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THE CLASS MESOMYCETOZOEA: A Heterogeneous Group of Microorganisms at the Animal-Fungal Boundary*

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■ **Abstract** When the enigmatic fish pathogen, the rosette agent, was first found to be closely related to the choanoflagellates, no one anticipated finding a new group of organisms. Subsequently, a new group of microorganisms at the boundary between animals and fungi was reported. Several microbes with similar phylogenetic backgrounds were soon added to the group. Interestingly, these microbes had been considered to be fungi or protists. This novel phylogenetic group has been referred to as the DRIP clade (an acronym of the original members: *Dermocystidium*, rosette agent, *Ichthyophonus*, and *Psorospermium*), as the class Ichthyosporea, and more recently as the class Mesomycetozoea. Two orders have been described in the mesomycetozoeans: the Dermocystida and the Ichthyophonida. So far, all members in the order Dermocystida have been pathogens either of fish (*Dermocystidium* spp. and the rosette agent) or of mammals and birds (*Rhinosporidium seeberi*), and most produce uniflagellated zoospores. Fish pathogens also are found in the order Ichthyophonida, but so are saprotrophic microbes. The Ichthyophonida species do not produce flagellated cells, but many produce amoeba-like cells. This review provides descriptions of the genera that comprise the class Mesomycetozoea and highlights their morphological features, pathogenic roles, and phylogenetic relationships.

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INTRODUCTION

Led by Haeckel's proposal that the metazoans may have had an ancestor within the unicellular protists, numerous studies utilizing morphology as well as molecular and phylogenetic analyses have supported his concept (18, 48, 51, 56). Those studies were validated in 1993 when Wainright et al. (95) used phylogenetic analyses of small subunit ribosomal DNA (18S SSU rDNA) sequences to conclude that the metazoans were a monophyletic group that shared ancestry with the choanoflagellates. They also reported that animals and fungi might have had a more recent common ancestor than either group had with plants, alveolates, or stramenopiles (14, 95). First Spanggaard et al. (84) and then Ragan et al. (77) reported that a new group of parasitic and saprotrophic protists had been found near the animal and fungal divergence. Later, others verified their findings (8, 18, 19, 30, 39, 44). These investigators confirmed that previously unclassified animal parasites and saprotrophic microbes grouped together as a new protistan monophyletic clade located near the point where the animals had diverged from the fungal boundary (Figure 1). Early on Ragan et al. referred to those microorganisms as the DRIP clade (an acronym for *Dermocystidium*, rosette agent, *Ichthyophonus* and *Psorospermium*). Later Cavalier-Smith placed them in the class Ichthyosporea (18), and more recently Mendoza et al. (57) established the class Mesomycetozoea to accommodate them. The location of this group at the divergence between animals and fungi was significant because it indicated that this unique group of microorganisms arose near the time that animals had diverged from fungi (Figure 1), providing additional organisms for comparative studies that could reveal the nature of the progenitor of the animal and the fungi, two of the kingdoms of multicellular and macroscopic organisms (17, 39, 77).

Examination of Figure 1 shows that the class Mesomycetozoea is a monophyletic group composed of two strongly supported clades, the orders Ichthyophonida and Dermocystida. However, as often is the case, the relationships of the Mesomycetozoea to other broad taxa are poorly supported in molecular phylogenetic analyses. Incorporating phenotypical data, Cavalier-Smith (17, 18) held that the ancestors of the animals and fungi were not mesomycetozoeans but unicellular flagellate organisms in the choanoflagellates. He based his conclusions on the facts that the mesomycetozoeans did not possess chitin or flagellate stages, a concept that recently was proven to be incorrect.

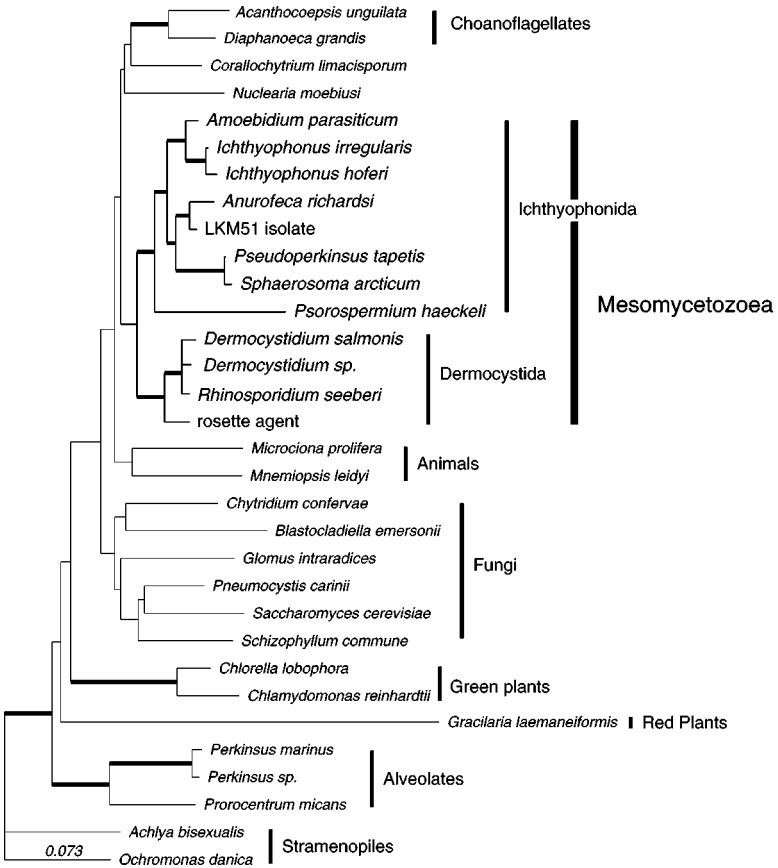

Figure 1 A phylogenetic analysis made by neighbor joining in Phylogenetic Analysis Using Parsimony (PAUP) using distances estimated by maximum likelihood (transitions/transversions estimated by ML; emperical nulceotide frequencies among site variation allowed comparable to the HKY85 model). The thickened branches are supported by 90% or greater of 1000 bootstrapped datasets. The topologies and support are similar to those found with parsimony analysis, with the exception of the placement of *Nuclearia moebiusi*, which can move as far as the branch subtending animals + choanoflagellaes + Mesomycetozoea. The scale for percent nucleotide substitution per nucleotide is given on the branch to *Ochromonas danica*.

Originally, Cavalier-Smith included the mesomycetozoeans in the class Ichthyosporea (18) (Table 1). This class was based on the fact that the original members of the DRIP clade described by Ragan et al. (77) were all fish parasites. However, new members of the group including *Amoebidium parasiticum*, *Anurofeca richardsi*, *Pseudoperkinsus tapetis*, *Rhinosporidium seeberi*, *Sphaerosoma*

TABLE 1 Current classification of the class Mesomycetozoea including new members

Kingdom Protozoa (Cavalier-Smith 1997)
Subkingdoms
1-Archezoa (*Giardia*, *Chilomastix*, *Retortamonas*)
2-Eozoa (*Trichomonas*)
3-Neozoa (four Infrakingdoms)
a) Sarcodina (*Amoeba*, *Acanthamoeba*, *Entamoeba*, *Dictyostelium*)
b) Alveolata (*Perkinsus*, *Eimeria*, *Plasmodium*, *Babesia*, *Paramecium*, *Tetrahymena*)
c) Actinopoda (*Acanthocystis*, Radiozoa)
d) Neomonada, Cavalier-Smith 1997
Phylum Neomonada
Subphyla
a) Apusozoa (*Apusomonas*)
b) Isomita (*Nephromyces*)
c) Mesomycetozoa (four Classes) stat. nov.
Class 1 Choanoflagellatea (*Sphaeroeca*)
Class 2 Corallochytrea (*Corallochytrium*)
Class 3 Mesomycetozoea (em. Mendoza et al. 2001)–(Ichthyosporea, Cavalier-Smith 1998)

Order Dermocystida	Order Ichthyophonida
a) *Dermocystidium* spp.	a) *Amoebidium parasiticum*
b) *Rhinosporidium seeberi*	b) *Anurofeca richardsi*
c) Rosette agent	c) *Ichthyophonus* spp.
	d) *Pseudoperkinsus tapetis*
	e) *Psorospermium haeckeli*
	f) *Sphaerosoma articum*
	g) LKM51

