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Amoebidium parasiticum is a protozoan, not a Trichomycete

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Abstract: Classification of the Amoebidiales (Trichomycetes, Zygomycota) within the Fungi is problematical because their cell walls apparently lack chitin and they produce amoeboid cells during their life cycle. A nearly full length fragment of the nuclear small subunit (SSU) rRNA of *Amoebidium parasiticum* was amplified by the polymerase chain reaction (PCR) and sequenced to examine its phylogenetic relationships. Results of a BlastN search of GenBank revealed that the *A. parasiticum* SSU rRNA sequence was most closely related to that of *Ichthyophonus hoferi,* an ichthyosporean in the Protozoa near the animal–fungal divergence. Maximum parsimony analysis of ichthyosporean and fungal SSU sequences, using sequences of choanoflagellates to root the 18S rDNA gene trees, resolved *A. parasiticum* as a strongly supported sister of *I. hoferi* within the Ichthyophonida clade of the protozoan class Ichthyosporea. In contrast to other members of this class, which are mostly obligate or facultative parasites of various animals, *A. parasiticum* and other members of the Amoebidiales are only known to be arthropodophilous symbionts. The results also provide the first evidence that mitochondrial cristae types exhibit homoplastic distributions within the Ichthyosporea.

Key Words: Amoebidiales, 18S rRNA, Ichthyosporea, mitochondrial cristae, phylogeny, Zygomycota

INTRODUCTION

Amoebidium Cienkowski and *Paramoebidium* Léger & Duboscq are genera of unusual fungal-like microorganisms traditionally classified within the Trichomycete order Amoebidiales (Zygomycota, Fungi) (Lichtwardt 1986). With 4 and 7 species, respectively, these arthropodophilous symbionts are typically found in fresh water in many parts of the world on Crustacea and Insecta (Lichtwardt 1986, 1997, Lichtwardt et al 1999), bloodworms (Lichtwardt and Williams 1992) and *Daphnia* spp. (Lichtwardt 1986), or in the hindgut or larval gills of invertebrates (Lichtwardt 1986, Lichtwardt and Arenas 1996, Lichtwardt and Williams 1992, Williams and Lichtwardt 1990). Species of *Amoebidium* produce unicellular thalli that are typically attached externally to a host by an acellular holdfast, whereas thalli of *Paramoebidium* are attached to the cuticle of the hindgut, or to other Trichomycetes inhabiting the host hindgut.

Study of the Amoebidiales has been advanced significantly by the axenic culture of *Amoebidium parasiticum* (Whisler 1962), which enabled Whistler (1963) and Trotter and Whisler (1965) to determine that the cell walls were not composed of cellulose or chitin. Culturing also has facilitated sequencing the mitochondrial genome, which at ca 300 kbp, is significantly larger than that reported for any fungus (Lang BF, Burger G. http://megasum.bch.umontreal.ca/ogmp/projects/apara/gen.html). In addition, Whisler (1968) was able to induce the amoeba-cyst phase of the life cycle. These findings indicate the Amoebidiales are unique among organisms classified as Fungi in the autapomorphic production of amoeboid cells. Amoebae encyst to form (cysto)spores which develop into new thalli. In contrast to *Paramoebidium,* species of *Amoebidium* also form sporangiospores.

The rationale for classifying Amoebidiales as Trichomycetes has been based on putative similarity in thallus morphology and a shared symbiotic association with arthropods. Whistler (1963) and Lichtwardt (1986), however, have theorized that this order may represent a nonfungal evolutionary lineage derived from a protozoan ancestor. This conclusion was based on the premise that similarities between the Amoebidiales and other Trichomycetes may be due to convergent evolution. Efforts to examine evolutionary relationships of the Amoebidiales have included phylogenetic analysis of 5S ribosomal RNA (Walker 1984), serological analysis (Sanger et al 1972) and comparison of ribosomal RNA molecular

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masses (Porter and Smiley 1979). Collectively, results of these studies, together with those of Trotter and Whistler (1965), suggest that the Amoebidiales are not closely related to other Zygomycota; however, they do not resolve the phyletic affinities of the order. Because phylogenetic analyses of nuclear small subunit (SSU) 18S rRNAs have helped resolve evolutionary relationships among fungi (Bruns et al 1992) and pseudofungi (Gunderson et al 1987), we generated a nearly full length SSU sequence for *A. parasiticum* of 1720 bp to which we added sequences from 19 taxa obtained from GenBank based on the results of a BlastN search (Benson et al 1999).

