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Phylogenetic Analysis of *Rhinosporidium seeberi*'s 18S Small-Subunit Ribosomal DNA Groups This Pathogen among Members of the Protoctistan Mesomycetozoa Clade

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For the past 100 years the phylogenetic affinities of *Rhinosporidium seeberi* have been controversial. Based on its morphological features, it has been classified as a protozoan or as a member of the kingdom Fungi. We have amplified and sequenced nearly a full-length 18S small-subunit (SSU) ribosomal DNA (rDNA) sequence from *R. seeberi*. Using phylogenetic analysis, by parsimony and distance methods, of *R. seeberi*'s 18S SSU rDNA and that of other eukaryotes, we found that this enigmatic pathogen of humans and animals clusters with a novel group of fish parasites referred to as the DRIP clade (*Dermocystidium*, rossete agent, *Ichthyophonus*, and *Psorospermium*), near the animal-fungal divergence. Our phylogenetic analyses also indicate that *R. seeberi* is the sister taxon of the two *Dermocystidium* species used in this study. This molecular affinity is remarkable since members of the genus *Dermocystidium* form spherical structures in infected hosts, produce endospores, have not been cultured, and possess mitochondria with flat cristae. With the addition of *R. seeberi* to this clade, the acronym DRIP is no longer appropriate. We propose to name this monophyletic clade Mesomycetozoa to reflect the group's phylogenetic association within the *Eucarya*.

Rhinosporidium seeberi is the hydrophilic agent of rhinosporidiosis (3). This granulomatous disease of humans and animals is characterized by the development of polyps that primarily affect the nostrils and the ocular conjunctiva of its hosts. Diagnosis is essentially based on the histological detection in tissues of *R. seeberi*'s pathognomonic endosporulating sporangia in various stages of development. These sporangia, which are the only known phenotypic structures produced by this pathogen, range from 60 to 450 μm or more in diameter. The mature sporangia have been estimated to contain up to 12,000 endospores (7 to 15 μm in diameter) that are discharged through a pore. The liberated endospores lodge in the host's tissue and mature into endosporulating sporangia, repeating their in vivo life cycles. Early claims that *R. seeberi* has been isolated in culture (4, 20) were never confirmed. The infections caused by this organism, therefore, have not been experimentally reproduced in animals, and its etiologic agent has yet to be cultured (4).

The taxonomy of *R. seeberi* has always been controversial. Seeber, who first described rhinosporidiosis in 1900, considered the sporangium of this enigmatic organism to be a sporozoan allied to the coccidia (23). Ashworth (5), in his monograph on rhinosporidiosis, concluded that "the nearest relatives of *Rhinosporidium* are not the Sporozoa but the lower fungi (Phycomycetes) such as the Chytridineae in which, suborder, near the Olpidiaceae, *Rhinosporidium* is provisionally placed." Dodge (9) interpreted the sporangia of *R. seeberi* to be multispored asci and classified it as an ascomycetous fungus. Interestingly, early investigators also noticed the similarities that *R. seeberi* has with such aquatic parasites as species of *Ichthyophonus* (19) and *Dermocystidium* (7, 10), both fish pathogens that did not have a clear taxonomic background at that time. Since then, numerous investigators have incorrectly considered *R. seeberi* to be a protozoan, a fungus, and more recently a cyanobacterium (1) and a carbohydrate waste product (2).

For 100 years, *R. seeberi* has been the center of taxonomic controversies. This unsettling confusion has stemmed in part from the frustrating fact that this human and animal pathogen is intractable to culture. Thus, its life history and phylogenetic affinities have remained unknown. We report in this study that phylogenetic analysis, with the 18S small-subunit (SSU) ribosomal DNA (rDNA) of *R. seeberi* and 23 other microorganisms, placed the etiologic agent of rhinosporidiosis within a recently described group of fish parasites known as the DRIP clade, which we propose to rename the Mesomycetozoa.

