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GENETICS, EVOLUTION, AND PHYLOGENY - ORIGINAL PAPER

Filling gaps in the microsporidian tree: rDNA phylogeny of *Chytridiopsis typographi* (Microsporidia: Chytridiopsida)

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Abstract

Microsporidia are intracellular eukaryotic parasites of animals, characterized by unusual morphological and genetic features. They can be divided in three main groups, the classical microsporidians presenting all the features of the phylum and two putative primitive groups, the chytridiopsids and metchnikovellids. Microsporidia originated from microsporidia-like organisms belonging to a lineage of chytrid-like endoparasites basal or sister to the Fungi. Genetic and genomic data are available for all members, except chytridiopsids. Herein, we filled this gap by obtaining the rDNA sequence (SSU-ITS-partial LSU) of *Chytridiopsis typographi* (Chytridiopsida), a parasite of bark beetles. Our rDNA molecular phylogenies indicate that *Chytridiopsis* branches earlier than metchnikovellids, commonly thought ancestral, forming the more basal lineage of the Microsporidia. Furthermore, our structural analyses showed that only classical microsporidians present 16S-like SSU rRNA and 5.8S/LSU rRNA gene fusion, whereas the standard eukaryote rRNA gene structure, although slightly reduced, is still preserved in the primitive microsporidians, including 18S-like SSU rRNA with conserved core helices, and ITS2-like separating 5.8S from LSU. Overall, our results are consistent with the scenario of an evolution from microsporidia-like rozellids to microsporidians, however suggesting for metchnikovellids a derived position, probably related to marine transition and adaptation to hyperparasitism. The genetic and genomic data of additional members of Chytridiopsida and Rozellomycota will be of great value, not only to resolve phylogenetic relationships but also to improve our understanding of the evolution of these fascinating organisms.

Keywords Microsporidia · Chytridiopsida · *Chytridiopsis* · Rozellomycota · Microsporidia-like organisms

Introduction

Microsporidia are obligate intracellular eukaryotic parasites of virtually all animals, including humans, and of some protists such as ciliates and gregarines (Alveolata). They are characterized by a distinct structure of the spore with its invasion apparatus, an extreme genome compaction and high genetic mutation rates (Corradi 2015; Vávra and Lukeš 2013; Han and Weiss 2017). Microsporidia are highly dependent on their host cell for metabolic processes and energy supply, because of massive nuclear gene losses and reduction of mitochondria to DNA-free mitosomes during their evolution (Katinka et al. 2001; Williams et al. 2002; Keeling et al. 2010; Cuomo et al. 2012). They steal ATP from the host through nucleotide translocases of bacterial origin that were integrated by an ancient horizontal transfer from the energy parasites Chlamydiae (Tsaousis et al. 2008).

The infective stage is a unicellular walled spore that presents a peculiar invasion apparatus consisting of a polar filament attached to a polar sac containing an anchoring disk and

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associated membraneous structures such as a polaroplast and a posterior vacuole. After an appropriate stimulus, the membranes swell and push the filament, which is rapidly everted towards the outside to pierce the host cell plasma membrane/phagosome, allowing the passage of the spore content (sporoplasm) (Franzen 2004; Xu and Weiss 2005). In the host cell, the sporoplasm begins a series of divisions (merogony), and the progeny finally enters a maturation phase with the formation of thick-membraned sporonts from which originate the sporoblasts, which will successively form new spores (sporogony) (Vávra and Lukeš 2013; Han and Weiss 2017).

Such distinct cytology, coupled to ultrastructural and developmental characteristics, founded the historical basis of the high-level classification of Microsporidia. However, this latter has been modified several times, and it still does not fit well with the genetic data. Current molecular phylogenies based on the small subunit rRNA gene (SSU rDNA) sequences allowed to identify five major groups, which include most of the known species (over 1500 in about 200 genera) presenting the typical characteristics of the phylum, the so-called classical Microsporidia. The remaining microsporidians include two poorly studied groups, the metchnikovellids and the chytridiopsids (each comprising less than 30 species included in a dozen of genera in total), that have unusual features and are commonly considered to be primitive (Larsson 2014).

At the same time, the morphological uniqueness of Microsporidia has rendered difficult to find clear affinities with any other lineage, so that their origin and evolution have remained enigmatic for a long time. Various studies have suggested some relationships with different fungal groups, but only recently, it was possible to establish a reliable link with *Rozella*, which are chytrid-like endoparasites of water molds and algae forming the most basal lineage of flagellated fungi (James et al. 2013). This evolutionary link was subsequently strengthened by the discovery within the Rozellomycota of microsporidia-like organisms that have strong morphological similarities with Microsporidia while keeping genetic characteristics of fungi: *Paramicrosporidium* spp. and *Nucleophaga* spp., both endonuclear parasites of free-living amoebae (Amoebozoa) (Corsaro et al. 2014a, b, 2016), and *Mitosporidium daphniae*, an intracytoplasmic gut parasite of the water flea *Daphnia magna* (Crustacea) (Haag et al. 2014). Phylogenetic analyses based on the rDNA indicate that the Rozellomycota are paraphyletic and that the Microsporidia have evolved from them, *Nucleophaga* being their closest relative (Corsaro et al. 2014a, 2016). Genome-scale studies are congruent with this scenario and show that multiple adaptations to intracellular parasitism have occurred in the group (Quandt et al. 2017). The microsporidia-like *Mitosporidium* (Haag et al. 2014) and *Paramicrosporidium* (Quandt et al. 2017) both have mitochondria that are probably functional although not visible, but with differentially derived mitogenomes. The one of *Mitosporidium* is slightly reduced and very similar to that of *Rozella* (James et al. 2013) while that of *Paramicrosporidium* being complete for mitochondrial respiration (Quandt et al. 2017). Also, both *Mitosporidium* and *Paramicrosporidium* are lacking genes for the nucleotide translocase that *Rozella* shares with all Microsporidia (James et al. 2013; Haag et al. 2014; Quandt et al. 2017). The translocase genes, however, are lacking also in the (partial) genome of *Amphiamblys* (Metchnikovellida), which otherwise appears to have a typical microsporidial mitosome (Mikhailov et al. 2016). The metchnikovellids are considered to be the more primitive microsporidians, and in the multigene tree, *Amphiamblys* indeed forms the sister group of the classical microsporidians while *Mitosporidium* branches earlier (Mikhailov et al. 2016).

