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ORIGINAL PAPER

Molecular identification of *Nucleophaga terricolae* sp. nov. (Rozellomycota), and new insights on the origin of the Microsporidia

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Abstract Microsporidia are widespread endoparasites of animals, including humans. They are characterized by highly modified morphological and genetic features that cause difficulties in elucidating their enigmatic origin and evolution. Recent advances, however, indicate that the Microsporidia have emerged from the Rozellomycota, forming together either the most basal lineage of the Fungi or its closer relative. The Rozellomycota comprise a huge diversity of uncultured environmental clones, with a very few known species endoparasitic of algae and water moulds, like the chytrid-like *Rozella*, and of free-living amoebae, like *Nucleophaga* and the microsporidia-like *Paramicrosporidium*. A possible ancestral microsporidium, *Mitosporidium*, has recently been described from the water flea *Daphnia*, since the phylogenomic reconstruction showed that it branches to the root of the microsporidian tree, while the genome analysis revealed a fungal-like nuclear genome and the persistence of a mitochondrial genome. Here we report the 18S rDNA molecular phylogeny of an additional microsporidium-like endoparasite of amoebae, which has a developmental cycle almost identical to that of *Nucleophaga amoebae*. Our results show that the endoparasite is closely related to *N. amoebae*, forming a distinct species, for which we propose the name *Nucleophaga terricolae*. Furthermore, the *Nucleophaga* lineage is recovered as sister to the Microsporidia while *Mitosporidium* turns out to be member of a well-supported group of environmental clones. These results raise the question about the actual ancestry of the Microsporidia within the Rozellomycota. A precise and robust phylogeny will require further comparative genomic studies of these various strains, and should also consider the primitive microsporidia, for which genetic data are still lacking, because all these organisms are essentially morphologically similar.

Keywords Rozellomycota · *Nucleophaga* · Amoebae · Microsporidia · Endoparasite

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Introduction

Microsporidia are microbial eukaryotes, widespread as intracellular parasites of virtually all animals, including humans, as well as of ciliates and gregarines (Alveolata). Beside their importance as pathogens, mainly for economically relevant animal species and for humans, especially with immunocompromised status, Microsporidia have attracted attention as a model of highly evolved intracellular organisms. They are indeed characterized by extreme morphological and genomic adaptations to intracellular parasitism, such as the loss of the

flagellum, mitochondria reduced to DNA-free mitosomes, a high genome compaction and plasticity, and accelerated gene mutation rates (Vávra and Lukeš 2013). Microsporidia have developed a peculiar infection apparatus, characterized by the polar filament, a syringe-like hollow tubular structure arranged into the spore, with a unique extrusion mode to inject the spore content into the host cell (Franzen 2004). The polar filament, absent in the active stages, is formed from an atypical Golgi apparatus. Once inside the host cell, the parasites proliferate by repeated fissions (merogony), then they enter into a successive stage (sporogony) to develop a new generation of spores. Some Microsporidia have the smallest eukaryote genomes (~2.2 Mb), with genome size variations up to ~51 Mb, which are however essentially due to the expansion of intergenic regions and/or the number of transposable elements (Parisot et al. 2014; Corradi 2015). These organisms have been variously interpreted as microscopic yeasts, primitive amitochondriate protists, sporozoa or degenerate zygomycotan fungi. Recent studies however showed that Microsporidia are specifically linked to *Rozella*, the earliest branch of zoosporic fungi (chytrids) (James et al. 2006a, b, 2013).

Rozella spp. are unicellular endoparasites of others chytrids (Chytridiomycota, Blastocladiomycota), oomycetes (Chromista) and some green algae (Plantae), with a life cycle comprising a motile uniflagellate stage (zoospore) which swims towards the host, encysts on its surface and forms a penetration tube to infect the host cell (Held 1981). Unlike other chytrids, *Rozella* grows intracellularly as a naked thallus possibly phagotrophic (Powell 1984), and forms walled zoosporangia and resting spores but acquiring wall matter from the host cell (Held 1981), its ability to develop walls being limited to immature stages (James et al. 2013).

Molecular phylogenies based on the 18S rDNA showed that *Rozella* belongs to a large clade comprising several uncultured environmental sequences, and microscopical observations evidenced that chytrid-like cells, i.e. zoospores and cysts on host cells, mainly diatom algae, may be associated with at least three distinct subgroups (clades C, LKM11 and LKM46) (Jones et al. 2011; Ishida et al. 2015). Rozellids appeared thus largely similar to other chytrids, as well as to aphelids, unicellular true phagotrophic endoparasites of green algae and chromistan algae, which also emerge at the base of the fungal tree comprising a huge diversity of uncultured lineages and showing a possible relationship with the Microsporidia (Karpov et al. 2013).