Class 4 Cristidiscoidea, Order Nucleariida (*Nuclearia*)

arcticum, and the isolate LKM51 are not fish parasites, which renders the term Icthyosporea inappropriate. In addition, the finding that *Ichthyophonus hoferi* has chitin in its cell walls (83) and that *R. seeberi* possesses at least one of the chitin synthase genes (40) suggested that this group of microbes may also have chitin in its cell walls, as do the fungi and some stramenopilans (60). This result did not come as a surprise because several investigators had previously suggested the presence of this polymer in the cell walls of some mesomycetozoeans (40, 83). The main problem has been that this polymer is not abundant in Mesomycetozoeans' cell walls. Thus it was difficult to demonstrate its presence in this group of protista (61). Moreover, chitin has always been associated with fungi but not with the protista, which in part discouraged some investigators from seeking it in organisms other than fungi (9). Mulisch (61) defied this trend by reporting that several protists possess chitin in their cell walls including the filamentous stramenopilans (60, 84). This finding strongly supported the reports of the presence of this polymer in *I. hoferi* and *R. seeberi*, and by extrapolation in all of the other mesomycetozoeans (9, 40, 83). Based on these facts, we recently emended Cavalier-Smith's original proposal and introduced the new class Mesomycetozoea (57), a name more suitable for this group of microbes (Figure 1). Accordingly, we use this term throughout our

review. The subphylum Choanozoa was also emended to subphylum Mesomycetozoa. Because the choanoflagellates were the only group between animals and fungi at that time, the epithet Chonozoa was previously introduced (17). With the inclusion of related nearby microbes, the term Mesomycetozoa (between animals and fungi), originally proposed by Herr et al. (39), was considered to be more appropriate.

Based on phylogenetic analyses of 18S small subunit rDNA genes, the class Mesomycetozoea comprises 10 different parasitic and saprophytic microbes. They are members of the genera *Amoebidium*, *Anurofeca*, *Dermocystidium*, *Ichthyophonus*, *Pseudoperkinsus*, *Psorospermium*, *Rhinosporidium*, *Sphaerosoma*, and two as yet unnamed microbes, the "rosette agent" and "clone LKM51" (Figure 1). Each of the species in these genera has several morphological characteristics in common with species in the other genera of the clade, but each has unique characteristics as well (Table 2). Although members of this group are typically thought to be aquatic pathogens of fish, there are exceptions: *R. seeberi*, the only member of the class

TABLE 2 Members of orders Dermocystida and Ichthyophonida and the only species of the class Corallochytrea (Cora) with highlights of their key attributes

Taxa	Mitocondrial cristae	Life cycle traits	Hosts
Dermocystida			
Dermocystidium spp	Flat	Cysts with endospores uniflagellate zoospores	Fish
Rosette agent	Flat?	Spherules with endospores uniflagellate zoospores	Fish
Rhinosporidium seeberi	Flat	Sporangium with endospores uniflagellate zoospores?	Mammals, Birds
Ichthyophonida			
Amoebidium parasiticum	Flat	Sporangium, sporangiospores amoebic stage	Insects Crustaceans
Ichthyophonus hoferi	Tubular	Hyphae, plasmodium, spores amoebic stage	Fish
Psorospermium haeckeli	Flat	Ovoid shell-bearing spores amoebic stage	Crayfish
Anurofeca richardsi	Flat?	Spherules with endospores	Anural larvae
Pseudoperkinsus tapetis	Unknown	Spherules with spores uniflagellate zoospores?	Clams
Sphaerosoma articum	Flat	Spherules with endospores	Saprotrophic?
Isolate LKM51	Unknown	Unknown	Saprotrophic?
Corallochytrea			
Corallochytrium limacisporum	Flat?	Spherules with spores amoebic stage	Saprotrophic

? = not clear.

known to cause infections in mammals and birds; *Amoebidium* species, a genus of saprotrophic organisms; *A. richardsi*, whose role in diseases of anuran larvae is not clear; *Ps. tapetis*, a possibly nonpathogenic species associated with clams; *S. arcticum*, usually a saprotrophic organism; and the isolate termed LKM51, a putatively saprotrophic eukaryote found in phytoplankton.

Nothing is known regarding Mesomycetozoea's geographical distribution or their relationships with their natural environments. Thus, their epidemiological features and their interactions with other microbes in their ecological niches are also largely unknown. Because most mesomycetozoeans are animal parasites, what we do know about their cell cycles was learned from studies conducted on their parasitic stages. These studies have been of pivotal importance for the partial construction of their life cycles (5, 64, 83, 92, 97) (Figure 2). For instance, it was demonstrated that the species of the genera *Amoebidium*, *Ichthyophonus*, and *Psorospermium* developed amoeba-like cells in vitro. Based on these studies it was speculated that the amoeba-like cells could be the infecting propagules in nature (83, 92, 97) (Figure 2). Likewise, in vitro the *Dermocystidium* spp. and the rosette agent developed uniflagellated zoospores, indicating that they could serve as the infectious propagules [(37, 65) K.D. Arkush, personal communication] (Figure 2). The major contribution of these studies is the finding that during their parasitic stages, at least one phenotypic form of the mesomycetozoean species could initiate a new cycle outside their hosts.

In spite of these findings, their true life cycles in nature remain a mystery. Sexual development in the Mesomycetozoea has yet to be reported. This is due in part to the fact that most mesomycetozoeans have only been studied in their parasitic stages rather than in culture. Thus, sexual fusion, gamete formation, meiosis, and other major important genetic traits have yet to be found or induced. It is important to note that some investigators reported the presence of multiple nuclei during the parasitic stages of some mesomycetozoeans during the formation of new spores. In addition, little is known about their feeding habits. It has been found, however, that during their parasitic stages *D. salmonis* (65), *I. hoeferi* (45), *P. haeckeli* (89, 91), and *R. seeberi* (59) absorb nutrients from the hosts through the mesomycetozoean's cell walls, a finding that might suggest a similar behavior during their environmental stages. However, more studies are necessary to validate this assumption.

The epithets used to identify the phenotypic stages of the members of the class Mesomycetozoea varied according to the type of microorganism the investigators thought them to be. For instance, mycological terminology was used to identify the structures of *A. parasiticum*, *A. richardsi*, *I. hoferi*, and *R. seeberi*, all of them studied by mycologists (5, 21, 38, 62, 98, 99). The terms they used included endospores, hyphae, sporangia, spores, sporangiospores, thalli, and others. In contrast, protozoological names such as amoeba, cyst, plasmodium, sporocyst, and zoospore were used by protistologists to identify the structures formed by *A. parasiticum*, *D. salmonis*, *I. hoferi*, *P. haeckeli*, and the rosette agent (14, 24, 42, 62, 64, 75). With their inclusion in the class Mesomycetozoea, standardization of the names

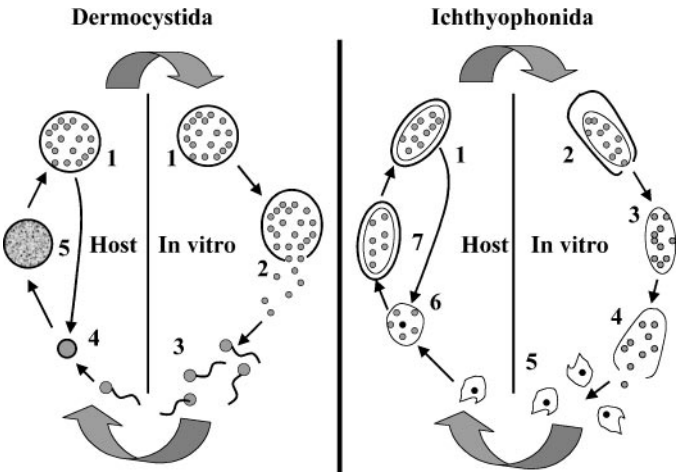

Figure 2 Depiction of the putative life cycle of members of the orders Dermocystida (*left*) and Ichthyophonida (*right*). Members of the order Dermocystida develop spherical cells with endospores (stage 1). In vitro the released endospores (stage 2) give rise to uniflagellated zoospores (stage 3). When the zoopores (infecting units) infect the host, they encyst (stage 4) and increase in size (stages 4, 5) and undergo cleavage into endospores (stage 1). The endospores can also be directly released within the host's infected tissues, and the cycle is repeated inside the hosts (stages 1, 4, 5, 1). Members of the order Ichthyophonida develop spherical (*Ichthyophonus* spp.) or ovoid cells in infected tissues (*Psorospermium haekeli*) or on its hosts (*Amoebidium parasiticum*) (stage 1). In vitro (stage 1) the hatching of spore receptacles occurs from the ovoid cells (stage 2); the receptacles containing spores (stage 3) then rupture and release their endospores (stage 4), which develop into motile amoeboid cells (infecting units) (stage 5). The amoeboid cell reach the hosts and develops into a small receptacle (stage 6) that later generates a hard cell wall with endospores (stages 7, 1). The endospores can also be released within their hosts, repeating the cycle (stages 1, 6, 7, 1). Note that in the genus *Ichthyophonus*, the development of hyphae that produce spherical cells with endopores (in vitro) is a feature, so far, not encountered in the other members of the order (62, 64, 83). For the order Ichthyophonida the cell cycle was adapted from Vogt & Rugg (92).

used to describe their phenotypic stages would be of importance in studying the members of this class. In this review, however, we continue to use the traditional nomenclature until a consensus is reached regarding their terminology.

