. DMF are likely to be abundant across the entire tree, but are

* Corresponding author. Institute for Biochemistry and Biology, Potsdam University, Potsdam, Germany.
E-mail address: hgrossart@igb-berlin.de (H.-P. Grossart).
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particularly common on the early diverging branches of the fungal tree of life, many of which presumably represent zoosporic fungi thriving in aquatic ecosystems. These fungi have been interpreted as saprotrophic and/or parasitic organisms. Yet, in reality very little is known about their ecological functions, such as their role in food web dynamics and biogeochemical cycling of organic matter, nutrients and energy. The documentation of DMF in the early diverging branches of the fungal tree prompts a re-evaluation of how well we know the phylogeny and ecology of this group.

In this opinion paper, thus, we focus on the exploration of new, recently discovered zoosporic fungal lineages, i.e. Rozellomycota and Chytridiomycota, and their implications for ecology, evolution, and biogeography of the rapidly growing fungal tree. We will discuss possible ecological roles of basal fungi and propose future research directions in light of new methodological approaches, including genomics and stable isotope probing.

Discovery of new fungal lineages and implications for our perception of fungal ecology

In the past 10 years, several new fungal phyla, classes and orders have been discovered and placed at the base of the fungal tree. Five morphological types can be distinguished: (a) Microsporidia (Microsporidiomycota); (b) flagellated fungi (Chytridiomycota, Monoblepharidomycota, Neocallimastigomycota, Blastocladiomycota and the asexual genus *Olpidium*; Hibbett et al., 2007; Voigt et al., 2013; Powell and Letcher, 2014); (c) the formerly aggregated group of Zygomycota with non-motile walled spores, which has now been separated into several fungal phyla and subphyla; (d) Rozellomycota (Cryptomycota) (Corsaro et al., 2014b; Jones et al., 2011); and (e) Aphelida (Karpov et al., 2013). We have only limited knowledge of these basal fungi which account for a very small proportion of described species (~2300 species; Kirk et al., 2008). All these lineages are "old" in evolutionary terms and diverged from the remaining fungi 710–1060 million years ago (Lücking et al., 2009) before the evolution of higher land plants and their fungal symbionts (Glomeromycota). In particular, the recent establishment of the fungal phylum currently named Rozellomycota (Corsaro et al., 2014b) or Cryptomycota (Lara et al., 2010; Jones et al., 2011) has had an enormous impact on fungal taxonomy. This group appears to be hyper-diverse, but has been documented almost exclusively through environmental DNA surveys. This phylum is considered the most basal branch to all other fungal phyla, and thus may have retained some of the most ancestral characters shared throughout the fungal kingdom. Importantly, aquatic habitats have been reported to harbor many taxa of Rozellomycota (James and Berbee, 2012; Livermore et al., 2013; Wurzbacher et al., 2014; Lazarus and James, 2015; Ishii et al., 2015).

Taxonomic matters are complicated because there are convincing arguments for placing the well-known Microsporidia (intracellular parasites of about 1300 described species) within the Rozellomycota (James et al., 2013; Corsaro et al., 2014a; Haag et al., 2014). There are currently only a handful of described species in the Rozellomycota and they are distributed among three genera (Fig. 1). Interestingly, a recently described microsporidian, *Mitosporidium daphniae* (Haag et al. (2014), placed in the LKM15 clade in Fig. 1 along with the species described in Yajima et al. (2013)), has a number of ancestral features that make it more like the Rozellomycota, including unfused rRNA genes, and a mitochondrial genome. From the perspective of gross morphology, *Mitosporidium* is very microsporidian-like, and whole genome phylogenomic analyses place it together with Microsporidia, instead of with *Rozella*, though with limited taxonomic sampling (Haag et al., 2014). In the more densely sampled rRNA analyses, it is placed in the LKM15 group of Rozellomycota (Fig. 1), as is the species described by Yajima et al. (2013) as an endoparasite of plasmodial slime molds. The intermediate phylogenetic nature of *Mitosporidium* bolsters the argument to merge Microsporidia and Rozellomycota into a single fungal phylum. Both groups seem to have intracellular stages in common and have an injection apparatus that may be homologous (James et al., 2013; Yajima et al., 2013; Corsaro et al., 2014a, 2014b; Haag et al., 2014). Another interesting fact is that both groups can have a very thick, presumably chitinous, cell wall (Yajima et al., 2013).