MATERIALS AND METHODS

Collection of tissue with *R. seeberi*'s sporangia and endospores. Biopsied tissue, containing *R. seeberi*'s sporangia and endospores, were obtained from two Sri Lankan men with rhinosporidiosis. The tissues were aseptically collected and transported without fixatives to the laboratory. The sporangia and endospores from both men were processed by two different methods. In the first method, the sporangia and endospores were physically dissected from the tissues, further purified by centrifugation to remove human cells (~50 *R. seeberi* cells/μl), and then disrupted with glass beads. The second method involved the use of 100 mg of human tissue containing *R. seeberi*'s sporangia. The tissues were placed in a mortar and ground under liquid nitrogen.

Genomic DNA isolation, PCR amplification, and sequencing of *R. seeberi*'s 18S SSU rDNA. For both of the above cases, the samples were placed in two Eppendorf tubes, treated with sodium dodecyl sulfate and proteinase K, and then extracted with phenol and chloroform. The amplification of the 18S SSU rDNA gene was by PCR first with the oligonucleotide primer NS1 (13) and, from the same study, the reverse primer NS8. The PCR conditions were as described by Gargas and DePriest (13). Since no PCR amplicons were obtained with this set

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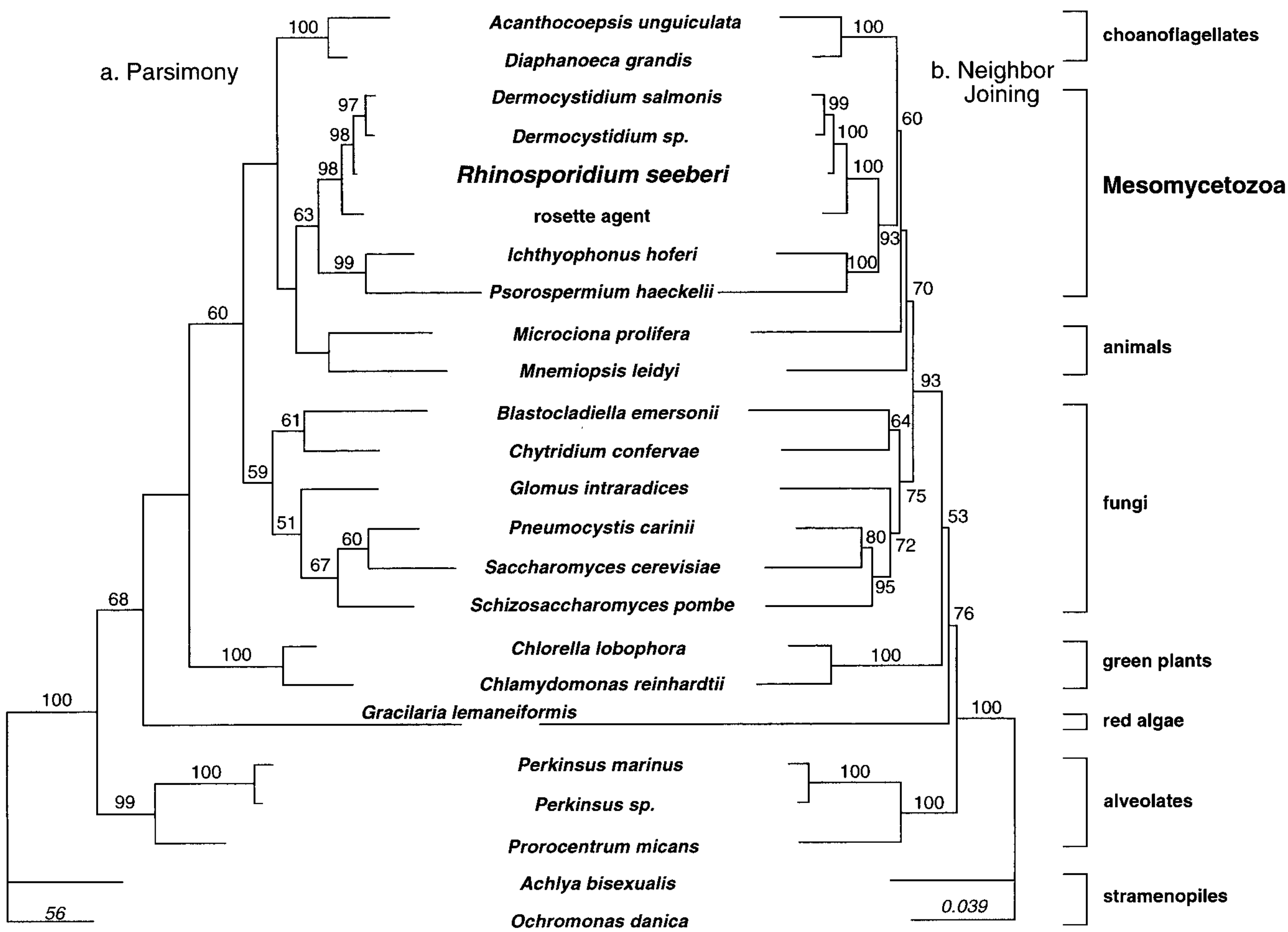

