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SYSTEMATICS OF PROTOSTELOID AMOEBAE

SYSTEMATICS OF PROTOSTELOID AMOEBAE

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

By

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Northeastern State University
Bachelor of Science in Biology, 2003

December 2011
University of Arkansas

ABSTRACT

Because of their simple fruiting bodies consisting of one to a few spores atop a finely tapering stalk, protosteloid amoebae, previously called protostelids, were thought of as primitive members of the *Eumycetozoa sensu* Olive 1975. The studies presented here have precipitated a change in the way protosteloid amoebae are perceived in two ways: (1) by expanding their known habitat range and (2) by forcing us to think of them as amoebae that occasionally form fruiting bodies rather than as primitive fungus-like organisms. Prior to this work protosteloid amoebae were thought of as terrestrial organisms. Collection of substrates from aquatic habitats has shown that protosteloid and myxogastrian amoebae are easy to find in aquatic environments. Also, prior to this work the *Eumycetozoa sensu* Olive 1975, was a supposedly monophyletic taxon that included protosteloid amoebae as basal to *Myxogastria* and *Dictyostelia.* Three studies presented here erode this idea. These studies include a brief review of the diversity in nucleolar ultrastructure present among eumycetozoans in which new transmission electron micrographs of the protosteloid amoeba *Echinosteliopsis oligospora* are presented. Further, phylogenetic analyses of protosteloid amoebae based on the gene sequences of the small subunit of the ribosomal RNA (SSU rDNA) show that protosteloid amoebae are polyphyletic within the eukaryote supergroup, *Amoebozoa*, and that some are deeply embedded within well characterized lineages of nonfruiting amoebae, *i.e.*, vannellids and acanthamoebids. As a result, we now call these simple, fruiting amoebae 'protosteloid amoebae' as opposed to 'protostelids' since the latter implies (incorrectly) that these organisms comprise a natural phylogenetic group. These molecular phylogenetic analyses suggest that isolate LHI05 represents a new protosteloid species that branches among the *Acanthamoebidae*, an amoebozoan taxon in which fruiting amoebae have never previously been described. Thus a new genus and species, *Luapeleamoeba hula* g. *ad interim* sp. *ad interim*, are proposed to accommodate protosteloid isolate LHI05.

Suggestions for additional taxonomic revision are presented as are suggestions for future research particularly with respect to molecular phylogenetic, phylogenomic, and evolutionary/developmental biological approaches. Finally, this work has prompted critical thinking about the origins and evolution of simple fruiting bodies and complex life cycles among amoebozoans, and these basic biological questions are discussed.

This dissertation is approved for recommendation
to the Graduate Council

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DEDICATION

This is dedicated to all those who see the intrinsic beauty and importance of ALL organisms, especially those people who marvel at the tiny world that can be seen only through the lens of a microscope, and to my sons John Miles and Samuel Marshall.

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Lindley LA, Stephenson SL, Spiegel FW (2007) Protostelids and myxomycetes isolated from aquatic habitats. Mycologia 99(4): 504-509.

Lindley LA, Edwards SM, Spiegel FW (2006) Variations in nucleolar morphology in eumycetozoans. Revista Mexicana de Micologia 23: 75-81.

Shadwick LL, Spiegel FW, Shadwick JDL, Brown MW, Silberman JD (2009) Eumycetozoa = amoebozoa?: SSUrDNA phylogeny of protosteloid slime molds and its significance for the amoebozoan supergroup. PLoS ONE 4(8): e6754. doi:10.1371/journal.pone.0006754

CHAPTER 1

INTRODUCTION

GENERAL BACKGROUND

Protosteloid amoebae, formerly called protostelids, are amoebae that make small, simple fruiting bodies. A protosteloid fruiting body consists of an acellular stalk and a spore, though in some species there are up to eight spores per stalk. Protosteloid amoebae had been classified as a paraphyletic assemblage of slime molds in the class *Eumycetozoa* (Olive 1975). Olive considered protosteloid amoebae to be primitive eumycetozoans (Olive 1967, Olive 1970, Olive 1975, Olive and Stoianovitch 1976). In fact, it was the accidental discovery of protosteloid amoebae by Lindsay S. Olive and Carmen Stoianovitch (Olive and Stoianovitch 1960), that ultimately prompted Olive (1975) to revive the taxon *Eumycetozoa* (Zopf 1885) to include the protosteloid amoebae and two major lineages of slime molds with large, complex, multicellular fruiting bodies—the dictyostelids (Raper 1984) and the myxogastrids (Martin and Alexopoulos 1969). Olive's (1975) concept of *Eumycetozoa* included only those fruiting amoebae with acutely pointed subpseudopodia and tubular mitochondrial cristae (Olive 1975, Dykstra 1977). Thus it was less inclusive than Zopf's (1885) *Eumycetozoa* which inlcuded free living, fruiting amoebae, *i.e.*, most of the fruiting amoebae that had been previously described (*Copromyxa*, *Guttulina*, *Acrasis*, dictyostelids, and myxogastrids).