Although the current status and naming of the Rozellomycota and relatives remains under discussion, the increasing number of observations and described species point towards the unification of Microsporidia and Rozellomycota. Karpov et al. (2014b) have already proposed unifying the Rozellomycota, Microsporidia and the Aphelida (parasites of algae) into a new superphylum, the Opisthosporidia, as a sister clade to the Fungi. In contrast, other authors such as James and Berbee (2012) consider Rozellomycota to belong to the Eumycota. The phylogenetic joining of Rozellomycota and Microsporidia into a single basal branch has received increasing support as more and more genomic data becomes available and sampling of taxa intensifies. However, the consideration of the clade as Eumycota challenges traditional morphological and biochemical definitions of the kingdom. One of the most commonly accepted circumscriptions of the fungal kingdom relies on the occurrence of chitin in their cell wall. Both Rozellomycota and Microsporidia have chitinous spores, but it is as yet unknown if they take up nutrients across a chitinous cell wall. It therefore remains controversial as to whether Rozellomycota, Microsporidia, and Aphelida should be considered a part of Eumycota or not (Fig. 2A).

Current ecological knowledge of DMF

DMF are common in aquatic ecosystems, and numerous amplicon based metagenomic surveys find that uncultured lineages of Chytridiomycota are more common than the well characterized ones (Monchy et al., 2011; Jobard et al., 2012; Lefèvre et al., 2012). Is it possible to assign any of these environmental species to formally described species? Although there are taxonomic keys to chytrids (e.g., Sparrow, 1960), their simple morphology (Powell, 1993; McLaughlin et al., 2009) makes it very difficult to identify and classify them at the genus let alone species level solely by microscopic techniques (Rasconi et al., 2012). Chytrid identification increasingly relies on a combination of ultrastructure and molecular data. This approach has revealed that many traditional chytrid genera

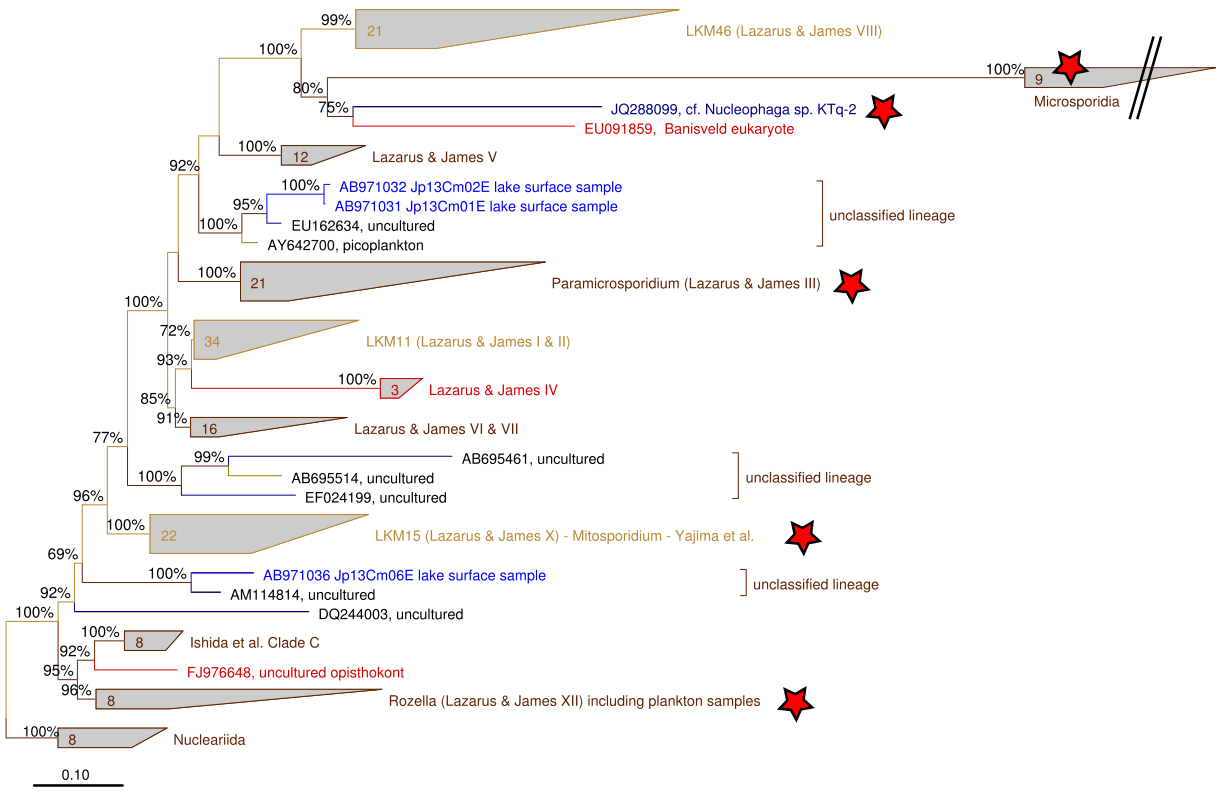

Fig. 1 – Rozellomycota lineages. Lineages and species representatives of basal fungi including Microsporidia and Rozellomycota based on an SSU rRNA gene phylogeny. The red stars mark lineages with described species. Red lineages and sequences have bad pintail values which point to potential artifacts (http://www.arb-silva.de/documentation/faqs/). Groups IX and XI of Lazarus and James (2015) were excluded. For references see: Lazarus and James (2015); Ishida et al. (2015).