FIG. 1. Phylogenetic comparison of 18S SSU rDNA from *R. seeberi* and 23 other organisms showing that this human and animal pathogen is a member of the Mesomycetozoa clade. (a) Parsimony tree (length = 1,688 steps; consistency index = +0.53; retention index = 0.51; homoplasy index = 0.47) based on a heuristic search with 1,000 random taxon input orders. The numbers on the internal branches are percentages of trees based on 1,000 bootstrapped data sets possessing the branch. The branch lengths reflect the length in steps. An italicized scale is given above the *Ochromonas danica* branch. (b) Neighbor-joining distance tree with Kimura's 3-parameter correction for multiple hits. The numbers on the internal branches are percentages of trees based on 1,000 bootstrapped data sets possessing the branch. The branch lengths reflect corrected distances. An italicized scale is given above the *O. danica* branch. The GenBank accession numbers of the organisms are as follows: *Acanthocoepsis unguiculata*, L10823; *Diaphanoeca grandis*, L10824; *Dermocystidium* sp., U21336; *Dermocystidium salmonis*, U21337; *I. hoferi*, U43712; *Psorospermium haeckelli*, U33180; the rosette agent, L29455; *R. seeberi*, AF118851; *Microciona prolifera*, L10825; *Mnemiopsis leidyi*, L10826; *Blastocladiella emersonii*, X54264; *Chytridium confervae*, M59758; *Glomus intraradices*, X58725; *Pneumocystis carinii*, X12708; *Saccharomyces cerevisiae*, J01353; *Schizosaccharomyces pombe*, X58056; *Chlorella lobophora*, X63504; *Chlamydomonas reinhardtii*, M32703; *Gracilaria lemaneiformis*, M54986; *Perkinsus* sp., L07375; *Perkinsus marinus*, X75762; *Achlya bisexualis*, M32705; *O. danica*, M32704; and *Prorocentrum micans*, M14649.

of primers, the NS8 primer was degenerated per the method of Issakainen et al. (17): 5′TCCGCAGGTTCACC(TA)ACGGA3′. The amplicons were subcloned into pCR 2.1-TOPO plasmids (Invitrogen, Carlsbad, Calif.), purified, and then sequenced by using BigDye Terminator chemistry in an ABI Prism 310 genetic analyzer apparatus (Perkin-Elmer, Foster City, Calif.). Before DNA extraction the sterility of the samples was always investigated by culture.

Transmission electron microscopy (TEM) studies. Both of the infected human tissues, containing the sporangia and endospores of *R. seeberi*, were fixed in 2.5% glutaraldehyde with 0.05 M sodium cacodylate-buffered saline (pH 7.4), at room temperature for 2 h with gentle agitation. The primary fixation was followed by three 0.05 M sodium cacodylate-buffered saline washes for 20 min each. The samples were then placed into a 1% OsO_4 and water solution at room temperature for 4 h with mild agitation. OsO_4 postfixation was followed by three 20-min distilled-water washes and dehydration in acetone. The samples were transferred to 33% followed by 66% Spurr resin in acetone solutions for 30 min in each concentration. The samples were then transferred to 100% Spurr resin for 5 h and then overnight, with resin changes at the end of each period. Ultrathin sections were made by using an ultramicrotome and sectioning the samples into 100-nm sections. The grids with the ultrathin sections were poststained with uranyl acetate for 30 min followed by lead as described by Hanaichi et al. (14) for 3 min. After the poststaining procedure, a thin layer of carbon was evaporated onto the surfaces of the grids. Examination of ultra-thin-sectioned material was with a Philips CM-10 electron microscope.

