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ORIGINAL ARTICLE

Clarifying the Relationships between Microsporidia and Cryptomycota

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Keywords

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ABSTRACT

Some protists with microsporidian-like cell biological characters, including *Mitosporidium*, *Paramicrosporidium*, and *Nucleophaga*, have SSU rRNA gene sequences that are much less divergent than canonical Microsporidia. We analysed the phylogenetic placement and environmental diversity of microsporidian-like lineages that group near the base of the fungal radiation and show that they group in a clade with metchnikovellids and canonical microsporidians, to the exclusion of the clade including *Rozella*, in line with what is currently known of their morphology and cell biology. These results show that the phylogenetic scope of Microsporidia has been greatly underestimated. We propose that much of the lineage diversity previously thought to be cryptomycotan/rozellid is actually microsporidian, offering new insights into the evolution of the highly specialized parasitism of canonical Microsporidia. This insight has important implications for our understanding of opisthokont evolution and ecology, and is important for accurate interpretation of environmental diversity. Our analyses also demonstrate that many opisthokont (aphelid+rozellid+microsporidian) SSU V4 OTUs from Neotropical forest soils group with the short-branching Microsporidia, consistent with the abundance of their protist and arthropod hosts in soils. This novel diversity of Microsporidia provides a unique opportunity to investigate the evolutionary origins of a highly specialized clade of major animal parasites.

MICROSPORIDIA are conventionally considered as highly derived parasitic protists sister to *Rozella* or diverging as the next branch below the fungi (James et al. 2013). Microsporidia, Balbiani 1882 display a suite of distinctive cell biological characters related to their obligate parasitic lifestyle, including a characteristic spore-extrusion apparatus (represented most conspicuously by the polar filament and its terminal anchoring disc) (Franzen 2004; Vávra and Lukeš 2013), unwallied intracellular trophic (meront)

stages, and multiwalled spores produced by merogony or other forms of proliferation (Vávra and Larsson 1999). Microsporidia lack canonical Golgi apparatus (Bez-noussenko et al. 2007; Vávra and Larsson 1999; Vávra and Lukeš 2013) and their mitochondria have been highly reduced to mitosomes (reviewed in Dean et al. 2016). These mitosomes are unable to generate their own ATP through oxidative phosphorylation, requiring energy to be imported from the host via nucleotide transporters.

Microsporidia also lack flagella and an apparent capacity for phagocytosis. The known diversity of Microsporidia is large, comprising approximately 1,300 described species (Vávra and Lukeš 2013), forming a long-branched clade (hereafter referred to as LB-Microsporidia) in SSU rRNA and multigene phylogenies (James et al. 2013). Metchnikovellids (e.g. *Amphiblyps*, *Amphicantha*) have traditionally been referred to as atypical, “primitive” microsporidians, but share many characters with LB-Microsporidia, and were recently shown to branch as sisters to them (Mikhailov et al. 2017).

Microsporidia are known primarily as parasites of invertebrates and vertebrates (including humans), but are also known as endosymbionts of ciliates (Fokin 2012; Fokin et al. 2008), and hyperparasites in protists: metchnikovellids are parasites of gregarines, protistan gut symbionts of many invertebrates, and *Hyperspora aquatica* is a hyperparasite of the paramyxid *Marteilia cochillia*, a serious pathogen of European cockles (Stentiford et al. 2017). *Rozella* species are zoosporic biotrophic parasites of oomycetes, chytrids, and Blastocladiomycota (Spatofora et al. 2017). Increased attention has recently been given to a large diversity of lineages shown by phylogenetic analyses including environmental sequences to be related to Microsporidia, rozellids, and aphelids. The first to highlight this diversity were Lara et al. (2010) and Jones et al. (2011), who showed a large diversity of environmental sequences which, in the absence of microsporidian sequences, group with *Rozella* in phylogenetic trees. These have been referred to as Rozellida (Fig. 1 in Lara et al. 2010), Rozellomycota (Corsaro et al. 2014a,b), and Cryptomycota (Fig. 1 in Jones et al. 2011).

