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Eccrinales (Trichomycetes) are not fungi, but a clade of protists at the early divergence of animals and fungi

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Abstract

The morphologically diverse orders Eccrinales and Amoebidiales have been considered members of the fungal class Trichomycetes (Zygomycota) for the last 50 years. These organisms either inhabit the gut or are ectocommensals on the exoskeleton of a wide range of arthropods—Crustacea, Insecta, and Diplopoda—in varied habitats. The taxonomy of both orders is based on a few micromorphological characters. One species, *Amoebidium parasiticum*, has been axenically cultured and this has permitted several biochemical and phylogenetic analyses. As a consequence, the order Amoebidiales has been removed from the Trichomycetes and placed in the class Mesomycetozoea. An affinity between Eccrinales and Amoebidiales was first suggested when the class Trichomycetes was erected by Duboscq et al. [Arch. Zool. Exp. Gen. 86 (1948) 29]. Subsequently, molecular markers have been developed to study the relationship of these orders to other groups. Ribosomal gene (18S and 28S) sequence analyses generated by this study do not support a close association of these orders to the Trichomycetes or to other fungi. Rather, Eccrinales share a common ancestry with the Amoebidiales and belong to the protist class Mesomycetozoea, placed at the animal–fungi boundary.
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1. Introduction

The Eccrinales have been placed in the class Trichomycetes (Zygomycota), which are fungal associates of various Arthropoda. The class has traditionally included four orders: Amoebidiales, Asellariales, Eccrinales, and Harpellales. This group of symbionts inhabits a wide range of hosts—Crustacea, Insecta, and Diplopoda—in varied habitats (marine, freshwater, and terrestrial). The order Eccrinales is characterized by unbranched, non-septate, multinucleate thalli, and sporangiospore production (Figs. 1A–C and F). They live attached to the cuticle lining of the digestive tract of their hosts by a secreted, basal holdfast. Asexual reproduction is by sporangiospores, which form basipetally from the thallus apex, a feature found only in the kingdom Fungi. Sexual reproduction has not been observed, except possibly in one species, *Enteropogon sexuale*, where scalariform conjugation was reported (Hibbits, 1978). The fact that the Eccrinales share a very specialized ecological niche, the arthropod gut, with a group of well-known fungi, Harpellales (Trichomycetes), in combination with the paucity of distinctive morphological characters, have been the key criteria used in their classification within the Trichomycetes. The Eccrinales is the only order of Trichomycetes with marine representatives; out of 17 genera, seven have species that inhabit crustaceans that live submerged in seawater. The distribution of the eccrinids ranges from tropical forests (White et al., 2000) to hydrothermal vents (Van Dover and Lichtwardt, 1986). They inhabit arthropods in almost all parts of the world in every habitat that is suitable for their hosts.

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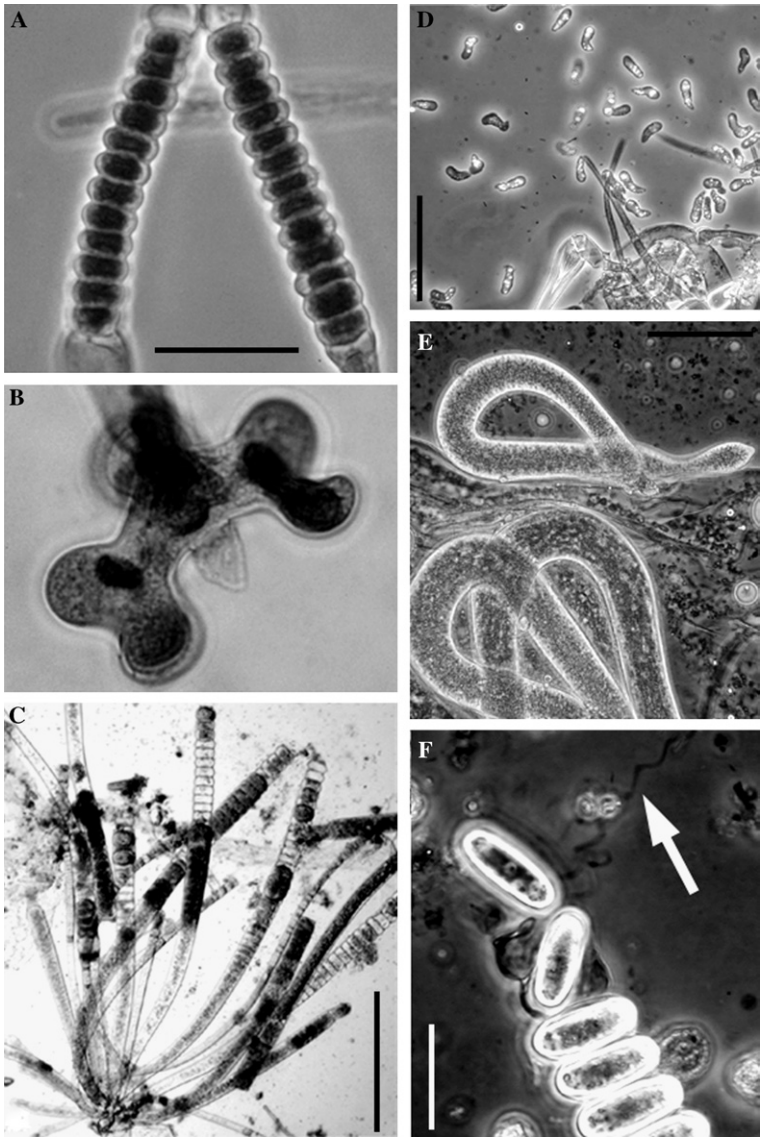

Fig. 1. *Alacrinella limnoriae* (A) sporangiospores at the thallus apex. (B) Holdfast area, lobed base. *Enteromyces callianassae* (C) young thalli sharing a multiple holdfast system. *Paramoebidium* sp. (D) swarm of released amoebae. (E) Mature thalli attached to the hindgut on a mayfly (Ephemeroptera). *Astreptonema gammari* (F) sporangiospore being released with one appendage noticeable (arrow). Scale bars A, B = 25 μm, C–F = 50 μm.

Eccrinales are the oldest described group of Trichomycetes. The first species, *Enterobryus elegans*, was described from the millipede *Narceus americanus* (Leidy, 1849). Duboscq et al. (1948) officially named the class Trichomycetes (meaning "hair-like" fungi) in their monograph referring to the Amoebidiales and Eccrinales.

Because of our inability to culture members of the Eccrinales, their classification has received little attention. Whisler (1963) provided early hints of some odd characteristics for the Eccrinales; he reported a lack of chitin, a characteristic feature of fungi, in his studies of cell-wall composition. In more recent years, there have been some reports on the ultrastructure of a few species of the group (Manier, 1979; Manier and Grizel, 1972; Mayfield and Lichtwardt, 1980; Moss, 1975, 1999; Saikawa et al., 1997; Tuzet and Manier, 1967). The lack of a septal pore and associated plug and the presence of dictyosomes have raised some questions about the true affinity of the Eccrinales with the Trichomycetes (Moss, 1999), and with fungi in general.