TAXONOMY

Taxonomically, protosteloid amoebae have been treated as a group, specifically either as the order *Protostelida* (Olive and Stoianovitch 1966b), the subclass *Protostelia* in class *Eumycetozoa* (Olive 1975), or as the class *Protostelida* (Spiegel 1990). Within this taxon, there are three formally named families of protosteloid amoebae (Olive 1975). These are (1) the *Cavosteliidae* (Olive 1964a), comprised of species with microscopic fruiting bodies and some flagellate trophic cells; (2) the *Ceratiomyxidae* (Schroeter 1889), comprised of species with macroscopic, mass fruiting bodies; and (3) the *Protosteliidae* (Olive & Stoianovitch 1966b), comprised of species with microscopic fruiting bodies and exclusively non-flagellate trophic cells. Within these three families, 17 genera and 36 species have been described (Table 1). Protosteloid amoebae were shown to be diverse by examination of the morphology of the amoebal stages of the different protosteloid amoeba life-cycles (Figures 1, 2) (Spiegel 1981a, Spiegel 1981b, Spiegel and Feldman 1985, Spiegel and Feldman 1988, Spiegel and Feldman 1989, Spiegel 1991, Spiegel and Feldman 1991, Spiegel *et al.* 1994). Based on this diversity, Spiegel (1990) divided the protosteloid amoebae into seven taxonomically informal morphological groups pending large-scale taxonomic revision (Table 1). Five of Spiegel's (1990) morphological groups were considered to belong to the monophyletic taxon *Eumycetozoa sensu* Olive 1975. The sixth group consisted of *Eumycetozoa incertae sedis*, while the seventh group consisted of species that had been excluded from the taxon *Eumycetozoa sensu* Olive 1975 based on morphology. Thus, prior to this dissertation, the systematics of eumycetozoans (Figure 3) was considered to comprise the monophyletic *Myxogastria* (myxogastrids or myxomycetes) and the monophyletic *Dictyostelia* (dictyostelids) embedded among a paraphyletic assemblage that included five morphology-defined groups I-Vb of protosteloid amoebae, and a few species of protosteloid amoebae that Spiegel (1990) thought were probably eumycetozoans even though they were not morphologically similar to other species (group VI: *Eumycetozoa incertae sedis*) illustrated in Figures 1 and 2 (Olive 1970, Olive 1975, Spiegel 1990, Adl *et al.* 2005). Finally, protosteloid species that might not be eumycetozoans were called “protostelid mimics” and placed in group VII (Spiegel 1990) pending taxonomic revision.

Table 1: Species of protosteloid amoebae arranged by major morphological group (adapted from Spiegel 1990). Note that while group Vb includes all myxogastrid amoebae, only one myxogastrian amoeba (*Echinostelium bisporum*) is said to have a protosteloid fruiting body. The family is listed in accordance with Olive (1975).

Group (Spiegel 1990)	Family (Olive 1975)	Species
I	*Protosteliidae*	*Protostelium mycophaga*
I	*Protosteliidae*	*Protostelium nocturnum*
I	*Protosteliidae*	*Protostelium okumukumu*
I	*Cavosteliidae*	*Planoprotostelium aurantium*
I	*Protosteliidae*	*Protostelium pyriformis*
II	*Protosteliidae*	*Schizoplasmodium cavostelioides*
II	*Protosteliidae*	*Schizoplasmodium obovatum*
II	*Protosteliidae*	*Schizoplasmodium seychellarum*
II	*Protosteliidae*	*Nematostelium gracile*
II	*Protosteliidae*	*Nematostelium ovatum*
II	*Cavosteliidae*	*Ceratiomyxella tahitiensis*
III	*Protosteliidae*	*Soliformovum irregularis*
III	*Protosteliidae*	*Soliformovum expulsum*
IV	*Protosteliidae*	*Schizoplasmodiopsis pseudoendospora*
IV	*Protosteliidae*	*Schizoplasmodiopsis vulgare*
IV	*Protosteliidae*	*Schizoplasmodiopsis reticulata*
IV	*Protosteliidae*	*Schizoplasmodiopsis micropunctata*
IV	*Cavosteliidae*	*Cavostelium apophysatum*
Va	*Cavosteliidae*	*Clastostelium recurvatum*
Va	*Cavosteliidae*	*Protosporangium articulatum*
Va	*Cavosteliidae*	*Protosporangium fragile*
Va	*Cavosteliidae*	*Protosporangium bisporum*
Va	*Cavosteliidae*	*Protosporangium conicum*
Va	*Ceratiomyxidae*	*Ceratiomyxa fruticulosa*
Va	*Ceratiomyxidae*	*Ceratiomyxa morchella*
Va	*Ceratiomyxidae*	*Ceratiomyxa sphaerospherma*
Va	*Ceratiomyxidae*	*Ceratiomyxa hemispherica*
Vb	*Cavosteliidae*	*Echinostelium bisporum*
VI	*Protosteliidae*	*Protosteliopsis fimicola*
VI	*Protosteliidae*	*Echinosteliopsis oligospora*
VI	*Protosteliidae*	*Schizoplasmodiopsis amoeboidea*
VI	*Protosteliidae*	*Microglomus paxillus*
VII	*Protosteliidae*	*Endostelium amerosporum*
VII	*Protosteliidae*	*Endostelium zonatum*
VII	*Protosteliidae*	*Protostelium arachisporum*

LIFE CYCLES

Among the protosteloid amoebae, life cycles vary dramatically (Figures 1, 2). All include both an amoeboid trophic stage and a fruiting stage (Figures 1, 2); however, some species also include flagellates and cysts. A few definitions are given below.

Definitions

Amoeboflagellate: an amoeba that can produce one or more flagella or an amoeba that has lost this ability yet retains the overall morphology of an amoeboflagellate (See Spiegel 1982 for a comparison between the flagellate cell of *Planoprotostelium aurantium* and the virtually identical nonflagellate cell of *Protostelium mycophaga;* and Spiegel *et al.* 1995a for an explanation of the concept of the nonflagellate amoeboflagellate).

Complex life cycle: A life cycle that consists of one amoeboid state that develops into another where the second morphologically distinctive amoebal state cannot revert to the first by a simple physiological change [*e.g.* the flagellate/nonflagellate transition when water is added or removed].

Cyst: resistance structures that lack a stalk.

Obligate Amoeba: an amoeba that cannot produce a flagellum and is morphologically distinct from any known amoeboflagellate (Spiegel and Feldman 1985); in life cycles with two amoebal stages, these are the amoebae that differentiate into fruiting bodies.