The order Metchnikovellida comprises two families (Amphiacanthidae and Metchnikovellidae) of hyperparasites of gregarines, the latter being all epicellular parasites of body cavities of mostly marine annelids (eunicids, phyllodocids, opheliids, capitellids, echiurids, and sipunculids) (Larsson 2014). Metchnikovellids have unusual structural and developmental features that suggest their possible ancestral status to be treated as a monotypic group, the class Rudimicrosporea (Sprague 1977). They have indeed a spore with a short polar filament without the coiled region (manubroid) that penetrates a polar sac devoid of anchoring disk and no polaroplast and posterior vacuole. The life cycle lacks a merogonic phase and includes two sporogonic sequences, a free sporogony producing free spores within the host gregarine cell, and an enveloped or sac-bound sporogony where spores accumulate in thick-walled cysts or spore sacs that are thus released from the gregarine host (Larsson 2000; Larsson and Køie 2006; Sokolova et al. 2013, 2014).

Many of these atypical features are also present in the order Chytridiopsida, which includes four families (Burkeidae, Buxtehudiidae, Chytridiopsidae, and Hesseidae) of microsporidians infecting mainly terrestrial arthropods like insects, mites and myriapods, or annelids (De Puytorac and Tourret 1963; Burke 1970; Beard et al. 1990; Larsson et al. 1997; Radek et al. 2015). The polar filament is usually short and coiled and attached to a simple polar sac, the posterior vacuole is lacking, and the polaroplast is highly reduced or missing. The life cycle generally lacks merogony but includes always an enveloped sporogony (spores in a sporocyst), some taxa (*Buxtehudea*, *Chytridiopsis*) having also a free sporogony (spores free in the host cell). Another feature common to both metchnikovellids and chytridiopsids is the spore formation in the enveloped sporogony. Spores are formed through vacuolation of sporont mother cells (endogenous sporogony) where the plasma membrane of the sporont will remain as the inner layer of the cyst while vacuoles will form the plasma membrane of the sporoblasts. In classical microsporidians, the plasma membrane of the sporoblasts originates from that of the sporont. All these features suggest that the Metchnikovellida and the Chytridiopsida are distinct but likely related groups and that together they are separated from the classical Microsporidia (Weiser 1977; Larsson 1993, 2000).

Recently, SSU rDNA sequences and even the genome of Metchnikovellida have been obtained, leaving Chytridiopsida as the sole group for which genetic data are lacking. Since Metchnikovellida and Chytridiopsida, both have overall similarities with the microsporidia-like organisms, clarifying the phylogenetic position of Chytridiopsida by molecular analyses may have significant implications to elucidate relationships among basal microsporidians. The representative genus of the group, *Chytridiopsis* (Chytridiopsidae), comprises mostly pathogens of insects, with *Chytridiopsis typographi* being a common pathogen of midgut epithelial cells of bark beetles and widely distributed throughout Europe (Purrini and Weiser 1985; Wegensteiner and Weiser 1996; Wegensteiner et al. 2015). Herein, we obtained the rDNA sequence of *Chytridiopsis typographi* from naturally infected bark beetle hosts. We used SSU and rDNA unit (SSU + 5.8S + LSU) to infer molecular phylogeny of microsporidians and allied lineages, we compared the internal transcribed spacer (ITS) structures, and we discussed evolutionary hypotheses on the origins and evolution of microsporidians.

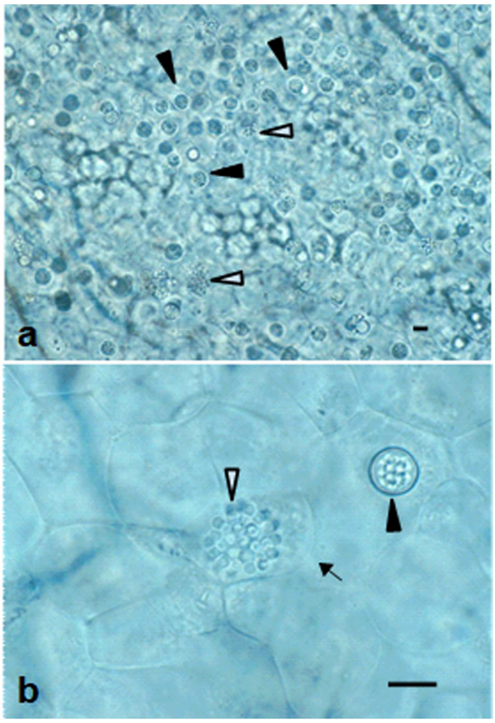


Fig. 1 *Chytridiopsis typographi* infecting the bark beetle *Ips typographus* (histological section). **a** *C. typographi* developing in midgut epithelium cells of *I. typographus*. **b** Spores of *C. typographi* free in an epithelial cell (arrow) and enclosed in a sporocyst. Free spores and sporocysts are indicated by white and black arrowheads, respectively. Toluidine blue stain. Scale bar = 10 μm

Material and methods

Sample origin and processing

Individuals of the European spruce engraver *Ips typographus* (Coleoptera: Curculionidae), an important conifer forest pest insect, were collected on July 11–13, 2016, at sites in Poland close to Serpelice (Mazowieckie) (52°18′41″ N 23°01′21″ E), and Białowieża (Podlaskie) (52°40′12″ N 23°46′12″ E). Infection by *C. typographi* was revealed by microscopic analysis of intestines (Fig. 1) (Wegensteiner et al. 1996). Midgut samples from infected individuals were stored in ethanol and processed for DNA extraction.

Spores of *C. typographi* were isolated from altogether six infected midguts. In brief, the ethanol was removed, and the samples were dissolved in 200 μl sterile dH_2O (Sigma-Aldrich, Vienna, Austria). Subsequently, the midgut tissue was disrupted by 3 cycles of freeze-thawing and the spores were separated from the host tissue cells by differential centrifugation. Both the supernatants and the pellets were collected for further analysis. The pellets were dissolved in 200 μl of sterile dH_2O, homogenized using a Precellys bead homogenizer (PeqLab, Erlangen, Germany), and subjected to DNA isolation using the QIAmp DNA isolation kit (Qiagen, Vienna, Austria) following the procedure for tissue. From the supernatants, DNA was isolated using the Precellys Bacterial/Fungal DNA isolation kit (PeqLab, Erlangen, Germany) following the procedure for fungal spores.