The enormous morphologic gap existing between the chytrid-like morphotype of these organisms and the Microsporidia has been first filled by the discovery of *Paramicrosporidium* spp., microsporidia-like rozellids (Rozellomycota) that are endonuclear parasites of free-living amoebae (Corsaro et al. 2014a). Indeed, *Paramicrosporidium* spp. have, like Microsporidia, a non-flagellate infectious chitinous walled spore with inside a (atypical) polar filament. However, in 18S rDNA trees, *Paramicrosporidium* spp. emerge as a distinct lineage of the Rozellomycota, and they differ from the Microsporidia by having rRNA genes of typical eukaryote size and structure (Corsaro et al. 2014a). Successively, another microsporidium-like organism, *Mitosporidium daphniae*, a gut parasite of the water flea *Daphnia magna* (Crustacea), was described as the ancestral microsporidium, as it emerges at the root of the Microsporidia but it retains fungal genomic features, including a mitochondrial genome (Haag et al. 2014). Recently, an additional endonuclear parasite of free-living amoebae, *Nucleophaga amoebae*, was shown to form a new lineage within the Rozellomycota, with an apparent intermediate morphotype between the microsporidia-like organisms and *Rozella* (Corsaro et al. 2014b). *Nucleophaga* is an old 'chytrid' (Dangeard 1895), formerly included in the family *Olpidiaceae* (Chytridiales) along with *Rozella* and some other unicellular endoparasites (Sparrow 1960) such as *Olpidium*, which is currently affiliated to the zygomycotan fungi (James et al. 2006a; Sekimoto et al. 2011), and *Sphaerita*, still enigmatic.

We recovered another endonuclear parasite of free-living amoebae, resembling *N. amoebae* during the growing stage, and producing non-flagellate walled spores containing putative polar filaments and anchoring discs (Michel et al. 2012). Here, we show by 18S rDNA molecular phylogeny that this strain, called KTt-1, belongs to the genus *Nucleophaga*, for which we propose the name *Nucleophaga terricolae*, and furthermore, that *Nucleophaga* species emerge as sister to the Microsporidia. After reexamination, microsporidia-like structures are also identifiable in spores of *N. amoebae*. Overall, these results open new exciting questions on the real ancestry of the Microsporidia.

Materials and methods

Strain origin and culture

The amoeba host, infected with the endonuclear parasite KTt-1, was recovered from the bark of a sycamore tree, *Platanus occidentalis* (Plantae, Angiospermae, Proteales), in Germany (Michel et al. 2012) and identified morphologically as *Thecamoeba terricola* (strain Tt-1) according to Page (1988). A laboratory strain of *Thecamoeba quadrilineata* (strain Dch-1) was also used for further cultures of the endoparasite. All amoebae were cultivated on bacterized non-nutritive agar at room temperature (Michel et al. 2012). Electron microscopy was performed according to previous studies (Michel et al. 2009a, 2012).

DNA extraction and phylogenetic analysis

Endoparasites from infected *Thecamoeba* were recovered by membrane filtration as described (Michel et al. 2009b; Corsaro et al. 2014a). Several eukaryotic primers were applied to amplify the 18S rDNA of endoparasites as described (Corsaro et al. 2014a, b). Sequencing of obtained products, however, resulted in only very short sequence fragments, closely related to *Nucleophaga* KTq-2, that were not suitable for phylogenetic reconstructions. Therefore, a new endoparasite-specific PCR forward primer nucleoF 5′-TTG AGA GTT GGA TTT ACA TG-3′ was designed on the sequences obtained from bands after a fingerprint analysis. Briefly, whole DNA was extracted from *T. quadrilineata* Dch-1 cells both uninfected and infected with endoparasites (MasterPure Complete DNA and RNA Purification Kit, Epicentre, WI, USA). The universal eukaryotic reverse primer Euk516rGC (Díez et al. 2001) was used to amplify partial 18S rDNA gene in combination with a series of forward eukaryotic primers: EukA (Medlin et al. 1988), 18S-For-n2 (Wylezich et al. 2012), 25F (Bass and Cavalier-Smith 2004) and EK82F (López-García et al. 2001). To separate parasite and host sequences from each other, a denaturing gradient gel electrophoresis (DGGE) of the fragments amplified was done as described by Wylezich and Jürgens (2011). Several bands unique to the sample containing KTt-1 were excised and re-amplified with the primer Euk516r (without GC clamp). The primers nucleoF and 18S-Rev-n (Wylezich et al. 2002) were used to amplify the nearly complete 18S rRNA gene of the endoparasite KTt-1. Sequencing was carried out externally (LGC Genomics, Germany) using the same primers as in the PCR and different internal sequencing primers (Wylezich et al. 2002). The obtained sequence fragments were carefully corrected and assembled in BIOEDIT (Hall 1999). The 18S rRNA gene sequence of KTt-1 was deposited in GenBank (acc. no. KX017226).

All sequences were aligned using MAFFT and manually refined using BIOEDIT to exclude ambiguous sites. Phylogenetic trees were built as described previously (Corsaro et al. 2014a).