are polyphyletic (e.g., Letcher et al., 2012; Karpov et al., 2014a). Often there are obvious mismatches between morphological descriptions based on drawings and photographs and similarities of ribosomal DNA sequences (Ishida et al., 2015). Therefore, inference on a chytrid's ecological role based on the literature is elusive due to the difficulty of assigning names to environmental sequences that match poorly to the databases. In addition to such mismatches, the great majority of Chytridiomycota sequences from aquatic habitats has rarely been cultured and studied, and is thus classified as "uncultured". This has resulted in a number of environmental clades based on the ribosomal SSU marker (Jobard et al., 2012; Ishida et al., 2015), and three for the ribosomal LSU marker (Lefèvre et al., 2012). Some of these lineages can be very prominent in aquatic surveys (see summary of Lefèvre et al., 2012), suggesting that it is imperative that a better way of characterizing them is found.

Likewise for Rozellomycota, Jones et al. (2011), Livermore et al. (2013), and Lazarus and James (2015) clearly demonstrated their almost ubiquitous distribution in all habitat types. The biome where they were discovered reflects their phylogenetic structure in terms of frequency, but there seem to be no strictly habitat-specific clades for freshwater, soil, or marine ecosystems (Livermore and Mattes, 2013). Some of the more peculiar habitats, where their presence has been documented, include waste water (and extreme waters such as Rio Tinto in Spain or the anoxic hydrocarbon rich Zodletone spring in USA), animal guts, anoxic freshwater and marine habitats, deep sea cold-seeps (Nagahama et al., 2011), and aquifers (Livermore and Mattes, 2013). As with Microsporidia (Stentiford et al., 2013), aquatic sources are still the richest sources for Rozellomycota where they show the highest abundance and diversity (James and Berbee, 2012; Livermore et al., 2013; Lazarus and James, 2015) or where they may even reach absolute dominance in sequencing libraries compared to other protists (Taib et al., 2013; Debroas et al., 2015). Gleason et al. (2012) discussed the potential ecological role of Rozellomycota as hyperparasites of zoosporic fungi in plankton communities (see also Held, 1980 for the known host range of the genus *Rozella*, which includes for example non-fungal Oomycetes). In comparison, Lepère et al. (2010) found them to be most abundant in the hypolimnion and metalimnion or close to the benthic layer, which argues against hyperparasitism as their dominant ecological niche. However, information on their ecology is scarce. An analysis of the data for a global soil fungal metagenomic survey by Tedersoo et al. (2014) shows very little differentiation of Rozellomycota by biome as opposed to Chytridiomycota (Fig. 3). This finding points to a non-specific mechanism of dispersal without significant dispersal limitations. The highest rank explanatory

