Parasitology Research (2018) 117:157–167
https://doi.org/10.1007/s00436-017-5685-6

ORIGINAL PAPER

New insights from molecular phylogenetics of amoebophagous fungi (Zoopagomycota, Zoopagales)

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Received: 20 April 2017 / Accepted: 15 November 2017 / Published online: 23 November 2017

Abstract

Amoebophagous fungi are represented in all fungal groups: Basidiomycota, Ascomycota, Zygomycota, and Chytridiomycota. The amoebophagous fungi, within the zygomycota (Zoopagales, Zoopagomycota), mainly affect naked amoebae as ectoparasites or endoparasites. It is rather difficult to isolate members of the Zoopagales, because of their parasitic lifestyle, and to bring them into culture. Consequently, gene sequences of this group are undersampled, and its species composition and phylogeny are relatively unknown. In the present study, we were able to isolate amoebophagous fungi together with their amoeba hosts from various habitats (moss, pond, bark, and soil). Altogether, four fungal strains belonging to the genera *Acaulopage* and *Stylopage* plus one unidentified isolate were detected. Sequences of the 18S rDNA and the complete ITS region and partial 28S (LSU) rDNA were generated. Subsequent phylogenetic analyses showed that all new isolates diverge at one branch together with two environmental clonal sequences within the Zoopagomycota. Here, we provide the first molecular characterization of the genus *Stylopage*. *Stylopage* is closely related to the genus *Acaulopage*. In addition, taxonomy and phylogeny of amoebophagous fungi and their ecological importance are reviewed based on new sequence data, which includes environmental clonal sequences.

Keywords Free-living amoebae · Amoebophagous fungi · *Acaulopage* · *Stylopage* · Zoopagales · Zygomycetes

Introduction

Fungi are important players in the cycles of matter because of their ability to mineralize organic material. They live mostly as saprotrophs on dead and decaying living matter, but also in mutualistic symbiosis with plants and animals, or as parasites of various organisms. The carnivorous or predaceous fungi are a peculiar and heterogeneous group of parasitic fungi. These fungi have developed trapping structures, such as adhesive spores, hyphae, or rings that may be constricting or non-constricting, to capture soil-inhabiting and water-inhabiting microinvertebrates like nematodes and rotifers, as well as testate and naked free-living amoebae (Drechsler 1941; Duddington 1956, 1973).

The nematode-trapping fungi are the best studied, because of their value as potential biocontrol agents for nematode diseases in plants and animals (Braga and Araújo 2014; Li et al. 2015). Many of these fungi belong to the Orbiliomycetes (Ascomycota) (Yang et al. 2012; Jiang et al. 2017).

Little is known about the amoebophagous fungi, by contrast, although their initial description dates back to the mid-late nineteenth century (Drechsler 1941; Duddington 1956). Various anamorphic genera assumedly belonging to the Ascomycota were described by Drechsler as predators of testate euglyphid (Cercozoa) and arcellinid (Amoebozoa) amoebae, e.g., *Pedilospora dactylopaga* (Drechsler 1934), *Tridentaria* spp. (Drechsler 1937, 1961, 1964), and *Triposporina* (Drechsler 1961). Parasites of naked amoebae were also reported, e.g., the basidiomycetes *Pagidospora*

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amoebophila (Drechsler 1960) and *Tulasnella zooctonia* (Drechsler 1969). In addition, some Basidiomycota and Ascomycota dimorphic fungi (i.e., growing either as yeast or as mycelium) are lethal to amoebae once ingested (Steenbergen et al. 2001, 2004; Bidochka et al. 2010). Zygomycetous fungi also comprise several amoebophagous as well as zoophagous members (Drechsler 1941; Duddington 1973). Other fungi known to parasitize amoebae are the chytrids, e.g., *Rhizophydium amoebae* and a few other species (Chytridiomycota, Rhyzophidiales) (Karling 1946), *Nucleophaga* and *Sphaerita* (Dangeard 1895), and *Paramicrosporidium* (Rozellomycota, Paramicrosporidiales) (Corsaro et al. 2014a). *Nucleophaga*, formerly included in the Chytridiales, was recently shown to belong to the rozellids (Rozellomycota, Nucleophagales) (Corsaro et al. 2014b). Rozellids form an early chytrid lineage comprising endoparasites of water molds (*Rozella*) as well as of various amoebae (Yajima et al. 2013; Corsaro et al. 2014a, 2014b). The Microsporidia have evolved from the rozellids (James et al. 2013; Corsaro et al. 2014a, 2016).

Zygomycotans are non-flagellated filamentous fungi characterized by a peculiar sexual meiospore, called a zygospore, derived from the fusion of conjugating hyphae. They represent the first terrestrial radiation of the fungal kingdom, intermediated therefore between the early diverging and mostly aquatic flagellated chytrids (Rozellomycota, Chytridiomycota, Blastocladiomycota) and the non-coenocytic multicellular Dikarya (Ascomycota, Basidiomycota) with complex fruiting bodies. Molecular phylogenetic studies indicate that zygomycotans might actually comprise distinct paraphyletic lineages (Tanabe et al. 2005; White et al. 2006; Liu et al. 2009). Recently, the group was reorganized into two phyla, the Mucoromycota and Zoopagomycota (Spatafora et al. 2016). While the Mucoromycota are sister to the Dikarya and form symbiotic associations with plants, the Zoopagomycota emerge as the first lineage of terrestrial fungi and interact with other fungi, animals, and amoebae. All the amoebophagous zygomycotans belong to the Zoopagales, the sole order in the subphylum Zoopagomycotina (Zoopagomycota). Zoopagales include ectoparasitic and endoparasitic fungi, such as *Amoebophilus* and *Cochlonema* that mainly attack naked amoebae. Dangeard (1910) proposed the genus *Amoebophilus*, by recognizing the misidentified uroidal structures described in some amoebae such as *Ouramoeba* (Leidy 1878) and *Longicauda* (Korotneff, 1879) as ectoparasitic fungal infections. The Zoopagales consist of obligate parasites, including those of fungi and invertebrates, which renders their isolation and culture complicated, explaining also in part the low number of species studied by biomolecular methods. By contrast, its sister group, the Kickxellomycotina, is well sampled (Tretter et al. 2014; Spatafora et al. 2016). The traditional phenotypic-based classification of the Zoopagales has been challenged by molecular studies (Tanabe et al. 2000; Köhsler et al. 2007; Michel et al. 2015). The data set includes however only 8 out of 23 described genera currently ascribed to the order (Benny et al. 2016).