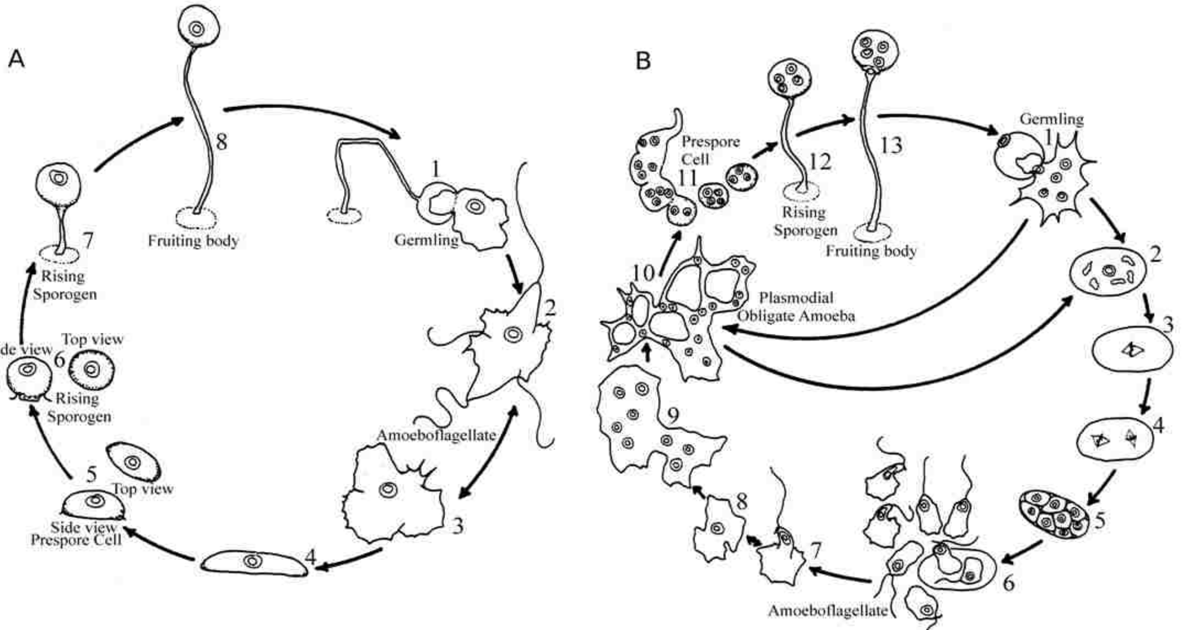
Plasmodium: a specialized type of obligate amoeba with many, sometimes hundreds, of nuclei (Spiegel and Feldman 1985).

Prespore cell: the beginning of the transition from amoeba or plasmodium to a fruiting body.

Sporocarp = Fruiting body: one or more resistant dispersal structures (*Spore* in this context) subtended by a subaerial stalk.

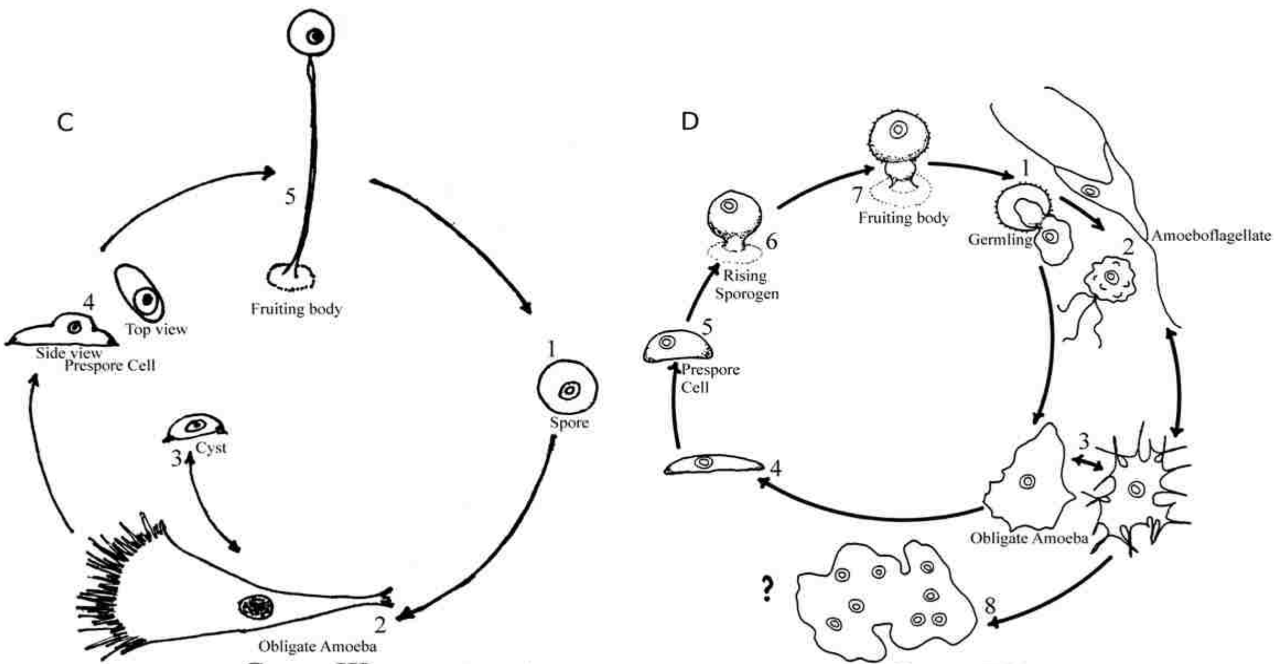
Sporogen: an immature fruiting body where the stalk is still elongating and the cell secreting the stalk has not matured into a spore.