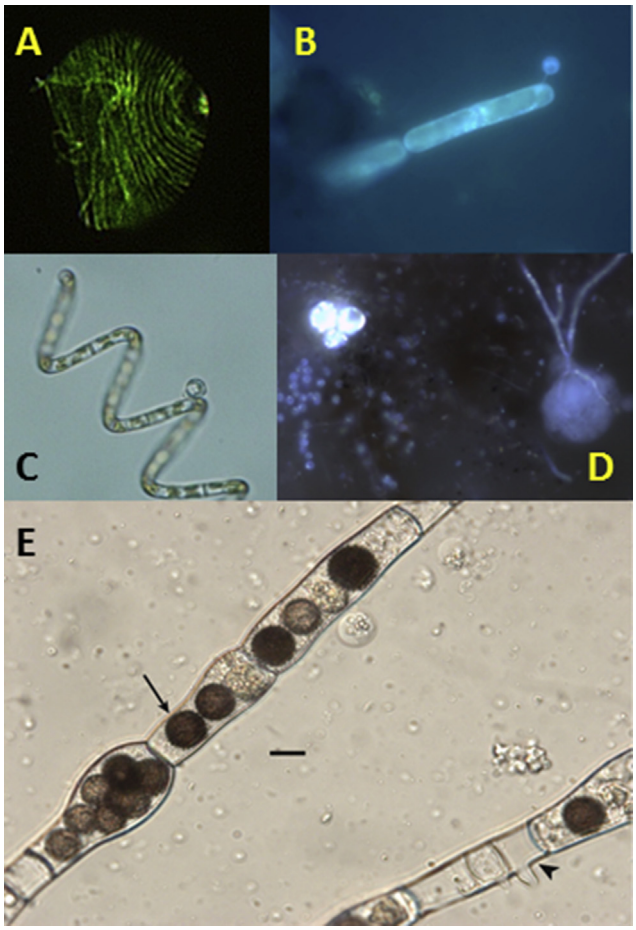

Fig. 2 – Dark matter fungi in the environment. (A) A planktonic chytrid sporangium (15 μm diameter) stained with the CBD chitin-staining procedure (Wurzbacher and Grossart, 2012) revealing single chitin fibrils; (B) A chytrid-like sporangium (4 μm diameter) sitting on an unknown rod-shaped cell (possibly Leptomitales, Oomycota) both stained by Calcofluor White; (C) Single spore analysis (6 μm diameter) revealed that the fungus attached to the diatom *Aulacoseira ambigua* belonged to Rozellomycota (Nozaki et al., unpublished, Clade C in Fig. 1). Photo was taken by Daiki Nozaki. (D) Various saprotrophic fungi, including a chytrid-like sporangium, in a lake sedimentation trap stained with Calcofluor White. (E) *Rozella allomycis* parasitizing the Blastocladiomycete *Allomyces macrogynus*. Hyphal form is produced by the host, and the endoparasite converts cells into numerous spiny, brown–red, resting sporangia (arrow) and zoosporangia (arrowhead) with a discharge papillum.

variable was nitrogen for Rozellomycota and pH for Chytridiomycota (Tedersoo et al., 2014), which may be related to the correlation of lake fungi with nitrate (Mangot et al., 2013). Overall, basic knowledge on the ecology of DMF is still lacking, but it is likely that the Rozellomycota and Chytridiomycota behave similarly to picoplanktonic eukaryotes (or micro-eukaryotes, i.e. eukaryotes smaller than 5 μm; e.g., Mangot et al., 2013; Lepère et al., 2013). For example, Lepère et al. (2013) found that regional lacustrine microeukaryote communities show a pronounced area–species relationship (similar to an island colonization pattern) and that geographical distance is the most important factor for changes in community composition. This raises important questions on micro-diversification of the Rozellomycota in freshwater systems and might explain their large contribution to rare taxonomic units in environmental surveys (Mangot et al., 2013; Taib et al., 2013; Debroas et al., 2015). Especially for aquatic habitats, there is still a huge knowledge gap and future research needs to target phylogeny, physiology and ecology of this interesting and ecologically relevant fungal group.

The "mycoloop" concept

The Chytridiomycota have attracted the attention of mycologists due to their potential for negatively affecting phytoplankton communities, and altering phytoplankton dynamics throughout the season (Canter and Lund, 1953). More recently, Chytridiomycota have been shown to effectively channel organic matter and energy to higher trophic levels, a mechanism which has been termed the "mycoloop" (Kagami et al., 2014). Chytridiomycota zoospores are grazed by zooplankton, especially by *Daphnia* (Kagami et al., 2004). They provide an excellent food source for zooplankton in lakes, due to their nutritional quality (e.g., high contents of PUFAs and cholesterols) and their high abundance (ranging from 10^1–10^9 spores l^{-1}) (Kagami et al., 2014). Zoospores may become particularly important for *Daphnia* when inedible food sources predominate (such as large phytoplankton, cyanobacteria and pollen). The chytrid zoospores derived from these sources provide the "mycoloop" by transferring material from inedible particles to zooplankton. In this way zoosporic fungi play an important role in shaping aquatic systems by altering food web dynamics, sinking fluxes or system stability (Kagami et al., 2014). The parasitic chytrids infecting cyanobacteria potentially improve the nutritional quality of cyanobacteria by adding sterols (Kagami et al., 2007), or by rendering them edible by fragmenting large filaments or colonies (reviewed in Sime-Ngando et al., 2012; Kagami et al., 2014). Such interactions may also occur in marine ecosystems. For example, the parasitic chytrid *Dinomyces arenysensis* is known to infect dinoflagellates (some of them toxic, such as *Alexandrium* spp.) in coastal areas and could serve as food for marine zooplankton (Lepelletier et al., 2014). Some Rozellomycota taxa have a similar life cycle to Chytridiomycota with a free-swimming stage (Jones et al., 2011) and infect various organisms (Fig. 2B) including large diatoms (Ishida et al., 2015, Fig. 2C). In addition, saprotrophic Chytridiomycota that decompose pollen have the potential to facilitate zooplankton growth in lakes where resource subsidies including pollen have a significant impact (Masclaux et al., 2011, 2013). Another important aspect is that most zoospores can only survive a couple of days (at least of some Chytridiomycota, Bruning, 1991), yet, it needs to be determined to what extent the non-grazed zoospores contribute to the dissolved organic matter (DOM) and detritus pool in aquatic systems and how this material flows through aquatic food webs. Further studies with advanced molecular tools may unravel the ecological role of zoosporic fungi in various aquatic ecosystems.