Free-living amoebae are a polyphyletic assemblage of microbial eukaryotes, mostly belonging to the phylum Amoeobozoa. They constitute an ecologically and morphologically very rich group of protists, abundant in all terrestrial and aquatic habitats and playing an important role as predators in controlling microbial and small invertebrate populations (Rodríguez-Zaragoza 1994; Yeates and Foissner 1995). The vegetative stage (trophozoite) moves and feeds by cytoplasmic extrusions (pseudopods), and it can be naked (naked amoebae) or shelled (testate amoebae). Additional stages may be present such as resistant cysts or flagellate forms. Some naked species, e.g., *Acanthamoeba* (Amoebozoa: Discosea) and *Naegleria* (Excavata: Heterolobosea), are important pathogens for vertebrates (Visvesvara et al. 2007), while testate amoebae are useful bioindicators in ecology and paleoecology (Mitchell et al. 2008).

In our study, we performed 18S rDNA characterization of four additional amoebophagous zoopagalean fungi, as well as molecular phylogenetic analyses that allowed us to identify several uncultured and unidentified sequences as belonging to the Zoopagales. We also identified the amoebal preys, usually poorly documented in the mycological literature.

Materials and methods

Samples

Fungi and amoebae investigated herein derived from mud/decaying plant material within aquatic habitats or soil samples. They were obtained after repeated co-cultivations on 1.5% non-nutrient agar (NNA) plates with addition of 0.1% sea salt covered with *Enterobacter cloacae* or *Escherichia coli*, incubated at room temperature as previously described (Köhsler et al. 2007; Michel et al. 2014). Amoebophagous fungi are rather rarely found in amoebae; their percentage in our samples was below 5%. Four fungal strains were initially identified morphologically (Drechsler 1935, 1942; Dayal 1973) as *Acaulopage tetraceros*, strain At-LEMO, isolated in August 2007 from a moss sampled at Staffanstorp (Scania) in the south of Sweden, preying on an *Acanthamoeba* sp. genotype T11 (Amoebozoa, Centramoebida), and *Acaulopage dichotoma*, strain Ad-Rom, isolated in February 2015 from a pond located in Heimbach-Weis (Neuwied District, Rhineland-Palatinate), Germany, and strain Ad-Syc, isolated in September 2013 from the bark of a sycamore tree at Andernach (Mayen-Koblenz District, Rhineland-Palatinate), Southwestern Germany. Strain Ad-Rom was initially isolated with a *Vannella* sp. strain Vs-ash (Amoebozoa, Vannellida) then

Table 1 Amoebal prey spectrum of Zoopagales

Fungal parasite	Amoebal prey			
Gen/sp./strain	Original isolate	Permissive	Non-permissive	Reference
Amoebophilus simplex	*Mayorella* spp. (Dermamoebida, D)	*Mayorella penardi*, *M. vespertilioides* (Dermamoebida, D)	*Korotnevella* spp. (Dactylopodida, D) *Stenamoeba stenopodia* (Thecamoebida, D)	Mrva (2008, 2011)
Cochlonema euryblastum ECM	*Thecamoeba quadrilineata* (Thecamoebida, D)	*Thecamoeba quadrilineata* (other strains)	*Thecamoeba striata* (Thecamoebida, D) *Sappinia diploidea*, *Sappinia* sp. (Thecamoebida, D) *Vannella placida*, *Vannella* sp. (Vannellida, D) *Saccamoeba lacustris*, *S. stagnicola* (Euamoebida, T)	Michel and Wylezich (2005)
Acaulopage tetraceros				
At-Blent	*Vannella* sp. (Vannellida, D)	*Vannella miroides*, *V. placida* *T. quadrilineata*, *T. striata*, *T. terricola* *Sappinia diploidea* (Thecamoebida, D) *Acanthamoeba* T4 (Centramoebida, D) *Hartmannella cantabrigensis* (Euamoebiba, T) *Saccamoeba lacustris* SL3 (Euamoebiba, T) *Dictyostelium* (Dictyostelea, M) *Physarum* (Myxogastrea, M) *Naegleria clarcki* (Heterolobosea) *Willaertia magna* (Heterolobosea)	*Saccamoeba lacustris* SL2 (Euamoebiba, T)	Michel et al. (2014, 2015)
At-LEMO	*Acanthamoeba hatchetti* T11	*Acanthamoeba* spp. (Centramoebida, D)		This study
Acaulopage dichotoma				
Ad-Rom	*Vannella* sp. (Vannellida, D)	*Vannella* sp. (Vannellida, D) *Ripella platypodia* (Vannellida, D) *Acanthamoeba* spp. (Centramoebida, D) *Stenamoeba* sp. (Thecamoebida, D)		This study
Ad-Syc	*Thecamoeba striata* (Thecamoebida, D)	*Thecamoeba* spp. (Thecamoebida, D) *Ripella platypodia* (Vannellida, D)		
Stylopage areae SA-ET	*Acanthamoeba* sp. (Centramoebida, D)	*A. castellanii* T4, *A. lenticulata* T5 *Vannella miroides* (Vannellida, D) *Stemonitis* sp. (Myxogastrea, M) *Willaertia magna* (Heterolobosea)		Michel et al. (2014); This study