We provide a brief description of the genera that are currently in the class Mesomycetozoea, highlighting their similarities and differences, with the intention of covering the latest known developments of the mesomycetozoeans and introducing the microbiological community to this novel class of microorganisms and their morphological, pathogenic, and phylogenetic relationships.

MEMBERS OF THE CLASS MESOMYCETOZOEA

Order Ichthyophonida

AMOEBIDIUM PARASITICUM The genus *Amoebidium* (21) is composed of four species: *A. australiense* (52), *A. colluviei* (53), *A. parasiticum* (98), and *A. recticola* (54). One of the well-studied species of this genus is *A. parasiticum*. This arthropodophilous symbiont is frequently found on crustaceans and insects that inhabit fresh water ponds (21, 52, 98). *A. parasiticum* shares with the other members of the class Mesomycetozoea its historical inclusion within the kingdom Fungi. The inclusion of this apparently nonpathogenic microorganism within the fungi was based on its production of a unicellular fungal-like thallus (sporangium), which is externally attached to the host. Like some mesomycetozoeans, *A. parasiticum* has been difficult to isolate in culture. It also possesses morphological characteristics different from most members of the group (Figure 3) (96–98). For instance, most mesomycetozoeans have spherical spores, but *A. parasiticum* has cigar-shaped spores (sporangiospores), from which amoeba arise, and also elongated thalli (sporangium) when attached to crustacea and insects (Figure 3).

Similarly, early morphological studies of *A. parasiticum* isolates showed that this microbe possesses unique features not found within the fungal Trichomycetes (Zygomycota), where it had long been classified (86, 97) (Figure 2). These facts

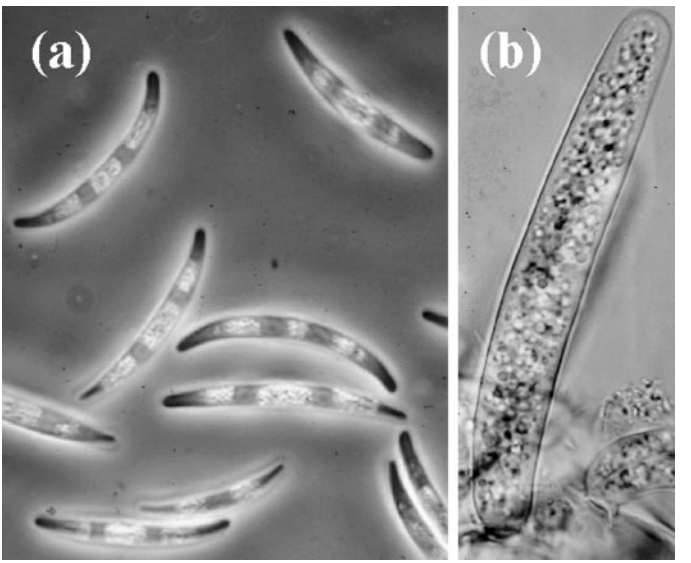

Figure 3 (*a*) Colorless cigar-shaped sporangiospores of *Amoebidium parasiticum* from which the amoeba phase developed (X 1000). (*b*) Sporangium-like thallus detached from its host's body (X 1250) (courtesy of R.W. Lichtwardt).

prompted some investigators to hypothesize that *A. parasiticum* represents a nonfungal evolutionary lineage possibly derived from a protozoan ancestor (54, 96). The finding of Whisler (97) that *A. parasiticum* forms amoebal cysts that give rise to several motile amoebas, which later encyst to form sporangium-like structures, provided the strongest evidence for this hypothesis. Although other investigators supported this new position (74, 85), those studies did not resolve the phylogenetic connection of this genus with other living organisms.

The phylogenetic affinities of *A. parasiticum* were almost simultaneously resolved when first Ustinova et al. (86) and later Benny & O'Donnell (12) sequenced the 18S SSU rDNA of *A. parasiticum*. Using phylogenetic analyses, these two independent groups of researchers found that *A. parasiticum* is a member of the class Mesomycetozoea and far from the fungal class Trichomycetes of the kingdom Fungi. In their phylogenetic trees, *A. parasiticum* was always closely related to *I. hoferi*. Interestingly, ultrastructural studies have indicated that *A. parasiticum* possesses mitochondria with flat cristae (9, 98), a finding that contrasts with the tubular cristae found on *I. hoferi*'s mitochondria (77). Our phylogenetic analyses confirm that *A. parasiticum* is the sister clade to *I. hoferi* and *I. irregularis* (Figure 1). These independent analyses showed that *A. parasiticum* belongs to the order Ichthyophonida, family Ichthyophonae in the class Mesomycetozoea (Table 1). Unfortunately, the other three species of the genus *Amoebidium* have not been phylogenetically investigated and are yet to be classified.

ANUROFECA RICHARDSI *Anurofeca richardsi* was originally held to be an alga and described under the name *Prototheca richardsi* (11, 99). It was first reported by Richards in 1958 (79) in feces of the larval state of *Rana pipiens* and implicated as a gut parasite of anural larvae (10, 79). Indeed, *A. richardsi* is isolated only from the guts of anuran larvae (99). Although the occurrence of *A. richardsi* is correlated with the presence of amphibian larvae in ponds, its role as a pathogen of anuran larvae is still unclear (8).

The genus *Anurofeca* was proposed by Baker et al. in 1999 (8). *Anurofeca richardsi* produces nonpigmented spherical cells 2–20 μm in diameter. Several small, round endospore-like cells develop within the spherical cells and are released after the cell wall breaks open. The inclusion of *A. richardsi* within the algal genus *Prototheca* was originally motivated by its morphological resemblance to members of that genus (Figure 4). Some investigators, however, noticed several inconsistencies. For instance, *A. richardsi* grows weakly on *Prototheca* isolation media, whereas the *Protheca* species can be easily cultured on that medium (73). It was also noticed that while other *Prototheca* species antigenically cross-reacted with each other, *A. richardsi* showed weak or no cross-reactions (99). Nonetheless, the final position of this algal-like organism was resolved by neither its morphological nor by its antigenic features.

A. richardsi's phylogenetic connection with the class Mesomycetozoea recently was revealed by Baker et al. (8). Using molecular approaches, they found that *A. richardsi* formed the sister clade to *I. hoferi* and was closely related to *P. haeckeli*,

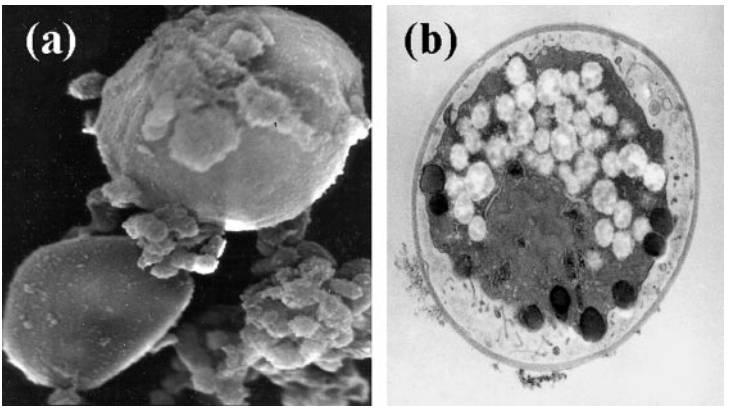

Figure 4 (*a*) Scanning electron microscopy of *Anurofeca richardsi* showing its spherical phenotype. Bacteria and debris are observed around the cells (X 5000). (*b*) Transmission electron microscopy of *A. richardsi* showing numerous vesicles within its cytoplasm (X 8720) (courtesy of T.J.C. Beebee).

far away from the algae. In our phylogenetic tree *A. richardsi* is closely related to the clone LKM51 isolate, and together they are the sister clade of *Pseudoperkinsus tapetis* plus *Sphaerosoma arcticum*. Mendoza et al. (57) placed *A. richardsi* within the phylum Neomonada, subphylum Mesomycetozoa (previously known as subphylum Choanozoa), class Mesomycetozoea, order Ichthyophonida, family Ichthyophonae (Table 1) (Figure 1).