A few other sequences branching between rozellids and LB-microsporidia represent microsporidia-like protists which have been morphologically characterized: *Nucleophaga* (Corsaro et al. 2014a, 2016), *Paramicrosporidium* (originally described as a microsporidian; Michel et al. 2000, 2009), and *Mitosporidium* (Haag et al. 2014).

These three genera clearly share some features with classical LB-Microsporidia, including forms of polar filaments (not necessarily functional as extrusion apparatus), unwallied intracellular meront stages, and nonflagellated spores; but in other respects, they are dissimilar, *Nucleophaga* and *Paramicrosporidium* being the least structurally similar to LB-Microsporidia. *Mitosporidium*, sometimes referred to as the earliest branching microsporidian (Mikhailov et al. 2017; Quandt et al. 2017), has a mitochondrion, albeit lacking Complex I of the oxidative phosphorylation pathway. *Paramicrosporidium* has a canonical fungal mitochondrial genome, and shares more gene content with distantly related fungi than with its closest relatives (Quandt et al. 2017). *Mitosporidium* and *Nucleophaga* have possibly nonhomologous finger-like extensions in naked intranuclear trophic stages similar to those of *Rozella* (Corsaro et al. 2014b; Haag et al. 2014).

Most published phylogenetic analyses that include Crypto/Rozellomycota/rozellid environmental sequences do not also include LB-Microsporidia. Those that do (Corsaro et al. 2016 (Fig. 3); Tedersoo et al. 2017) suggest that

Rozella is sister to a highly diverse clade comprising LB-Microsporidia, the microsporidian-like protists described above, and a large diversity of uncharacterized environmental sequences. In this study we investigate these relationships further, integrating morphological, phylogenetic, and sequence diversity data, to determine the phylogenetic and taxonomic boundaries of microsporidia and their immediate relatives.

MATERIALS AND METHODS

The nr nucleotide GenBank database was blastn searched using seed SSU rRNA gene sequences of characterized microsporidia-like protists, metchnikovellids, deeply branching LB-Microsporidia (clades 2 and 3 Stentiford et al. 2017), and the phylogenetic diversity of “Cryptomycota” and aphelids in Karpov et al. (2014). The top 50 matches for each sequence were downloaded, aligned with mafft e-ins-i (Katoh and Standley 2013), deduplicated, and a preliminary tree constructed on the basis of which the number of closely related sequences was reduced, retaining the longest possible sequences. The shorted branched LB-Microsporidia *Janacekia*, *Trichonosema*, and *Bacillidium* were selected to represent LB-Microsporidia in order to reduce the possibility of phylogenetic artefacts caused by LBA. Published phylogenies (e.g. Stentiford et al. 2017) show that these form a very robust monophyletic group with all other LB-Microsporidia, so it is reasonable to use them as a proxy for the whole group in this study. A Bayesian phylogeny was inferred under the CAT+GTR+Gamma (4) model in PhyloBayes-MPI 1.7 (Lartillot et al. 2013). Convergence among four MCMC chains was assessed by comparing the discrepancies in bipartition frequencies and in a range of continuous model parameters, along with the effective sample sizes of the continuous parameters. A consensus tree was built once all discrepancies were < 0.1, with sample sizes > 100. A maximum likelihood phylogeny was estimated under the GTR+Gamma(4)+F model in IQ-Tree (Nguyen et al. 2015), with 200 traditional nonparametric bootstraps. The distribution of microsporidian characters (Table 1) was constructed from the literature. OTUs assigned to any of “Opisthosporidia” (Karpov et al. 2014); “Cryptomycota” (Jones et al. 2011), “Holomycota” (Liu et al. 2009), and “Microsporidia” and otherwise unassigned “fungi” by the taxonomic assignment algorithms of each study were extracted from environmental amplicon sequencing data of tropical forest soils clustered into OTUs by Swarm v2.1.5 (Mahé et al. 2015) and European coastal water and sediment samples (Logares et al. 2014). We inferred a maximum likelihood tree from our reference database using RaxML v8.2.8 (Stamatakis 2014). The OTUs were then aligned to the reference database using PaPaRa (Berger and Stamatakis 2011) and placed on the tree by RaxML-EPA (Berger et al. 2011). The distribution of placements (Fig. 2, 3) was created with Genesis (<http://genesis-lib.org/>) and visualized with FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>). Based on the 49 flagellar toolkit proteins assembled for a previous study (Torruella et al. 2015), we searched by