Major groups of Amoebozoa are indicated as D (Discosea) and T (Tubulinea) for subphylum Lobosa and M (Mycetozoa) for subphylum Conosa transferred to plates with *Stenamoeba* sp. SP1 (Amoebozoa, Thecamoebida). Strain Ad-Syc was isolated together with a strain of *Thecamoeba striata* (Amoebozoa, Thecamoebida). *Stylopage araea* strain SA-ET was isolated in July 2011 from a moss sample collected within a small valley at Engelsbachtal near Rengsdorf (Neuwied District, Rhineland-Palatinate), Southwestern Germany, associated with an *Acanthamoeba* sp. and then transferred to plates with *Stemonitis* sp. strain BuP (Amoebozoa, Mycetozoa). A fifth strain, Ac-zygo, isolated in October 2010 from a garden soil at Nancy (Lorraine), Northeastern France, preying on *Acanthamoeba* sp. genotype T4, was not identified morphologically.

DNA extraction, amplification, and sequencing

Fungal DNA was extracted from high-density growing cultures using a commercial kit (QIAmpDNA, Qiagen), and a set of eukaryotic primers was used to amplify and to sequence fragments of the ribosomal RNA unit, i.e., the 18S ribosomal RNA gene (18S rDNA) and the internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2) plus partial 28S rDNA (ITS-LSU) as previously described (Corsaro et al. 2014a; Michel et al. 2015). The 18S rDNA of the amoebae was obtained in the same manner, starting from parallel cultures containing only amoebae. All sequences were deposited in GenBank and are available under accession numbers KY934455–KY934460 and KY937193–KY937196.

Molecular phylogeny

For the fungal 18S rDNA phylogeny, representatives of the main groups of Fungi and relatives were retrieved from GenBank. Close relatives of the fungal sequences obtained here were searched via the BLAST server. All sequences ($n = 73$) were aligned using MAFFT and manually refined to exclude ambiguous sites (1259 retained sites) using BIOEDIT, and maximum likelihood (ML), distance matrix (neighbor-joining (NJ), Kimura-2P), and maximum parsimony (MP) trees (1000 replicates) were built as described (Corsaro et al. 2015). Another multiple alignment, including sequences ($n = 78$) from Zoopagomycota only, was prepared as described above (1289 retained sites). Three and six partial sequences were added to the fungal and the zoopagomycotan trees, respectively, without affecting the overall topology.

Because we obtained also ITS and partial LSU sequences from four of our strains, a distinct alignment including this region of the ribosomal operon (ITS-LSU) was prepared (305 retained sites) by including selected members of the Zoopagomycota.

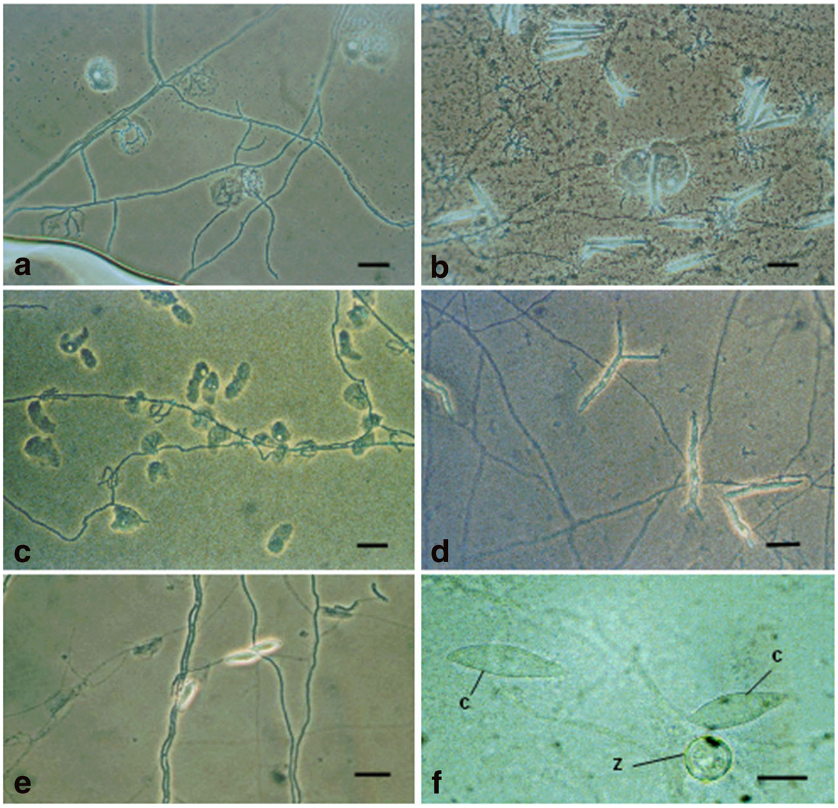

Fig. 1 Amoebal predation by *Acaulopage dichotoma* (**a**–**d**) and *Stylopage araea* (**e**, **f**). **a** Hyphal network of *A. dichotoma* strain Ad-Syc growing between trophozoites of *Ripella platypodia*. Hyphae penetrating within at least three amoebae are clearly visible. **b** Strain Ad-Syc showing typical bifurcated conidia, one of which is accidentally located in between two trophozoites of *Thecamoeba striata*. **c** Trophozoites of *Ripella platypodia* invaded by hyphae of *A. dichotoma* strain Ad-Rom. **d** Bifurcated conidia of strain Ad-Rom emerging from a network of hyphae. **e** Conidia of *S. araea* emerging from hyphal network preying on *Vannella miroides* (Van-Aun). **f** Two conidia (*c*) and a zygospore (*z*) of *S. araea*. Scale bars = 20 μm (**a**–**e**) and 10 μm (**f**)