Phylogenetic analysis

The rRNA operon was amplified and sequenced by using a set of eukaryotic primers to cover the complete SSU, the ITS, and partial large subunit (LSU) rDNA, as described previously (Corsaro et al. 2014a). The forward and reverse primers that worked for the metchnikovellids (Mikhailov et al. 2016) were also used. The various fragments obtained were analyzed by BLAST and partial tree-building to exclude DNA sequences originating from the beetle or other possible organisms. Fragments from distinct samples were virtually identical (> 99.9%) in their overlapping portions. Thus, a consensus sequence was generated. The finished rDNA sequence of *Chytridiopsis* was deposited in GenBank (ID MH728789).

The SSU rDNA sequences of representatives of main groups of the microsporidians and their relatives were aligned using MAFFT and manually refined to exclude ambiguous sites using BIOEDIT. A distinct alignment was prepared as above by concatenating SSU, 5.8S, and LSU sequences of selected members of the analyzed taxa. We used MrBayes (Ronquist and Huelsenbeck 2003) and maximum likelihood (ML) for phylogenetic analyses. The MrBayes analysis was performed using the GTR model of substitution (Lanave et al. 1984) and gamma-shaped distribution of rates of substitution among sites with eight rate categories (GTR + Γ:8). The Bayesian analysis was performed for 20,000,000 generations and sampled every 2000 generations for four simultaneous MCMC chains (burn-in = 1000). The ML tree was built using a GTR + Γ + I:4 model with Treefinder (Jobb et al. 2004), with 1000 bootstraps.

Secondary structure analyses

Secondary structures of SSU rRNA, ITS-2, and 5.8S/LSU interactions were inferred manually and with the help of Mfold following literature data (Coleman 2007; Vaughn et al. 1984; Wuyts et al. 2001; Corsaro et al. 2014a). Final structures were drawn using RnaViz (De Rijk et al. 2003).

Results

Molecular phylogeny

As for *Paramicrosporidium*, trials with microsporidia-specific primers yielded no results and the rDNA (SSU-ITS-partial LSU) of *Chytridiopsis* was obtained using eukaryotic primers (Corsaro et al. 2014a). For the SSU rDNA, sequence similarity values of *Chytridiopsis* are ~ 61 and 57% with *Amphiamblys* and *Amphiacantha*, respectively, and slightly higher (~ 65%) with *Nucleophaga*. In the SSU rDNA tree (Fig. 2), the main fungal phyla are generally recovered as highly supported lineages, and the Microsporidia form a clade with Rozellomycota, as shown previously (James et al. 2013; Corsaro et al. 2014a, 2016; Mikhailov et al. 2016). As expected, classical microsporidians and metchnikovellids both form long branches each grouping into fully supported holophyletic lineages. Within the classical microsporidians, four of the five clades are well recovered, while clade II members form two distinct lineages. Surprisingly, *Chytridiopsis* emerges as the most basal branch of Microsporidia, metchnikovellids and classical microsporidians are sisters, and the environmental clone BAQA65 from a brackish anoxic sediment, which was shown previously to be related to Microsporidia (Dawson and Pace 2002), forms an intermediate lineage. *Nucleophaga* appears to be the closest relative to Microsporidia, in agreement with previous studies (Corsaro et al. 2016; Grossart et al. 2016; Mikhailov et al. 2016; Stentiford et al. 2017), which also show that among the Rozellomycota, the microsporidia-like organisms (i.e., *Mitosporidium*, *Nucleophaga*, *Paramicrosporidium*) never form a clade but are instead intermingled with distinct groups of environmental clones some of which (i.e., LKM11, LKM46) have a flagellate chytrid-like stage (Corsaro et al. 2014a, b, 2016; Grossart et al. 2016; Stentiford et al. 2017).

The rDNA unit (SSU + 5.8S + LSU) tree (Fig. 3) confirms the sisterhood of classical microsporidians and metchnikovellids (*Amphiamblys*), and the basal position of *Chytridiopsis*, whereas the position of *Nucleophaga* as the closest relative is less supported, presumably because only a short portion (< 300 nt) of the LSU was obtained.