BLAST in available early-branching microsporidian predicted protein sets from genomic data, in *Mitosporidium daphniae* (Haag et al. 2014), *Paramicrosporidium* (Quandt et al. 2017), and *Amphiblysis* sp. (Mikhailov et al. 2017), using an e-value threshold of 1e-10 and manual scrutiny.

RESULTS AND DISCUSSION

An expanded Microsporidia

We constructed an SSU rRNA gene sequence alignment including a comprehensive selection of the microsporidian-like protists and their related environmental sequences, metchnikovellids, and representatives of short-branch LB-Microsporidia (see Methods), apheids, rozellids, and related unclassified groups. Maximum Likelihood and Bayesian phylogenetic analyses (Fig. 1) showed that the expected metchnikovellid+LB-Microsporidia clade emerges from within a diverse and well-supported (Bayesian posterior probability (BPP) = 0.98; Maximum Likelihood bootstrap 96%) clade including *Paramicrosporidium*, *Nucleophaga*, *Mitosporidium*, LKM-11, and many other environmental sequences referred to as rozello-/cryptomycotans. *Rozella* formed a clade with high support with a relatively small number of environmental sequences.

It is well recognized that SSU rRNA gene data alone are unable to resolve deep phylogenetic relationships. However, although not a substitute for multigene data, the high taxon sampling afforded by SSU provides phylogenetic information currently inaccessible for phylogenomic analyses (Berney et al. 2004; Cavalier-Smith 2004). We calculated a range of trees of varying taxon samplings (e.g. Fig. 2; other data not shown), and although the branching order within the clade was not consistent between them (although many subclades were consistently recovered), the microsporidian clade as marked on Fig. 1 was invariably and strongly recovered.

These analyses provide additional evidence for an expanded and strongly supported microsporidian clade, including all of the LB-Microsporidia, (metchnikovellids, the “microsporidian-like” protists discussed above, and almost all of the environmental “crypto/rozellomycotan” diversity indicated in Lara et al. 2010; Jones et al. 2011; Corsaro et al. 2014a,b, 2016; Karpov et al. 2014; Lazarus and James 2015; Tedersoo et al. 2017), but excluding rozellids (= *Rozella*), NAMA-KO-37, and NCLC1 (Basal Clone Group 1). We therefore propose that this clade including the large diversity of environmental sequences, are all actually microsporidia, and we refer to them (excluding LB-Microsporidia) here as short-branched Microsporidia (SB-Microsporidia). For the purposes of this study we also exclude metchnikovellids from the definition of SB-Microsporidia as sequenced metchnikovellids have significantly longer branches than the SB-Microsporidia shown on Fig. 2. However, it is likely that as more related lineages are discovered a more gradual continuum of branch lengths between SB-Microsporidia such as *Nucleophaga* (Fig. 2), metchnikovellids, and LB-Microsporidia will be revealed.

This “expanded” Microsporidia concept is consistent with the original descriptions of *Paramicrosporidium* (Michel et al. 2000, 2009) as “microsporidian” or “microsporidian-like”, and *Mitosporidium*, which exhibits merogony and a coiled polar filament, the latter exclusive to Microsporidia, being “profoundly morphologically similar to Microsporidia” (Haag et al. 2014). This phylogenetically broader circumscription of Microsporidia is morphologically distinct because all characterized lineages in the microsporidian clade possess the key morphological features of Microsporidia: spores with multilayered cell walls containing polar filament apparatus (Richards et al. 2017; Vávra and Lukeš 2013), and merogony, whereas *Rozella*, apheids, and fungi do not possess these characters.