Representative life cycles of each of Spiegel's five proposed monophyletic groups are depicted in Figures 1 and 2. The simplest type of these life cycles is one in which the obligate amoeba emerges from the spore and then feeds and divides until its progeny either make cysts or prespore cells and fruiting bodies, as in Figure 1C. In some cases two different amoebal morphologies are present within the same life cycle (Figures 1B7/1B9, 1D2/1D3, 2A1/2A3, 2B5/2B7, 2C4/2C7, 2D4/2D5) (Spiegel and Feldman 1985, Spiegel and Feldman 1988). In these cases, the amoebae alternate between an amoeboflagellate state (1B7, 1D2, 2A1, 2B5, 2C4, 2D4) and an obligate amoeba (Figure 1B9, 1D3, 2A3, 2B7, 2C7, 2D5) (Spiegel and Feldman 1985). In species with both amoeboflagellates and obligate amoebae, amoeboflagellates usually develop associated with spore germination, and it is the obligate amoebae that form fruiting bodies (Figure 1, 2). When life cycles of protosteloid amoebae are discussed, as in Spiegel (1990), they are generally lumped together with the implication that less complex life cycles like those of *Protostelium mycophaga* (lacks stage 2 Figure 1A, group I) or *Nematostelium gracile* (lacks stages 2-9 Figure 1B, group II) are simply reductions of the more complex life cycles like those of *Planoprotostelium aurantium* or *Ceratiomyxella tahitiensis*, respectively (Olive and Stoianovitch 1971a, 1971b; Spiegel 1990).



Group I
Planoprotostelium aurantium

Group II
Ceratiomyxella tahitiensis



Group III
Soliformovum irregularis

Group IV
Cavostelium apophysatum

Figure 1: Life cycles of protosteloid amoebae representative of groups I-IV (Spiegel 1990). (A) *Planoprotostelium aurantium,* all other species in group I, *Protostelium* spp., differ in that they lack stage 2. (B) *Ceratiomyxella tahitiensis*; all other species in group II, *Schizoplasmodium* spp. and *Nematostelium* spp., differ from *C. tahitiensis* in that they lack stages 2 through 9. (C) *Soliformovum irregularis*, the other group III species *S. expulsum* has an identical life cycle. (D) *Cavostelium apophysatum,* all other group IV species, *Schizoplasmodiopsis* spp. and *Tychosporium acutostipes*, lack stage 2. *Schizoplasmodiopsis pseudoendospora* has a plasmodial obligate amoeba. Life cycles were drawn by Frederick W. Spiegel except for *Soliformovum irregularis*, which was drawn by Lora L. Shadwick, who also added numbers and labels.