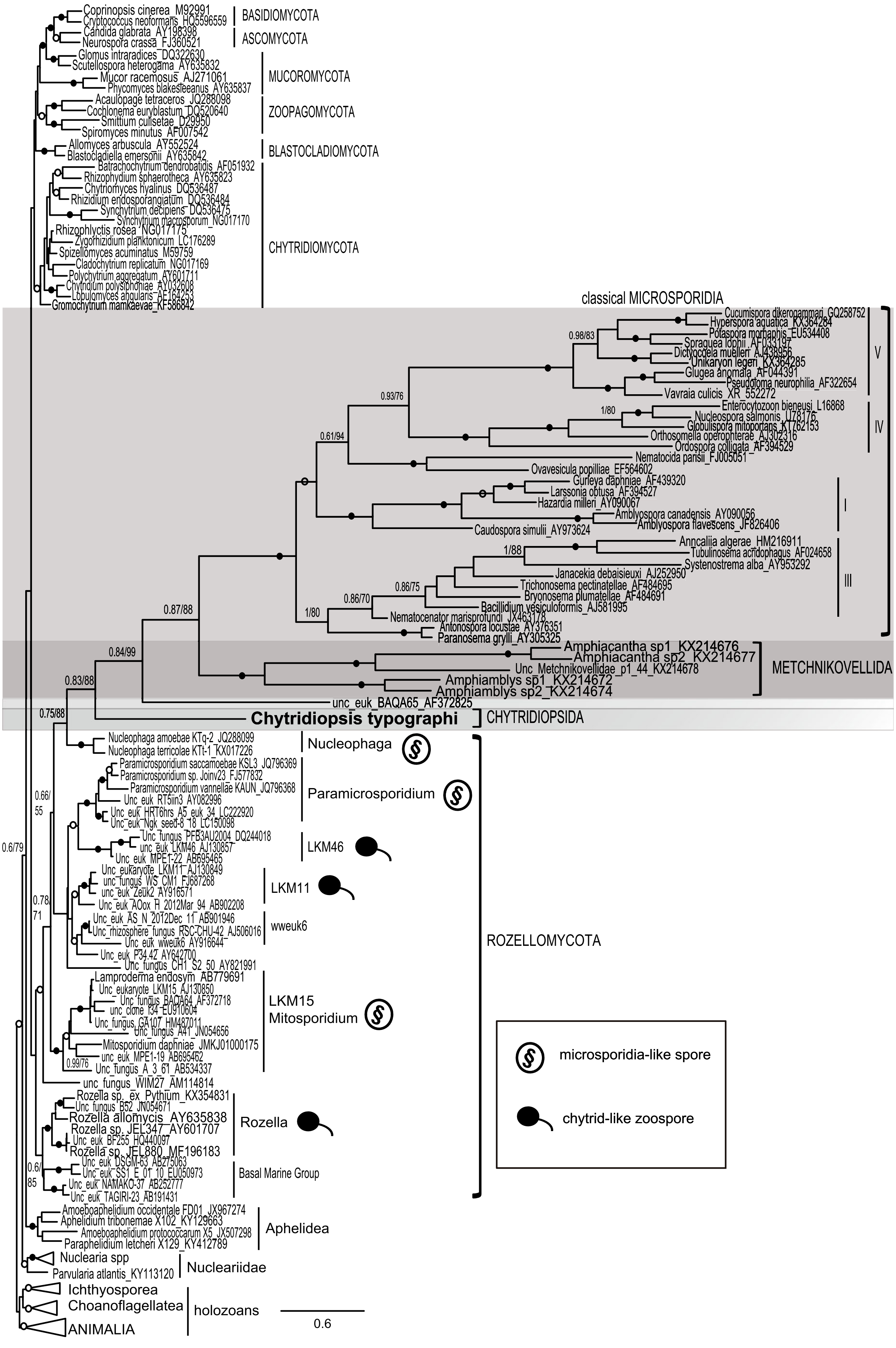
SSU rRNA secondary structure

Secondary structures for the SSU rRNA of *Chytridiopsis* and the metchnikovellids *Amphiacantha* and *Amphiamblys* were determined and compared with those of classical microsporidians available at the Comparative RNA Web site (http://www.rna.icmb.utexas.edu).

Microsporidian rRNA sequences are known for their drastic reduction in the eukaryotic core structure helices (Wuyts et al. 2001). Notably, in their SSU rRNA, H10 is almost always missing, and when E23 is present, it has only one expansion (E23/1). Overall, these modifications seem to be in agreement with the phylogenetic position of classical microsporidians, basal groups (e.g., *Bacillidium*, *Bryonosema*) having often less reduced structures compared to advanced groups (e.g., *Nosema*, *Orthosomella*, *Vairimorpha*).

The *Chytridiopsis* SSU rRNA, although shorter (1557 nt) than a typical eukaryotic sequence, keeps the entire core structure helices, except for H27 that is missing, and it also presents up to five expansion loops in E23 (Fig. 4). A similar structure is present in *Amphiacantha* (1690 nt), which also keeps all helices, but H27 shows four expansion loops in E23 and also two others between H43 and H44 (E43/1 and E43/2) (Suppl. Fig. 1). The structure of the other available *Amphiacantha* (1460 nt) is almost identical, lacking H46 and E43 and having only two loops in E23. The shorter *Amphiamblys* SSU (~ 1370 nt) presents a slightly reduced structure, lacking H10, H18, and H44 but possessing H27 and only E23/1 (Suppl. Fig. 2). On the other hand, the SSU rRNA sequences of *Mitosporidium*, *Nucleophaga*, and *Paramicrosporidium* have typical eukaryotic sizes (~ 1800 nt) and are easily alignable with that of *Rozella*, all having a complete set of eukaryotic structure helices.

Fig. 2 SSU phylogenetic tree of Fungi and relatives (holomycotans). The ▶ main fungal phyla are indicated in uppercase, and the main subgroups of Microsporidia are highlighted by distinctly shaded areas. For the classical Microsporidia, clades I, III, IV, and V are indicated. The recovered sequence for *Chytridiopsis* is in bold. The tree is rooted with members of holozoans (collapsed). In Rozellomycota, subgroups are named according to Corsaro et al. (2016), and icons mark those for which the spore type is known. Support values for Bayesian posterior probability and maximum likelihood bootstraps (1000 replicates) are indicated at nodes, with values 1/100% or 0.90/95% (filled or open circles, respectively)