Phylogenetic analysis. The 18S SSU rDNA sequence of *R. seeberi* was aligned by visual inspection with sequences previously analyzed by Spanggaard et al. (27) and Ragan et al. (22) with the Sequence Navigator (Perkin-Elmer/Applied Biosystems). Regions of ambiguous alignment were excluded from phylogenetic analysis, and gaps introduced to facilitate alignment were treated as missing data. Phylogenetic analysis was by a distance method (neighbor joining with Kimura's 3-parameter multiple-hit correction) (27) and parsimony. Support for internal branches was assessed by using 1,000 bootstrapped data sets. Parsimony analyses were conducted with the computer software program PAUP (PAUP: Phylogenetic Analysis Using Parsimony, version 3.1; D. L. Swofford, Illinois Natural History Survey, Champaign, Ill.). Parsimony analysis employed 1,000 heuristic searches with randomized orders of taxon entry to increase the chance of finding the shortest trees. Trees were drawn with stramenopiles and alveolates as the outgroups, based on previous phylogenetic studies (18, 22, 27).

Nucleotide sequence accession number. The sequence for *R. seeberi*'s 18S SSU rDNA has been submitted to GenBank under accession no. AF118851.

RESULTS

Phylogenetic analysis with *R. seeberi*'s 18S SSU rDNA. The NS1 and the modified reverse primers amplified 1,790 bp of the gene from both cases, nearly the full length of *R. seeberi*'s