Results

Morphological features

Amoebophagous fungal strains were successfully cultivated together with a wide variety of prey amoebae (Table 1) as previously reported (Michel et al. 2014), in which they corresponded morphologically to *Acaulopage dichotoma* and *Stylopage araea*. On agar plates covered by amoebae, both *Acaulopage* and *Stylopage* develop a network of vegetative hyphae that grow between the amoebal trophozoites (Fig. 1). The adhering hyphae attack single trophozoites by penetrating them and developing bush-like haustoria (Fig. 1a, c, e) causing the final death of the amoebae. The amoeba cysts are generally more resistant to predation, but in some occasions, they also may be penetrated by hyphae, including also very resistant cysts such as those of *Acanthamoeba*. This is consistent with our recent observations (Michel et al. 2014). Production of conidia (asexual reproduction) is frequently observed with nearly all amoebae, usually when most of the amoebae are destroyed. Conidia emerge from the hyphal network as typically bifurcated for *A. dichotoma* (Fig. 1b, d) and ovoid for *S. araea*, which also produces zygospores (sexual reproduction) (Fig. 1e, f).

Almost identical features were exhibited by *Acaulopage tetraceros* (Michel et al. 2014, 2015). Whereas *Cochlonema euryblastum* forms a coil-shaped thallus in the cytoplasm of the parasitized amoeba which germinates from a conidium engulfed by the amoeba itself. Then, the thallus produces

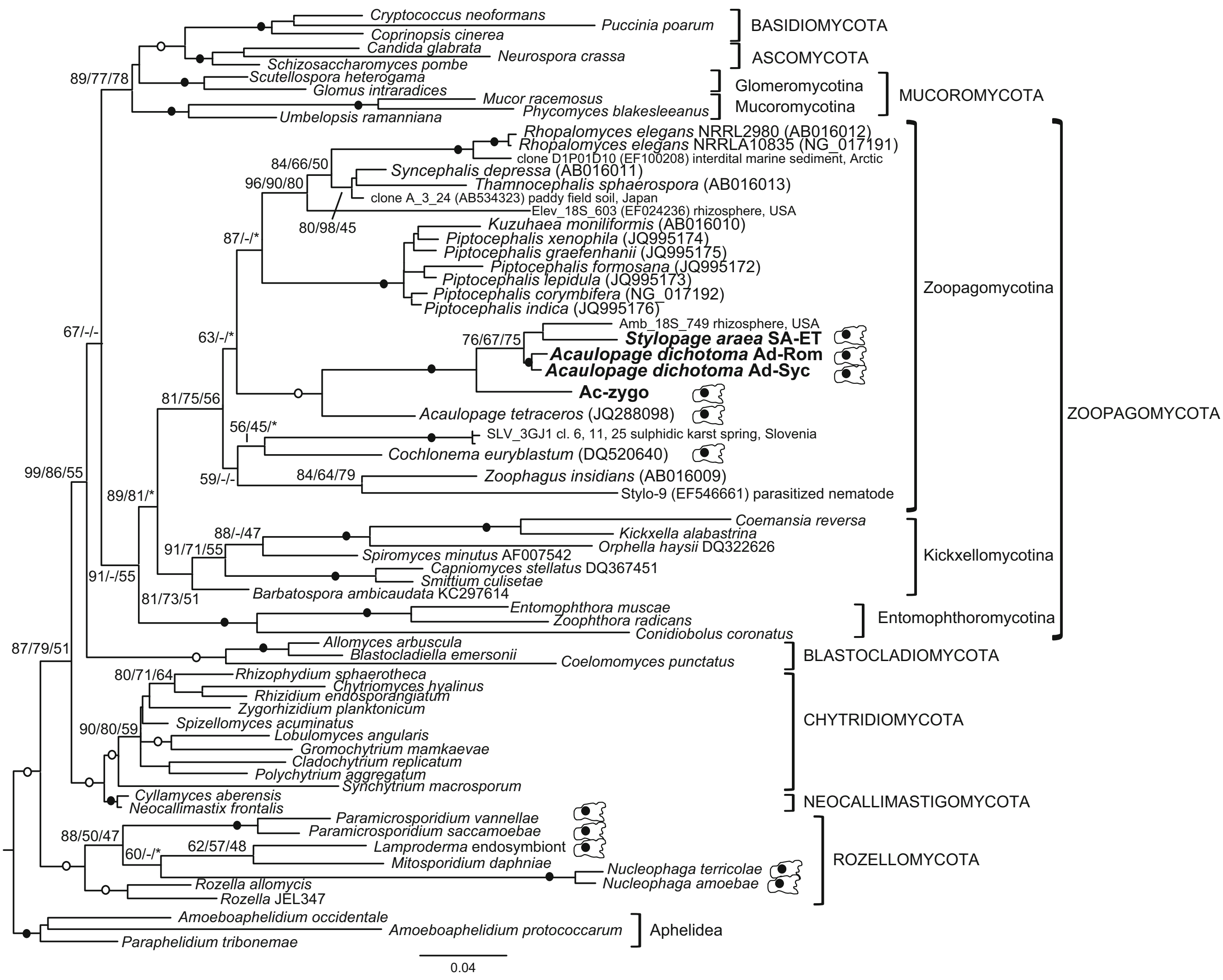

Fig. 2 All-fungal 18S rDNA ML phylogenetic tree, with the main fungal phyla indicated in uppercase. The tree is rooted with Nucleariidae (not shown). The recovered amoebophagous strains in the Zoopagomycotina are in bold. An amoeba icon marks fungi known to parasitize free-living amoebae. Note that free-living amoebae may be parasitized also by a few Chytridiomycota and various filamentous/dimorphic fungi in Basidiomycota and Ascomycota (not marked in the figure). SLV clones (Genbank IDs KT072123, KT072099, KT072109). Bootstrap values (BVs) after 1000 replicates for ML/NJ/MP are indicated at nodes. Nodes with 100 or > 95% BV support with all methods (filled and open circles, respectively); node supported but BV < 40% (asterisk); node not supported (hyphen)

new hyphae which rupture the pellicule of the amoeba and form new conidia externally (Köhsler et al. 2007). In the case of *Amoebophilus simplex*, a conidium adheres to the surface of the amoeba, germinates, and enters the amoebal cytoplasm forming a globular haustorium. The conidia are produced in chains, and it is ectoparasitic (Barron 1983; Mrva 2008).