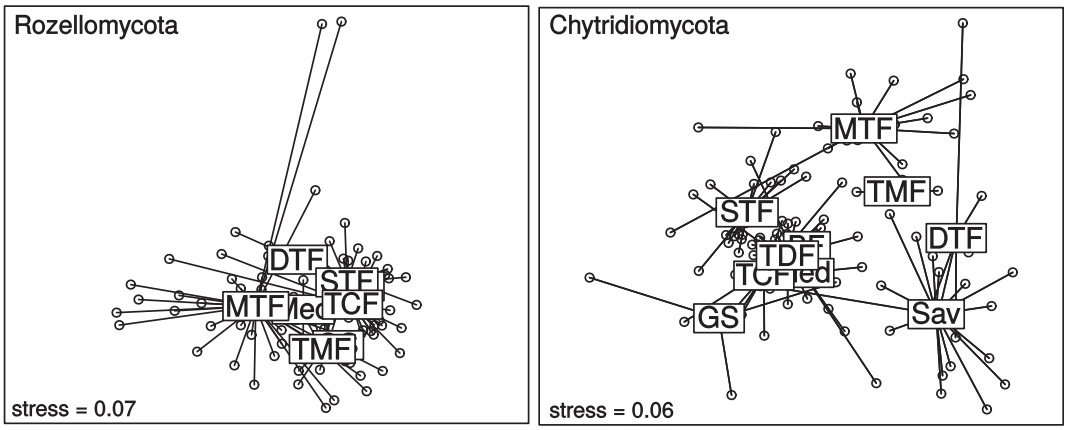

Fig. 3 – The effect of habitat on community structure of zoosporic fungi. Reanalysis of the data from Tedersoo et al. (2014) obtained from terrestrial biomes at low resolution (20–338 sequences per sample). Non-metric multidimensional scaling based on the Bray–Curtis distance of percentage normalized community matrices for Rozellomycota and Chytridiomycota classified operational taxonomic units. BF: boreal forest, DTF: dry tropical forest, GS: grassland and shrubland, Med: Mediterranean, MTF: moist tropical forests, Sav: savannas, STF: southern temperate forests, TCF: temperate coniferous forests, TDF: temperate deciduous forests, TMF: tropical montane forests.

Quantifying matter and energy flows of the mycoloop *in situ*, and comparing its relative importance to the "microbial loop", which is mainly based on the heterotrophic metabolism of bacteria, remains challenging. For more accurate comparison of their respective ecological relevance, linear inverse modeling analysis can be applied (Niquil et al., 2011), which requires comparing the relative abundances of heterotrophic nanoflagellates (HNF) and *Chytridiomycota* zoospores via CARD (catalyzed reporter deposition) – or TSA (tyramide signal amplification)-FISH (fluorescence in-situ hybridization), real-time qPCR, or various biomarkers and antibodies (Lefèvre et al., 2007; Mangot et al., 2013, Jobard et al., 2010; Wurzbacher and Grossart, 2012). Direct quantification of *Daphnia* zoospore consumption is another suitable approach to evaluate the ecological importance of the mycoloop in the field. Quantifying the amount of zoospores in *Daphnia* guts by RT qPCR is now possible (M. Kagami, unpubl. data) and can be used to reliably determine the relative importance of the mycoloop. Experimental manipulations together with isotopic analysis and labeling can be combined with various molecular approaches to more reliably detect and quantify matter and energy flow through the mycoloop.

Interactions between Chytridiomycota and their hosts

The roles of Chytridiomycota as parasites are becoming more and more appreciated, such as parasitism of cyanobacteria (Gerphagnon et al., 2013) and as causative agents of the global amphibian decline (e.g., Longcore et al., 1999). In general, obligately parasitic Chytridiomycota are considered to be highly species- and even cell type-specific (Kagami et al., 2007). In the case of the diatom parasite *Zygorhizidium formosa*, the adaptation of a laboratory strain to a particular host genotype has been observed after serial passaging (de Bruin et al., 2008). Agha et al. (2014) also confirmed that cyanobacteria (*Microcystis*) consist of different genotypes (strains) which may differ in their susceptibility to Chytridiomycota infection. Interestingly, this genotype specificity to chytrid infection could be also detected in a lake ecosystem (Gsell et al., 2013). The authors suggest that a high prevalence of chytrid parasitism can promote genetic diversification of natural populations of clonal diatom hosts (*Asterionella formosa*), and thus leads to selection of host genotypes that are not susceptible to the prevailing parasite genotype. Hence, genotype-specific parasitism represents an important mechanism to maintain a high-genotypic diversity even of clonal organisms. It is, however, still unclear how host specificity is controlled or how it has evolved. There is no doubt that host specificity is already relevant at the stage of finding and attaching to the host (chemotaxis) and later during encystment of the zoospores (Kagami et al., 2007). The decline of cyanobacteria populations and their susceptibility to microbial infections was related to the loss of surface lectins (Müller and Sengbusch, 1983), which may serve as recognition patterns for parasites. Recently, toxic substances, e.g. cyanobacterial microcystins, were shown to affect Chytridiomycota virulence of highly host-specific strains (Rohrlack et al., 2013). On the other hand, Chytridiomycota with low host specificity have been discovered on diatoms, i.e. certain strains of Chytridiomycota can infect several diatom hosts (Canter and Jaworski, 1978; Kagami et al., 2007; Ishida et al., 2015). Conversely, several diatom species (hosts) can be infected by several parasitic fungi, such as Chytridiomycota, Rozellomycota and other DMF (Ishida et al., 2015). We can thus postulate that several parasitic strategies exist, which vary in their host specificity and life cycle. Host-parasite interactions, including coevolution and defense mechanisms, may need to be examined when considering the function of DMF. Single cell PCR together with parasite isolation and cross infection experiments may be essential to unravel specific host/parasite subpopulations with different ecological traits. Paleolimnological analysis can