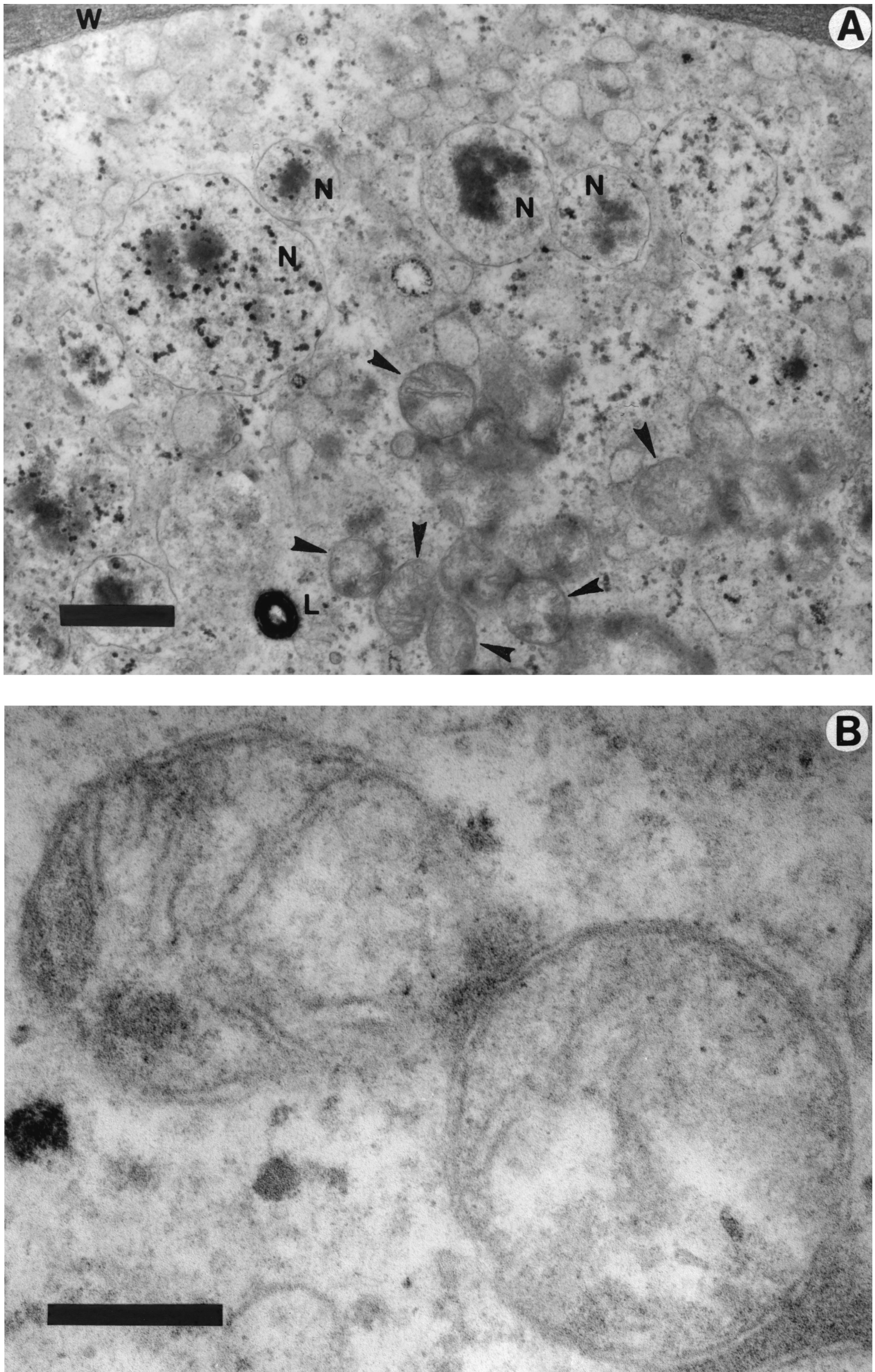

FIG. 2. (A) TEM photograph showing an intermediate sporangium of *R. seeberi* with a prominent cell wall (W), several nuclei (N), numerous mitochondria (arrowheads), and a laminated body (L). Bar, 1.0 μm. (B) Enlargement of two mitochondria with flat cristae within an intermediate sporangium. Bar, 0.250 μm. The features of *R. seeberi*'s flat mitochondrial cristae are analogous to those of its sister taxon, *Dermocystidium* species, reported by Ragan et al. (22).

18S SSU rDNA sequence. Sequence analysis of *R. seeberi*'s 1,790-bp amplicons showed that the DNA sequences from the rhinosporidiosis cases were identical. Phylogenetic analysis by neighbor joining and parsimony, with *R. seeberi* and 23 other 18S SSU rDNA sequences, supported very similar phylogenetic trees (Fig. 1). In these trees, *R. seeberi* was always the sister taxon to the two *Dermocystidium* species used in this analysis. The position of the sister taxon was strongly supported with bootstrap searches in both parsimony and distance analyses. *R. seeberi* also clustered closer to other members of the DRIP clade, the rosette agent, *Ichthyophonus*, and *Psorospermium*, used in this study. Parsimony and neighbor analyses also showed that the DRIP group and *R. seeberi* are localized near the choanoflagellates and between the kingdoms Animalia and Fungi (Fig. 1).

TEM analysis. TEM analysis from both tissues showed the typical sporangial phenotype that characterized *R. seeberi*'s infections. Sporangia at different stages of development were observed throughout the infected tissues. *R. seeberi*'s mitochondria were difficult to find in any of the phenotypic stages except the intermediate sporangia. The intermediate sporangial stage was characterized by the presence of a prominent cell wall, multiple nuclei, numerous mitochondria, and laminated bodies (Fig. 2A). Our TEM analysis showed that *R. seeberi* has mitochondria with flat cristae identical to those of the *Dermocystidium* spp. reported by Ragan et al. (22) (Fig. 2B).