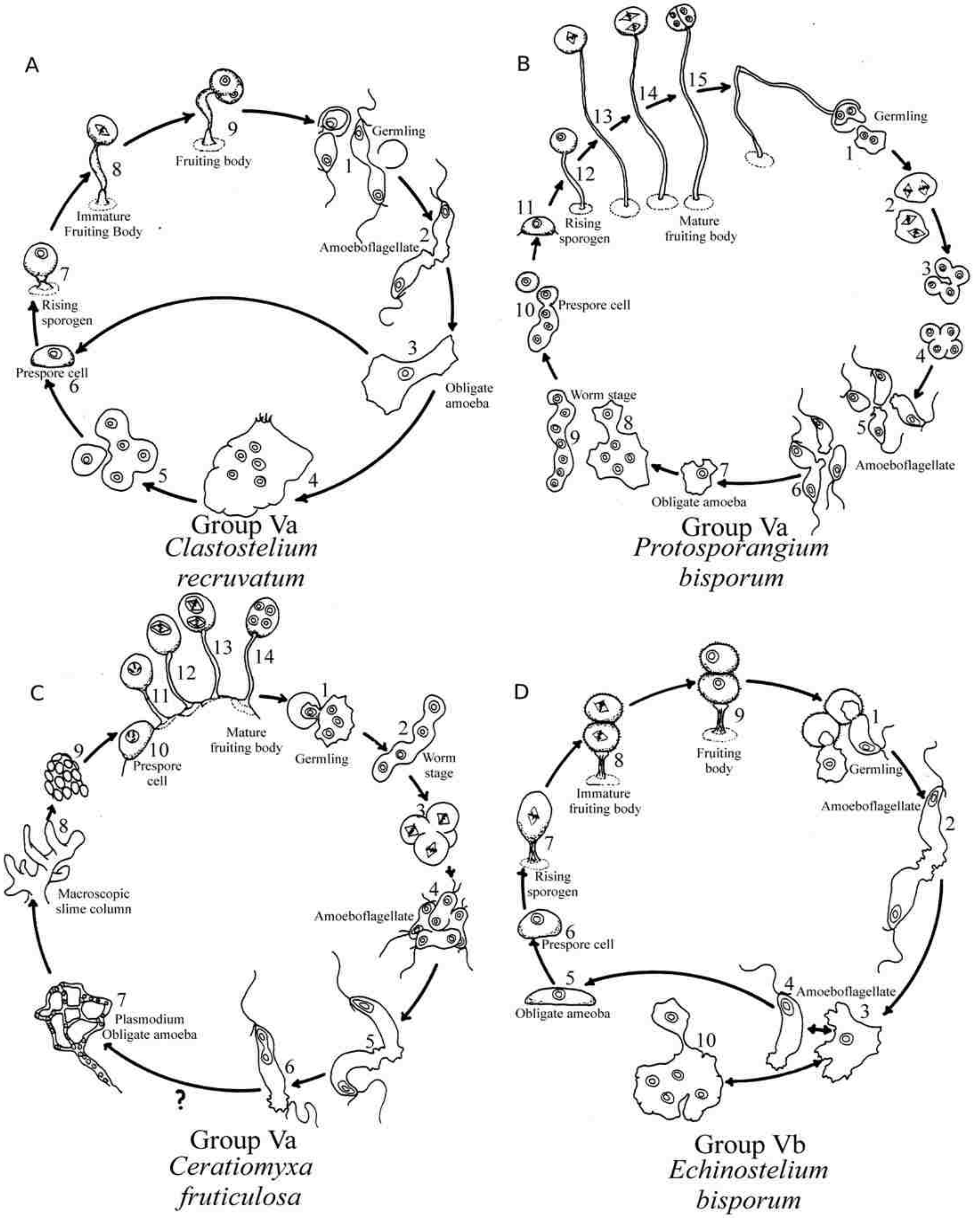

A
9
Fruiting body
Germling
1
8
Immature
Fruiting Body
2
Amoeboflagellate
7
Rising
sporogen
3
Prespore cell
6
Obligate
amoeba
5
4
Group Va
Clastostelium
recruvatum
B
13
14
15
Germling
1
12
11
Rising
sporogen
Mature
fruiting body
2
3
10
Prespore cell
4
Worm stage
8
9
5
Amoeboflagellate
7
6
Obligate amoeba
Group Va
Protosporangium
bisporum
C
11
12
13
14
1
10
Prespore
cell
Mature
fruiting body
Germling
2
Worm
stage
9
3
8
Macroscopic
slime column
4
Amoeboflagellate
7
Plasmodium
Obligate amoeba
5
6
?
Group Va
Ceratiomyxa
fruticulosa
D
9
Fruiting
body
1
Germling
8
Immature
fruiting body
Amoeboflagellate
2
7
Rising
sporogen
6
Prespore cell
5
Obligate ameoba
4
Amoeboflagellate
3
10
Group Vb
Echinostelium
bisporum

Figure 2: Life cycles of group V protosteloid amoebae. (A) *Clastostelium recurvatum* (B) *Protosporangium bisporum,* (C) *Ceratiomyxa fruticulosa,* and (D) *Echinostelium bisporum.* Other group Va protosteloid amoebae include additional members of the genera *Protosporangium* and *Ceratiomyxa* (Table 1). Small variations in the life cycles of *Protosporangium* species are noted in the species descriptions (Olive & Stoianovitch 1972, Bennett 1986a). These include variations in the exact timing of mitosis, and duration of amoeboflagellate, obligate amoebal, and worm stages (Olive & Stoianovitch 1972, Bennett 1986a). Group Vb also includes all other myxogastrian amoebae; these generally have a plasmodial obligate amoeba (Martin and Alexopoulos 1969). Life cycles were drawn by Frederick W. Spiegel. Numbers and labels were added by Lora L. Shadwick.

Obligate Amoebae

There is a wide variation in the amoebae that precede fruiting body formation among the protosteloid amoebae, and the homology of these amoeboid feeding stages has been questioned (Spiegel and Feldman 1985, Spiegel and Feldman 1991, Bortnick 1993). It is generally assumed that a highly complex life cycle that includes both flagellates and obligate amoebae such as in *Ceratiomyxella tahitiensis* is a primitive character in the *Eumycetozoa sensu* Olive (1975) (Figure 1) (Olive and Stoianovitch 1966b, Olive and Stoianovitch 1971b, Spiegel 1990). Under this assumption, Spiegel put forth the obligate amoeba hypothesis that suggests that the varied morphologies of protosteloid amoebae are a result of loss of some life cycle stages and subsequent differentiation of the remaining life cycle stages (Spiegel and Feldman 1985; Spiegel and Feldman 1988; Spiegel, Lee, and Rusk 1995). This allows us to assume homology between amoeboflagellates but not between obligate amoebae, except where clear cases of homology can be demonstrated as within major morphological groups (Spiegel 1990). This supposition is highly dependent on the evolutionary history of the group, namely (1) that *Eumycetozoa sensu* Olive (1975) is indeed a monophyletic group, (2) that amoeboflagellates are homologous and derived, and (3) that the majority of protosteloid amoebae do in fact belong to this group. In addition, Bortnick (1993) showed that in several cases where there is only a single nonflagellate amoeba (see Table 1) present in the life cycle of a protosteloid species, as in (Figure 2 C2), it is unknown whether this amoeba is simply a modified amoeboflagellate that has lost its flagellum and is thus homologous to the other protosteloid amoeboflagellates or if it is an independently derived obligate amoeba. Where amoebae of different species are morphologically indistinguishable, as in *Soliformovum irregularis* and *S. expulsum,* these amoebae are assumed to be homologous, and the species are thought to be closely related (Spiegel 1990, Spiegel *et al.* 1994).

Flagellates

Since their discovery in protosteloid organisms (Olive 1964), flagellate states have been strongly emphasized in the study and perception of fruiting amoebae. Olive thought that protosteloid amoebae evolved from free-living flagellates (Olive 1970, also depicted in Olive 1967). This is at least partly because many characteristics of amoeboflagellates were thought to be homologous, both within the *Eumycetozoa* (Olive 1975) and with other eukaryotes (Spiegel and Feldman 1985, Spiegel 1991). Spiegel concluded that the flagellate protosteloid species and myxogastrians were members of a monophyletic group, based on the proposed synapomorphies among their flagellar apparatuses (Spiegel 1981a). Among protosteloid amoebae, *Ceratiomyxella tahitiensis* has the most complex type of flagellar apparatus described, and it was interpreted as similar to the ancestral flagellar apparatus (Spiegel 1981a). Other, less complex, flagellar apparatus types were interpreted as reductions of a complex flagellar apparatus similar to that of *C. tahitiensis* (Spiegel 1981a). For example, the two protosteloid amoebae with a single basal body per flagellum, *Cavostelium apophysatum* and *Planoprotostelium aurantium,* were interpreted as being reduced from the state of having two basal bodies per flagellar apparatus (Spiegel 1981a).

Several genera have been noted to represent distinct lineages based on the type of flagellar apparatus they possess. For instance, *Ceratiomyxa* represents a distinct line of decent among protosteloid amoebae and is not a myxogastrid (Spiegel 1981b). A recent phylogeny based on molecular data supported the assertion that *Ceratiomyxa* is distinct from the myxogastrids (Fiore-Donno *et al.* 2010). Spiegel also established that *Planoprotostelium* represents a distinct lineage from other protosteloid amoebae and myxogastrids (Spiegel 1982). This is only partially corroborated by recent molecular phylogenies which have shown that the genera *Planoprotostelium* and *Protostelium* are intermingled, and that together these two genera represent a lineage that is distinct from myxogastrids (L. Shadwick *et al.* 2009, J. Shadwick 2010).