18S rDNA molecular phylogeny

18S rDNA sequences were obtained from strains Ad-Rom, Ad-Syc, Ac-zygo, and SA-ET but not from At-LEMO. The 18S rDNA fungal tree (Fig. 2) recovered the various zygomycetous groups as paraphyletic lineages, those now defined as Mucoromycota and Zoopagomycota being sister to the Dikarya or forming an opposite more basal branch, respectively. This is congruent with previously reported studies (White et al. 2006; Spatafora et al. 2016). Among the chytrids, the Blastocladiomycota emerge as sister to the terrestrial fungi, while the Chytridiomycota/Neocallimastigomycota and Rozellomycota form the two more basal lineages. The putative "opisthosporidia," that would unite the rozellids and aphelids, were never recovered, even including several other

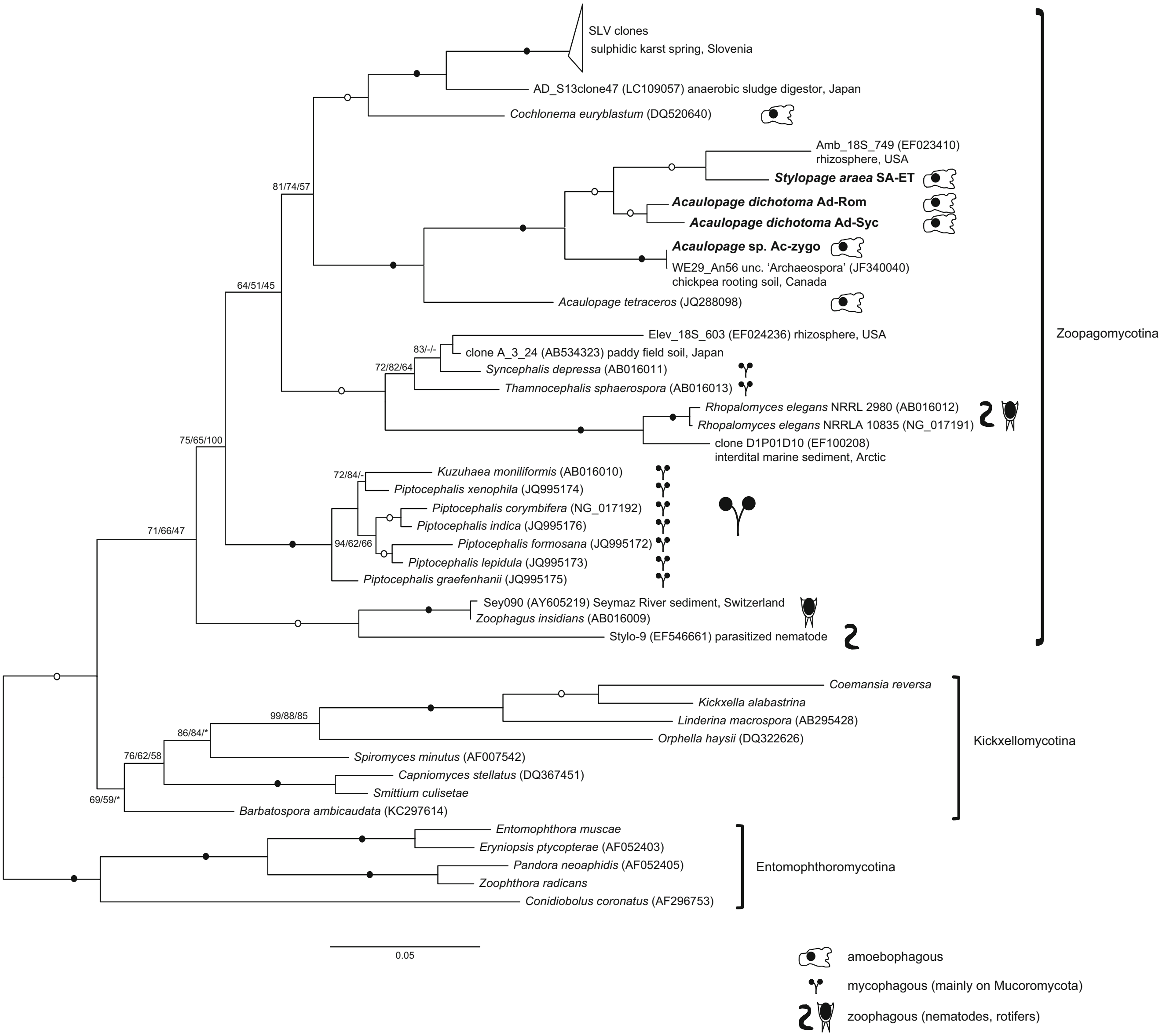

Fig. 3 Zoopagomycota-only 18S rDNA phylogenetic tree, which indicated the three subphyla. The tree is rooted on the Entomophthoromycotina. For the Zoopagomycotina, fungi parasites of different preys are marked by specific icons. SLV clones collapsed and include 39 almost identical sequences (Genbank IDs KT072125, KT072128, KT072129, KT072132, KT072133, KT072136–KT072139, KT072143, KT072145–KT072155, KT072157–KT072160, KT072162–KT072164, KT072173, KT072176, KT072183, KT072184, KT072187, KT072188, KT072194, KT072198). The amoebophagous fungi recovered in this study are in bold. At nodes, BV for ML/NJ/MP (1000 replicates), as for Fig. 2

sequences and also Microsporidia, as already shown (Corsaro et al. 2014a, 2016). Within the Zoopagomycota, the Zoopagomycotina, that include the sole order Zoopagales, are sister to the Kickxellomycotina, forming a well-supported clade, sister to the Entomophthoromycotina.