Lieberkühn (1856) and Schenk (1858) described amoebae-producing organisms otherwise morphologically similar to the Eccrinales attached to the exoskeleton of freshwater arthropods in Europe, which Cienkowski (1861) later named *Amoebidium parasiticum*. Léger and Duboscq (1929) described a second amoebae-producing genus, *Paramoebidium* (Figs. 1D and E), which they found in the hindgut of *Nemura variegata* (Plecoptera). Together with *Amoebidium*, they placed *Paramoebidium* in the order Amoebidiales within the class Trichomycetes.

Amoebidium was the first trichomycete to be cultured (Whisler, 1960), which permitted more comprehensive studies on its biology and its phylogenetic relationships (Trotter and Whisler, 1965; Whisler, 1962, 1968; Whisler and Fuller, 1968). The production of amoebae is not otherwise present in the kingdom Fungi; thus this character alone raised many questions about the position of the Amoebidiales (Lichtwardt, 1986). Other features were described that further caused researchers to question this relationship. Trotter and Whisler (1965) reported that *A. parasiticum*, like members of the Eccrinales, lacked chitin in its cell wall. Ultrastructural studies indicated the presence of stacked dictyosomes (Whisler and Fuller, 1968), which have not been found in fungi (Beckett et al., 1974; Bracker et al., 1996). Serological affinities (Sangar et al., 1972), rRNA weight comparisons (Porter and Smiley, 1979), immunological studies (Peterson and Lichtwardt, 1987), 5S DNA sequencing (Walker, 1984), and isozyme studies (Grigg and Lichtwardt, 1996) have shown little affinity between Amoebidiales and culturable members of the Trichomycetes. Recent molecular sequence data have proven very useful in establishing the true affinity of the Amoebidiales, now a member of the Protista (Benny and O'Donnell, 2000; Mendoza et al., 2002; Ustinova et al., 2000). Here, I present results from phylogenetic analyses based on molecular data (rDNA) that include unculturable taxa of Eccrinales and Amoebidiales.

2. Materials and methods

2.1. Specimen collection

The material for this study was obtained from many regions and habitats (Table 1). Marine arthropods were collected from mud flats and tide pools. Freshwater crustaceans and dipterans were collected in streams and lakes, and terrestrial millipedes and isopods were sampled in many places. Collections were made in the field following standard procedures (Lichtwardt et al., 2001; White et al., 2001). Specimens were usually kept on ice in coolers or shaded with minimal native vegetation or substrate to maintain specimens alive until returned to the laboratory. Under a dissecting microscope gut fungi were removed from the digestive tract, with as little host tissue as possible, by manipulation with fine needles. Thalli were slide-mounted for identification. Preferably, wet mounts of gut fungi were photographed live using a compound microscope or after infiltration of the specimens with lactophenol-cotton blue (0.05% w/v). Coverslips were sealed by ringing them with clear fingernail polish, rendering the slides semi-permanent. Slide-mounted specimens were kept as vouchers. Alternatively, once the fungi had been identified and photographed, selected samples were preserved in microcentrifuge tubes containing 500 µl CTAB buffer (Hillis et al., 1996) for subsequent DNA extraction. Storage at −20 °C from several weeks to three years yielded DNA suitable for PCR amplification.

2.2. DNA extraction

First, thalli were broken by several cycles of freezing and thawing. Microcentrifuge tubes were dipped in liquid nitrogen and then thawed in a heat block at 65 °C; this procedure was repeated at least three times. One volume of chloroform was added, vortexed briefly, and centrifuged for 10 min at maximum speed. When samples contained large amounts of debris, the chloroform extraction was repeated. After recovering the supernatant, DNA was precipitated using an equal volume of isopropanol overnight or for 48 h at −20 °C. After centrifugation at maximum speed for 15 min, the resulting pellet was washed twice with 70% ethanol and dried in a speed vac at 65 °C. The DNA was resuspended in 35 µl of TE/10 (10 mM Tris, 0.1 mM EDTA, pH 8.1) and aliquots were diluted (1:50) in ddH_2O for PCR amplification.

2.3. PCR amplification

Universal fungal primers from White et al. (1990), for nuclear ribosomal genes, were the starting point for amplification procedures. Portions of the ribosomal 18S gene were amplified using different combinations of these primers (NS1–NS8). Several Eccrinales-specific primers were designed using this fragmented information and the sequence of *A. parasiticum* as a reference (GenBank AF274051). PCR amplification of the 18S rRNA gene was then performed in two separate reactions to obtain overlapping fragments, using combinations of universal and specific primers (NS1-ECT1R and ECT1-NS8) (ECT1 5′-GAGCGTGGGCGGAGTTCG GGAC-3′, ECT1R 5′-GTCCCGAACTCCGCCCACG CTC-3′), respectively. Amplification of the first two variable domains (D1, D2) of the 28S molecule was performed either using the primers, NL1 and NL4, from O'Donnell (1996), or a forward eccrinid-specific primer (Ecc28F 5′-CGGACGCYTRTKTGGAYGAYGGTG-3′) in combination with NL4. Primers were tested on DNA extracted from a culture of *A. parasiticum* (isolate FRA-1-14). The amplification reactions were standardized for a total final volume of 20 µl. A 2× master mix containing 0.25 mM of each primer, 0.225 mM dNTPs, and a 10% solution of 10× buffer (M190A, Promega, Madison, WI) was used with variable $MgCl_2$ concentration (1.5–3.5 mM). A typical reaction cocktail contained 10 µl of master mix, 2 µl of 5× HiSpec Additive (Bioline USA, Randolph, MA) and 8 µl of diluted DNA sample (1:50). Cycle parameters for PCR were adjusted depending on primer pairs, but mostly followed the protocol of Gottlieb and Lichtwardt (2001).

Table 1
List of taxa collected in this study for molecular work (sorted by order and genus name)

Species name	Source[a]	Host	Location	Collector[b] and date	Accession Nos.	
					18S	28S
Amoebidiales						
Amoebidium parasiticum	A1a*	*Daphnia* sp., Cladocera	California, USA	HCW 1959		AY336692
Amoebidium parasiticum	FRA-1-14*	*Daphnia* sp., Cladocera	Herault, France	RWL Jun 68	AF274051	AY336691
Paramoebidium sp.	KS-61-W6^{+}	Ephemeroptera	Breidenthal Biological Reserve, Douglas Co., Kansas, USA	MMW May 98	AY336708	
Eccrinales						
Alacrinella limnoriae	MA-8-W4^{+}	*Limnoria* sp, Isopoda	Woods Hole, Massachusetts, USA	MMW Mar 98	AY336703	
Astreptonema gammari	MN-3-W6^{+}	*Gammarus* sp., Amphipoda	Pine Needle Preserve, Minnesota, USA	LCF Mar 00	AY336709	
Astreptonema sp.	WA-3-C3	Sphaeromatidae, Isopoda	Eagle Cove, San Juan Island, Washington, USA	MJC Mar 99	AY336706	
Eccrinidus flexilis	SPA-10-C2	*Glomeris* sp., Diplopoda	St. Llorens del Munt, Barcelona, Spain	MJC Aug 02	AY336698	
Eccrinidus flexilis	SPA-11-C45	*Glomeris* sp., Diplopoda	Punta de la Mora, Tarragona, Spain	MJC Aug 02	AY336700	
Enterobryus halophilus	CA-11-C4	*Emerita analoga*, Anomura	Salmon Creek Beach, Bodega Bay, California, USA	MJC Jul 01		AY336694
Enterobryus oxidi	KS-79-W2^{+}	Diplopoda	Rice Woodland Tract, KU Ecological Reserve, Douglas, Kansas, USA	MMW Aug 99	AY336710	
Enterobryus sp.	SPA-10-C6	Iulidae, Diplopoda	St. Llorens del Munt, Barcelona, Spain	MJC Aug 02	AY336711	
Enterobryus sp.	SC-3-C2	Diplopoda	Clemson University, South Carolina, USA	RWL Oct 98		AY336693
Enterobryus sp.	MA-11-C1	Diplopoda	Peach's Point, Essex, Massachusetts, USA	MJC Feb 02	AY336701	
Enteromyces callianassae	CA-12-C4b	*Callianassa californiensis*, Anomura	Walker Creek Marsh, Tomales Bay, Marin, California, USA	MJC Jul 01		AY336696
Enteropogon sexuale	WA-1-C5	*Upogibia pugettensis*, Anomura	False Bay, San Juan Island, Washington, USA	MJC Mar 99	AY336705	
Palavascia patagonica	ARG-D1-C15	*Exosphaeroma studeri*, Isopoda	Puerto Deseado, Santa Cruz, Argentina	MJC Dec 98	AY682845	AY336695
Taeniella carcini	WA-1-C37	*Hemigrapsus nudus*, Decapoda	False Bay, San Juan Island, Washington, USA	MJC Mar 99	AY336707	
Taeniellopsis sp.	MA-5-C17	*Orchestia* sp., Amphipoda	Nobska Point, Barnstable Co., Massachusetts, USA	MJC Mar 98	AY336704	AY336697