Results

Morphological features

The life cycle and morphology of strain KTt-1 were described previously (Michel et al. 2012). Briefly, the parasite infects amoebae of the genus *Thecamoeba* (Amoebozoa, Thecamoebida). The infectious stage is a non-flagellate walled spore (1.5–1.8 μm), which is engulfed by amoebal phagocytosis. The parasite thus invades the nucleus of the host amoeba, inside to which it grows at the expense of the endosome as an early naked unicellular trophont (1–2 μm). During the growth, the early throphic stage enlarges (up to ~7 μm) and develops finger-like extensions at the surface, in intimate contact with the host karyoplasm. Inside the late large stage (unicellular sporangium), various sporogenic areas (~0.8–1 μm), usually >3, are then formed, to develop a new generation of spores (endogenous sporogony).

KTt-1 shows a developmental cycle very similar to *N. amoebae* KTq-2 (Michel et al. 2009a; Corsaro et al. 2014b), from which it differs by having apparently more polymorphic, amoeboic early trophonts and slightly larger late stages (7 vs. 5.5 μm). Also, the number of sporogenic areas observed in KTt-1 (up to 8) is higher with respect to KTq-2 (usually <3); thus, KTt-1 should produce more spores.

Microsporidia-like structures, i.e. a prominent anchoring disc and an atypical polar filament, are present in the mature spores of KTt-1 (Fig. 1a–c), along with dark bodies and a membrane-bounded body containing virus-like particles, of unknown nature (Michel et al. 2012). Reexamining pictures of KTq-2, these unknown bodies seem to be absent; however, similar anchoring disc and atypical polar filament structures may be seen in mature spores (Fig. 1d). Thus, KTt-1 and KTq-2 share very similar developments and mature spores with apparently microsporidia-like structures.

Molecular phylogeny

We performed a first SSU rDNA-based molecular analysis, by including members of all the opisthokont lineages (i.e. animals, fungi and their protistan relatives), except microsporidia. In the opisthokont 18S tree (Fig. 2), *Rozella*, *Paramicrosporidium*, *Nucleophaga* and *Mitosporidium* emerge as separate lineages within the highly supported holophyletic Rozellomycota, sister to the other Fungi, with Aphelidea as an outer basal lineage and Nucleariidae as the basalmost lineage of the holomycota (Fungi and protistan relatives). Our tree is largely congruent with previous studies, which also recovered rozellids (and microsporidians) as the early branch of Fungi and aphelids as an outer basalmost lineage (James et al. 2006b, 2013; Corsaro et al. 2014a, b; Karpov et al. 2014).

The strain KTt-1 clusters tightly with *N. amoebae* KTq-2, forming a deep and highly supported holophyletic lineage (Fig. 2). KTt-1 is 94.5 % identical with KTq-2, and both strains have similar genetic distances with *Rozella* spp. and *Paramicrosporidium* spp. (~80.5 %) and with *M. daphniae* (~79.5 %). The *Nucleophaga* lineage (KTq-2 and KTt-1) shows only a moderate relationship with the group LKM46, comprising clones prevalently originating from freshwater samples, while *Mitosporidium* emerges robustly within the group LKM15.

As recent studies showed the Microsporidia to be evolutionarily related to rozellids (James et al. 2013; Corsaro et al.