MATERIALS AND METHODS

Material examined.—Amoebidium parasiticum (FRA-1–14 = NRRL 20524 = ATCC 32708) was grown in a shallow layer of distilled water covering one-tenth strength brain heart infusion agar in 60 × 15 mm plastic petri dishes at room temperature (ca 25 C) as recommended by Lichtwardt (1986). After 1 wk cells were harvested and lyophilized overnight.

Molecular biology.—Total genomic DNA was isolated from the lyophilized cellular material by the phenol/chloroform procedure described by O'Donnell et al (1997) for herbarium specimens. Polymerase chain reaction (PCR) and sequencing protocols were done according to the procedure described by O'Donnell et al (1998), using primers described by White et al (1990), and NS21d 5′–TTGATAGGGCAGAAATTTG and NS41g 5′–CCAACTGTCCCTATTAATCAT.

Dataset construction and phylogenetic analysis.—The SSU rDNA sequence of *Amoebidium parasiticum* was submitted to a BlastN search (vers. 2.0.10) of GenBank (Benson et al 1999). The SSU sequence of *Ichthyophonus hoferi* (Ichthyosporea, Protozoa) yielded the highest score (Spanggaard et al 1996). Based on the results of this search, we downloaded 19 SSU 18S rRNA from GenBank or the TreeCon website (http://rrna.uia.ac.ve/ssu/index.html). These included ichthyosporean sequences (Baker et al 1999, Cavalier-Smith 1998a, Herr et al 1999, Ragan et al 1996) and representatives of the major clades of fungi including the Harpellales (Trichomycetes). In addition, sequences of two choanoflagellates, *Acanthocoepsis unguiculata* and *Diaphanoeca grandis* (see FIG. 1), were selected for rooting the tree by the outgroup method. GenBank accession numbers for the 20 terminals are as follows: *Acanthocoepsis unguiculata* L10823, *Amoebidium parasiticum* AF274051, *Anurofeca richardsi* Wong & Beebee AF070445.1, *Aspergillus fumigatus* Fres. M60300.1, *Capnomyces stellatus* S. W. Peterson & Lichtw. AF007531.1, *Chytridium confervae* (Wille) Minden M59758.1, *Dermocystidium* sp. U21336.1, *Dermocystidium salmonis* U21337.1, *Diaphanoeca unguiculata* Ellis L10824, *Ichthyophonus hoferi* Plehn & Mulsow U25637, *Neocallimastix* sp. LM-2 M59761.1, *"Perkinsus" atlanticus* Azevedo AF192386.1 (the generic name is placed in quotes because authentic *Perkinsus* spp. are nested within the alveolates), *Psorospermum haeckelii* Hilgendorf U33180, *Rhinosporidium seeberi* Wernicke AF118851, rosette agent L29455, *Smittium culisetae* Lichtw. AF007540.1, *Sphaerosoma arcticum* Y16260.2 (cited in GenBank as Joestensen JP, Johansen S, Sperstad S, Landfald B. *Sphaerosoma arcticum,* a new member of a clade of protists near the animal-fungal divergence: systematic position, in vitro growth characteristics, and gross biochemical composition), *Spizellomyces acuminatus* (D.J.S. Barr) D.J.S. Barr M59759.1, Unknown ichthyosporean AJ130859, *Ustilago maydis* (DC.) Corda X62396.1.

Sequences were aligned using ClustalX (Thompson et al 1997) and then by eye using SemWare Editor Professional/32 vers. 2.80b (SemWare Corporation, Marietta, Georgia). The dataset was analyzed by equally weighted maximum parsimony using PAUP*4.0b2 (Swofford 1999). Phylogenetic analysis employed a heuristic search, with gaps treated as missing data, 1000 random addition sequences with MULPARS on and TBR branch swapping. Clade stability was assessed by 1000 parsimony bootstrap replications, using 10 random addition sequences per replicate, and Bremer support (= BS, Bremer 1988) using TreeRot (Sorenson 1996). The PAUP* file has been deposited in TreeBASE as S491.