[a] *, Culture from the University of Kansas Mycological Culture Collection; +, dissection and CTAB preservation by MMW.
[b] HCW, Howard C. Whisler; LCF, Leonard C. Ferrington; MJC, Matías J. Cafaro; MMW, Merlin M. White; RWL, Robert W. Lichtwardt.

2.4. Cloning and sequencing

Following PCR amplification, if a single band was observed on a 1% agarose gel run in 1× TAE buffer, products were cleaned using QIAquick PCR purification kit (Qiagen, Valencia, CA). When multiple products were present, the bands were separated by electrophoresis in a low melt agarose (Fisher Scientific, Pittsburgh, PA) gel and purified using QIAquick Gel extraction kit (Qiagen, Valencia, CA). All products in this study were cloned using the pGEM-T Easy Vector System II cloning kit (Promega, Madison, WI). Positive bacterial clones were grown in 3 ml of LB broth + ampicillin (10 mg/ml) overnight and their plasmids were extracted with QIAprep Spin Miniprep kit (Qiagen, Valencia, CA). Sequencing reactions were performed using Thermo Sequenase Fluorescent labeled primer cycle sequencing kit (Amersham–Pharmacia Biotech, Piscataway, NJ). Primers (IR-labeled) for the cloning vector were used in the reactions (T7 promoter and M13Rev) by using simultaneous bi-directional sequencing (SBS) in a LI-COR 4200L IR^2 DNA sequencer (LI-COR, Lincoln, NE).

2.5. Data analyses

GenBank BLAST searches were performed for raw sequences to determine whether the data corresponded to the desired eccrinid sample or to another gut inhabitant, food source (i.e., algae, bacteria, dinoflagellates, etc.) or host tissue. Contaminating DNA sequences were excluded from further analysis. Sequence fragments were combined, edited, and aligned using Sequencher 4.2 (Gene Codes, Ann Arbor, MI). The resulting alignment data were edited by eye using MacClade 4.06 (Maddison and Maddison, 2003). Outgroup sequences were downloaded from GenBank. Two datasets were constructed with 18S and 28S sequences. The small subunit (18S) dataset contains members of several kingdoms: Animalia: *Beroe cucumis* (D15068), *Microciona prolifera* (L10825), and *Mnemiopsis leidyi* (AF293700); Fungi: *Chytriomyces hyalinus* (M59758), *Smittium culisetae* (AF007540), *Capniomyces stellatus* (AF007531), *Kickxella alabastrina* (AF007537), *Gigaspora gigantea* (Z14010), *Tricholoma matsutake* (U62538), and *Xylaria carpophila* (Z49785); Stramenopila: *Achlya bisexualis* (M32705); Protista: *Monosiga brevicollis* (AF100940), *Diaphanoeca grandis* (L10824), *Sphaerothecum destrudens* (AY267345), *Rhinosporidium seeberi* (AF118851), *Dermocystidium salmonis* (U21337), *Sphaeroforma arctica* (Y16260), *Anurofeca richardsi* (AF070445), *Ichthyophonus hoferi* (U25637), and 12 new complete sequences for the Eccrinales and two for the Amoebidiales (see Table 1 for GenBank accession numbers). The large subunit (28S) dataset contains newly generated sequences for two strains of *A. parasiticum* from different locations (France and USA) and five representatives of the Eccrinales plus 14 outgroup sequences: Animalia: *Beroe ovata* (AY026369), *Leucosolenia* sp. (AY026372), and *M. leidyi* (AF026373); Fungi: *S. culisetae* (AF031072), *C. stellatus* (AF031073), *Furculomyces boomerangus* (AF031074), *K. alabastrina* (AF031064), *Linderina pennispora* (AF031063), *Coemansia reversa* (AF031067), *Actinomucor elegans* (AF157173), *Saccharomyces cerevisiae* (J01355), and *T. matsutake* (U62964); Protista: *I. hoferi* (AY026370), *M. brevicollis* (AY026374). The two datasets were analyzed independently. The alignments were inspected for the presence of ambiguously aligned regions caused by gap insertions. An equally weighted parsimony analysis was conducted on the unambiguously aligned regions using PAUP* 4.0 (Swofford, 1999) via heuristic searches (MULTREES in effect, branches collapsed if maximum branch length is zero, 1000 random stepwise additions, TBR branch swapping and using gaps as missing characters). In a second analysis, the ambiguously aligned regions were unequivocally coded to form a new set of characters replacing these regions in the phylogenetic analyses. Coding of ambiguous regions and step matrices calculations were performed with the program INAASE 0.2c1 (Lutzoni et al., 2000). Unambiguous regions were also subjected to step matrices taking into consideration the frequency of each class of possible changes (Fernández et al., 1999; Miadlikowska et al., 2002) with help of the program STMatrix 2.2 (Lutzoni and Zoller, Dept. of Biology, Duke University). Maximum parsimony (MP) analyses were performed using PAUP* 4.0 via heuristic searches (MULTREES in effect, branches collapsed if maximum branch length is zero, 1000 random stepwise additions, TBR branch swapping and using gaps as fifth character state). Bootstrap (BP) support was calculated for internal branches after 1000 pseudoreplicates and one random stepwise addition per pseudoreplicate.

Both datasets (with ambiguous regions removed) were analyzed under Bayesian inference using Mr. Bayes 3.0b4 (Huelsenbeck and Ronquist, 2001), which calculates posterior probabilities using a Metropolis-coupled Markov chain Monte Carlo (MCMCMC) analysis. All of the analyses employed one cold chain and three incrementally heated chains, where the heat of the ith chain is $B = 1/[1 + (i-1)\,T]$ and $T = 0.2$, when $i = 1$, $B = 1$ corresponding to the cold chain. The general time reversible (GTR) model was selected under a gamma distribution for the among-site rate variation. Starting trees for each chain were generated at random; burn-in was set to 2000 trees after verifying convergence to stationarity of parameters α, $\ln L$, and TL. The run was set to two million generations with sample frequency of trees every 100 generations. The prior distribution for the substitution rates was set to a flat model.