In order to increase the resolution within the Zoopagales, we built a Zoopagomycota-only 18S tree (Fig. 3). Here, the branching pattern within the Zoopagales is sligthly modified, as the branch leading to *Cochlonema*, poorly supported in the all-fungal tree (Fig. 2), is indeed recovered with high support as sister to the *Acaulopage* clade, congruent with a recent study (Michel et al. 2015). The two groups *Syncephalis*/*Thamnocephalis*/*Rhopalomyces* (STR clade) and *Kuzuhaea*/*Piptocephalis* already identified by Tanabe et al. (2000) are always recovered. Their position as sister in the all-fungal tree (Fig. 2) is poorly supported, while their paraphyletic branching shown in the zoopagomycotan tree (Fig. 3) is stronger. Interestingly, all-fungal and zoopagomycotan 18S rDNA trees both recovered *Zoophagus* as belonging to the Zoopagales in contrast with previous studies (White et al. 2006; Köhsler et al. 2007; Michel et al. 2015).

The search for additional sequences, and the few species already available from the Zoopagales, allowed us to identify environmental clones that could be representative of at least seven new lineages. Phylogenetic analyses appear to be consistent with the other data available on these sequences.

Stylopage araea strain SA-ET forms a clade with the rhizosphere clone Amb_18S_749 (92.7% similarity), sister to the closely related (98.5% similarity) *A. dichotoma* strains Ad-Rom and Ad-Syc that cluster robustly together in both the 18S and ITS trees (Figs. 2, 3, and 4). The unidentified strain Ac-zygo emerges within the *Acaulopage* clade and possibly represents a distinct species. It clusters with the soil clone WE29_An56. The latter was misidentified as an uncultured *Archaeospora* (Glomeromycotina), whose partial sequence is almost identical (99.4%) to the sequence of our strain *Acaulopage* sp. Ac-zygo. The *Acaulopage* clade is well supported, with *A. tetraceros* strain At-Blent (Michel et al. 2015) as basal lineage. The *Acaulopage*-*Stylopage* clade is sister to the *Cochlonema* clade.

Cochlonema euryblastum, an endoparasite of the soil amoeba *Thecamoeba quadrilineata*, was isolated from an

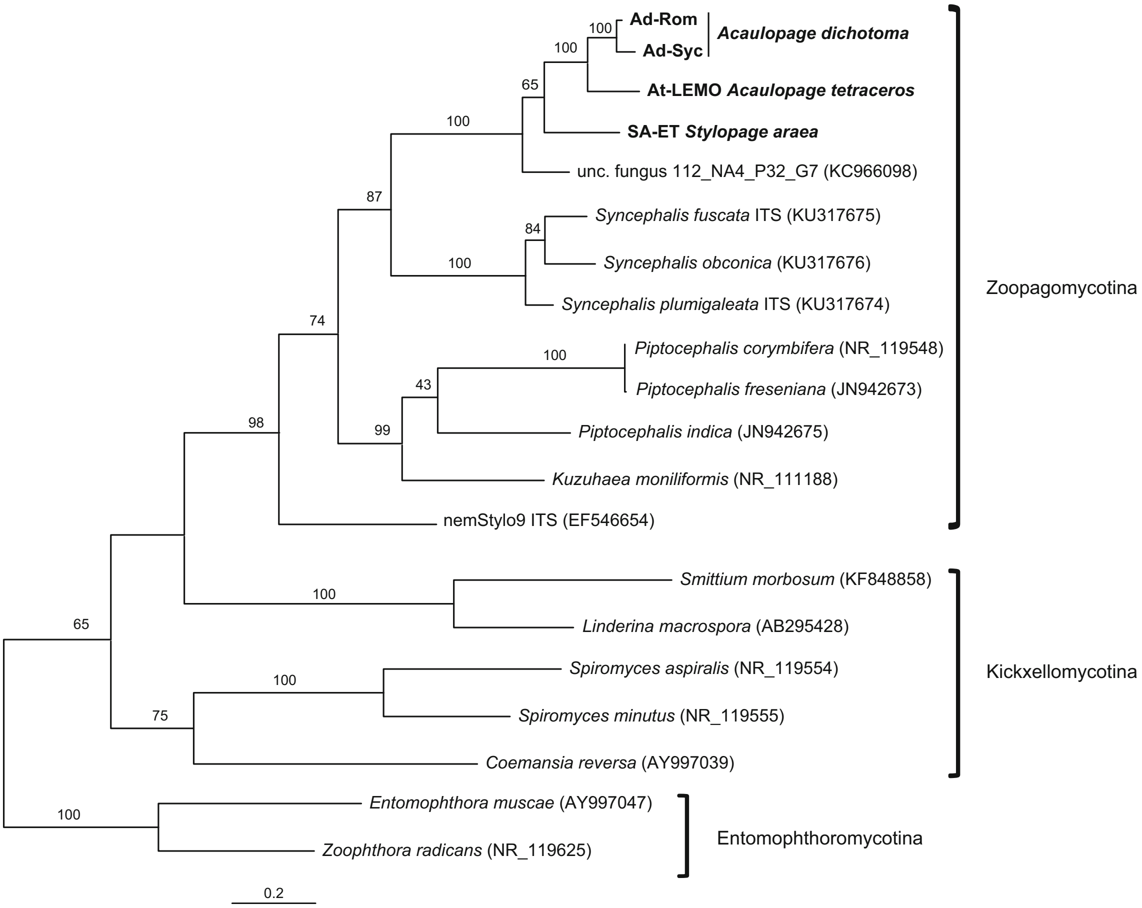

Fig. 4 ML phylogenetic tree of the ITS region for selected representatives of the Zoopagomycota. The tree is rooted on the Entomophthoromycotina. The recovered amoebophagous strains in the Zoopagomycotina are in bold. BV after 1000 replicates