RESULTS

The dataset consisted of 20 aligned sequences 2081 bp in length, but 732 ambiguously aligned characters were excluded from the analyses. Equally weighted maximum parsimony analysis of the 1349 included characters, 150 of which were phylogenetically informative, yielded 3 most-parsimonious trees 527 steps in length (FIG. 1) (consistency index = 0.712, retention index = 0.709, rescaled consistency index = 0.505). With this dataset, *Amoebidium parasiticum* was deeply nested within the Ichthyophonida (bootstrap = 90%, BS = 7), a monophyletic sister-group of the Dermocystida (bootstrap = 99%, BS = 7). The latter clade contained *Rhinosporidium seeberi,* the etiological agent of rhinosporidiosis of humans and other animals (Herr et al 1999). As indicated by the BlastN search of GenBank, *A. parasiticum* was strongly supported as a sister to *Ichthyophonus hoferi* (FIG. 1) (bootstrap = 94%, BS = 5). However, relationships of major lineages within the Ichthyophonidia and fungi were incompletely resolved by the SSU 18S rDNA data as evidenced by one node within each of these clades with a bootstrap score of < 50%.

DISCUSSION

Results of the present study confirm Whistler's (1963) and Lichtwardt's (1986) hypothesis that *Amoebidium parasiticum* (Amoebidiales) is a protozoan rather than a Trichomycete (Zygomycota). A BlastN search of GenBank and the present phylogenetic reconstruction based on parsimony analysis of SSU 18S

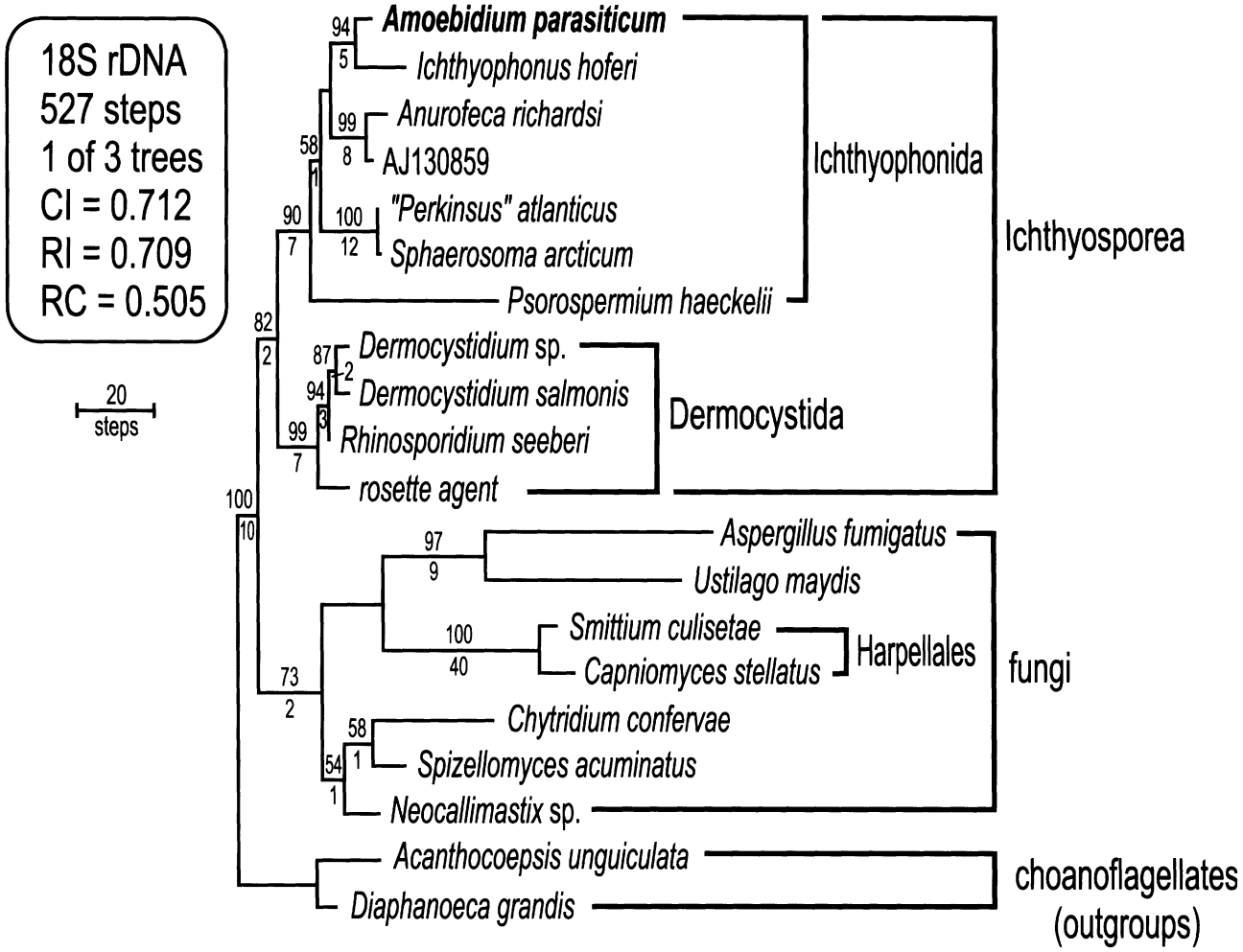

FIG. 1. One of three most-parsimonious phylograms 527 steps in length found by PAUP*, using the heuristic search option with 1000 random addition sequences. Sequences of two choanoflagellates were used to root the tree. Bootstrap intervals ≥50% are indicated above nodes; numbers below nodes represent Bremer support calculated with TreeRot (Sorenson 1996).