ICHTHYOPHONUS HOFERI AND *I. IRREGULARIS* *Ichthyophonus hoferi* (72) was until recently considered to be a species of unknown taxonomic placement. Owing to its spherical in vivo morphologic features, *I. hoferi* was considered to be either a fungus or a protozoan, a taxonomic history that it shares with the other members of the class Mesomycetozoea. At least three other species, *I. gastrophilum* (20), *I. intestinalis* (50), and *I. lotae* (49), have also been described. More recently, however, *I. gastrophilum* was found to be synonymous with *I. hoferi*, whereas *I. intestinalis* and *I. lotae* were found to be related to the order Enthomophthorales in the kingdom Fungi (49, 62). This placement partly explains why early investigators considered *I. hoferi* to be a zygomycetous fungus.

Since the early twentieth century many reports of infections caused by *I. hoferi* in freshwater and marine fishes have been published. At least 14 marine and 6 freshwater fishes were found infected with *I. hoferi* worldwide. This microorganism frequently affects the internal organs of the infected animals such as the heart, liver, muscles, and spleen. *I. hoferi* occurs in infected tissues as spherical, thick-walled, multinucleated cells referred to as cysts, spores, and/or resting spores (45) (Figure 5). This organism can easily be recovered in pure culture from tissue samples of infected hosts (Figure 5).

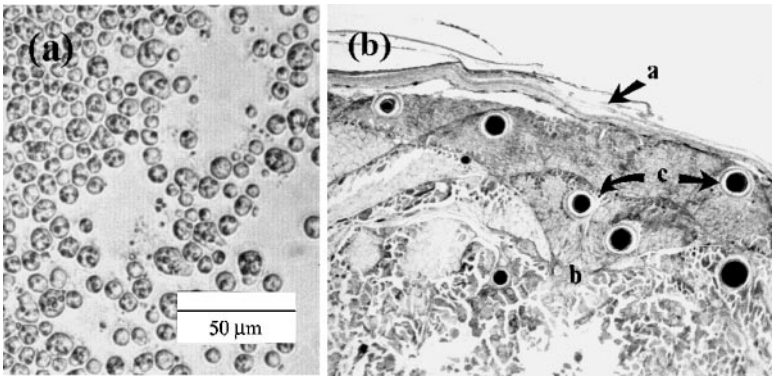

Figure 5 (A) *Ichthyophonus hoferi*'s "microspores" released from the tips of its hyphae. (*B*) Histological section of herring infected with *I. hoferi* depicting (*a*) epidermis, (*b*) body musculature, and (*c*) *I. hoferi* spores (X 100) (courtesy of R.M. Kocan).

Ichthyophonus hoferi has been the only species of the genus *Ichthyophonus* recognized to cause disease in fish. However, it had been reported for some time that a variety of fishes were infected with an *Ichthyophonus*-like organisms (38, 75). Based on morphological criteria and phylogenetic DNA sequences, Rand et al. (76) found that an unusual species of *Ichthyophonus*, morphologically different from *I. hoferi*, had been recovered from yellowtail flounders (*Limanda feruginea*) in Nova Scotia. When the 18S SSU rDNA from this particular isolate was sequenced, it was found to be distinct from two previously sequenced and geographically distant isolates of *I. hoferi* recovered from infected fish. These investigators proposed that their isolates were a new species, which they named *Ichthyophonus irregularis*. This revealed that the genus *Ichthyophonus* comprises more than one species and that all may be fish pathogens.

The controversy over the phylogenetic placement of *I. hoferi* came to an end when Spanggaard et al. (84) isolated the 18S SSU rDNA from this pathogen and found that it was related to the choanoflagellates. Later, when Ragan et al. (77) included more taxa in their analysis, it was found that *I. hoferi* was not a choanoflagellate but a member of the class Mesomycetozoea. In contrast to some members of this class, *I. hoferi* can be easily cultured (64, 75, 76, 83), and it possesses mitochondria with tubular cristae (9, 77). Cultivation studies have shown that at a low pH the organism developed hyphal forms, but when shifted to higher pHs (7–9) it developed motile amoeba-like forms (64). The studies were later confirmed by others (83). This finding indicated that the infecting units of this pathogen could be its amoeba-like form. The ability to develop motile amoeba-like forms was also found in some other members of the order Ichthyophonida (89, 91, 92, 97), but so far not in members of the order Dermocystida.

PSEUDOPERKINSUS TAPETIS *Pseudoperkinsus tapetis* was originally described as *Perkinsus atlanticus* in samples isolated from clams infected with a *Perkinsus*-like microorganism [(29) B. Novoa & A. Figueras, personal communication]. Although the morphological features that separate *Ps. tapetis* from *Perkinsus* spp. were relatively minor, with the use of molecular techniques it was evident that *Ps. tapetis* belongs to the new class Mesomycetozoea, phylogenetically far from the alveolate genus *Perkinsus* (9, 17, 18, 71) (Table 1). With this information, Figueras et al. (29) recommended a new genus to accommodate the organism originally named *P. atlanticus* by his group. He proposed the genus and the species *Pseudoperkinsus tapetis* (29). Confirming evidence that *Ps. tapetis* is distinct from the members of the genus *Perkinsus* came also from molecular studies using 5.8S ribosomal RNA (7, 31, 41, 46) on *P. marinus*, *P. atlanticus*, and other *Perkinsus* species. Those studies revealed that the *Perkinsus* spp. clustered together with the alveolates and that they were all different from the DNA sequences of *Ps. tapetis* deposited in the GenBank by Figueras et al. (29).

Although morphologically the *Ps. tapetis* and *Perkinsus* spp. are indistinguishable, the fact that *Ps. tapetis* did not cause high mortality in species of clams susceptible to *Perkinsus* spp. provided a phenotypic difference that correlated with the molecular divergence (B. Novoa & A. Figueras, personal communication). However, the virulence of *Ps. tapetis* to several other species of clams is still under investigation (68, 69). *P. tapetis* is spherical with several round vesicles in its cytoplasm (Figure 6). It can be recovered easily from clams and cultivated in synthetic media. It is interesting to note that in thioglycolate, this organism increases in size and produces spherical inclusions within its cytoplasm, similar to those observed in the hypnospores of the species classified in the genus *Perkinsus*. The production

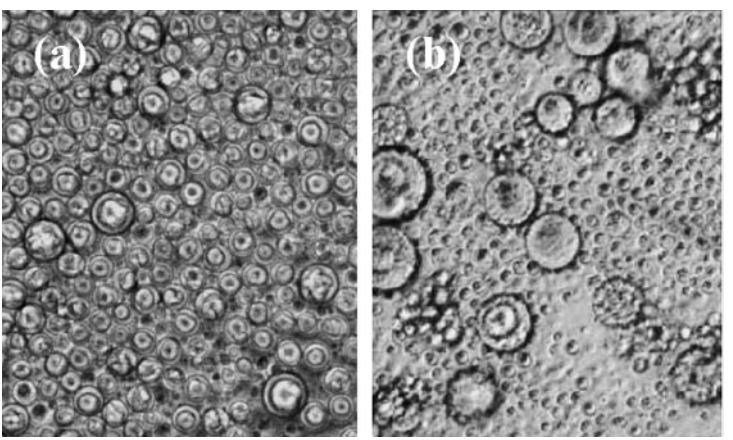

Figure 6 (*a*) Fresh mount preparation of *Pseudoperkinsus tapetis* from Spanish clam cultures (X 250). (*b*) Detail of the spherules developed by *Ps. tapetis* from culture samples (X 400) (courtesy of A. Figueras & B. Novoa).

of amoeba-like cells, typical of the order Ichthyophonida, has not yet been found in *Ps. tapetis* (B. Novoa & A. Figueras, personal communication). However, Ordás & Figueras (67) reported that one of their isolates, identified initially as *P. atlanticus* and later considered to be *Ps. tapetis*, produced uniflagellated zoospores. If confirmed, this report would be the first evidence for flagellated cells in the order Icthyophonida. The observation of flagella in *Ps. tapetis*, however, is controversial because *Perkinsus* spp. are well known to develop uniflagellated zoospores, raising the possibility that the *Ps. tapetis* cultures had been contaminated with a *Perkinsus* sp. Contamination is plausible because, as mentioned above, both microbes are morphologically indistinguishable, and they often have been recovered simultaneously in culture (B. Novoa, personal communication). Nonetheless, the finding of uniflagellated cells in this microbe (67) warrants further study.

Pseudoperkinsus tapetis was found to belong in the class Mesomycetozoea when its 18S SSU rDNA sequence was used for phylogenetic analysis (29). Figueras and colleagues found that *Ps. tapetis* is part of the class Ichthyophonida in the phylum Neomonada and the sister taxon of *S. arcticum*, as is also shown in our analysis (Table 1) (Figure 1). More importantly, its DNA sequences were found to possess a 99.5% identity with *S. arcticum*, a finding that may indicate that *Ps. tapetis* is a member of the genus *Sphaerosoma* and not in a separate genus. Additional data, however, from other molecules are needed to validate this claim.