Among protosteloid amoebae that have an amoeboflagellate state, there are at least two morphologies that can be adopted. These are (1) comma-shaped myxogastrid-like amoeboflagellates that swim in a jerking spiral such as *Protosporangium* (Spiegel *et al.* 1986) and (2) gliding amoeboflagellates such as *Planoprotostelium* (Spiegel 1982) and *Cavostelium* (Spiegel 1981a). *Planoprotostelium* and *Cavostelium* swimming cells are not comma-shaped like myxogastrid amoeboflagellates, and their flagellar rootlets are not linked with the nucleus (Spiegel 1981a, 1982). In *Protosporangium articulatum* flagellate cells, the flagella associated with the nucleus are capable of generating a myxogastrid-like jerking helical swim, but the supernumerary flagella that are dissociated from the nucleus are not capable of generating this type of force (Spiegel *et al.* 1986). This suggests that attachment to the nucleus is important for generating the jerking helical swim (Spiegel *et al.* 1986).

Spiegel used flagellates to unite the so-called myxomycete-like protostelids (Spiegel 1981b, Spiegel *et al.* 1986, Spiegel and Feldman 1988, Spiegel 1991). The myxomycete-like protostelids (Spiegel and Feldman 1988; Spiegel 1991) include the genera *Protosporangium* (Spiegel *et al.* 1986), *Clastostelium* (Spiegel and Feldman 1988), and *Ceratiomyxa* (Spiegel 1981b). Proposed synapomorphies of this group include (1) microtubule array-3 contains 2 microtubules; (2) stalk of microtubule array-1 when present, is striated; (3) identical amoeboflagellate cell coat; (4) short lived flagellate state; and (5) meiosis in the prespore cell (Spiegel *et al.* 1986, Spiegel and Feldman 1988, and Spiegel 1991). Synapomorphies shared with myxogastrids include the architecture of the flagellar apparatus and the details of the posterior parakinetosomal structure (PPKS) (Spiegel 1991).

Spiegel (1991) proposed a phylogeny of flagellated protosteloid amoebae that was based primarily on characters of the flagellar apparatus, flagellate cells, and fruiting. It proposed a close sister group relationship between the myxomycete-like protostelids (*Clastostelium recurvatum*, *Protosporangium articulatum,* and *Ceratiomyxa fruticulosa*) and the myxogastrid *Echinostelium bisporum*. In addition, it proposed a close relationship between the two protosteloid amoebae, *Cavostelium apophysatum* and *Planoprotostelium aurantium,* with the simplest type of flagellar apparatus. Spiegel's (1991) phylogeny placed the protosteloid amoeba with the most complex flagellar apparatus and a highly complex life cycle *Ceratiomyxella tahitiensis,* as the sister group to the taxa (*Planoprotostelium* and *Cavostelium*) with a single basal body per flagellum.

Spiegel's (1991) proposed phylogeny of flagellated protosteloid amoebae contained several limitations. These were (1) nonflagellates could not be included in the tree, (2) it was heavily weighted with characters of the flagellate cells, and (3) the only outgroup used was the oomycete *Phytophthora* (no amoeboid outgroups, flagellate or nonflagellate, were included so comparisons with amoeboid organisms could not be made), and 4) the tree appears to be artificially rooted with *Phytophthora* (Spiegel 1991).

Spiegel's (1990) five morphologically defined groups of protosteloid amoebae include four groups with at least one flagellate member; therfore, I could overlay his morphogroups onto the flagellate phylogeny presented in Spiegel (1991) to yield an expected phylogeny of protosteloid amoebae (Figure 3). This expected tree is also compatible with several early molecular phylogenetic analyses (Spiegel *et al.* 1995; Baldauf and Doolittle 1997; Baldauf *et al.* 2000), but not with the more taxon rich analysis (Zaman *et al*. 1999), which cast some doubt on protosteloid amoebae, myxogastrian amoebae, and dictyostelid amoebae as a monophyletic clade.