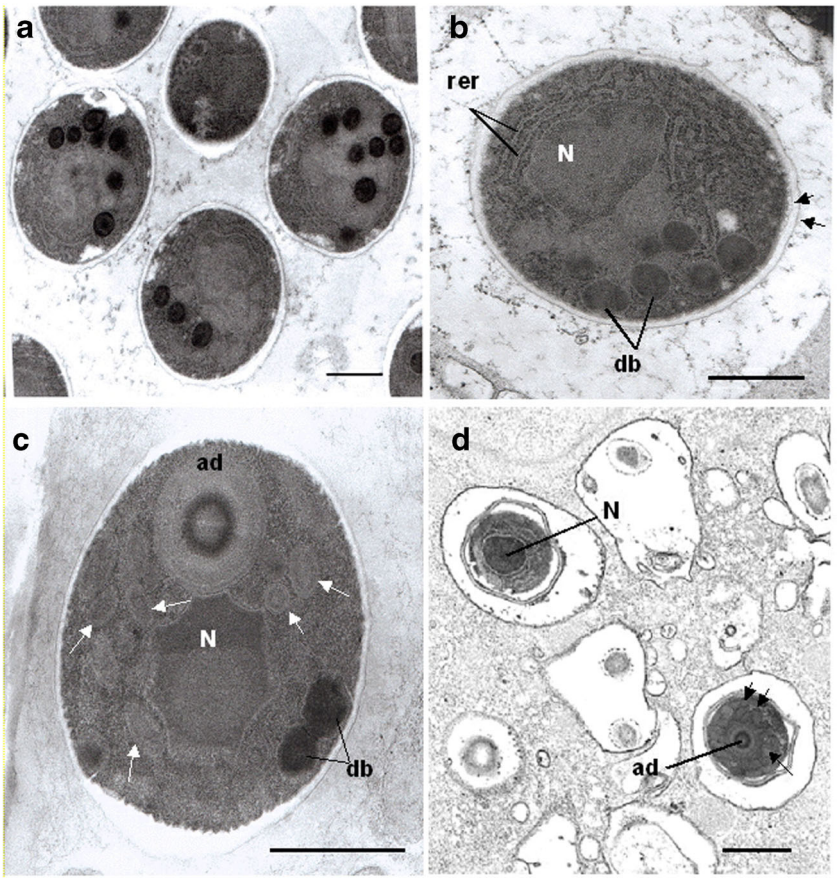

Fig. 1 Spores of *Nucleophaga*. **a** Spores of strain KTt-1 in group, each showing dark bodies. **b** Single spore of strain KTt-1, enveloped by endospore and exospore layers (*arrows*), with visible rough endoplasmic reticulum (*rer*) surrounding the nucleus (*N*). Several dark bodies (*db*) visible. **c** Spore of KTt-1 showing a central nucleus (*N*), a prominent anchoring disc (*ad*), and different section planes of the polar filament (*arrows*). Also visible are dark bodies (*db*). **d** Two spores of strain KTq-2. After optical brightening, microsporidia-like traits like an anchoring disc (*ad*) and polar filaments (*arrows*) become visible in some spores (*right side*). Within most spores, only the nucleus (*N*) can be observed (*left side*). *Scale bar* 500 nm

2014a; Haag et al. 2014) and aphelids (Karpov et al. 2013; Letcher et al. 2013), the basal holomycotan groups (i.e. Nucleariidae, Aphelidea and Rozellomycota) and putative basal microsporidians were used to build a distinct SSU molecular tree. In the basal holomycotan tree (Fig. 3), Aphelidea are holophyletic and sister to the Rozellomycota, from which emerge the Microsporidia. Unexpectedly, the latter have *Nucleophaga* as sister-group, while *Mitosporidium* remains tightly within the group LKM15 (Fig. 3). Also clustering within the group LKM15 is the sequence SAPA100012 (~99 % identical with LKM15 and closely related clones), obtained from the cyst-like fungal bodies found as endobiotic in fruiting bodies of the nivicolous myxomycete *Lamproderma* (Amoebozoa, Mycetozoa) (Yajima et al. 2013). By excluding the microsporidian sequences, the relationships recovered are similar to those showed in Fig. 2, with *Mitosporidium* strongly within the group LKM15 and *Nucleophaga* moderately sister to the group LKM46. While, by excluding *Nucleophaga*, Microsporidia are now clustering with the group LKM15, but not as sister to *Mitosporidium*, and however the relationship is not supported by ML (Fig. 4). The sisterhood relationship between *Nucleophaga* and the Microsporidia was recently recovered by Letcher et al. (2015), who however omitted *Mitosporidium* and all uncultured sequences, but also by Grossart et al. (2016), who analysing both *Mitosporidium* and many uncultured rozellids, but not aphelids, obtained a result overall similar to ours.

Taxonomy summary

Kingdom Fungi

Phylum Rozellomycota CORSARO and MICHEL, 2014, MB807390

Genus *Nucleophaga* DANGEARD 1895, MB20374

Species *Nucleophaga amoebae* DANGEARD 1895, MB172878

Description (Corsaro et al. 2014b, em.): Unicellular, with aflagellated infective spores with chitinous wall; anchoring disc and atypical polar filament present; mitochondria absent. Intranuclear parasites of amoebae as unwalled cells, finger-like projections at cell surface. Parasitic stage up to 5 μm, spores of 1.2–1.5 μm in diameter (Michel et al. 2009a).

Species *Nucleophaga terricolae* sp.nov. CORSARO et al., 2016, MB816522

Etymology: *terricolae*, species name, Lat. gen., of (*Thecamoeba*) *terricola*. Type strain: KTt-1 (to be deposited); natural host: *Thecamoeba terricola* (Amoebozoa, Longamoebia, Thecamoebidae).

Description: Unicellular, with aflagellated infective spores with chitinous wall; anchoring disc and atypical polar filament