To test hypotheses that competed with the traditional view that the Eccrinales are Fungi, parametric boot-

strapping (Goldman et al., 2000; Huelsenbeck et al., 1995) was performed for both datasets. Constrained trees were imposed on the datasets under MP analysis and tree length was calculated. The difference in tree length between constrained and unconstrained trees is the parameter to be tested statistically. A constrained model tree under the maximum likelihood criterion was built in PAUP* 4.0 after establishing the model of sequence evolution using ModelTest 3.06 (Posada and Crandall, 1998). This tree and the ML model were used to simulate evolution of DNA characters using the program Mesquite 1.01 (Maddison and Maddison, 2004). The likelihood ratio test implemented in ModelTest 3.06 indicated the general time reversible model with a proportion of invariant sites and a gamma distribution for the among-site rate variation (GTR + I + Γ) for the 18S dataset and Tamura–Nei model with a proportion of invariant sites and a gamma distribution for the among-site rate variation (TrN + I + Γ) for the 28S dataset. The simulation was run for 600 data matrices. The program plots the distribution of the statistic and allows to compare the observed valued of the test statistic (tree length) to the distribution of the statistic expected under the model as determined by the simulations (for more details, see Maddison, 2004).

3. Results

3.1. PCR amplification and sequencing

The products amplified for the 18S gene varied in size ranging from 1950 to 2045 bp. For the 28S gene, all products were approximately 900 bp. At least two clones per amplified product were sequenced. It was difficult to obtain PCR products for most of the isolates collected in the field. Few samples were successfully amplified, with only 23 products out of 97 samples giving positive results. For the small subunit, 13 almost complete sequences were obtained while there were only seven for the large subunit. In some cases, only partial 18S gene sequences were obtained due to lack of amplification of one half of the gene and were not included in the analyses (GenBank AY336699, AY336702, AY336712, and AY336713).

3.2. Phylogenetic analyses

The 18S sequences generated in this study BLASTed with *A. parasiticum* and other Mesomycetozoea. The close relationship of Eccrinales to this group was unexpected. To verify the hypothesis that the Eccrinales are closely related to the Amoebidiales, phylogenetic analyses of representatives of several phyla of eukaryotes were performed. The 18S matrix includes 33 taxa; of these 12 new complete sequences belong to the Eccrinales and one new sequence to *Paramoebidium* sp. (Amoebidiales). The final length of the alignment was 2220 characters, of which 637 were excluded due to ambiguous alignment. *A. bisexualis* (Stramenopila) was used to root 18S trees. The 28S alignment was 1224 characters in length, of which 785 were excluded due to ambiguous alignment. It is also worth mentioning here that the 28S gene sequences were extremely difficult to align because of the variability within the D1/D2 region. *M. brevicollis* (Protista) was used to root 28S trees.

Bayesian analysis of the 18S dataset yielded a tree that is almost fully resolved except for the relationships between the Mesomycetozoea, the animals, and the choanoflagellate protists, which nonetheless collectively form a weakly supported monophyletic group (PP 0.55, Fig. 2) sister to the Fungi. This analysis supports the hypothesis that the Eccrinales belong to the protist class Mesomycetozoea (PP 0.95), more specifically to the order Ichthyophonida (PP 1.00). *Paramoebidium* sp. groups, as expected, with *A. parasiticum*, hence forming a well-supported Amoebidiales clade (PP 1.00) closely related to the Eccrinales (PP 0.97). Within the Eccrinales, three well-supported notable clades are present: a taxonomically diverse core eccrinid clade forms a strongly supported monophyletic group (PP 1.00, clade **a**), two *Enterobryus* spp. form another group (PP 1.00, clade **b**), and basal to both is *Eccrinidus flexilis* (PP 1.00, clade **c**). The Harpellales (Trichomycetes) (PP 1.00) are nested within the Zygomycota (Fungi) sharing a common ancestor with the Kickxellales (PP 0.95), as previously reported (O'Donnell et al., 1998).

Equally weighted MP analysis of the 18S dataset matrix consisted of 2220 characters, of which 637 were excluded, 986 were constant, and 204 were variable but parsimony uninformative. This analysis of 407 parsimony informative characters yielded one most parsimonious tree 4286.46 steps long (CI = 0.570, RI = 0.605) (Fig. 3A). This tree is very similar to the one from Bayesian analysis, except for the Fungi, which fail to form a monophyletic group. According to these data, the Eccrinales belong to the class Mesomycetozoea, more specifically to the order Ichthyophonida (BP 99%). The three reported clades within the Eccrinales were also recovered, but clade **b** is not highly supported (BP 68%). Amoebidiales form a well-supported clade (BP 100%) closely related to the Eccrinales. Again, the relationship between the Mesomycetozoea, the animals and the choanoflagellates is not resolved as previously reported for the 18S gene (Ragan et al., 2003).

The MP analysis of the 18S dataset including INAASE characters resulted in a data matrix of 2234 characters after adding 14 ambiguously aligned regions recoded using the INAASE program. Only 456 characters were used in the analysis (637 excluded, 901 constant, and 240 parsimony uninformative), which yielded one most parsimonious tree 5226.5 steps long (CI = 0.557, RI = 0.598)