Morphological vs. genomic evolution in Microsporidia

Corresponding morphological and genomic datasets are available for only a very small proportion of lineages representing SB-Microsporidia and metchnikovellids. Until very recently this applied to only *Mitosporidium* (Haag et al. 2014) and *Amphiblysis* (Mikhailov et al. 2017), but the addition of nuclear and mitochondrial genomes of *Paramicrosporidium saccamoebae* (Quandt et al. 2017) have provided several intriguing new perspectives on microsporidian evolution. It is increasingly apparent that, although all SB-Microsporidia exhibit microsporidian-defining morphological characters, their genomic evolution appears far more mosaic (Quandt et al. 2017); Table 1. *Rozella* and LB-Microsporidia both have horizontally acquired *Rickettsia*-like NTT ATP/ADP transporters, but metchnikovellids, *Mitosporidium* and *Paramicrosporidium* do not (Table 1). LB-Microsporidia lack mitochondrial genomes, which are present in *Mitosporidium* and *Paramicrosporidium*. The mitochondrial genomes of *Mitosporidium* and *Rozella* lack Complex I of the oxidative phosphorylation pathway, are degenerate, and AT-rich, but that of *Paramicrosporidium* includes all genes of that pathway typically found in fungi; in fact, the total gene complement of both *Paramicrosporidium* and *Mitosporidium* have more in common with fungi than with its closest relatives (Haag et al. 2014; Quandt et al. 2017). The very fast rates of sequence evolution (and so long branches in the phylogeny) observed for LB-Microsporidia correlate with extensive cellular and genomic reduction, including strong mutational bias to AT and the loss of some DNA replication and repair genes that, in other eukaryotes, help to promote genome stability (Williams et al. 2016). While genome data are currently very sparse, the slower evolutionary rates observed for the 18S genes of SB-Microsporidia suggest that, while these organisms are also parasites, reductive evolution has not proceeded to the same extreme degree as in the LB clade. Another instance of potentially horizontally acquired genes (the distribution of which may understandably not correlate with phylogeny) are thymidine kinases found in *Rozella* and LB-Microsporidia, but not *Paramicrosporidium* (Alexander et al. 2016; Quandt et al. 2017). *Paramicrosporidium* possesses a full set of meiosis genes and significant evidence for diploidy, more similar to LB-Microsporidia than is known to be the

Table 1. Molecular and genomic characteristics of Microsporidia and their relatives. Two long-branch microsporidia infecting nematodes (*Nematocida parisii*) and vertebrates (*Encephalitozoon cuniculi*) and *Rozella* (Rozellida) are shown in comparison. Dashes represent unavailable data. ✓/X indicate presence/absence of a character; where uncertain these are shown in parentheses. Note that, following ancestral reduction, some LB-Microsporidia have experienced independent secondary expansions in genome size (e.g. up to 51.3 Mb in *Edhazardia aedis* Desjardins et al. 2015), but the additional material largely comprises noncoding and repetitive sequences, without a concomitant increase in cellular or metabolic complexity. The polar filament was reported as absent in *Nucleophaga amoebae*, although a form of it is present in the very closely related *N. terricolae*. It is possible that it has been lost in *N. amoebae* or was simply not seen in the cells investigated by Corsaro et al. (2014b)