PSOROSPERMIUM HAECKELI Haeckel (33) first reported crayfish infections caused by *P. haeckeli* in 1857 (33), but he did not propose a name for the etiologic agent. In 1883 Hilgendorf (42) named the organism *P. haeckeli* as a tribute to Haeckel. This organism mainly infects crayfish and has been reported in the Americas, the majority of European countries, as well as in Siberia (23, 47, 62, 90, 93). Originally *P. haeckeli* was considered to be either a fungus, a protist, or an alga based on its morphological similarities with fungi and algae (63). Owing to the taxonomic uncertainties of *P. haeckeli* it has always been referred to as "the enigmatic parasite of freshwater crayfish."

Psorospermium haeckeli may not be the only member of the genus. In addition, *Psorospermium orconectis* (24) and a *Psorospermium* sp. from Siberia (93) have been described, although the morphological features of *P. orconectis* were similar to those of *P. haeckeli* and the two may be conspecific. Based on size, morphology, and histology, Vogt (89) recently stated that the genus *Psorospermium* is probably composed of at least six morphotypes, each one perhaps representing different pathogenic species.

Psorospermium haeckeli affects the connective tissues around the gut of infected crayfish. It also has been reported to affect muscles and other tissues. In the infected areas *P. haeckeli* appears as a 45×90 μm–diameter ovoid and elongated spore with a refractive cell wall enclosing refringent globules of different sizes in their cytoplasm (Figure 7). Its oval morphology contrasts with the other members of the class, which have spherical phenotypes. The development of a binucleate amoeba-like stage has been used in the past to classify this parasite within the Protozoa

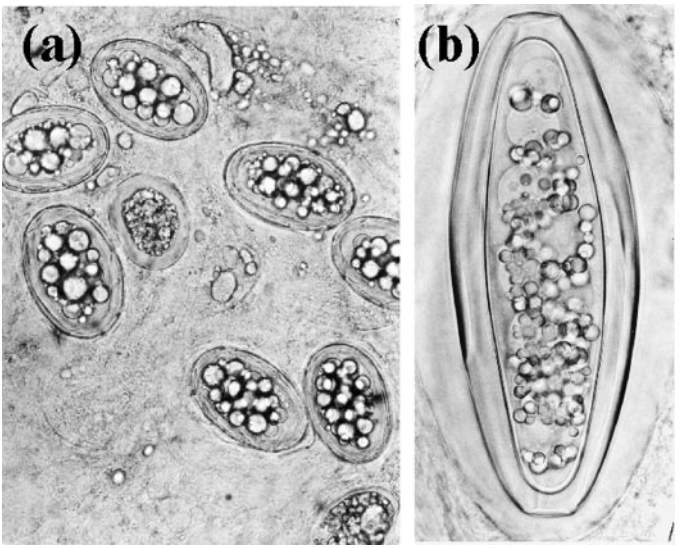

Figure 7 (*a*) Fresh mount preparation of connective tissue from crayfish infected with the ovoid cells of *Psorospermiun haekeli* (X 400). Note several globular vesicles within the cell's cytoplasm. (*b*) Elongate spore of *P. haekeli* from the connective tissue of a crayfish (X 1000) (courtesy of G. Vogt).

(81, 32). This feature was corroborated by Vogt (89), who also induced the hatching of slowly moving amoeboid cells from oval spores obtained from crayfish tissue infected with *P. haeckeli*. *P. haeckeli* in all constructed phylogenetic trees has been found to be closely related to *I. hoferi*. The two species have amoeba-like bodies and pathogenicity for marine animals. They differ, however, in that *P. haeckeli* possesses flat mitochondrial cristae rather than the tubular form found in *I. hoferi* (92). Mitochondrial ultrastructure was considered to be a key characteristic prior to the development of molecular phylogenetics, but it seems that the morphological character is not as stable as was thought, judging from the apparent shift from flat to tubular cristae in the case of *I. hoferi* (87, 101).

The mystery of its taxonomic affinities was resolved when Ragan et al. (77) amplified the 18S SSU rDNA molecule from this pathogen. They found that *P. haeckeli* was not a fungus but that it was part of the DRIP clade. In that study *P. haeckeli* clustered close to *I. hoferi*, a finding confirmed later by Cavalier-Smith (18), Baker et al. (8), Herr et al. (39), Fredericks et al. (30), and others. In our phylogenetic tree *P. haeckeli* lies at the base of the order Ichthyophonida, with the whole order resting on a well-supported branch (Figure 1).

SPHAEROSOMA ARCTICUM *Sphaerosoma arcticum* is a new member of the Mesomycetozoea. The story of how this organism was first found is an example

of the rapid expansion of our understanding of the Mesomycetozoea. During an expedition to the high Arctic Dr. Bjarne Landfald and collaborators, while investigating unusual bacteria in cold marine habitats, isolated a microorganism from the amphipod *Gammarus setosus*. They recovered in culture a spherical eukaryotic microorganism (B. Landfald, personal communication). Because the host from which the organism had been isolated did not show pathological changes, it was not clear if *S. arcticum* was a parasite, a harmless protist, or that perhaps *G. setosus* had only an accidental association with this spherical organism, e.g., a food organism.

In culture *S. arcticum* develops spherical structures containing several individual cells, which at maturity release ~100 smaller cells (B. Landfald, personal communication). Electron microscopic studies of *S. arcticum* showed that the spherical bodies, with well-defined internal spherical cells, were similar to the morphological features of the algal species of *Chlorella* and *Prototheca* (Figure 8). The internal cells contained mitochondria with flat cristae and a well-defined nucleus. Attempts to infect related amphipod species with *S. arcticum* were unsuccessful (B. Landfald, personal communication). However, the fact that *S. arcticum*'s 18S SSU rDNA sequence is 99.5% similar to the *Ps. tapetis* sequence suggests

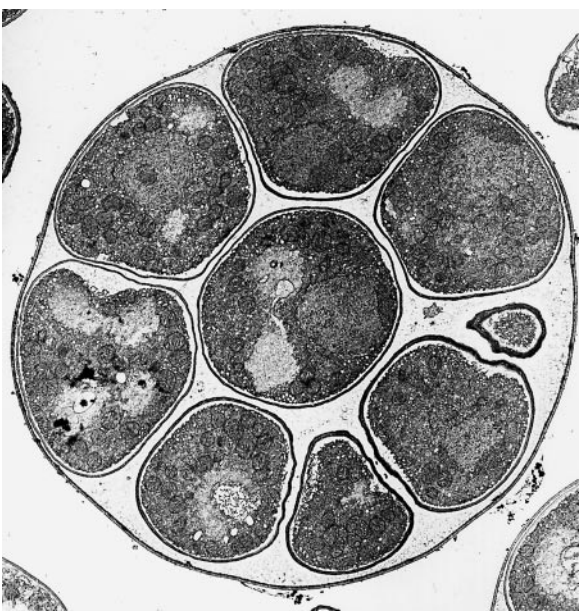

Figure 8 Transmission electron microscopy section of *Sphaerosoma arcticum* from cultured samples. The presence of numerous spores within the cytoplasm is its main phenotypic characteristic. The morphological features that *S. arcticum* shares with the species of the algal genera *Prototheca* and *Chlorella* are strikingly similar (X 26,400) (courtesy of B. Landfald).

not only that *Ps. tapetis* may be perhaps a species of the genus *Spaerosoma* but also that *S. arcticum* may be a microbe associated with clams. Therefore, one can speculate that *S. arcticum* may be a nonpathogenic species associated with clams, as in the case of *Ps. tapetis*. This might explain in part why Landfald could not infect amphipods with *S. arcticum*. Phylogenetic analysis of the *S. arcticum*'s 18S SSU rDNA showed that this organism is a member of the class Mesomycetozoea (B. Landfald, personal communication). In our analysis *S. arcticum* and *Ps. tapetis* form the sister clade to *A. richardsi* plus the LKM51 isolate, and both groups are well supported within the order Ichthyophonida (Figure 1).