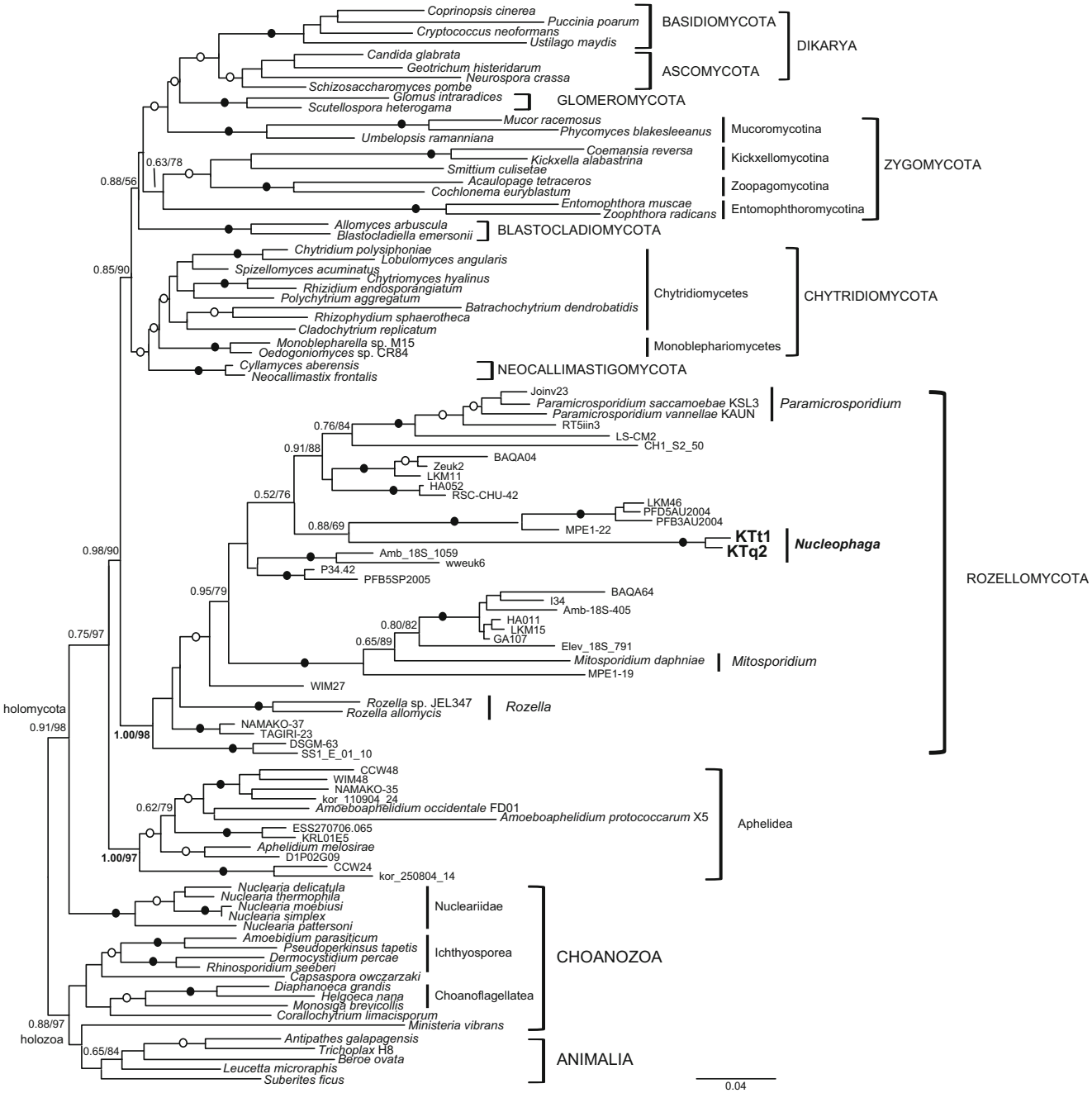

Fig. 2 Opisthokont phylogenetic tree (Microsporidia excluded). 18S rDNA tree of Fungi and relatives (holomycotans), rooted on holozoans (Animalia and part of Choanozoa). *Nucleophaga* strains are in *bold*. Support values are for Bayesian posterior probability and Maximum Likelihood (1000 bootstraps). *Filled and open circles* represent values of 1/100 % and of at least 0.90/90 %, respectively

present; mitochondria absent. Intranuclear parasites of amoebae as unwalled cells, finger-like projections at cell surface. Parasitic stage up to 7 μm, spores of 1.5–1.8 μm in diameter (Michel et al. 2012).

Remarks and differential diagnosis: *N. amoebae* strain KTq-2 and *N. terricolae* strain KTt-1 were recovered from distinct *Thecamoeba* species, *T. quadrilineata* and *T. terricola*, respectively, and both are able to successfully develop in various other *Thecamoeba* spp. The only difference observed was with the binucleate amoeba *Sappinia*, another member of Thecamoebidae. Albeit *Sappinia* spp. were highly susceptible to the infection with both strains, full development with formation of mature spores was observed only for KTq-2, which infects the two nuclei of *Sappinia* separating them from each other (Michel et al. 2009a). Strain KTt-1 appeared less virulent for *Sappinia*, infecting a lower number of trophozoites which died without releasing mature spores (Michel et al. 2012). *N. terricolae*