DISCUSSION

Our phylogenetic analysis indicated that *R. seeberi* is a member of the DRIP clade (22), renamed Mesomycetozoa in this study. This clade comprises previously unknown related organisms at the most basal branch, between the animal-fungal divergence, and close to the choanoflagellates, the lowest branch of the kingdom Animalia (18, 27, 28). Internal branches in the Mesomycetozoa (DRIP) clade showed strong bootstrap support by both parsimony and distance analyses, but the basal branch was strongly supported only in distance analysis.

Interestingly, members of the Mesomycetozoa (DRIP) group have several features in common with *R. seeberi*. For instance, (i) all possess spherical parasitic stages (some have endospore-like structures) with fungus-like characteristics, which explain in part their long history of inclusion within the fungi (11, 12). (ii) With the exception of *Ichthyophonus hoferi* (26), they are intractable to laboratory culture; therefore, their complete life histories are unknown (8, 15, 16). (iii) All are parasites related to aquatic habitats (3, 4, 6, 8, 12, 19, 26). All of these are biological and morphological characteristics that further support our phylogenetic analysis. In addition, the morphological similarities between the mitochondria with flat cristae of *R. seeberi* and *Dermocystidium* spp. also support our phylogenetic analysis.

Our analysis indicated that the two *Dermocystidium* species used in our study are a sister taxon of *R. seeberi* (Fig. 1). This molecular analogy is striking since their common morphological features were first noted by Carini in 1940 (7). In that study, Carini suggested that the morphological features of a spherical frog parasite had similarities with *R. seeberi*. Because of its unusual host, however, he believed that it was different from *R. seeberi* and created the new genus and species *Dermosporidium hylarum* (erroneously referred to as *Dermosporidium hylae* in later studies). Based on Carini's morphological description, however, we believe that the *D. hylarum* and *Dermosporidium granulosum* infections described later (6) were more likely caused by *R. seeberi* in frogs. We based our assumption on the fact that *Dermocystidium* spp. and *R. seeberi* share some phenotypic features but their habitats are different. For instance, *R. seeberi* has been found in wet terrestrial habitats, while *Dermocystidium* spp. are found on fish in both fresh and marine waters. Although we have not examined the 18S SSU rDNA sequence of *Dermosporidium* spp. or *Dermocystidium* spp. growing on amphibians, we speculate that they would be better classified in the genus *Rhinosporidium*, perhaps even as *R. seeberi*, based on their described morphological features and their capacity to cause infection on terrestrial animals.

The use of 18S SSU rDNA sequences to determine the evolutionary relatedness between eukaryotic organisms with incomplete life cycles and tenuous morphological affinities has been very successful. The usefulness of the SSU rDNA molecule in the phylogenetic analysis of eukaryotic and prokaryotic organisms has been reviewed by Olsen and Woese (21). In our study, we used 18S SSU rDNA because the frequencies of compositional changes at different positions allow the molecule to be used for comparisons over a wide spectrum of phylogenetic distances. In addition, the abundance of other 18S SSU rDNA sequences in databases enables a better comprehensive analysis.

Moreover, phylogenetic analysis with this molecule has provided data to suggest the demise of an entire phylum (24, 25) or to support the creation of new clades (22). In some cases, new relationships have led to such names as the DRIP clade, an acronym derived from the names of the organisms that comprise the group. The inclusion of new members in this clade, however, renders this acronym inappropriate. Based on previous phylogenetic analyses (22, 27) and the data presented in this study, we are proposing the term Mesomycetozoa (between animals and fungi) to accommodate the DRIP group, *R. seeberi*, and any future-described organisms with identical phylogenetic characteristics.

The finding that *R. seeberi*'s 18S SSU rDNA sequences amplified from the tissues of both our cases were identical suggested that *Rhinosporidium* is a monotypic genus. However, new 18S SSU rDNA sequences from other humans and animals with rhinosporidiosis have to be evaluated to validate our study. It is anticipated that the inclusion of a human and animal pathogen within the Mesomycetozoa will encourage more studies of this clade. It is our hope that the results of such studies will unveil more details about the ecology, biology, and molecular aspects of this unique group of microorganisms, recently suggested as the possible ancestors from which the fungi and the animals may well have originated.

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