	Flagellate stage	Genome size (Mb)	Gene density genes/kbp	Number of protein coding genes	ATP/DP translocases (HGT)	Mitochondrial genome		Polar filament			Spore	
						Electron transport chain	Electron transport	Regular	Atypical	Absent	Exo-tendospore	Posterior vacuole
<i>Encephalitozoon cuniculi</i> (LB-Microsporidia) ^{1,2}	x	2.9	0.81	1,997	✓	x	x	✓				✓
<i>Nematocida parisii</i> ERTm1 (LB-Microsporidia) ^{3,4}	x	4.1	0.65	2,661	✓	x	x	✓				✓
<i>Amphibmlys</i> sp. WSBS 2006 (metchnikovellid) ⁵	x	5–7	0.66	2,529 (nr)	x*	x	x	-	-	-	-	-
<i>Amphibmlys capitellides</i> (metchnikovellid) ⁶	x	-	-	-	-	-	-	✓				x
<i>Mitosporidium daphniae</i> (SB-Microsporidia) ⁷	x	5.64	0.585	3,300	x	✓	Partial	✓				x
<i>Paramicrosporidium saccamoebae</i> (SB-Microsporidia) ^{8,9}	x	7.3	-	3,750	x	✓	✓	✓				x
LKM11-01; CM1.1 (SB-Microsporidia) ¹⁰	✓	-	-	-	-	-	-	-	-	-	-	-
<i>Nucleophaga terricolae</i> (SB-Microsporidia) ^{11,12}	x	-	-	-	-	-	-	✓				x
<i>Nucleophaga amoebae</i> (SB-Microsporidia) ¹³	x	-	-	-	-	-	-		✓?		‡	x
<i>Rozella allomyces</i> (rozellid) ¹⁴	✓	11.9	0.535	6,350	✓	✓	Partial	✓				x

nr, nonredundant. 1. Katinka et al. (2001), 2. Karpov et al. (2014), 3. Cuomo et al. (2012), 4. Troemel et al. (2008), 5. Mikhailov et al. (2017), 6. Larsson and Koie (2006), 7. Haag et al. (2014), 8. Corsaro et al. (2014a), 9. Quandt et al. (2017), 10. Jones et al. (2011), 11. Corsaro et al. (2016), 12. Michel et al. (2012), 13. Corsaro et al. (2014b), 14. James et al. (2013), 15. Letcher and Powell (2005), and 16. Ajello (1948).

*Incomplete genome (estimated 90% complete).

†Undetected or potential loss.

‡Two-layered cell wall.