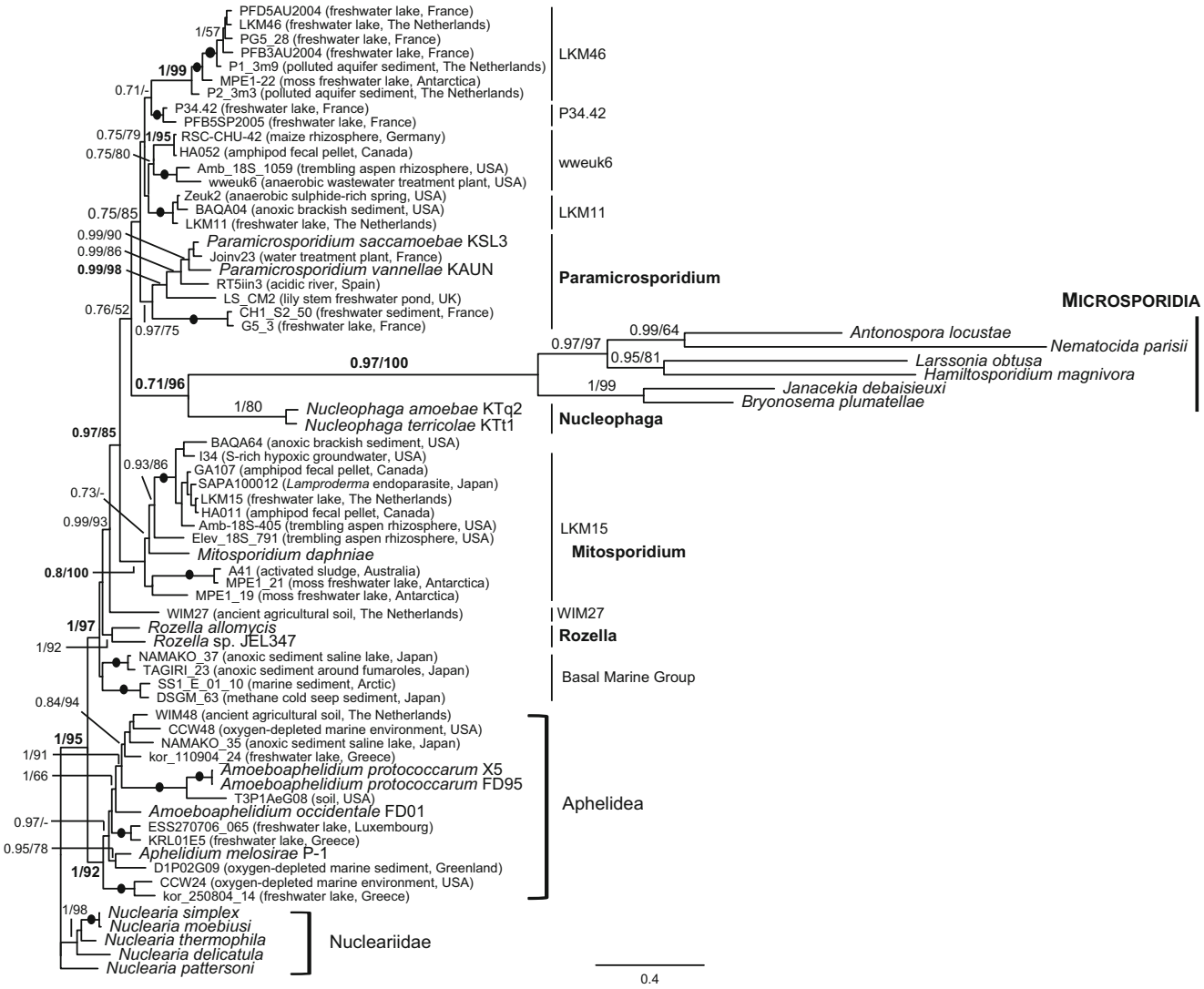

Fig. 3 Basal holomycotan tree. 18S rDNA tree of Aphelidea, Rozellomycota and Microsporidia, rooted on Nucleariidae. For Rozellomycota, major clades are indicated. Support values are for Bayesian posterior probability and Maximum Likelihood (1000 bootstraps). *Filled circles* represent values of 1/100 %; hyphen, node not supported

KTt-1 shows a life cycle identical to that of *N. amoebae* KTq-2, with very similar developmental stages and spores. *N. terricolae* KTt-1 differs from *N. amoebae* KTq-2 by their 18S rDNA (similarity 94.5 %) and molecular phylogeny (Figs. 2 and 3).

Discussion

Previous molecular studies identified at least two new lineages, rozellids and aphelids, basal to the fungal tree, both comprising a huge diversity of almost exclusively uncultured lineages. While Jones et al. (2011) were unable to recover any relationship between rozellids (cryptomycotans) and Microsporidia, already suggested by a comprehensive multigene study (James et al. 2006b), subsequent studies revealed the possible existence of an Aphelids-*Rozella*-Microsporidia clade (Karpov et al. 2013; Letcher et al. 2013) and especially of a *Rozella*-Microsporidia relationship (James et al. 2013). However, it is still unclear whether these lineages form really a separate group, and whether they should be considered as basal to the Fungi or as their closest relatives. Recent advances in the molecular characterization of aphelids, and especially the discovery of various microsporidia-like organisms, are currently allowing to better define these lineages and to resolve the enigmatic origin of the Microsporidia.