Coprinopsis cinerea_M92991
Cryptococcus neoformans_HQ5596559
BASIDIOMYCOTA
Candida glabrata_AY198398
Neurospora crassa_FJ360521
ASCOMYCOTA
Glomus intraradices_DQ322630
Scutellospora heterogama_AY635832
Mucor racemosus_AJ271061
Phycomyces blakesleeanus_AY635837
MUCOROMYCOTA
Acaulopage tetraceros_JQ288098
Cochlonema euryblastum_DQ520640
Smittium culisetae_D29950
Spiromyces minutus_AF007542
ZOOPAGOMYCOTA
Allomyces arbuscula_AY552524
Blastocladiella emersonii_AY635842
BLASTOCLADIOMYCOTA
Batrachochytrium dendrobatidis_AF051932
Rhizophydium sphaerotheca_AY635823
Chytriomyces hyalinus_DQ536487
Rhizidium endosporangiatum_DQ536484
Synchytrium decipiens_DQ536475
Synchytrium macrosporum_NG017170
Rhizophlyctis rosea_NG017175
Zygorhizidium planktonicum_LC176289
Spizellomyces acuminatus_M59759
Cladochytrium replicatum_NG017169
Polychytrium aggregatum_AY601711
Chytridium polysiphoniae_AY032608
Lobulomyces angularis_AF164253
Gromochytrium mamkaevae_KF586842
CHYTRIDIOMYCOTA
classical MICROSPORIDIA
0.98/83
Cucumispora dikerogammari_GQ258752
Hyperspora aquatica_KX364284
Potaspora morhaphis_EU534408
Spraguea lophii_AF033197
Dictyocoela muelleri_AJ438956
Unikaryon legeri_KX364285
Glugea anomala_AF044391
Pseudoloma neurophilia_AF322654
Vavraia culicis_XR_552272
V
0.93/76
1/80
Enterocytozoon bieneusi_L16868
Nucleospora salmonis_U78176
Globulispora mitoportans_KT762153
Orthosomella operophterae_AJ302316
Ordospora colligata_AF394529
IV
0.61/94
Nematocida parisii_FJ005051
Ovavesicula popilliae_EF564602
Gurleya daphniae_AF439320
Larssonia obtusa_AF394527
Hazardia milleri_AY090067
Amblyospora canadensis_AY090056
Amblyospora flavescens_JF826406
Caudospora simulii_AY973624
I
1/88
Anncaliia algerae_HM216911
Tubulinosema acridophagus_AF024658
Systenostrema alba_AY953292
Janacekia debaisieuxi_AJ252950
0.86/75
Trichonosema pectinatellae_AF484695
Bryonosema plumatellae_AF484691
0.86/70
Bacillidium vesiculoformis_AJ581995
Nematocenator marisprofundi_JX463178
1/80
Antonospora locustae_AY376351
Paranosema grylli_AY305325
III
0.87/88
0.84/99
Amphiacantha sp1_KX214676
Amphiacantha sp2_KX214677
Unc_Metchnikovellidae_p1_44_KX214678
Amphiamblys sp1_KX214672
Amphiamblys sp2_KX214674
METCHNIKOVELLIDA
0.83/88
unc_euk_BAQA65_AF372825
0.75/88
Chytridiopsis typographi
CHYTRIDIOPSIDA
Nucleophaga amoebae KTq-2_JQ288099
Nucleophaga terricolae KTt-1_KX017226
Nucleophaga
§
Paramicrosporidium saccamoebae KSL3_JQ796369
Paramicrosporidium sp. Joinv23_FJ577832
Paramicrosporidium vannellae KAUN_JQ796368
Unc_euk_RT5iin3_AY082996
Unc_euk_HRT6hrs_A5_euk_34_LC222920
Unc_euk_Ngk_seed-8_18_LC150098
Paramicrosporidium
§
0.66/55
Unc_fungus_PFB3AU2004_DQ244018
unc_euk_LKM46_AJ130857
Unc_euk_MPE1-22_AB695465
LKM46
0.6/79
Unc_eukaryote_LKM11_AJ130849
unc_fungus_WS_CM1_FJ687268
unc_euk_Zeuk2_AY916571
Unc_euk_AOox_H_2012Mar_94_AB902208
LKM11
0.78/71
Unc_euk_AS_N_2012Dec_11_AB901946
Unc_rhizosphere_fungus_RSC-CHU-42_AJ506016
Unc_euk_wweuk6_AY916644
wweuk6
Unc_euk_P34.42_AY642700
Unc_fungus_CH1_S2_50_AY821991
ROZELLOMYCOTA
Lamproderma endosym_AB779691
Unc_eukaryote_LKM15_AJ130850
Unc_fungus_BAQA64_AF372718
unc_clone_I34_EU910604
Unc_fungus_GA107_HM487011
Unc_fungus_A41_JN054656
Mitosporidium daphniae_JMKJ01000175
unc_euk_MPE1-19_AB695462
0.99/76
Unc_fungus_A_3_61_AB534337
LKM15
Mitosporidium
§
§ microsporidia-like spore
● chytrid-like zoospore
unc_fungus_WIM27_AM114814
Rozella sp._ex_Pythium_KX354831
Unc_fungus_B52_JN054671
Rozella allomycis_AY635838
Rozella sp. JEL347_AY601707
Unc_euk_BF255_HQ440097
Rozella sp. JEL880_MF196183
Rozella
0.6/85
Unc_euk_DSGM-63_AB275063
Unc_euk_SS1_E_01_10_EU050973
Unc_euk_NAMAKO-37_AB252777
Unc_euk_TAGIRI-23_AB191431
Basal Marine Group
Amoeboaphelidium occidentale FD01_JX967274
Aphelidium tribonemae X102_KY129663
Amoeboaphelidium protococcarum X5_JX507298
Paraphelidium letcheri X129_KY412789
Aphelidea
Nuclearia spp
Parvularia atlantis_KY113120
Nucleariidae
Ichthyosporea
Choanoflagellatea
ANIMALIA
holozoans
0.6