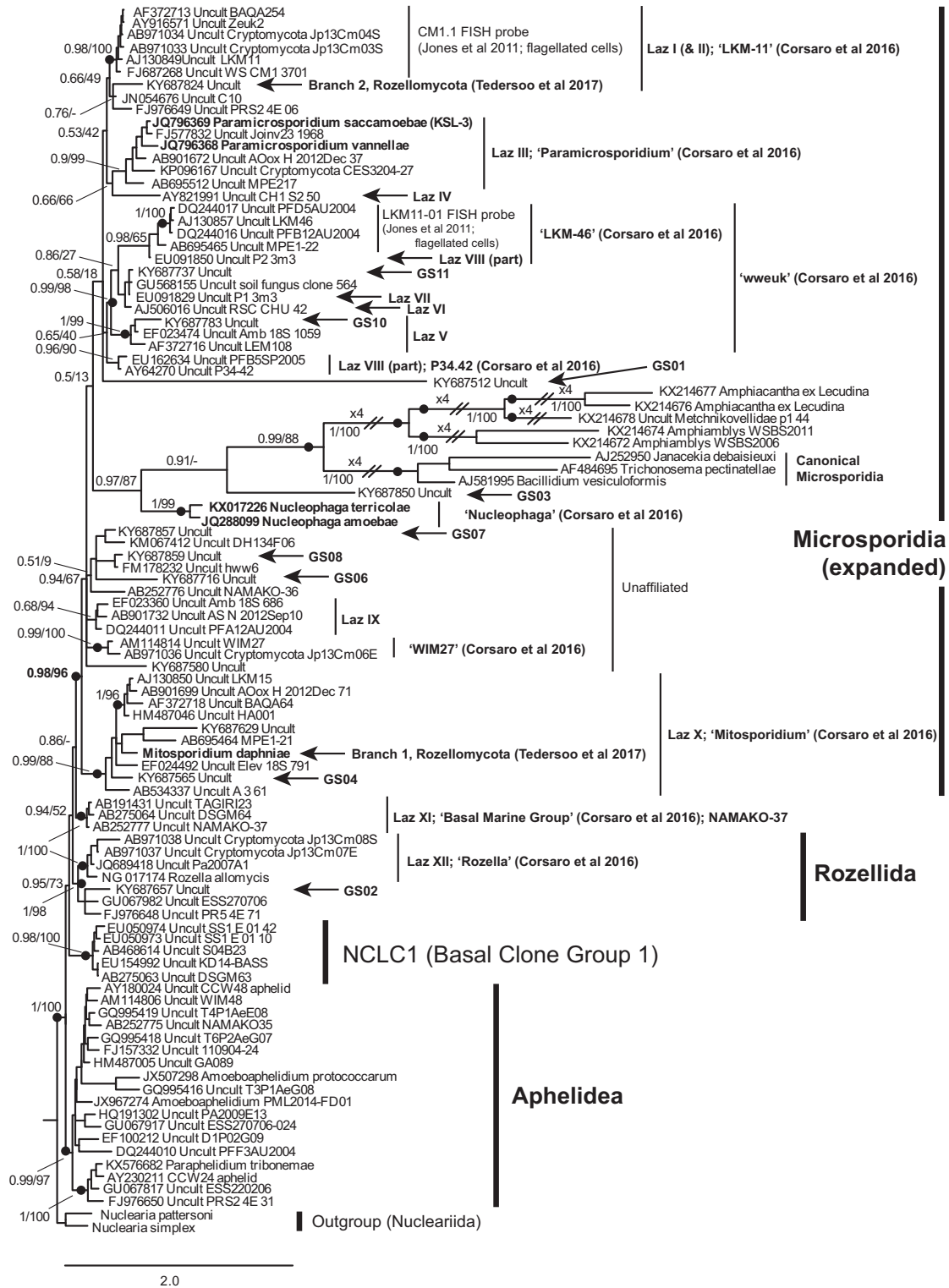


Figure 1 Phylogenetic relationships among canonical, long-branching (LB) Microsporidia, metchnikovellids, Rozellida, and a diversity of related short-branching lineages. LB-Microsporidia form a clade with a diversity of short-branching lineages (SB-Microsporidia) that share key cell biological characters defining the microsporidian clade. Lineages that have been labelled in other studies are labelled: GSxx from Tedersoo et al. (2017), Laz x from Lazarus and James (2015); others as marked. The extent of the expanded Microsporidia is shown by the bracket on the far right. The tree was inferred under the CAT+GTR model in PhyloBayes-MPI, on a final alignment of 1729 sites from the SSU rRNA gene. Black blobs indicate support values of > 0.96 BPP and > 95% ML bootstrap (actual values also shown). Branch lengths are proportional to the expected number of substitutions per site, as denoted by the scale bar.

2017; Spatafora et al. 2017; Richards et al. 2017; and examples cited throughout this study). Some authors include aphelids within Cryptomycota (e.g. Letcher et al. 2013), but more normally Crypto/Rozellomycota are used to encompass *Paramicrosporidium*, *Mitosporidium*, *Nucleophaga*, the lineages detected by FISH in Jones et al. (2011), the strongly supported clade containing *Rozella*, and the large diversity of environmental sequences branching around and among these lineages. This is the most frequently used classification, informally described by Letcher et al. (2018) and shown in Jones et al. (2011), Lazarus and James (2015), and (excluding *Mitosporidium*, which is classified as microsporidian) Quandt et al. (2017).

We suggest that the frequently referred to “paraphyly of Rozello/cryptomycota” is both misleading and avoidable. The defining morphological characters of microsporidia are spores with multilayered cell walls containing polar filament apparatus homologs (not necessarily functioning in extrusion). The phylogenetic distribution of these characters is coincident with the clade containing only SB- and LB-Microsporidia, which is recovered by both SSU and multigene phylogenetic analyses. Therefore, all members of that clade are in fact Microsporidia, and there is no need for them to be regarded as belonging to a paraphyletic group. The closest known relatives of microsporidia, again according to SSU (Fig. 1) and multigene phylogenies, are rozellids, which are restricted to a robustly supported clade including the genus *Rozella*. *Rozella* shares some microsporidian-like features with the more distantly related LB-Microsporidia (e.g. horizontally acquired *Rickettsia*-like NTT ATP/ADP transporters, degenerate, and AT-rich mitochondrial genomes lacking Complex I of the oxidative phosphorylation pathway, thymidine kinases), but not with all of their shorter-branch relatives, and are highly morphologically distinct.