Indeed, albeit *Rozella* is evolutionarily linked to the Microsporidia (James et al. 2013), it is more similar either to other chytrids or to aphelids. A first indication of a possible missing link came by identifying *Paramicrosporidium* within the Rozellomycota in an 18S tree and by recovering it as sister to the Microsporidia in an rDNA unit tree (18S + 5.8S + 28S)

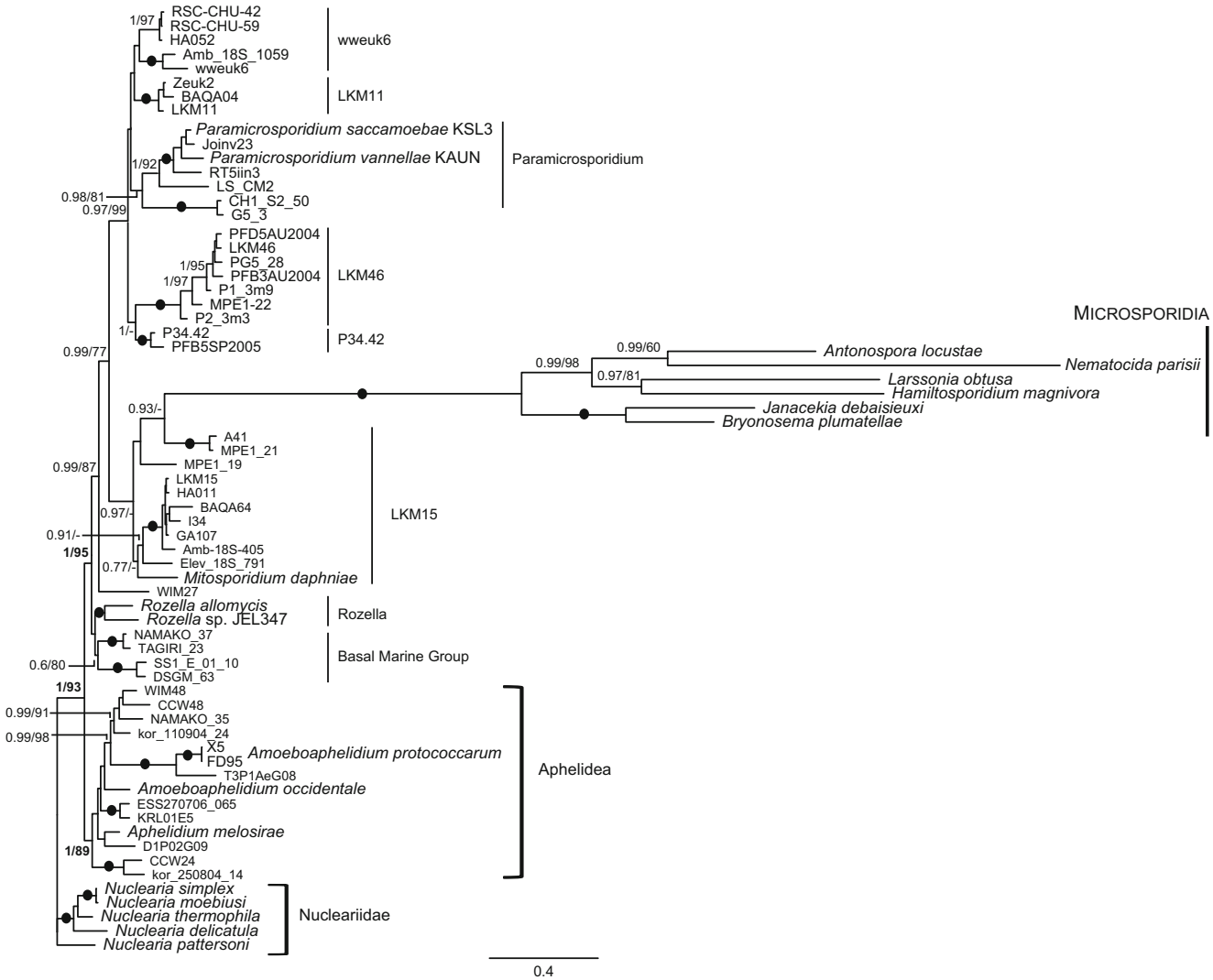

Fig. 4 18S rDNA tree of basal holomycotans, but excluding *Nucleophaga* sequences. Symbols as in Fig. 3