be used to examine long-term changes in host-parasite abundance and geno-/chemo-types in lake sediment cores (Kyle et al., 2015). This may also reveal past host-parasite coevolution in nature and unravel general mechanisms driving these patterns.

Disease dynamics

The severity of epidemics is determined by the densities of both host and parasite, since successful infection is density dependent. Environmental abiotic factors, such as temperature and nutrients, affect both density and growth of the host and parasite, and these factors influence the severity of epidemics (reviewed by Ibelings et al., 2003; Kagami et al., 2007). Therefore, anthropogenic changes, such as eutrophication and global warming, are expected to alter disease dynamics, yet little is known concerning the details of these interactions, which makes predictions difficult (Johnson et al., 2007; Ibelings et al., 2011). For instance, increasing temperature may not always increase the severity of the fungal infection, which renders the consequences of climate change idiosyncratic (Ibelings et al., 2011). The survival of the parasite depends not only on abiotic factors, but also on food web structure and biological interactions. Intensive predation by zooplankton on free-living *Chytridiomycota* zoospores reduces the risk of infection and hence mortality of the host. This has important implications for disease development and thus food web dynamics. *Daphnia* grazes zoospores not only of pathogens infecting phytoplankton, but also those of chytrids infecting amphibians (*Batrachochytrium dendrobatidis*) (Kagami et al., 2004; Hamilton et al., 2012; Searle et al., 2013). To predict the complex disease dynamics and tri-trophic interactions (host/chytrid/zooplankton), experimental manipulation and modeling approaches are required (Miki et al., 2011; Duffy et al., 2005). Johnson et al. (2007) examined the dynamics of *Daphnia pulicaria*, a keystone zooplankter in lake ecosystems, to explore the long-term causes and consequences of infection by a Blastocladiomycetes parasitoid (*Polycaryum laeve*). This study nicely showed that host-parasite interactions are determined by a complicated interplay between biological (e.g., food web interactions) and specific environmental factors (e.g., thermal stratification). Hence the ecological role of the newly discovered fungi can only be understood when also taking into account their multiple interactions within the food web, as well as their responses to several environmental parameters. To better unravel the ecological consequences of such interactions, modeling approaches taking into account the complex spatio-temporal dynamics of these interactions seem to be an appropriate tool (Johnson et al., 2007; Miki et al., 2011).

Methodological considerations to explore the ecological role of DMF in aquatic food webs

To explore the parasitic or saprotrophic nature of DMF – an important topic both in terms of fungal evolution and the flow of matter through aquatic and terrestrial food webs – new methodological approaches are needed. There seems to be no clear boundary between saprotrophic and parasitic lifestyles and the transition between saprotrophs and parasites may simply be determined by environmental parameters. For example, the well-known amphibian parasite *B. dendrobatidis* grows well on agar plates containing only tryptone (Longcore et al., 1999) and can persist for up to 6 weeks after inoculation into autoclaved lake water (Johnson and Speare, 2003), suggesting that it may be a facultative parasite.