rDNA sequence data identified the ichthyosporean intracellular fish parasite *Ichthyophonus hoferi* as its closest known relative. The present molecular phylogeny indicates that both taxa are nested within the order Ichthyophonida of the class Ichthyosporea (Cavalier-Smith 1998a). This finding is consistent with preliminary phylogenetic analysis of mitochondrial DNA that places *A. parasiticum* at the root of the animal–fungal clade as a sister taxon to choanoflagellates (Lang BF, Burger G. http://megasum.bch.umontreal.ca/ogmp/projects/apara/gen.html). Phylogenetic analysis of a more inclusive SSU 18S rDNA data set (data not shown) that included sequences of *Ginkgo biloba* and *Zamia pumila* as an outgroup did not resolve a possible relationship of the Ichthyosporea with the animals, fungi or choanoflagellates (Wainright et al 1993). The Ichthyosporea was initially identified near the animal-fungal divergence and informally named the DRIP clade composed of protozoan parasites of crustaceans and fish (Ragan et al 1996). Subsequently, five additional members of this clade were discovered using phylogenetics of 18S rRNA sequences: *Anurofeca richardsi* from tadpole larvae (Baker et al 1999), *Sphaerosoma arcticum* from a marine arctic invertebrate (B. Landfald pers comm), "*Perkinsus*" *atlanticus* from a marine clam, a cloned sequence from an experimental microbial community (van Hannen et al 1999), and *Rhinosporidium seeberi* from humans with rhinosporidiosis (Herr et al 1999). Like *Amoebidium parasiticum, R. seberi* and *I. hoferi* were thought to be fungi because their thalli and/or sporangia are fungal-like (reviewed in Herr et al 1999, Rand 1994); however, the latter two taxa were treated as *incertae sedis* by Hawksworth et al (1995).

Although all Ichthyosporea were thought to possess parasitic stages (Cavalier-Smith 1998a), there is no evidence that either *Amoebidium parasiticum* or

Anurofeca richardsi (Baker et al 1999) are parasitic. Furthermore, in addition to *Amoebidium parasiticum* (Whistler 1962), pure cultures have been established for *Anurafeca richardsi* (cited in van Hannen et al 1999), *I. hoferi* (Spanggaard et al 1995), *"Perkinsus" atlanticus,* and *S. arcticum* (B. Landfald pers comm), indicating that these taxa are not obligate parasites. Interestingly, all five ichthyosporeans that have been cultured axenically are members of the Ichthyophonida clade. Furthermore, the experimental conditions established in the microbial ecology study by van Hannen et al (1999) apparently supported growth of a free living ichthyosporean from which the SSU rDNA sequence AJ130859 was cloned. Nevertheless, because knowledge of their life cycles is incomplete, we cannot rule out the possibility that parasitic stages are present in all of the ichthyosporeans.

Results of the present molecular phylogeny support the classification of Cavalier-Smith (1998a) that recognizes the monophyletic sister clades Ichthyophonida and Dermocystida within the Ichthyosporea. Although tubulovesiculate and flat mitochondrial cristae were thought to characterize these respective sister orders (Ragan et al 1996, Herr et al 1999), cristae of *Amoebidium parasiticum* mitochondria are mostly flat (Whistler and Fuller 1968), indicating that this character is homoplasious. Ironically, in addition to erecting a protozoan class for the ichthyosporeans (Cavalier-Smith 1998a), Cavalier-Smith (1998b) also described the fungal class Enteromycetes to accommodate the Amoebidiales and Eccrinales (Lichtwardt 1986).

Results of the present study also highlight the need to further test the monophyly of the Trichomycetes. Fortunately, Lichtwardt (1973, 1986) and Moss and Young (1978) have published explicit testable hypotheses outlining putative phyletic relationships of the Trichomycetes, including the poorly known and uncultured Eccrinales and Asellariales for which no molecular systematic data are currently available.

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