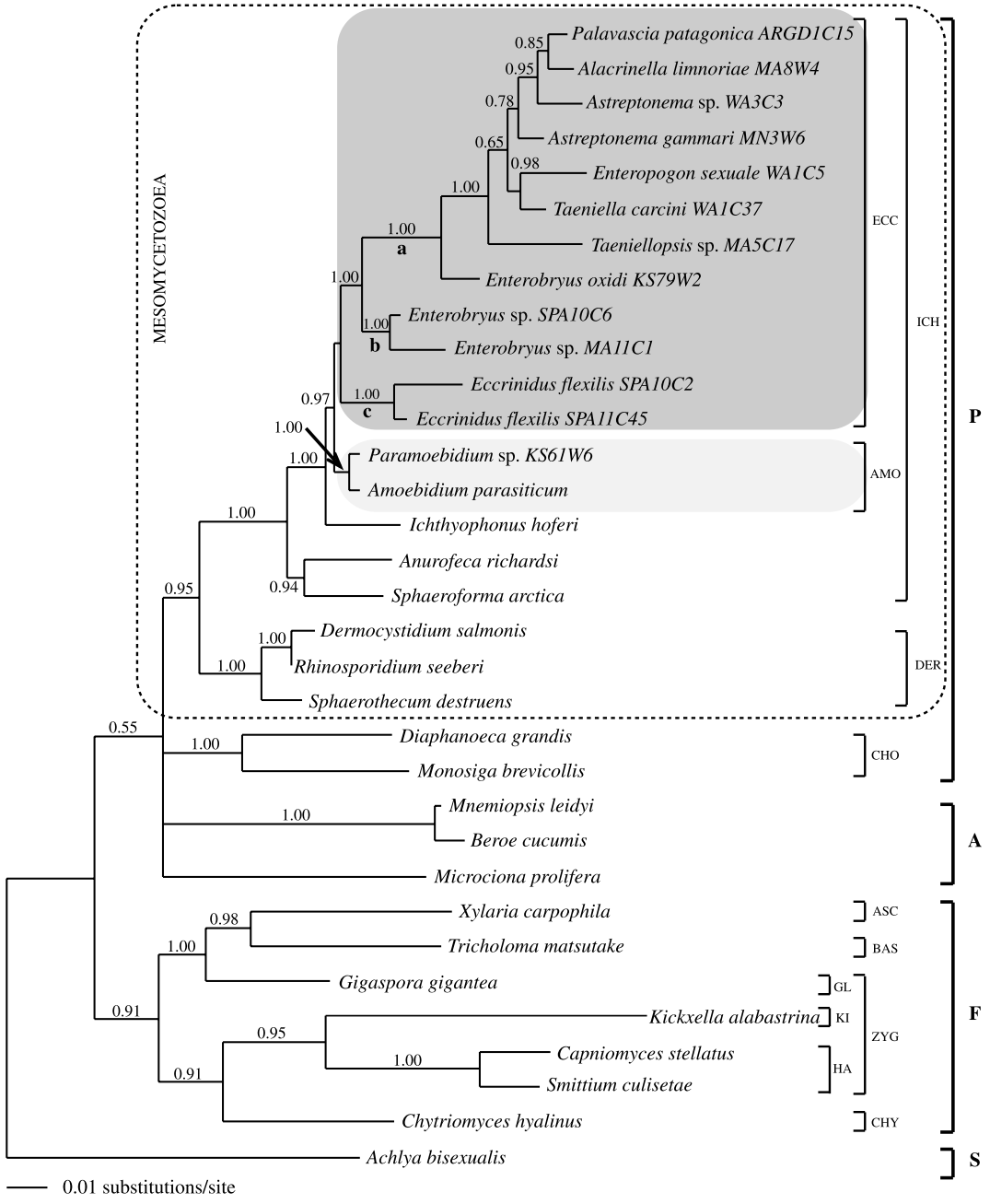

Fig. 2. Bayesian phylogenetic tree of 33 taxa from 18S dataset after two million generations of MCMCMC chains. Values on the nodes correspond to posterior probabilities (PP). ECC, Eccrinales; AMO, Amoebidiales; DER, Dermocystidia; ICH, Ichthyophonida; CHO, Choanoflagellates; GL, Glomales; KI, Kickxellales; HA, Harpellales; ASC, Ascomycota; BAS, Basidiomycota; ZYG, Zygomycota; CHY, Chytridiomycota; A, Animalia; F, Fungi; S, Stramenopila; and P, Protista.

(Fig. 3B). This tree is fully resolved with the Fungi forming a well-supported monophyletic group (BP 85%). The Eccrinales are not monophyletic, but the core eccrinid clade is well-supported (clade **a**, BP 100%). Amoebidiales fail to form a monophyletic group, though they are still closely related to other members of the Ichthyophonida. Though it is a resolved tree, major relationship between animals, protists, and fungi are not supported. The addition of recoded regions with INAASE into the analysis increases the resolution of the tree, but general support is lost throughout the tree. Although the posterior probabilities are not comparable to the bootstrap values (Alfaro et al., 2003), all the analyses support the placement of the Eccrinales within the Mesomycetozoea.

Bayesian analysis of the 28S dataset produced a tree (Fig. 4) that is in general concordance with the results obtained with the 18S gene, i.e., Eccrinales are part of the class Mesomycetozoea (PP 1.00). Although the Eccrinales appear as a monophyletic group there is low support (PP 0.53). This result might be due to poor taxon

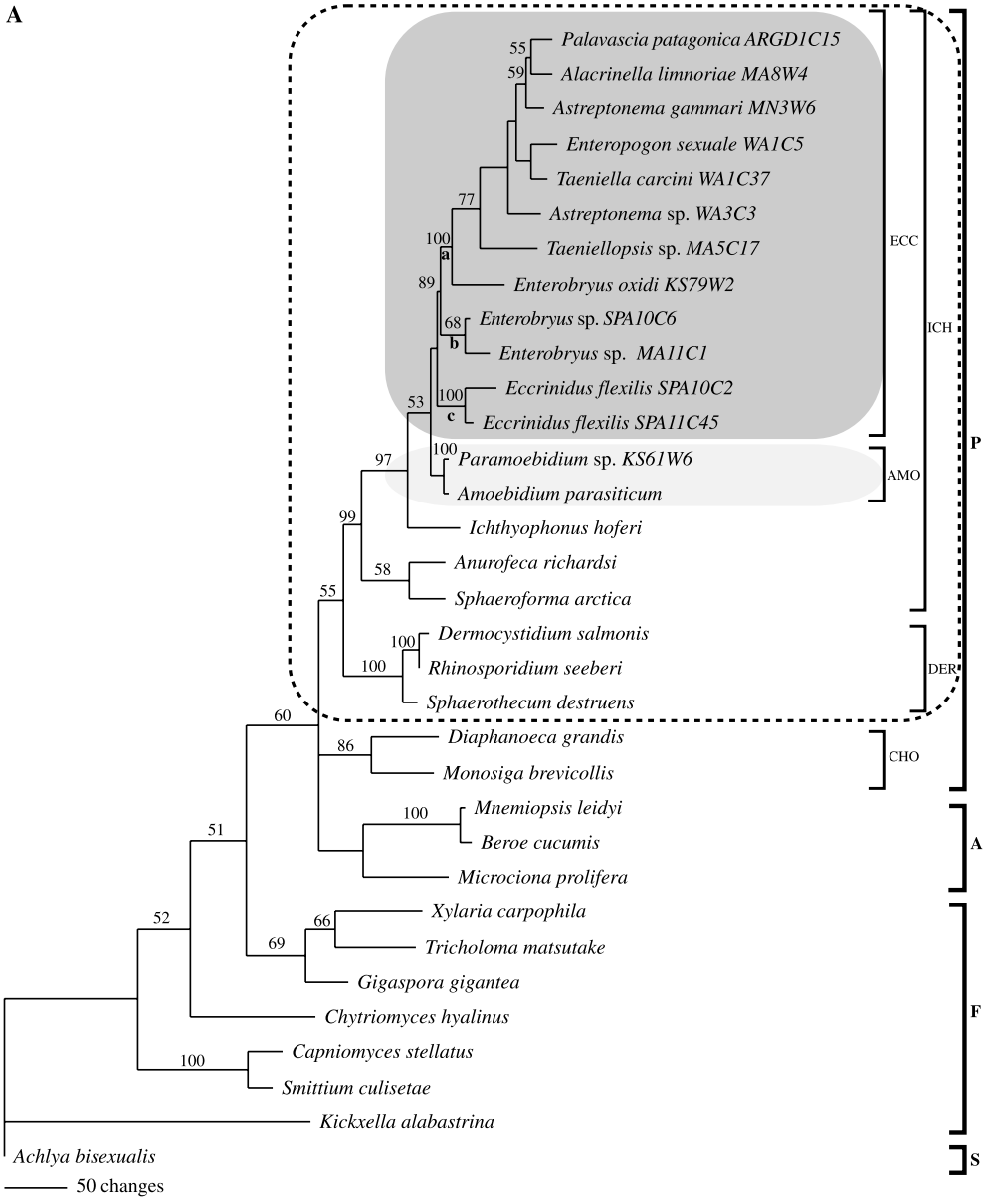

Fig. 3. (A) Phylogram derived from an equally weighted MP analysis with bootstrap values indicated at the nodes. (B) Phylogram derived from a MP analysis including recoded characters from INAASE program. For both trees, only MP BP > 50% are shown. ECC, Eccrinales; AMO, Amoebidiales; CHO, Choanoflagellates; A, Animalia; F, Fungi; and P, Protista.

sampling in both the ingroup and outgroup. The paucity of sequences for the 28S gene for the Mesomycetozoea in GenBank made it difficult to add an adequate representation of the class. According to these data, Mesomycetozoea is more closely related to the fungi than to the animals (PP 0.98) and *A. parasiticum* forms a monophyletic group with *I. hoferi* (PP 0.81). Again, within the fungi, the Harpellales and Kickxellales group together (PP 0.98).