On the other hand, many of the basal fungal lineages are known to be obligate parasites, which have led to their absence in bait and pure media culture studies, and consequently many of the DMF are likely to be parasites as well. For example, all formally described Rozellomycota, Microsporidia, and Zoopagales are obligate parasites. The absence of primary components of metabolism from the genomes of these early lineages shows that they are host-dependent for essential aspects of primary metabolism (James et al., 2013). In other groups, where obligate parasitism is not consistently observed, e.g. Chytridiomycota and Blastocladiomycota, being a facultative or obligate parasite may be a species-specific characteristic, and does not necessarily reflect broader trends within taxonomic families or orders. Since 2013, we know that certain species such as *Rhizidium phycophilum* (Chytridiaceae) can enter at least a facultative mutualistic relationship with their algal host (Picard et al., 2013), demonstrating the complexity and context dependent nature of symbiosis between zoosporic fungi and other organisms. Therefore, molecular sequences and their taxonomic assignments may not be sufficient to classify the discovered fungi either as parasites or saprotrophs under given environmental conditions. Even though single spore PCR analysis can be used to detect fungi attached to phytoplankton without culturing (Ishida et al., 2015), this does not differentiate between obligate parasites of diatoms and taxa that also possess a saprotrophic lifestyle. However, not only parasitic, but also saprotrophic fungal lifestyles can be of great ecological relevance. For example, saprotrophic Chytridiomycota and Rozellomycota are commonly found on pollen, which occurs in huge quantities in lakes, particularly during the clear-water phase in spring when organic matter and nutrients are low (Wurzbacher et al., 2014). These fungal groups are also found in sedimentation traps in the aphotic zone of lakes (Fig. 2D). Full and partial genomes from single cell genomics or metagenomes are powerful methodological tools to identify certain key functional genes indicative of either a saprotrophic or parasitic lifestyle, and together with meta-/transcriptome approaches one might be able to reliably determine potential nutritional modes on the basis of enzyme coding potential.

If metagenomes or single cell genomes can furnish near complete assemblies, the absence of many genes of primary metabolism may point to an obligately biotrophic lifestyle. For example, the genomes of *Rozella* and *Mitosporidium* species show varying degrees of loss of essential genes that are required for an independent lifestyle, such as synthesis of sterols and nucleotides (James et al., 2013; Haag et al., 2014). Parasites might also be identified through the presence of various proteins involved in pathogenesis. Although there are no smoking guns for parasitism, the CRN domain or Crinkler proteins are a class of effectors (proteins that are secreted into a host and modify their behavior) that are known only from parasites or biotrophic organisms (Sun et al., 2011; James et al.,

2013; Velasquez et al., 2014). Other potential marker genes for parasitism are the adhesins, known virulence factors involved in host attachment (Wizemann et al., 1999). Further, it might be possible to identify obligate fungal parasitism by analyzing genome-wide patterns of parasitism-relevant genes in order to search for a "two-speed genome", analogous to what has been done for the Oomycete plant pathogen *Phytophthora infestans* (Haas et al., 2009). Lastly, the presence of various types of carbohydrate active enzymes (i.e. CAZys; Lombard et al., 2014) and proteases can be involved both in attacking the host and in immune evasion (McKerrow et al., 2006), or can suggest potential types of hosts and substrates that may be utilized. Many examples exist where specific enzymes identified in genomes point to potential nutritional modes in fungi. For example, the amphibian pathogen *B. dendrobatidis* genome differs from its most closely related sister taxon *Homolaphlyctis polyrhiza* (a non-pathogenic species) by the copy numbers of three protease gene families (Joneson et al., 2011). Pectinases are compounds that are associated with the attack of plant cell walls, and among six zygomycete and chytridiomycete genomes investigated, only one of these (*Allomyces macrogynus*) contained genes encoding for this enzymatic function (Zhao et al., 2013). In a last example, the sucrose utilization enzyme invertase was found in essentially all genomes of endophytes, plant pathogens, and plant associated saprobes, but noticeably absent in mycorrhizas (Parrent et al., 2009). Future analyses will be needed to determine how conserved and useful the various types of proteins (e.g., effectors and enzymes) and analyses are for the distinction between parasitic and saprotrophic fungi.

Future research towards a better understanding of zoosporic fungal ecology

To explore the whole fungal diversity in hitherto understudied aquatic habitats, it will be important to develop molecular tools that allow reliable, simultaneous detection of all members of the fungal tree, in particular of DMF. Whereas methods based on specific primers are often biased by primer selectivity (false positive and false negative matches), universal eukaryotic primers offer the advantage of being less selective and allowing the detection of DMF. They also allow estimates of the relative proportion of fungi in relation to all eukaryotes in the studied habitat. Because of the increasing throughput of next-generation sequencing methods, one can discard up to 90% of a dataset (because it is non-fungal) and still retain large numbers of fungal DNA sequences for analysis. Moreover, universal eukaryotic primers facilitate the discovery of novel DMF lineages, some of which may diverge from Eumycota even earlier than Rozellomycota or between Rozellomycota and Chytridiomycota. Alternatively, a combination of ITS with LSU databases increases the phylogenetic resolution of the fungal primers. This approach would combine the phylogenetic information of the well-established, but rapidly evolving, ITS sequences with the much less often used LSU sequences to achieve a higher and more reliable phylogenetic inference. For Chytridiomycota, this approach has been well established (Letcher et al., 2008; Lefève et al., 2012). Further, a combined SSU and LSU approach could be useful for linking environmental clades from SSU and LSU studies to clarify the diversity of lineages in freshwater systems that have been separately studied with these two markers. Once high-quality fungal sequences have been generated, hybrid capture methods can further expand the diversity or may be used to cover the even more variable flanking parts of the ribosomal region (Tsangaras et al., 2014). These data can in turn be used to specifically study abundance and phylogenetic diversity of selected fungi by developing real time quantitative PCR (qPCR) assays.