ISOLATE LKM51 Isolate LKM51 is known only from an 18S SSU rDNA sequence obtained from DNA isolated from phytoplankton (86a). Thus, mesomycetozoeans have joined the many archaea, eubacteria, and uncultivated eukaryotic microbes whose presence is known only from environmental DNA, but which could well comprise 70% of the earth's microbiota (3, 43, 80, 94). Recently van Hannen et al. (86a), while investigating the correlation between the biomass of bacteria and the biomass of protozoans in phytoplankton samples, found that the affiliation of ~20% of the 18S SSU rDNA sequences investigated during this study could not be quickly determined. One of these sequences, clone LKM51, was later grouped with the mesomycetozoeans. Although the morphological features and other characteristics of the organism from which the DNA was isolated were not investigated, this was the first evidence that some mesomycetozoeans could be planktonic. Whether isolate LKM51 represents a free-living planktonic organism or simply part of its life cycle is not known, but were it shown to be free living, it would be the only mesomycetozoean that is not an obligate parasite. Phylogenetic analysis of the LKM51 clone showed that it is the sister clade of *A. richardsi* and well supported within the order Ichthyophonida (86a). Our analysis gave the same results (Figure 1).

Order Dermocystida

DERMOCYSTIDIUM SPP. The genus *Dermocystidium* is the sister taxon of *Rhinosporidium*. The latter genus comprises approximately 12 species, all of which cause deadly infections in fish and other marine animals (22, 27, 37, 66). The *Dermocystidium* spp. are characterized by their spherical sporangia (cysts) and the production of endospore-like structures (Figure 9). Such features are also encountered in the other members of the order Dermocystida (Table 1). These similarities could well explain why early investigators called attention to the morphological relationship between the *Dermocystidium* spp. and their sister taxon *R. seeberi* (39). Owing to their morphological features, the *Dermocystidium* spp. were previously considered to be either members of the haplosporeans, the fungi, or the apicomplexa groups (9, 26, 62, 70, 77, 88, 100).

Because of their intractability to cultivation, the ecological distribution of *Dermocystidium* spp. is poorly known. It is believed that fish are infected through

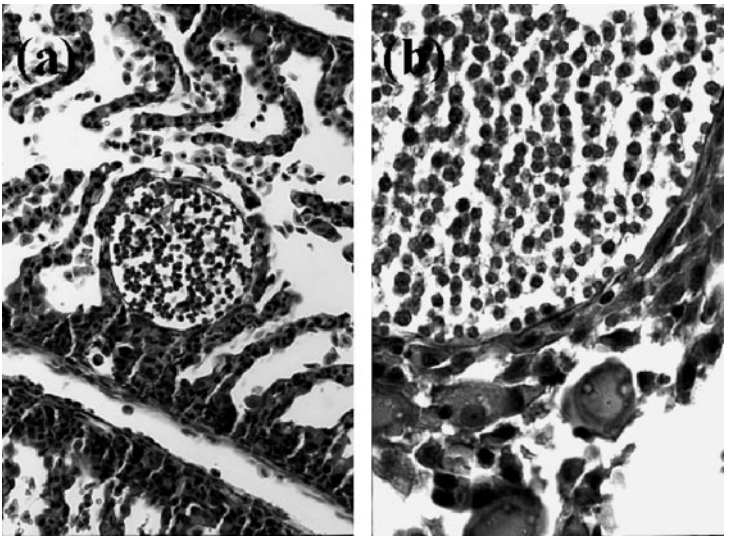

Figure 9 (*a*) Histological section of a fish's gills infected with a sporangium of *Dermocystidium salmonis* containing numerous endospores (X 400). (*b*) Histological section of an enlargement of *D. salmonis*'s sporangium. Note the presence of well-developed endospores, a feature also observed in *Rhinosporidium seeberi* (X 800) (courtesy of K.D. Arkush).

contact with the endospores released from infected fish. The spherical sporangia of *Dermocystidium* measure 200–400 μm in diameter and contain hundreds of endospores. They are readily observed in infected gills (Figure 9). These spherical sporangia are produced in great numbers to the point of making it impossible for the infected fish to take up oxygen and they finally succumb to their infections. However, the presence of uniflagellate zoospores in *D. salmonis* (65) suggests that motile zoospores could be the infective propagules of the *Dermocystidium* species. Recently it has also been found that the rosette agent develops uniflagellate zoospores (K.D. Arkush, personal communication). The rosette agent is also a member of the order Dermocystida and also causes a disease that primarily affects fish gills.

Early reports of *Dermocystidium* spp. suggested that the species of that genus could cause infections not only in fish but also in amphibians (14, 15, 25). Based on its spherical parasitic stage and the ecological distribution of its hosts, however, other investigators speculated that the true etiology of the spherical structures in amphibians were not the *Dermocystidium* species but members of a new genus (14). Accordingly, the genus *Dermosporidium* was created to accommodate the *Dermocystidium* spp. in infected amphibians (14). However, based on morphological characteristics, Herr et al. (39) speculated that *Dermosporidium granulosum* (14) and *Dermosporidium ranarum* (15) should be identified as *R. seeberi* in

amphibians rather than as species of the newly proposed genus *Dermosporidium* or species of the genus *Dermocystidium*. Nevertheless, the phylogenetic connection of the amphibian parasite with *R. seeberi* has yet to be established by comparison of DNA sequences. It is important to note that the species of *Dermocystidium* are also phenotypically similar to those of the genus *Perkinsus*. Thus, the *Perkinsus* species have been studied in the past as members of the genus *Dermocystidium*. A good example of misidentification is *P. marinus*, which was once erroneously included in the genus *Dermocystidium* as *D. marinus* (71). We now know, however, that members of both genera are phylogenetically distant (71).

The taxonomic affinities of the *Dermocystidium* spp. were resolved when Ragan et al. (77) sequenced their 18S SSU rDNAs. Those studies revealed that these fish pathogens possess mitochondria with flat cristae and were related to other fish parasites. Later, Herr et al. (39) found that the genus *Dermocystidium* was the sister taxon of *R. seeberi* and was closely related to the rosette agent. That study, in part, explained why the phenotypic features of the *Dermocystidium* spp. had been previously confused with *R. seeberi* in the tissues of their infected hosts (14, 15, 25). Cavalier-Smith (18) placed the genus *Dermocystidium* in the order Dermocystida along with the rosette agent; *R. seeberi* was added later (57) (Figure 1) (Table 1).

RHINOSPORIDIUM SEEBERI *Rhinosporidium seeberi* Seeber (82) is the etiologic agent of rhinosporidiosis, a cutaneous and subcutaneous disease of humans, other mammals, and birds, which is characterized by the formation of polypoidal masses in mucous membranes. *R. seeberi* appears in infected tissue as spherical structures referred to as sporangia. As is the case in the species of the genus *Dermocystidium*, these sporangia can grow up to 450 μm in diameter and can hold as many as several thousand endospores (Figure 10). Like some mesomycetozoeans, *R. seeberi* cannot be isolated in culture, and until recently it was classified both as a fungus and as

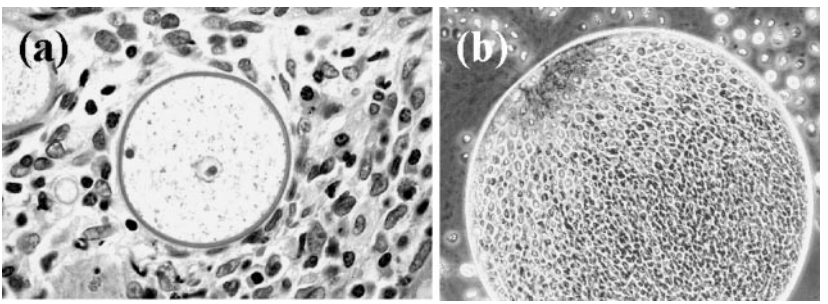

Figure 10 (*a*) Histological section of polypoidal tissue from a Sri Lankan man infected with *Rhinosporidium seeberi*, depicting an immature sporangium and inflammatory cells (X 400). (*b*) In vitro release of endospores from a mature sporangium. Note the large number of endospores within the sporangium (X 800).

a protozoan (5, 6, 23). The possible development of uniflagellated zoospores by *R. seeberi*, similar to those of *Dermocystidium* spp. and the rosette agent, is currently under investigation (D. McMeekin & L. Mendoza, unpublished data).

Light and electron microscopic analyses in the past 100 years have indicated that *R. seeberi* has a complex in vivo life cycle (5, 22) that is initiated with the release of endospores into its host's tissues from spherical bodies (100–450 μm) referred to as sporangia (Figure 10). Once implanted, the endospores increase in size and progressively develop into juvenile, intermediate, and finally mature sporangia with endospores (59). The endospores are then released and the in vivo cycle is reinitiated. These analyses, however, did not provide clues regarding *R. seeberi*'s taxonomic affinities. In 1999 a team of investigators from Michigan State University, Emory University, University of California, Berkeley, and the University of Paradeniya, Sri Lanka (39), using phylogenetic analysis, found that the 18S SSU rDNA of *R. seeberi* from humans with rhinosporidiosis clustered with the DRIPs, then a recently discovered group of fish parasites. Their data showed that *R. seeberi* was the sister taxon of two *Dermocystidium* spp. and that this trio was close to the rosette agent. This study was later corroborated by Fredericks et al. (30), whose 18S SSU rDNA sequence from a dog with rhinosporidiosis proved to be identical to that of the human isolate sequenced by Herr et al. (39). That finding strongly supported the view that *R. seeberi* may be a monotypic genus. Although Frederick et al. (30) reported that *R. seeberi* possessed mitochondria with tubular cristae, Herr et al. and Mendoza and colleagues (39, 58) demonstrated that *R. seeberi* did indeed have mitochondria with flat cristae.