What then are Cryptomycota (Jones et al. 2011) and Rozellomycota (Corsaro et al. 2014a)? On the basis that they refer to the same assemblage of lineages (which is usually the case) two names are unnecessary and confusing. The purpose of this study is neither to decide between them nor suggest an alternative, nor even to invalidate them. However, to be consistent with recent usage this label could be applied to an uncharacterized monophyletic group branching somewhere in the opisthosporidian clade, excluding aphelids, the expanded microsporidia, or the clade including *Rozella*. The latter already has order or class status, according to different authorities (Lara et al. 2010; Ruggiero et al. 2015). Figure 1 indicates the diversity revealed by general eukaryote-wide environmental sequencing studies, including those clades coded and labelled by Corsaro et al. (2016), Tedersoo et al. (2017), and the diversity detected by the targeted PCR approach of Lazarus and James (2015). Of this very substantial environmental diversity, only two groups within Opisthosporidia do not branch within the expanded microsporidian, rozellid, or aphelid clades: (1) the NAMAko-37 clade (which in Corsaro et al. (2016) branches outside of the microsporidian clade before the divergence of *Rozella*, in Jones et al. (2011) is sister to

Rozella, and in Fig. 1 of this study [under the best-fitting CAT+GTR phylogenetic model], and in Lazarus and James (2015) is sister to microsporidia), and (2) NCLC1 (Basal Clone Group I), which in Fig. 1 branches between NAMAko-37 and aphelids, but whose actual branching position is unresolved (Richards et al. 2015, 2017). On the basis of their phylogenetic position and existing provenance data we suggest that the last two groups are zoospore parasites of marine microbial eukaryotes.

We infer that the “unaffiliated” group (Fig. 1) is microsporidian (and therefore not cryptomycotan) based on tree topology and bipartition support. These cells could provide key insights into early microsporidian evolution. We hypothesize that they possess mitochondria, and possibly flagellar structures or/and simpler cell extrusion apparatus than in other SB-Microsporidia. The NAMAko-37 clade cannot be classified until more data are available. There is negligible support for their being microsporidian or belonging to any other recognized group, so the label “Cryptomycota” could be used for this monophyletic lineage, at least for the time being. However, the message of this article remains unchanged whether the whole Rozellida + Microsporidia clade is referred to as Rozello- or Cryptomycota or whether these names fall out of use, what the relationships between aphelids, rozellids, and microsporidia actually are, and where the boundary between fungi and other protists is. Crucially, acknowledging an expanded Microsporidia as an evolutionarily and morphologically coherent unit both unambiguously clarifies the taxonomy/classification of this very interesting clade in the eukaryote tree, and provides a clear framework for future research.

High diversity of SB-Microsporidia in neotropical soils

An important consequence of our analyses is that the taxonomic affiliations of large-scale SSU rRNA amplicon sequencing studies must now be revisited. Most of the sequences annotated as “crypto/rozellomycota” in taxonomically curated databases such as SILVA and PR² (Guillou et al. 2013) are likely to be SB-Microsporidia. The rozellid annotation should be restricted to the clade indicated on Fig. 1. This is not purely a matter of classification: Microsporidia and rozellids are each monophyletic and distinct in terms of biology and ecology, but are currently conflated in the major sequence databases. Clarifying the composition of the two clades will enable much more accurate and high-resolution analyses, and interpretation of microsporidian diversity and function.

To demonstrate the particular relevance of this more inclusive definition of microsporidia to the annotation and interpretation of environmental sequencing studies, we reevaluated a recent SSU rRNA high-throughput sequencing study of three Neotropical rainforest soils (Mahé et al. 2017), using the Evolutionary Placement Algorithm as implemented in RAxML (Stamatakis 2014) to place operational taxonomic units (OTUs) previously annotated as Opisthosporidia, crypto/rozellomycota, or unassigned fungi. We similarly analysed an OTU dataset from

Microsporidia, and in particular their propensity for direct parasitism of animals.

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