The MP analysis of the 28S data included seven ambiguously aligned regions recoded using INAASE. Of the 446 included characters 140 were constant and 62 variable, but parsimony uninformative. The analysis of 249 characters yielded one most parsimonious tree 2522.96 steps long (CI = 0.562, RI = 0.590) (Fig. 4). This tree has the same topology as the tree from Bayesian analysis except for the position of *A. elegans*. The Eccrinales form a well-supported monophyletic group (BP 98%) within the Mesomycetozoea (BP 79%). The fungi appear as a weakly supported monophyletic group (BP 57%) most closely related to the Mesomycetozoea (BP 88%).

Tree length differences between constrained and unconstrained analyses placing the Eccrinales with the fungi, rather than with the Mesomycetozoea, were significant for both datasets. Differences of 1042.28 and 40.14 steps for 18S and 28S datasets, respectively, were recorded. The null hypothesis that the Eccrinales form a monophyletic group with the fungi was rejected at

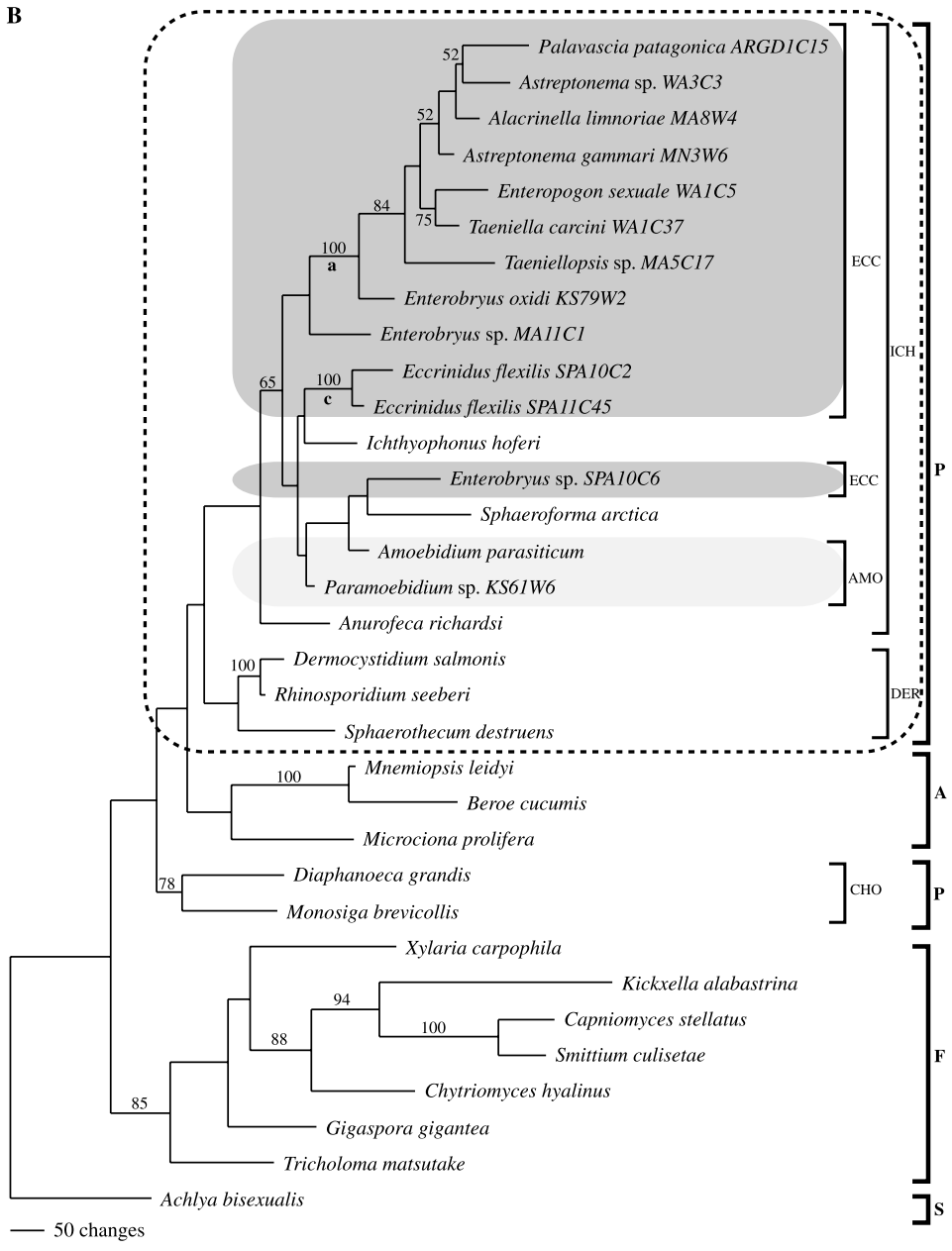

Fig. 3. (*continued*)

$P < 0.01$ in both datasets. Monophyly for Eccrinales + Amoebidiales + Ichthyophonida with fungi was also rejected ($P < 0.01$) for the 18S data.

4. Discussion

4.1. Phylogenetic relationships

The results of the present phylogenetic study strongly support the affiliation of the Eccrinales with the class Mesomycetozoea. Monophyly of the group was maintained in almost all the analyses, though not well supported in some cases. The Amoebidiales remained a monophyletic group following the addition of *Paramoebidium* sp. Amoebidiales and Eccrinales were identified as closely related within the order Ichthyophonida. Duboscq et al. (1948) recognized this relationship when they erected the Trichomycetes and established the Eccrinides for the Eccrinales and Amoebidiales. These authors based their grouping on rather unusual morphological characters that provided few clues concerning the identity of their relatives at that time. Cavalier-Smith (1998) placed the Eccrinales and Amoebidiales together in the class Enteromycetes based on morphology, ecology, and ultrastructure (presence of dictyosomes). Lichtwardt (1986) considered the Eccrinales to be the most derived order of the Trichomycetes, whereas Moss (1999) suggested that