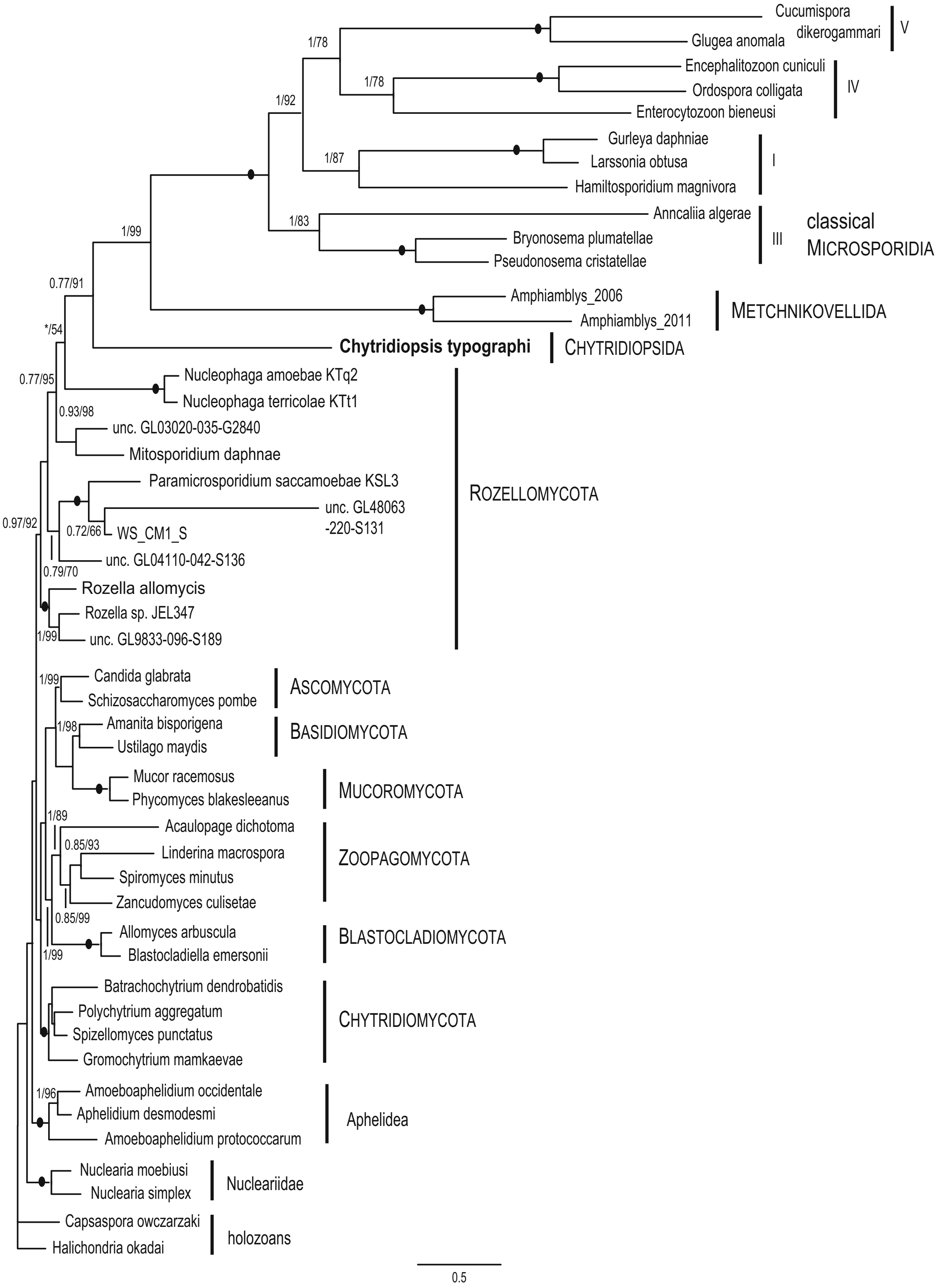


Fig. 3 Phylogenetic tree of Microsporidia and relatives based on rDNA unit (SSU + 5.8S + LSU). Symbols as in Fig. 2

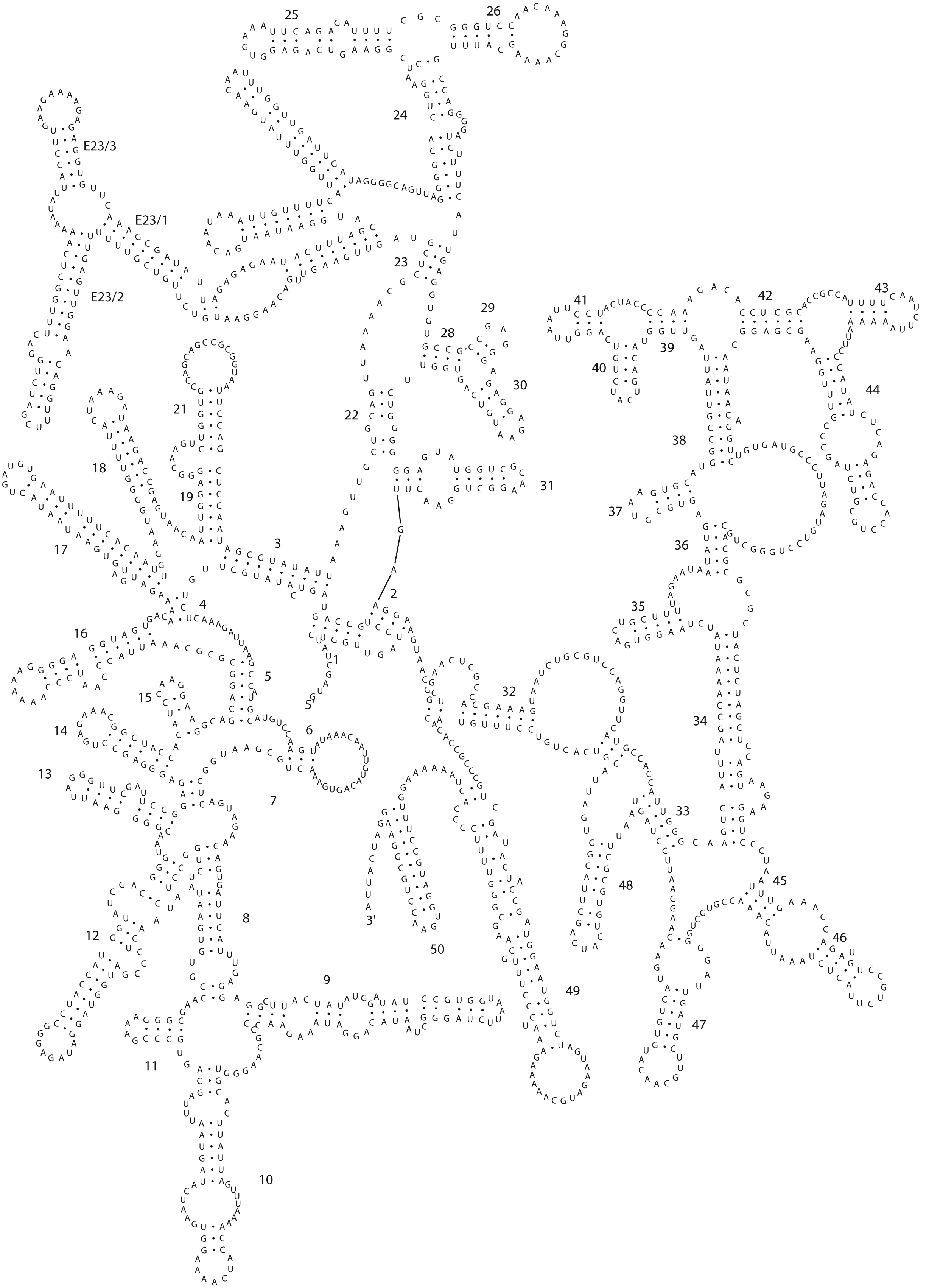


Fig. 4 Secondary structure of the SSU rRNA of *Chytridiopsis typographi*. Stems and helices were numbered according to Wuyts et al. (2001). The 5′-end (22 nt) was determined with the eukaryotic forward primer used to amplify SSU of metchnikovellids (Mikhailov et al. 2016)

ITS structure and 5.8S/LSU interaction

Microsporidia are characterized by lacking a separated 5.8S rRNA gene, which is almost always fused to the 5′-end of the LSU rDNA, a feature resembling the prokaryote condition but that originated by the loss of the ITS-2. We sequenced ITS and partial LSU rDNA of *Chytridiopsis*, in order to obtain the 5.8S rRNA gene, to verify the presence of ITS-2, and to model the 5.8S/LSU interaction. The various gene portions were analyzed according to previous studies (Coleman 2007; Vaughn et al. 1984; Corsaro et al. 2014a) and, for comparison, the analysis included available sequences of *Rozella allomycis*, *Paramicrosporidium saccamoebae*, and *Nucleophaga terricolae* (Corsaro et al. 2014a, 2016; this study) for Rozellomycota, *Amphiamblys* sp. (Mikhailov et al. 2016) for Metchnikovellida, and *Bryonosema plumatellae* (Genbank ID AF484691) (Canning et al. 2002) for classical Microsporidia.