The phylogenetic home of *R. seeberi* has been controversial for over a century. When Seeber described the first known infection caused by *R. seeberi* in 1900 (82), he believed that this spherical microorganism was a coccidium. Later, other investigators, using morphological and staining procedures, suggested that the pathogen was more closely related to members of the kingdom Fungi than to members of the kingdom Protoctista. Investigations mainly focused on the in vivo histopathological features of *R. seeberi* because it could not be cultivated. None of those investigations, however, provided clues as to the true nature of this pathogen. This frustration led other investigators to propose extreme views such as that *R. seeberi* was a carbohydrate waste product resulting from the ingestion of tapioca (1) or a cyanobacterium in the genus *Microcystis* (2). The recent finding that *R. seeberi*'s 18S SSU rDNA clustered with a novel clade of fish parasites in the divergence between animals and fungi ended 100 years of taxonomic uncertainties. *R. seeberi* differs from the other mesomycetozoeans in that it is the only member pathogenic to mammals and birds. Correspondingly, *R. seeberi* has several features in common with the other mesomycetozoeans: (*a*) It was previously classified as a fungus or a different type of protozoan; (*b*) it was associated with aquatic environments; (*c*) it produced spherical structures containing several daughter cells (endospores); and (*d*) *R. seeberi* and some other mesomycetozoeans are intractable to culture (Table 1).

ROSETTE AGENT This organism is an obligate intracellular fish parasite. The infections it causes were first described by Harrell et al. (35) in net-pen-reared chinook salmon (*Onchorhynchus tshawytscha*). No generic name was proposed at that time. It was only referred to as the rosette agent because of the false impression that it clustered as a six-celled organism, resembling a rosette, in infected tissues (28). Based on morphological findings, Harrell et al. (35) pointed out that this unique salmon pathogen could either be a fungus, a protozoan, or an alga.

Infected salmon develop severe anemia and lymphocytosis. Swollen kidneys and spleens occur. Gram-stained smears of the infected tissue showed gram-positive spherical structures ~5–7 μm in diameter. The spherical organisms were found within the macrophages of infected kidneys and spleens. Transmission electron microcopy of the infected areas revealed spherical cells with multilayered cell walls, vacuoles, and a prominent nucleus (Figure 11). More recently, morphological and molecular studies of several isolates of the rosette agent showed that their 18S SSU rDNAs were identical. That study suggested that the rosette agent represented a new protozoan genus and species. Details of this proposal will be published elsewhere (K. D. Arkush, personal communication).

Experimental infections using rosette agent endospores taken from infected salmon tissues failed. Recently, however, Arkush at the University of California, Davis, induced the production of uniflagellated zoospores by the cells

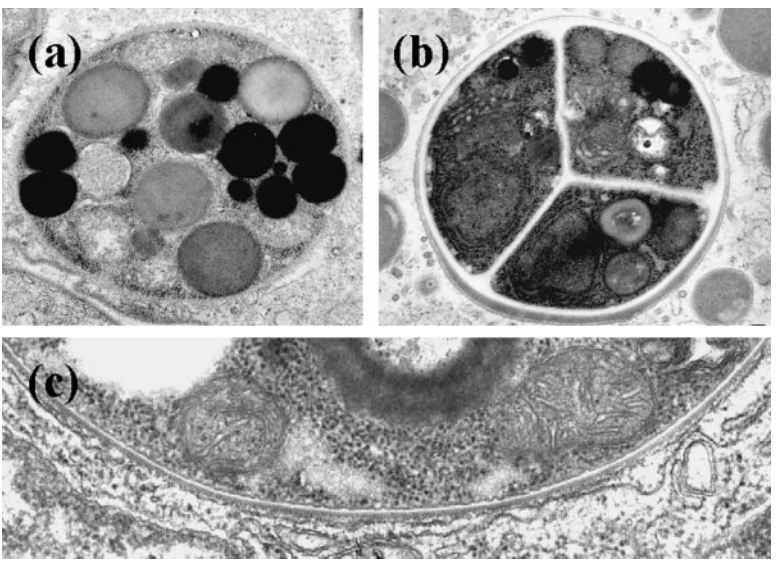

Figure 11 (*a*) Transmission electron microscopy section of a salmon kidney's interstitium infected with the rosette agent (X 27250). (*b*) Transmission electron microscopy showing the rosette agent undergoing division by progressive internal cleavage of its cytoplasm (X 24500). (*c*) Enlargement of a rosette agent's spherule showing two mitochondria with flat cristae (X 34024) (courtesy of R.A. Elston & K.D. Arkush).

of the rosette agent. The zoospores were approximately 1–2 μm in diameter and nearly spherical. They had only one flagellum $\sim$10 μm long with a typical $9 + 2$ configuration of microtubules. When the zoospores were used to infect salmon, the animals developed lesions comparable to those in natural infections (K. D. Arkush, personal communication). In addition, the rosette agent has been successfully cultured using the chinook salmon embryo cell line CHSE-214 at 15°C (4).

The rough phylogenetic relationships of the rosette agent were first revealed when Kerk et al. (44) characterized its18S SSU rDNA. Spanggaard et al. (84) corroborated this finding. Both studies revealed that the rosette agent was closely related to the choanoflagellates. Ragan et al. (77) established that this pathogen was part of a novel clade of parasites located between the divergence of fungi and animals, a finding corroborated by others (8, 12, 19, 29, 30, 39, 57, 101). In our phylogenetic analyses the rosette agent is the sister clade to *R. seeberi* and *Dermocystidium* spp., all possessing flat mitochondrial cristae and all closely related to other members of the order Ichthyophonida (Figure 1). Interestingly, Arkush et al. (4) found that the rosette agent possessed tubular mitochondrial cristae. However, a close evaluation of their electron microscopic figures and new photographic data indicated that the rosette agent might have mitochondria with flat cristae (Figure 11*c*).

RELATIONSHIPS OF THE MESOMYCETOZOEANS WITH THE OTHER CLADES ARISING NEAR THE ANIMAL-FUNGAL DIVERGENCE

The report of Ragan et al. (77) that the mesomycetozoeans form a monophyletic clade near the animal-fungal divergence was an unexpected finding. The exact position of this group, however, remains controversial. Ragan et al. (77) found that, depending on the sequences added to their phylogenetic trees, the mesomycetozoeans are either the sister group to the Animal kingdom plus the choanoflagellates or the sister group to the animals plus the choanoflagellates plus the fungi. Cavalier-Smith (18) argued that the mesomycetozoeans are the sister group to choanoflagellates plus *Corallochytrium*, and not to choanoflagellates plus both *Corallochytrium* and animals as inferred by Ragan et al. (77). Broader phylogenetic studies tend to confirm Cavalier-Smith's position (30, 39). Phylogenetic studies including additional members of the order Ichthyophonida, suggested that the mesomycetozoeans are the sister clade to the choanoflagellates and that together they are the sister group to animals and, with the inclusion of the animals, to the fungi (12, 29, 86). Our own phylogenetic analyses, using all the microbes in previous studies, indicated that mesomycetozoeans are indeed the sister group to the choanoflagellates plus *Corallochytrium* and the *Nuclearia* (Figure 1), thus supporting previous interpretations (18). However, it must be kept in mind that none of the deeper branches relating the Mesomycetozoea to other clades are well supported.

Based on the data published by several investigators (44, 77), Cavalier-Smith (18) proposed two different orders within the mesomycetozoeans, the

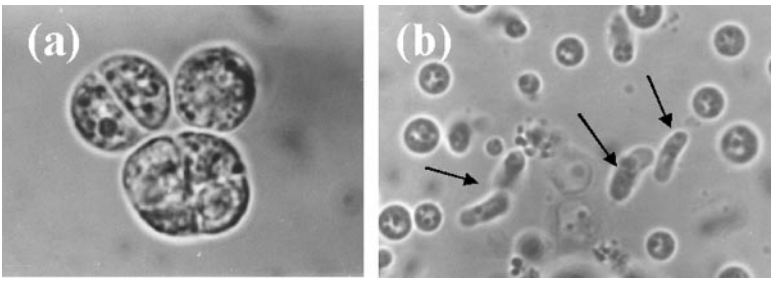

Figure 12 (*a*) Single, diad, and tetrad spherical vegetative cells of *Corallochytrium limacisporum* (X 1500). (*b*) Vegetative and elongated limax amoeba cells of *C. limacisporum* from cultures (*arrows*) (2000) (courtesy of S. Raghu-Kumar).