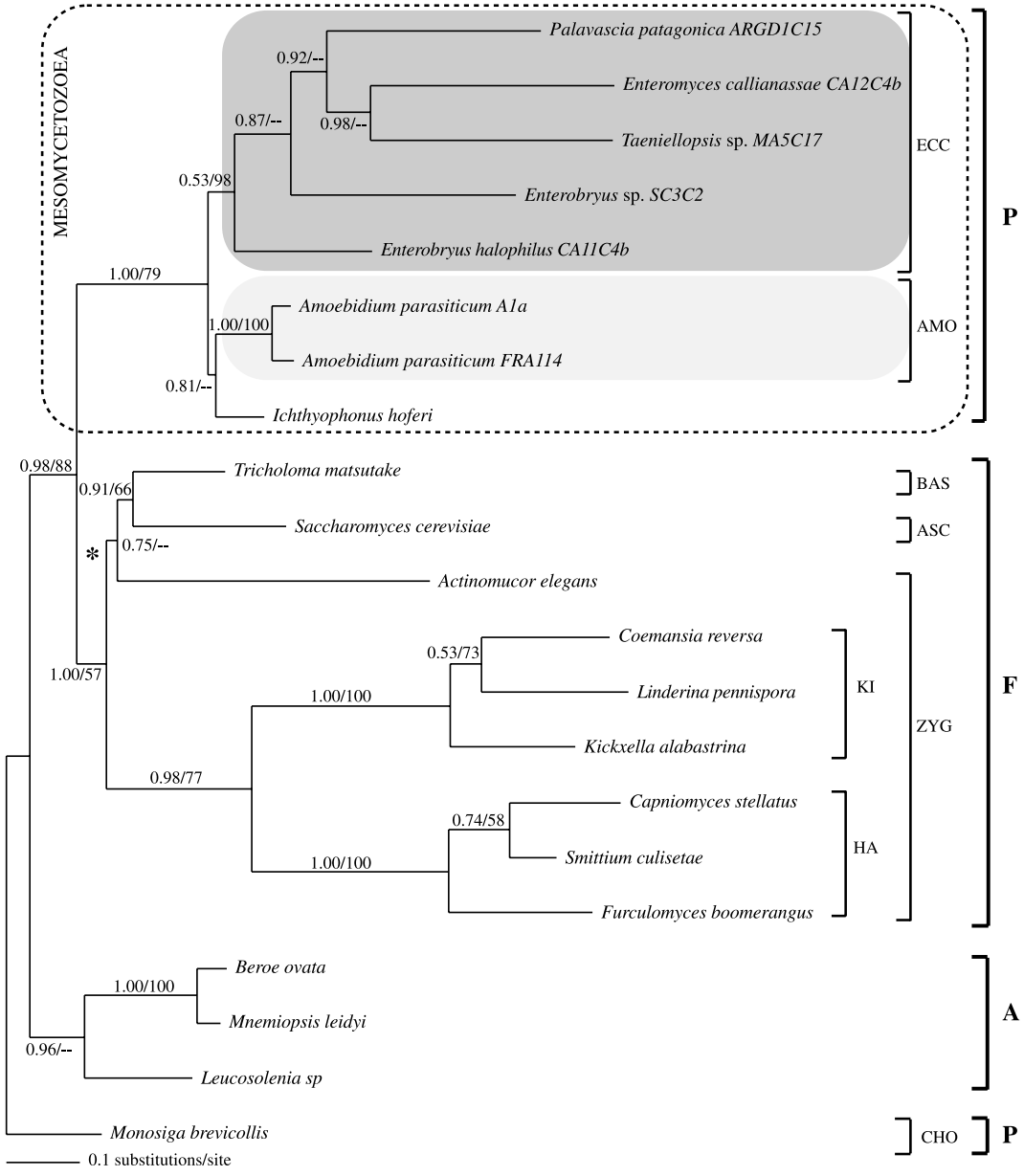

Fig. 4. Bayesian phylogenetic tree from 28S dataset after two million generations of MCMCMC chains. Values to the left and right of the slash correspond to posterior probabilities (PP) and parsimony bootstrap values (BP) derived from a separate analysis, respectively. Only maximum parsimony BP values >50% are shown; if support is lower, a dash is indicated. The asterisk indicates the only difference found in the MP tree (the relative position of *Actinomucor elegans*). ECC, Eccrinales; AMO, Amoebidiales; CHO, Choanoflagellates; ASC, Ascomycota; BAS, Basidiomycota; ZYG, Zygomycota; KI, Kickxellales; HA, Harpellales; A, Animalia; F, Fungi; and P, Protista.

there is no evidence, beyond similarity in habitat, to consider them part of this group. In the Eccrinales, the presence of dictyosomes, lack of chitin in the cell wall and the formation of septa without a plug support Moss' hypothesis. The septum that forms between sporangia is the only one present in the otherwise coenocytic thallus. The septum is initially perforated but occluded with wall material deposited at maturity, as described for *Astreptonema gammari* (Moss, 1999). The septa between sporangia do not appear to be homologous to the septa in Harpellales and Asellariales because of functional differences. These structures in the Eccrinales are form during reproduction, and their function within a coenocytic thallus, preventing loss of cytoplasmic content upon release of sporangiospores, is not equivalent to those in the other trichomycete orders. Asellariales and Harpellales have thalli that are septate throughout, with a defined septal pore structure that allows cellular continuity across the hyphae. The septum in Eccrinales is a complete barrier; no cellular continuity or migration of organelles occurs after its formation. Simple organization of the thallus, sporangiospores, and common association with arthropods are characters shared by the four traditional orders of

Trichomycetes, including Amoebidiales. The data presented here indicate that the Trichomycetes, as traditionally established, are polyphyletic.

4.2. Morphology, biology, and evolution

The Eccrinales are a morphologically diverse group, in part owing to the wide range of its hosts (Diplopoda, Crustacea, and Insecta) and the varied habitats of these hosts. The main characteristics of Eccrinales are that they are unbranched, unicellular, multinucleate thalli that produce sporangiospores by basipetal septation from the thallus apex (Figs. 1A–C and F). The Amoebidiales morphology is less diverse, but possesses the basic cylindrical shape like the Eccrinales. The two genera *Amoebidium* and *Paramoebidium* have coenocytic, multinucleate thalli that produce amoebae (Fig. 1D), which typically encyst and produce cystospores. *Paramoebidium* differs in its larger size and location in the host—it attaches to the hindgut lining—while *Amoebidium* spp. are attached to the exoskeleton. Another important intergeneric difference is that *Amoebidium* produces sporangiospores; hence the whole thallus is considered a sporangium.

Members of the Eccrinales produce two types of sporangiospores, a primary infestation type which is typically uninucleate, oval to ellipsoidal and thick-walled that can act as a resistant spore or dissemination unit, and a secondary infestation type, which is multinucleate, elongate and thin-walled. Secondary infestation spores are believed to germinate within the host gut thus serving to increase infestation levels within the same individual. There is considerable variation in shape and size of these sporangiospores, ranging from oval to ellipsoidal to allantoid. Most of the eccrinids live in hosts that are gregarious, exhibit parental care of the young, or live in lentic waters; all of these factors favor the possibility of ingestion of released spores by new hosts.

In marine and lotic freshwater environments, sporangiospores have evolved appendages that probably increase their ability to float and remain near the host population that gave origin to the spore, increasing the chances of being ingested. Four genera of Eccrinales produce appendaged spores (*Arundinula*, *Astreptonema*, *Palavascia*, and *Taeniella*). The appendages are formed within the sporangium and only become apparent following spore release. The appendages of *A. gammari* (Fig. 1F) are the only ones that have been studied in detail (Moss, 1975, 1979, 1999). They are extensions of an outer, mucilaginous, sporangiospore wall which is formed early in spore ontogeny by the extracellular deposition of material derived from dictyosome vesicles. Appendages have not been reported for any species of Amoebidiales. The presence of appendages in the Eccrinales and Harpellales (Trichomycetes) was used as a shared character to indicate common ancestry (Lichtwardt, 1986). The phylogenetic analyses do not support this relationship (Figs. 2–4).