To explore the metabolic potential of specific fungi, metagenomic and metatranscriptomic approaches should be applied, taking into consideration possible limitations of the knowledge of assigned functional genes (incompleteness and reliability of databases). Furthermore, single cell genomics may be even more powerful for linking genes into units that function together, i.e. species. Identifying the functionality of specific genes remains dependent on our knowledge of the precise physiological role of the detected genes and their products. These databases will inevitably become more informative as additional studies of physiological functions of specific genes or gene clusters are completed. In the interim, as new reference genomes of species of known ecological function are added to databases, clusters of orthologous genes that are specific to particular ecological guilds can help us refine possible functions of genes and species.

To study the ecological role of certain fungal groups in natural aquatic ecosystems, a combination of high resolution and high throughput sequence analyses and analyses of environmental features can be used. Applying network analyses can reveal co-occurrence of fungal taxa and correlations with specific physico-chemical and biological variables. Such dependencies can be experimentally tested by SIP (stable isotope probing) and/or nanoscale secondary ion mass spectrometry (NanoSIMS) in combination with FISH for assessing the metabolic capacities and physiology of fungal groups in the natural habitat. Again, the utility of these methods depends on the quality of available sequence information.

To explore the metabolic capacity of specific fungal groups involved in polymeric organic matter degradation, in particular of aromatic organic pollutants, the cultivation approach has frequently been used. A major advantage of working with fungal isolates is that it allows combining studies of specific sequences and metabolic features of the cultured organism. This knowledge allows more targeted exploration of gene expression, enzyme synthesis and metabolic pathways. For example, the addition of labeled substrates enables targeted studies of the metabolic potential, which has been previously assessed by gene expression and genome analyses. Generating more cultures of DMF is a future goal. Access to DMF genomes may reveal nutritional requirements that have thus far prevented the cultivation of these DMF.

To better define ecological niches of aquatic fungi in natural ecosystems, changes in environmental variables can be simulated by using fungal isolates in microfluidic devices and combining them with modern biochemical measurements, e.g., Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR-MS). This approach allows studying fungi at the cellular level under relatively natural conditions. Consequently, well characterized fungal isolates will facilitate

experimental studies exploring the full ecological potential of specific fungi or fungal groups. In particular, fungal isolates can be cultivated in the presence of other fungal taxa or organisms such as bacteria and microalgae. Co-cultivation and interaction experiments (Fig. 2E) can be designed to study metabolic connectivity and dependence between different fungal species and other micro- and/or macroorganisms.

Future experimental strategies should combine several of the above mentioned methods and approaches to improve exploration of fungal biodiversity, metabolic potential and ecological role in aquatic ecosystems.

Summary

Exploration of fungal biodiversity in aquatic habitats is gaining momentum, as new molecular tools and approaches have revealed an unexpected abundance of fungi with unknown ecological function and uncertain phylogenetic placement. Extending these investigations from biodiversity to ecological roles of aquatic fungi has revealed that some have purely parasitic, others purely saprotrophic, and some mixed lifestyles. Analyzing fungal genomes may allow us to study their physiology in greater detail and take into account their interactions with potential substrata or hosts. Baiting with living and non-living substrata will allow us to explore metabolic potentials and lifestyles of fungi under quasi-natural conditions. Parasitic fungi can have a tremendous impact on food web structure and dynamics by affecting primary as well as secondary producers, and saprotrophic fungi affect remineralization and fluxes of nutrients and organic matter in aquatic environments. Currently, we lack important information on the ecological roles of a number of fungal groups, in particular the recently discovered Rozellomycota. With today's methodology we have a variety of tools at our disposal to test a number of interesting hypotheses in the field:

- DMF in aquatic systems account for a large proportion of the overall biodiversity and metabolic potential.
- Parasitic and saprotrophic DMF affect organic matter mineralization and respiration and hence carbon and energy cycling in aquatic systems.
- Parasitic DMF have the potential to structure and control functioning of aquatic food webs.
- In humic matter-rich and acidic environments, fungi are key players in organic matter decomposition.
- Environments with high particle loads, e.g., coastal/littoral systems, favor high fungal abundance.

Today, we are fortunate that a variety of novel molecular, biogeochemical and experimental methods have become available, whose combination allows investigation not only of the diversity of fungi, but also their ecological roles in natural environments. We believe that the time is ripe to link the variety of available methodological tools with well-designed experiments exploring metabolic potential and more importantly the significance of fungi in food webs.

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