eaves gutter (Michel and Wylezich 2005; Köhsler et al. 2007). *Cochlonema euryblastum* clusters tightly with the clone AD_S13-47 from activated sludge of municipal sewage in Japan, and with an assemblage of virtually identical (> 99%) environmental sequences (SLV clones, $n = 39$) recovered from a sulfidic karst spring in Slovenia. Sequence similarities between the AD_S13-47 and SLV clones are about 91.5%, and they share with *Cochlonema* similarities of 89.9 and 88.4–88.9%, respectively.

Recovery of sequences belonging to the putatively amoebophagous Zoopagales should be expected in environments rich in amoebae, as the soil/rhizosphere, activated sludges, and sulfidic karst springs (Engel 2010).

Similarly, in the STR clade, we recovered two clones from the soil/rhizosphere clustering with *Syncephalis* and *Thamnocephalis* that are both mycoparasites mainly of soil and plant-associated Mucoromycota. Another clone from interdital marine sediment clusters with *Rhopalomyces* and shares with it high sequence similarity (94.5%). As known *Rhopalomyces* species are soil predators of nematodes and rotifers, this sequence may belong to a new species that preys upon aquatic invertebrates.

We did not recover environmental clones of the *Kuzuhaea*/*Piptocephalis* clade, an independent lineage of mycoparasites.

Finally, the sequence Stylo-9 from a fungus parasitizing a nematode clusters with the rotifer parasite *Zoophagus insidians*, forming the basal clade of the Zoopagales. This is congruent with the report that some *Zoophagus* species prey on nematodes (Benny et al. 2016). The partial sequence Stylo-9 of 650 bp shares only 86.7% similarity with *Z. insidians*; however, a high genetic diversity in the clade may be expected as another 400-bp partial sequence (no deposited in GenBank) of a *Zoophagus* sp. was reported to share only 91% similarity with *Z. insidians* (Pajdak-Stós et al. 2016).

ITS molecular phylogeny

We obtained the ITS (ITS1-5.8S-ITS2) and partial LSU sequences of four of our amoebophagous strains, *A. dichotoma* strains Ad-Rom and Ad-Syc, *A. tetraceros* strain At-LEMO, and *Stylopage araea* strain SA-ET, and an additional phylogenetic tree was build with selected members of the Zoopagomycota. The ITS-LSU tree topology (Fig. 4) is congruent with that obtained with 18S rDNA sequences, supporting the close relationship between *Acaulopage* and *Stylopage*, and the sister position of *Syncephalis* to the *Acaulopage* clade. Furthermore, the recovery of the ITS sequence of Stylo-9 as the basal branch of the Zoopagales is an additional proof supporting that *Zoophagus* belongs to the group. Indeed, this ITS sequence has been associated with a fungus infecting a nematode. Therefore, we assume that it originated from the same parasitic fungus for which also the 18S rDNA sequence is available and forms a very strong clade with *Zoophagus* (Figs. 2 and 3).

Discussion

The Zoopagales currently include 23 described genera and more than 200 species, divided into five families based on morphological and ecological criteria (Benny et al. 2016). The Piptocephalidaceae and Sigmoideomycetaceae are mycoparasites; the Cochlonemataceae and Helicocephalidaceae are ectoparasites/endoparasites of free-living amoebae or of microinvertebrates (nematodes, rotifers), respectively, whereas the Zoopagaceae include predators of both microinvertebrates and free-living amoebae.

This historical classification however turned out to be inconsistent with the results of the first 18S rDNA-based phylogenetic study (Tanabe et al. 2000). Indeed, in this study, while *Piptocephalus* and *Kuzuhaea* (Piptocephalidaceae) were found to be sister groups, the other genus of the family, *Syncephalis*, clustered with *Thamnocephalis* (Sigmoideomycetaceae) and *Rhopalomyces* (Helicocephaloidaceae) forming a robust clade. In addition, *Zoophagus* (Zoopagaceae) was recovered forming an independent branch of uncertain position. Subsequent molecular studies showed similar results and provided further support against the previous classification, by recovering a close relationship between *Cochlonema* (Cochlonemataceae) and *Acaulopage* (Zoopagaceae) (Köhsler et al. 2007; Michel et al. 2015).

In the present study, we analyzed additional amoebophagous Zoopagales including, for the first time, a *Stylopage* isolate and various environmental sequences. The results of the phylogenetic analyses presented herein (Fig. 3) suggest that in the Zoopagomycotina, the amoebophagous taxa have emerged all on a distinct branch of the Zoopagales after the radiation of the zoophagous and mycoparasitic lineages. The *Zoophagus* clade, which we show here, contrarily to previous studies, as belonging to the Zoopagales, is the most basal lineage, suggesting that the ancestral state of the group might have been zooparasitic.