Eccrinales present a great range of host types and habitats. Several families of Diplopoda consistently have eccrinids in their guts. Other terrestrial hosts include isopods and beetles. Crustaceans that bear Eccrinales inhabit terrestrial, freshwater, and marine environments from intertidal zones to deep oceans (Van Dover and Lichtwardt, 1986). Members of the Amoebidiales, in contrast, are limited to freshwater habitats and larval aquatic insects and small crustaceans. Results of the present study do not indicate any particular pattern associated with host type. It is possible that the Eccrinales radiated very early in their association with arthropods, thus resulting in a wide variety of hosts. Paleontological evidence supports an origin of arthropods in the Upper Proterozoic as suggested by Ediacaran rocks containing jointed-legged animals (Margulis and Schwartz, 2001). Recent fossil discoveries and molecular clock data suggest that arthropod diversification began in the Precambrian (Brusca, 2000). An "eccrinid-like" organism has been documented from silicified peat deposits collected in the Antarctica formation of Fremouw from the Middle Triassic (White and Taylor, 1989). The authors compared the organism to *Enterobryus*, pointing out the presence of holdfast, aseptate thalli, spores, and septal plugs. However, septal plugs are absent in extant eccrinids. This absence and the lack of an arthropod host associated with this fossil raise the question about the true affinity of this fossil organism. It is more probable that the common ancestor of Amoebidiales and Eccrinales was present in superficial—and probably facultative—associations with arthropods. Under this hypothesis, the Amoebidiales may be a very old group with members that attached to the exterior exoskeleton of arthropods. This interpretation would view colonization of the gut as an independent event in their evolutionary history. Lichtwardt (1986) presented a scenario in which *Amoebidium* evolved by first attaching externally and promiscuously to a wide range of different aquatic hosts because it does not appear to have special nutritional requirements. It is presumed that continual ingestion by some hosts of resistant spores from *Amoebidium* resulted in spore germination in the gut, and thus *Paramoebidium* -like organisms adapted to gut habitation. Given this scenario, the eccrinids could have been derived from this *Paramoebidium*-like ancestor before losing the sporangiospore phase or by reacquiring it. Further studies and data are needed to address these hypotheses.

4.3. Classification and position of Amoebidiales and Eccrinales within Mesomycetozoea

The class Mesomycetozoea was established by Mendoza et al. (2001) to accommodate a group of fish

and shellfish parasites, human and anuran pathogens, and *A. parasiticum*. The group was previously named DRIPs, an acronym for *Dermocystidium*, *Rhinosporidium*, *Ichthyophonus*, and *Psorospermium*, by Ragan et al. (1996), who recognized its monophyly based on 18S sequence data. The clade was positioned near the animal–fungal divergence based on molecular data (Ragan et al., 1996; Spanggaard et al., 1996). Cavalier-Smith (1998) proposed the class Ichthyosporea and divided the group into two orders, Ichthyophonida and Dermocystidia. Mendoza et al. (2001) emended the class by changing the name to Mesomycetozoea and recently, Mendoza et al. (2002) revised the current classification. The group has a long history of misidentifications and unclassified descriptions for animal parasites and saprotrophic microbes that comprise the class. The organisms included in the Mesomycetozoea have a diverse array of shared characters that suggest a common origin, such as flat mitochondrial cristae, symbiotic habit, formation of some kind of endospore, cyst or spherical resistant structure, and unicellular thalli. However, the strongest support for the Mesomycetozoea as a monophyletic group comes from molecular data (Medina et al., 2001; Ragan et al., 2003, 1996). The present study increases that support for the Mesomycetozoea by adding more members to the group and by showing the same monophyletic clade obtained with sequence data from the 28S ribosomal gene (Fig. 4). The Eccrinales are placed as sister taxon of the Amoebidiales in the order Ichthyophonida. This result will require a re-description of the class Mesomycetozoea in the future to include these symbionts of various arthropods.

The type of symbiotic relationship of the Eccrinales and Amoebidiales to their hosts has always been a subject of discussion (Lichtwardt, 1986; Moss, 1999). Traditionally, as Trichomycetes, they have been regarded as commensalistic in the absence of evidence to indicate that these organisms acted as parasites or mutualists. The lack of axenic cultures (except for the ectocommensal *A. parasiticum*) made it almost impossible to study the relationship between arthropod host and symbiont. Few studies have been attempted to answer this question in the Eccrinales (Charmantier and Manier, 1981). Recently, Kimura et al. (2002) studied the relationship between *Enteromyces callianassae* and the mud shrimp *Nihonotrypaea harmandi* in two populations one with and one without infestation. The authors concluded that a parasitic situation is unlikely but that the relationship could be either commensalistic, when nutrients are abundant, or mutualistic, when nutrient supply drops below an unknown threshold.

Ichthyophonus hoferi is the closest relatives of Amoebidiales and Eccrinales within the order Ichthyophonida (Figs. 2 and 4). Like *A. parasiticum*, *I. hoferi* has the ability to develop hyphal forms. Interestingly, *I. hoferi* produces hyphae at a low pH (Baker et al., 1999) and amoebae at higher pH (Okamoto et al., 1995). The symbiotic life style and basic morphology of *Ichthyophonus*, Amoebidiales and Eccrinales suggest that they are closely related. This hypothesis is supported by the phylogenies recovered in this study. Biochemical characters might help clarify this relationship further; for example, the reported lack of chitin in the cell wall of Amoebidiales and Eccrinales (Whisler, 1963) is apparently not the case in *Ichthyophonus* (Spanggaard et al., 1995). This important character suggests that the Mesomycetozoea may be more closely related to Fungi than to Metazoa. Recently, Ragan et al. (2003) concluded that Mesomycetozoea are members of the clade containing animals, fungi, and choanoflagellates based on elongation factor 1α sequences. Lang et al. (2002) sequenced the entire mitochondrial genomes of representatives of the choanoflagellates (*M. brevicollis*) and the Mesomycetozoea (*A. parasiticum*). The authors' results showed that the choanoflagellates are a sister taxon to the Metazoa and that the Mesomycetozoea is sister to these taxa. They placed them all together in a new group named Holozoa. The relationship of the Mesomycetozoea to animals and fungi is inconsistent in analyses incorporating one or few loci. A multiple nuclear protein-coding gene analysis may be needed to address this deep–node relationship (Baldauf et al., 2000).

Currently, the Eccrinales are divided into three families: Eccrinaceae, Palavasciaceae, and Parataeniellaceae. Target loci from members of the Parataeniellaceae failed to amplify and were not included in this study. Therefore, it is clear that a wider range of taxon sampling is both possible and needed to address the internal organization of the group. New or understudied groups of organisms, like the Eccrinales, might be found and added to the Mesomycetozoea in the future. Detailed comparisons of members of these groups are needed to define their internal relationships. In the case of the Eccrinales, it is necessary to gather sequences from more taxa and from other genes to establish if there is a pattern in their relationships regarding host, habitat, and/or taxonomic arrangements.

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