Dermocystida and Ichthyphonida. These two phylogenetic groups were found to differ from each other, not only on the basis of their 18S SSU rDNAs and internal transcriber spacers sequences but also on the morphological stages of their life cycles (Figure 12). For instance, the production of uniflagellate zoospores was the main feature of the *Dermocystidium* species and the rosette agent, both in the order Dermocystida. Flagellated cells are not known in *R. seeberi*, but as mentioned above, the matter is currently under investigation (Table 2). In contrast, most members of the order Ichthyophonida (*Amoebidium*, *Ichthyophonus*, and *Psorospermium* spp.) develop motile, amoeba-like cells. No such simple dichotomy exists when it comes to the morphology of mitochondrial cristae. Both *R. seeberi* and *Dermocystidium* spp. in the Dermocystida have mitochondria with flat cristae. The rosette agent was reported to have tubular mitochondrial cristae (4). However, the electron micrographs of the rosette agent's mitochondria described by Arkush et al. (4) had features compatible with flat mitochondrial cristae. Recent morphological studies of the rosette agent's mitochondria confirmed that it possesses flat cristae (Figure 11*c*). Alone among the mesomycetozoeans, *I. hoferi* seems to have tubular mitochondrial cristae. It may be that the morphology of cristae easily changed over evolutionary time or that interpretation of mitochondrial morphology is difficult in this group. As Zettler et al. (101) argued, the finding of different mitochondrial cristae types among microbes considered to be monophyletic should be carefully evaluated together with other morphological and physiological characteristics to avoid misinterpretations.

CLOSE RELATIVES OF THE MESOMYCETOZOEA AND THE EVOLUTION OF TRAITS COMMON TO ALL

The closest members of the mesomycetozoeans are *Corallochytrium limacisporum* and *Nuclearia* spp. in the classes Corallochytrea and Cristidiscoidea, and the choanoflagellates (Table 1). Study of these taxa may help us understand the origins

of the Mesomycetozoea. Although *C. limacisporum* is not a mesomycetozoean, it is included in this review for its phylogenetic proximity to that group.

Corallochytrium limacisporum is a novel type of saprotrophic marine protist (78). This organism was originally isolated from a coral reef in the Indian Ocean (78). Like some members of the class Mesomycetozoea, it is a spherical, single-celled organism, 4.5–20.0 μm in diameter that undergoes several binary fissions to later release numerous elongated daughter cells (up to 32 daughters per single cell) (Figure 12). *C. limacisporum* releases its endospores through one or more pores in its cell wall, recalling the behavior of *R. seeberi*. However, in *R. seeberi* there is only one exit pore (59). The elongated released spores are amoeba-like and have a slow sinusoidal movement (Figure 12). The production of an amoebic stage is a characteristic shared with mesomycetozoeans in the order Ichthyophonida. In that order *Amoebidium parasiticum* also produces elongated spores and has an amoeba-like stage. *C. limacisporum* apparently possess mitochondria with flat cristae (19), but photomicrographs depicting these organelles have not been published, and their morphology needs verification (Table 2).

Using phylogenetic analyses of 18S SSU rDNA, Cavalier-Smith & Allsopp (19) reported that *C. limacisporum* was closely related to the choanoflagellates and to the only member of the mesomycetozoeans known at that time, the rosette agent; it was not a thraustochytrid, the group in which it had previously been placed. Based on this result, Cavalier-Smith later created a new class, the Corallochytrea (18). Our phylogenetic analyses confirmed Cavalier-Smith's placement of this microbe outside of the mesomycetozoeans and close to the choanoflagellates (Table 1) (Figure 1).

Cavalier-Smith (16–18) placed the nucleariid amoeba in the phylum Neomonada, subphylum Mesomycetozoa (new subphylum that replaces the subphylum Choanozoa), class Cristidiscoidea, order Nucleariia, closely associated with *C. limacisporum* and the mesomycetozoeans (Table 1). According to the taxonomic classification of Cavalier-Smith (18), they all share the same phylum and subphylum (Table 1). This was confirmed by Zettler et al. (101) and by our own phylogenetic analyses (Figure 1). Members of the nucleariid amoeba are classified according to their morphological characteristics, especially those related to locomotion styles and feeding habits. Mitochondrial cristae morphology is important in the classification of nucleariid amoebae, which contain flat and discoidal mitochondrial cristae, the least common of the mitochondrial types. Based on phylogenetic analyses, Zettler et al. (101) speculated that the nucleariid amoeba might have developed discoidal mitochondrial cristae independently from other microbes that possess this type of cristae, again pointing out the evolutionary plasticity of this characteristic. The recent placement of the nucleariid amoeba near the mesomycetozoeans, choanoflagellates, and corallochytrians, and the rate at which new groups are being found at the animal-fungal boundary, suggest that more microbes at the same location will be found. Both *Corallochytrium* and *Nuclearia* spp. have amoeba or amoeba-like cells and neither have flagella, whereas animals, choanoflagellates, some fungi, and some Mesomycetozoea retain flagella.

Assuming that it is much easier to lose a flagellum than to gain one, it seems that the *Corallochytrium* and *Nuclearia* spp. must have lost flagella independently of similar losses in the Ichthyophonida and several phyla of the kingdom Fungi (87).

Amoebal motility, like the flagellar type, appears to be an ancestral trait found in amoebal-flagellates like the plasmoidal slime molds and their relatives (9, 18, 87). Even in the kingdom Fungi, when flagellated zoospores, especially those of the Blastocladiales, are trapped under cover glass and microscope slides, amoebal motility is observed (55). Cell walls also appear to be ancestral, being found in the choanoflagellates, fungi, the Mesomycetozoea, the *Corallochytrium*, and *Nuclearia* spp., but not in the animals. Surely, cell walls were lost in animals. The ancestors of all groups at the animal-fungal boundary must have been unicellular, and only in the animals and fungi did multicellularity develop and become widespread. In the Mesomycetozoea *I. hoferi* is filamentous, but the filaments are cenocytic and lack regular septa. They resemble the fungal hyphae in the Blastocladiales, Entomophthorales, Mucorales, Glomales, and other zygomycete orders. The members of those fungal orders similarly lack the multicellularity and macroscopic morphology typical of fungi in the Ascomycota and Basidiomycota. Multicellularity and macroscopic structures clearly developed independently in several lineages, including the green, brown, and red plants, in addition to fungi and animals. Further study may find macroscopic Mesomycetozoea. Most Mesomycetozoea are associated with animals as parasites or commensals. We speculate that *Sphaerosoma* and the LKM51 isolate are saprotrophic and free-living. It seems likely that other saprotrophic Mesomycetozoea will be found when methods of cultivating these organisms are applied to samples taken from nature.

FUTURE DIRECTIONS OF RESEARCH IN THE MESOMYCETOZOEA

What can we expect from the Mesomycetozoea in the near future, in addition to the discovery of more members? The cultivation of more members seems likely, and with it the possibility of more detailed comparisons of mesomycetozoean development with that of the other groups at the animal-fungal boundary. For example, the problem of mitochondrial form might be solved, as well as discovery of more flagellated species in the order Dermocystida. Cultivation also increases the possibility of gathering sequences from additional DNA regions in hopes of resolving the relationships among Mesomycetozoea, fungi, animals, and choanoflagellates. For example, Loytynoja & Milikovitch (55) have compared sequences of mitochondrial ADP-ATP carriers to question the relationship of fungi and animals as sister taxa, albeit without including the Mesomycetozoea or choanoflagellates. It would be worth extending the analysis to include these groups. The geological timing of divergence of the Mesomycetozoea, fungi, and animals is also an important topic. Several estimates of the date when fungi diverged from animals have been made, ranging from ~1 billion (13) to 1.6 billion yeas ago (36). Again, the class

Mesomycetozoea is not represented in these studies, and additional sequences might lead to new estimates of the timing of the divergence of these taxa. Cultivation also introduces the possibility of genomic analyses, and given the obvious interest in the origin of the Animal Kingdom, a strong case can be made for obtaining the genome of a mesomycetozoean for comparative genomics. Important contributions can be made, however, with traditional approaches. For example, the life cycle of no member of the Mesomycetozoea is known completely. Investigations to determine if gametes are produced, and the nuclear division needed to produce them, as well as the fusion of gametes to form zygotes, would be most welcome, as they could bring genetics to the class.

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