For *Chytridiopsis*, we found that 5.8S and LSU rRNA genes are separated by 102 nt, which is nearly twofold the lengths found in the two *Amphiamblys* spp. (47 and 64 nt). In our reconstructions (Fig. 5), insertion sequences of *Chytridiopsis* and both *Amphiamblys* spp. form structures with three or two helices, respectively. Pyrimidine-pyrimidine bulge typical of ITS-2 helix II (Coleman 2007) is only recognizable in the four-helix structure of *Rozella* as well as in three-helix models of *Paramicrosporidium* and *Nucleophaga*. The ITS region is not available for *Mitosporidium*. However, by considering its 5.8S and LSU rDNA portions, we can predict that *Mitosporidium* also has ITS-2. As expected, in *Bryonosema*, ITS-2 is lacking and secondary structures are formed only by the LSU and its 5′-end portion corresponding to the 5.8S.

Discussion

Classical microsporidians have been known since the middle of the nineteenth century and early recognized as a distinct group based on their peculiar spore with a coiled polar filament that discharges (Thélohan 1894). This feature, however, was not visible for metchnikovellids and chytridiopsids, also known from the end of the nineteenth century. Both groups were assigned to the Microsporidia only when, in the 1960s, electron microscopy studies allowed to show their microsporidian ultrastructure (Vivier 1965; Manier and Ormieres 1968). Nevertheless, metchnikovellids and chytridiopsids differ from classical microsporidians by sharing some structural and developmental traits presumably primitive, like very short polar filaments, polaroplast absent or poorly developed, and endogenous sporogony by vacuolation in the spore sac. They have thus been considered as primitive, usually placing metchnikovellids as the ancestral lineage and chytridiopsids as an intermediate group (Sprague 1977; Weiser 1977; Larsson 2000; Sokolova et al. 2014).

Results presented here, while confirming some affinity between metchnikovellids and chytridiopsids as well as their basal position, modify this scenario, suggesting that they are in a reversal place. In both SSU and rDNA unit (SSU + 5.8S + LSU) phylogenies (Figs. 2 and 3), metchnikovellids are clearly a distinct lineage, sister to classic microsporidians, as it was shown by previous phylogenomic analyses (Mikhailov et al. 2016), while *Chytridiopsis* branches earlier forming the most basal lineage of the Microsporidia. Such an unexpected phylogenetic result is, however, congruent with the structures of the rRNA genes of the different taxa we analyzed. Indeed, only classical microsporidians have the rRNA operon of prokaryote-like size and structure, i.e., 16S-like SSU and 23S-like LSU with 5.8S rRNA genes fused at 5′-end. By contrast, *Ampiacantha* and *Chytridiopsis*, both have rather a 18S-like SSU rRNA keeping the eukaryote core helices (Fig. 4, Suppl. Fig. 1). Although the SSU rRNA of *Amphiamblys* is reduced in size and structure, its rRNA operon, however, has the 5.8S separated from the LSU by a short (64 nt) ITS2-like fragment; a longer (102 nt) and more complex ITS2-like fragment is present in *Chytridiopsis* (Fig. 5). This is congruent with the progressive reduction in size and structure of the ITS-2 from the ~ 300 nt of chytrids (~ 250 nt for *Rozella*), to the ~ 150 nt of microsporidia-like rozellids (129 nt for *Paramicrosporidium*; 169 nt for *Nucleophaga*) until its complete disappearance in classical microsporidians leading to the 5.8S/LSU rRNA gene fusion (Corsaro et al. 2014a).

A relative rate test conducted on SSU rDNA sequences also indicates progressive value increasing with accelerated mutation rates starting with the primitive microsporidians. *Nucleophaga* evolves ~ 2.5 times faster than *Paramicrosporidium* and *Mitosporidium*, while *Chytridiopsis* and metchnikovellids (*Amphiacantha* and *Amphiamblys*) are 5–10 and 10–15 times faster than the three microsporidia-like rozellids, respectively. Metchnikovellids are only twofold faster than *Chytridiopsis*, suggesting, however, they are evolving more rapidly. As expected, classical microsporidians (*Bryonosema* and *Larssonia*) show the higher values, up to 20-fold with respect to the microsporidia-like rozellids, only 2–3-fold with respect to the primitive microsporidians. Obviously, future availability of protein-coding genes will allow a better calibration of such values.

Furthermore, the earlier branching of *Chytridiopsis* would also allow a better explanation of the transition from the rozellids to microsporidians. It is now clear that traits thought to be synapomorphic to Microsporidia (Vávra and Lukeš 2013) have appeared, perhaps more than once, in Rozellomycota (Corsaro et al. 2014a, 2016; Haag et al. 2014). These microsporidia-like rozellids, having a rather long albeit atypical polar filament, appear morphologically more