The number of sampled taxa is still too low compared to the number of known taxa in the Zoopagales. Although there are now data strongly supporting that the Piptocephalidaceae include only *Kuzuhaea* and *Piptocephalis*, no molecular data are available for the other five genera of mycoparasites or zooparasites assumedly close to *Thamnocephalis* or *Rhopalomyces*; these latter appear to be affiliated with *Syncephalis* to form an independent STR clade. Moreover, some amoebophagous genera, such as *Cystopage* and *Stylopage*, also include nematophagous species. Further efforts are therefore needed to obtain sequences of several of these missing taxa. This will fill possible gaps to better elucidate the relationships between the different lineages and to finally increase our knowledge of the ecology and evolutionary history of the group.

Of the amoebophagous taxa known to date, most prey upon free-living naked amoebae, mostly Amoebozoa, and a few species, e.g., of *Cochlonema* and *Zoopage*, on euglyphid testate amoebae (Cercozoa) forming zygospores within the amoebae's tests. A unique example is given by *Basidiolum* (White 2003), a possible zoopagalean ectoparasite on the amoeboid *Amoebidium* (Choanozoa, Ichthyosporea), an ectocommensal of freshwater arthropods.

Much of the literature on amoebal predatory fungi dates back to the early twentieth century, especially Drechsler's studies, when almost all naked amoebae were generally assigned to the genus *Amoeba*, a taxon representing in reality several distinct genera and families. Trying to identify some of these amoebae with the current genera would thus seem rather speculative, but might be possible in some cases. The current genera *Amoeba* and *Chaos* (Amoebozoa, Tubulinea, Euamoebida) and *Mayorella* (Amoebozoa, Discosea, Mayorellida) are probably the amoebal preys reported by Penard (1902) parasitized by caudal fungal chains, that Dangeard (1910) named *Amoebophilus*. The prey "*Pelomyxa vorax*" of Dangeard was also probably an euamoebid. While in other studies, Mrva (2008, 2011) found *Mayorella* spp. parasitized by *Amoebophilus* and was able to identify the fungus with *A. simplex* described by Barron (1983) and also the amoebae previously reported by Barron (1983) and Leidy (1879) as *Mayorella*.

It was possible not only to identify the amoebal prey, but also on various occasions, to analyze the prey spectrum (Table 1) of the amoebophagous fungi recovered during our research. *Cochlonema euryblastum* was able to parasitize only strains of *Thecamoeba quadrilineata* (Michel and Wylezich 2005), whereas *A. tetraceros* showed the broadest prey spectrum by parasitizing various amoebae belonging to distinct Amoebozoa groups as well as *Naegleria* and *Willaertia* (Heterolobosea) (Michel et al. 2014). The additional *Acaulopage* strains reported herein, *A. tetraceros* At-LEMO and *A. dichotoma* Ad-Rom and Ad-Syc, also show a relatively broad prey spectrum that includes *Acanthamoeba*, *Thecamoeba*, *Stenamoeba*, and *Vannella* spp. Similarly, *S. araea* SA-ET preys upon various strains of distinct genotypes of *Acanthamoeba* (T4, T5), the myxomycete *Stemonitis*, and the heterolobosean *Willaertia magna*.

All these amoebae and the different fungi with which they interact are widespread in various aquatic environments, as well as in soils and on vegetation. Their diversity and the complexity of their interactions are however still poorly known, as highlighted for example in recent studies on soils (Geisen et al. 2015, 2016). Amoebae may play a significant role also in aquatic environments. The Zoopagales are not strictly terrestrial, retaining some features of an aquatic life. In addition, although some chytrids have been described (Karling 1946) as parasites of amoebae, probably *Thecamoeba*, the true extent of this type of interaction has not been explored; most studies on chytrid parasitism focus only on phytoplankton. However, it appears that amoebae have an important role in the ecology of the Rozellomycota, as indicated by recently discovered species (Michel et al. 2000, 2009a, 2009b, 2012; Corsaro et al. 2014a, 2014b, 2016; Yajima et al. 2013) as well as by the recovery of other rozellid phylotypes within various amoebae (unpublished data).

The food web in water and soil is in reality highly complex and interwined by two-way functional relationships among the different groups of organisms. Small invertebrates such as nematodes, fungi, and protozoa like amoebae can be both predators and preys of each other. Large amoebae and ciliates may engulf nematodes and are themselves preyed on by various microinvertebrates (Sayre 1973; Yeates and Foissner 1995; Yeates et al. 1993). Fungi are attacked by mycophagous amoebae and nematodes (Old and Darbyshire 1978; Yeates et al. 1993), and they also trap amoebae, nematodes, and other small invertebrates (Drechsler 1941; Jiang et al. 2017). Most amoebae are polyphagous, capable of engulfing by phagocytosis any body of appropriate size. They can therefore feed on fungal spores and conidia and yeast cells, but not on hyphae. In some cases however, the potential prey turns out to be a parasite of the amoeba. This occurs, for example, when the amoeba ingests the spores or conidia, respectively, of certain Rozellomycota (e.g., *Paramicrosporidium*) and Zoopagales (e.g., *Cochlonema*) that develop as endoparasites.

The ingestion of spores and conidia with the final death of the amoeba occurs also in the case of some filamentous/dimorphic fungi belonging to the Basidiomycota (e.g., *Cryptococcus*) and Ascomycota, especially those from various orders in the Pezizomycotina (e.g., *Histoplasma*, *Fusarium*, *Metarhizium*). These fungi are all opportunistic pathogens of humans and other animals, and their interactions with amoebae resemble those with phagocytic cells of the immune system, suggesting a possible role of amoebae in the evolution of fungal virulence (Steenbergen et al. 2001, 2004; Bidochka et al. 2010; Van Waeyenberghe et al. 2013; Hillmann et al. 2015; Maisonneuve et al. 2016).

Acknowledgements We would like to thank Iveta Häfeli and Susi Glöckl from the Institute of Specific Prophylaxis and Tropical Medicine, Medical University of Vienna, for excellent technical assistance.

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