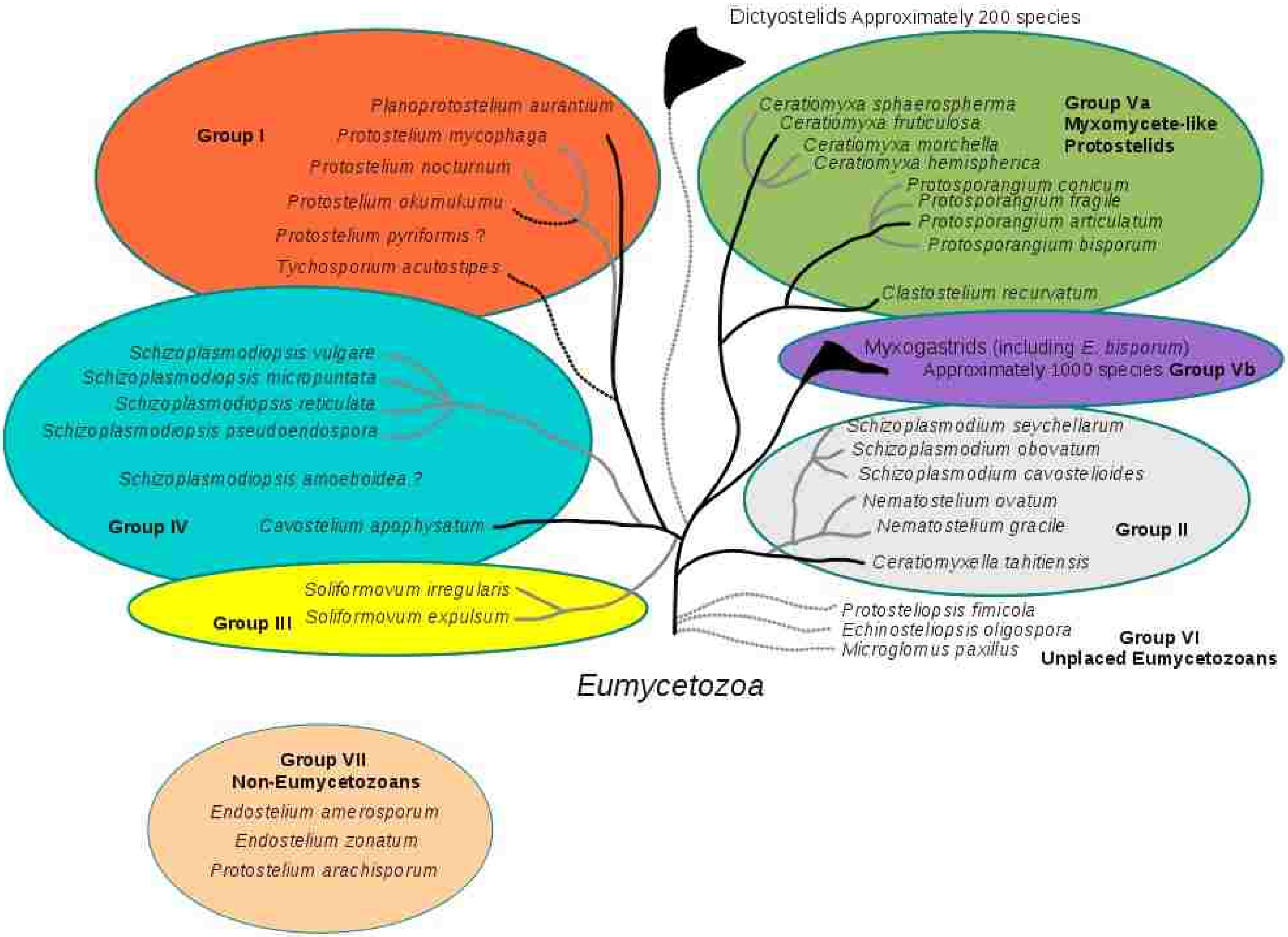

Dictyostelids Approximately 200 species
Group I
Planoprotostelium aurantium
Protostelium mycophaga
Protostelium nocturnum
Protostelium okumukumu
Protostelium pyriformis ?
Tychosporium acutostipes
Group Va
Myxomycete-like
Protostelids
Ceratiomyxa sphaerospherma
Ceratiomyxa fruticulosa
Ceratiomyxa morchella
Ceratiomyxa hemispherica
Protosporangium conicum
Protosporangium fragile
Protosporangium articulatum
Protosporangium bisporum
Clastostelium recurvatum
Myxogastrids (including *E. bisporum*)
Approximately 1000 species **Group Vb**
Group IV
Schizoplasmodiopsis vulgare
Schizoplasmodiopsis micropuntata
Schizoplasmodiopsis reticulata
Schizoplasmodiopsis pseudoendospora
Schizoplasmodiopsis amoeboidea ?
Cavostelium apophysatum
Group II
Schizoplasmodium seychellarum
Schizoplasmodium obovatum
Schizoplasmodium cavostelioides
Nematostelium ovatum
Nematostelium gracile
Ceratiomyxella tahitiensis
Group III
Soliformovum irregularis
Soliformovum expulsum
Protosteliopsis fimicola
Echinosteliopsis oligospora
Microglomus paxillus
Group VI
Unplaced Eumycetozoans
Eumycetozoa
Group VII
Non-Eumycetozoans
Endostelium amerosporum
Endostelium zonatum
Protostelium arachisporum

Figure 3: Depiction of phylogeny of protosteloid amoebae, based on characters of the flagellar apparatus (solid black lines) (Spiegel 1991), morphological groupings (solid gray lines) (Spiegel 1990), and species or genus descriptions (dotted black lines) (Spiegel *et al.* 1995b; Spiegel *et al.* 2006). Some species (*Protosteliopsis fimicola*, *Echinosteliopsis oligospora*, and *Microglomus paxillus*) or lineages (Dictyostelids) have no sister groups among the protosteloid amoebae and have been arbitrarily placed among the protosteloid amoebae in this tree (dotted gray lines) (Olive 1975, Spiegel 1990). The question marks after *Schizoplasmodiopsis amoeboidea* in group IV and *Protostelium pyriformis* in group I reflect suggested removal from their respective groups (Bortnick 1993; Spiegel *et al.* 1994). Branch lengths are arbitrary. Morphological groups are highlighted with colored balloons (Spiegel 1990). Proposed noneumycetozoans (Spiegel 1990) have been placed in a separate floating balloon.

EVOLUTIONARY HISTORY

Because there is no fossil record for protosteloid amoebae, little is known directly about their evolutionary history. What is known has been inferred from extant species and based heavily on Olive's (1975) concept of the *Eumycetozoa*. The term "protostelid" was coined to refer to the morphology of those eumycetozoans *sensu* Olive (1975) with simple fruiting bodies (Spiegel 1990). Fruiting body formation was thought to be a synapomorphy of the *Eumycetozoa sensu* Olive (1975, Spiegel *et al.*1995a), and presumably Olive thought that the ancestral dictyostelids and myxogastrian amoebae would have been able to form a protosteloid type of fruiting body (Olive 1975, Spiegel 1990). In addition, the ancestral protosteloid organism would have had amoeboid/flagellate feeding cells, tubular mitochondrial cristae, filose (*sensu* Olive) subpseudopodia, and a life cycle that included sex (Olive 1975, Spiegel *et al.* 1995a).

Possible relatives and phylogenetic outgroups for protosteloid amoebae have been debated. Olive thought that protosteloid amoebae evolved from free-living flagellates (Olive 1970, also depicted in Olive 1967), and that long stalked protosteloid amoebae evolved from short stalked ancestors (Olive 1970). Spiegel (1981a) suggested that it might have been possible for fruiting protosteloid amoebae to have arisen multiple times independently from non-fruiting amoebae with filose subpseudopodia.