(Corsaro et al. 2014a). Phylogenomic analyses then revealed *Mitosporidium* as an early divergent branch of Microsporidia within a very robust clade including also *Rozella allomycis*, replacing *Paramicrospodium* as their closest relative in the rDNA unit tree (Haag et al. 2014). In this tree, *Paramicrosporidium* emerged on a separate branch. These analyses are however limited by the very few rDNA unit and genomic data available for rozellids, in contrast to legions of 18S sequences. Nevertheless, the results are congruent with the overall morphological similarity of both organisms with the Microsporidia, and further suggest that some phenotypic traits previously considered unique to Microsporidia have already emerged in the rozellids (Corsaro et al. 2014a; Haag et al. 2014). Therefore, the placement of *Nucleophaga* near to the Microsporidia in rDNA trees, first by using only cultivated taxa (Letcher et al. 2015), and subsequently confirmed by including also a large number of environmental sequences (Grossart et al. 2016; this study), raises the question of the true ancestor of the Microsporidia. *Nucleophaga* appeared morphologically distinct from Microsporidia (Michel et al. 2009a; Corsaro et al. 2014b); however, new data indicate that structures resembling anchoring disc and atypical polar filament are present in its spores (Michel et al. 2012; this study). In the opisthokont 18S tree, *Nucleophaga* forms a deep branch with fast-evolving rDNA sequences (~1.8 and 1.3 faster with respect to *Paramicrosporidium* and *Mitosporidium*, respectively), and its clustering with Microsporidia in 18S trees may be due to a long branch attraction effect. Nevertheless, *Mitosporidium* is always recovered with high statistical support within the group LKM15. In the study of Grossart et al. (2016), who included a higher number of uncultured clones, the group LKM15 including *Mitosporidium* and the clade comprising Microsporidia, *Nucleophaga* and even the group LKM46, were recovered as very distinct branches.

These new microorganisms show similar morphological features, like atypical polar filaments, polaroplast absent or poorly

developed, and endogenous sporogony, all traits present in the 'primitive' microsporidia, the Metchnikovellidea (Rudimicrosporea), hyperparasites of gregarines infecting mainly marine worms (anellids), and the Chytridiopsida, parasites prevalently of terrestrial arthropods (Sprague 1977; Larsson 2014; Sokolova et al. 2014; Radek et al. 2015; Rotari et al. 2015). The simpler morphologies showed by metchnikovellids and chytridiopsids, as compared to classic microsporidians, suggested indeed that they may represent two basal nested lineages of the phylum. Therefore, the future availability of genetic data from them will be of great interest to clarify the relationships among all these lineages of putative basal microsporidia.

While in *Rozella* the penetration tube is formed once the parasite is attached to the host, in these various microsporidia-like organisms the invasion apparatus is preformed in the spore, like in the Microsporidia. *Paramicrosporidium* and *Nucleophaga* enter the amoebal cell by host phagocytosis; however, they might use the apparatus successively, e.g. to escape from the food vacuole or to penetrate the nucleus.

In contrast to Microsporidia, which have 16S-like SSU and 5.8S fused into the LSU, these new organisms also have a standard eukaryotic SSU size (18S) and rDNA unit structure (5.8S and LSU separated by ITS2). Furthermore, the active stages of *Nucleophaga* and *Mitosporidium* produce pseudopodia-like projections interdigitating with the nucleoplasm and cytoplasm, respectively, of the cell hosts. These projections, absent in Microsporidia, interestingly recall those of *Rozella* growing within the host's cytoplasm. Another peculiarity of *Mitosporidium* is its fungal-like nuclear genome and the persistence of functional mitochondria producing ATP with a reduced mitogenome very similar to that of *Rozella*, and the apparent lack of ATP translocase (s) (Haag et al. 2014). These proteins, present in Microsporidia and *Rozella*, allow to import ATP from the host cell, and their phylogeny indicates that all originated via horizontal gene transfer from the energy-parasitic bacteria Chlamydiae (James et al. 2013). A possible explanation could be that for *Mitosporidium* stealing the host ATP might have been unnecessary due to the conservation of still functional mitochondria, resulting then in the loss or non-integration of ATP translocase (Haag et al. 2014). Mitochondria have not been seen in *Paramicrosporidium* spp. and *N. amoebae*, but their presence in *Mitosporidium* was revealed by genome sequencing. In *N. terricolae*, enveloped structures were observed, which could be mitochondria/mitosomes containing 30-nm virus-like particles (VLP) (Michel et al. 2012). In the Microsporidia, similar VLP, of 25 or 50 nm, were only rarely seen in the sporont cytoplasm or in the spore nucleus (Larsson 1988; Lom and Pekkarinen 1999; Vávra et al. 1997). Genomic data from the amoebal endoparasites will help to clarify the evolution of ATP translocases and of mitochondria within the Rozellomycota. In this context, it is noteworthy that chlamydiae are widely present in the environment, and various of them are endosymbionts of free-living amoebae, including those infected by our microsporidia-like rozellids (Corsaro et al. 2010, 2013). The horizontal gene transfer(s) between chlamydiae and rozellids could have been facilitated by the sharing of the same host such as amoebae or other protists. The possibility that a rozellid was infected by a chlamydia should however not be excluded.

The microsporidia evolved from endoparasitic rozellids, in an aquatic environment, probably freshwater, almost certainly involving protists and invertebrates as hosts. Surely further studies on these organisms, involving multigene and/or comparative genome analyses, are needed to resolve the early evolutionary history of the Microsporidia. This will provide a better understanding of the evolution of intracellular parasitism, and will also contribute to a better definition of the fungal kingdom and its close relatives.

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