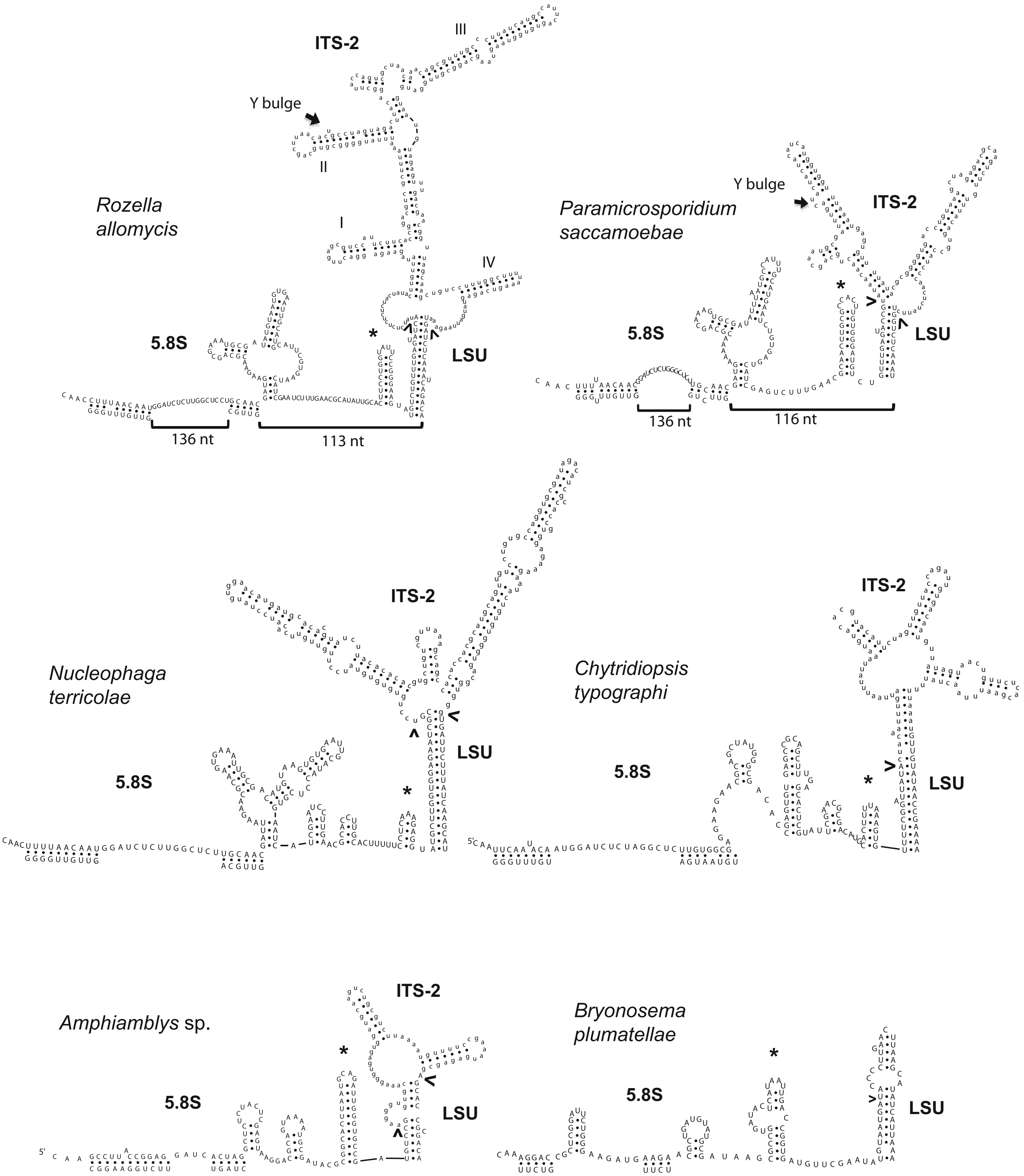


Fig. 5 Secondary structure models of the 5.8S/LSU rRNA interaction. Full sequences for 5.8S and ITS-2 and interacting portions only for LSU are shown. The ITS-2 sequences are in lowercase, with helices labeled by Roman numbers showed only for *Rozella*. Separations between genes are indicated by arrowheads. For the 5.8S, the typical GC-rich stem is marked by an asterisk

similar to *Chytridiopsis* than to the metchnikovellids, which are characterized by the manubrium. This is especially evident for *Mitosporidium*, which could have been classified as a member of Chytridiopsida in the absence of genetic data. All this implies that the typical characteristics of the Metchnikovellida, commonly thought to be primitive, could actually be the result of their evolution as hyperparasites of gregarines. The hypothesis that gregarines might have served as Trojan horses for the dispersal of microsporidians from marine and brackish water annelids to terrestrial arthropods (Sokolova et al. 2013) appears thus invalidated. It is easier to imagine that the evolutionary steps have occurred in the freshwater, an environment common to all the different groups, accompanied by adaptations to their different hosts. Free-living amoebae could have been a more suitable transfer host, as they are ubiquitous in both aquatic and terrestrial environments. Some amoebae (e.g., *Acanthamoeba*, *Malamoeba*, *Neoparamoeba*) can infect invertebrates, and various genera of Amoebozoa are known as hosts of the microsporidia-like *Paramicrosporidium* and *Nucleophaga*. Most of the environmental rozellid clones were recovered in freshwater habitats, and *Mitosporidium* infects *Daphnia*, a freshwater crustacean. The more basal lineage of the classical microsporidians (e.g., *Bacillidium*, *Bryonosema*, *Systenostrema*) also have as dominant hosts various freshwater invertebrates such as bryozoans, tubificid annelids, and aquatic stages of insects (Canning et al. 2002; Morris et al. 2005; Sokolova et al. 2006). Although chytridiopsids infect mainly terrestrial arthropods, a few species have been found in freshwater invertebrates: *Chytridiopsis aquaticus* and *Chytridiopsis trichopterae* infect aquatic larvae of insects, *Sheriffia brachynema* infects the freshwater snail *Biomphalaria glabrata*, and *Jiroveciana limnodrili* infects tubificid annelids (Larsson 1993, 2014). Another chytridiopsid, *Buxtehudea scaniae*, infects bristletails, primitive archeognathe insects living on seashores (Larsson 1980). Metchnikovellids evolved probably after a freshwater-to-marine transition, and the clone BAQA65 from a brackish anoxic sediment (Dawson and Pace 2002) could represent an intermediate step.

The genetic/genomic characterization of other members of the Chytridiopsida will be necessary to determine if they form a coherent group and to confirm their apparent basal position. In addition, significant efforts should be devoted to the isolation and characterization of members of the many rozellid lineages known only by their sequences. In this perspective, it may be interesting to note that, beside *Nucleophaga* (Dangeard 1895), several other parasitic "chytrids" have been described in the ancient literature (Sparrow 1960) that could belong to this group. Some like *Plasmophagus* and *Dictyomorpha*, which are parasites of green algae or oomycetes, respectively, are very similar to *Rozella*, with which they could be affiliated (Blackwell et al. 2016, 2017). And at least one other enigmatic organism, *Sagittospora*, parasite of ciliates, lacks a flagellate stage and shows some resemblance to Microsporidia (Lubinsky 1955). Whether these organisms are members of Rozellomycota or not, this indicates, however, that they are more diverse than expected and that they can be finally isolated. This will allow a finest knowledge of their real morphological diversity, and a better resolution of the relationships among microsporidia-like rozellids and basal microsporidians, avoiding forced interpretations (Bass et al. 2018). It is obvious that only genome-scale studies will allow to elucidate some fundamental steps like the origin of the polar filament or the loss of functional mitochondria, contributing to future works in the higher systematics of the entire lineage.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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