Molecular Phylogenetics

More recently, *Eumycetozoa sensu* Olive (1975) has been placed within the eukaryotic supergroup *Amoebozoa* (Cavalier-Smith 1998, Baldauf *et al.* 2000, Cavalier-Smith 2003, Adl *et al.* 2005). A few published molecular phylogenetic analyses included SSU rDNA gene sequences of protosteloid amoebae (Spiegel, Lee, and Rusk 1995, Baldauf and Doolittle 1997, Baldauf *et al.* 2000, Fiore-Donno *et al.* 2005, Schaap *et al.* 2006, and Brown *et al.* 2007). These analyses included only *Protostelium mycophaga* and/or *Planoprotostelium aurantium* as representative protosteloid species; thus, they provided no information about the relationships among protosteloid species or about the relationships between most protosteloid amoebae, myxogastrian amoebae, and dictyostelid amoebae. None of these analyses included broad taxon sampling of nonfruiting *Amoebozoa* (Spiegel *et al.* 1995a; Baldauf and Doolittle 1997; Baldauf *et al.* 2000; Fiore-Donno *et al.* 2005; Schaap *et al.* 2006), except Brown *et al.* (2007) which did not include the generally long-branched myxogastrid amoebae. Thus, the relationships among the majority of protosteloid amoebae and other *Amoebozoa* had not been illuminated by molecular phylogenetic analyses.

OVERVIEW

There are several hypotheses, questions, and goals presented in this dissertation. These are outlined below.

I. Does the perception that protosteloid amoebae and/or myxogastrian amoebae are terrestrial result in an underrepresentation of some of these organisms in ecological surveys?

  A. Do protosteloid amoebae and/or myxogastrian amoebae occur in aquatic habitats?

  B. For those protosteloid amoebae and/or myxogastrian amoebae that do occur in aquatic habitats, are they found on emergent substrates, submerged substrates, or both?

  C. What are the implications of finding protosteloid amoebae and/or myxogastrian amoebae in aquatic habitats?

II. What is the range of ultrastructural diversity within eumycetozoans *sensu* Olive (1975) with respect to the nucleolus?

  - **A.** What nucleolar morphologies are present within eumycetozoans?
  - **B.** How are these morphologies distributed among eumycetozoan species or major clades?
- **III.** What is the evolutionary history of protosteloid amoebae?
  - **A.** What are the phylogenetic relationships among protosteloid amoebae?
  - **B.** Do phylogenetic trees based on the small subunit ribosomal DNA support monophyly of the *Eumycetozoa sensu* Olive (1975)?
  - **C.** What are the relationships between protosteloid amoebae and nonfruiting amoebae?
- **IV.** How might one new isolate of protosteloid amoebae be characterized?
  - **A.** Is isolate LHI05 a new species?
  - **B.** Does isolate LHI05 represent a new genus?
  - **C.** To what taxon is isolate LHI05 most closely related?
- **V.** Does the current systematic, taxonomic, and nomenclatural system used for protosteloid amoebae adequately reflect what we have learned about these organisms?
- **VI.** What should be the directions of future research on protosteloid amoebae?

The remainder of chapter one introduces and summarizes chapters 2 through 6. Please note that my name has changed from Lora Ann Lindley (LAL) in chapters 2 and 3 to Lora Lindley Shadwick (LLS) in the remainder of the dissertation.

Chapter 2: Lindley LA, Stephenson SL, Spiegel FW (2007) Protostelids and Myxomycetes Isolated from Aquatic Habitats. Mycologia 99(4): 504-509.

Protosteloid amoebae (referred to as protostelids in this chapter) and myxogastrians (referred to as myxomycetes) had been suggested to have amoebozoan affinities when this was written (Baldauf and Doolittle 1997, Cavalier-Smith 1998, Baldauf *et al*. 2000). These organisms were traditionally considered to be terrestrial (being active in water films in soil or on decaying plants) (Stephenson and Stempen 1994; Spiegel *et al.* 2004), while many nonfruiting amoebae are aquatic (Page 1988). We decided to determine if fruiting amoebae could be found in aquatic environments.

If aquatic protosteloid and myxogastrian amoebae did exist, they would be generally overlooked by the ecological survey methods routinely used to study these organisms around the world (Spiegel *et al*. 2004, Spiegel *et al.* 2007). The first chapter is a published paper (Lindley *et al.* 2007) that came out of a class project in which the standard ecological survey methods for myxogastrian and protosteloid amoebae were adapted to aquatic systems. Some protosteloid and myxogastrian amoebae were found in these aquatic environments. The findings in this chapter demonstrate that these organisms are easy to isolate from aquatic environments. It also suggests that abundance of some species may be underestimated because aquatic environments are routinely overlooked in ecological surveys.

Author contributions: Conceived and designed the experiments: LAL SLS. Performed the experiments: LAL. Analyzed the data: LAL. Contributed reagents/materials/analysis tools: SLS FWS. Wrote the paper: LAL FWS SLS.

Chapter 3: Lindley LA, Edwards SM, Spiegel FW (2006) Variations in Nucleolar Morphology in Eumycetozoans. Revista Mexicana de Micologia 23: 75-81.

Persistence in aquatic environments is not the only feature that protosteloid amoebae share with many other amoebozoans; they also have wide variation in nucleolar morphology as do some archamoebae, thecamoebids, vannellids, and flabellinids (Page 1988). Most protosteloid amoebae have a single, central, round nucleolus; but several species have strikingly different nucleolar arrangements. The second chapter is a published paper (Lindley *et al.* 2006) that uses new electron microscopy images of the nucleoli of one protosteloid amoeba *Echinosteliopsis oligospora* (Reinhardt and Olive 1966), and reviews the various nucleolar arrangements found in other *Eumycetozoans sensu* Olive (1975). In this chapter, light and electron micrographs show that *Echinosteliopsis oligospora* has a nucleolar morphology that is unique compared to other *Eumycetozoans sensu* Olive (1975).

Author contributions: Conceived and designed the experiments: LAL SME FWS. Performed the experiments: LAL SME. Analyzed the data: LAL SME FWS. Contributed reagents/materials/analysis tools: FWS. Wrote the paper: LAL FWS SME

Chapter 4: Shadwick LL, Spiegel FW, Shadwick JDL, Brown MW, Silberman JD (2009) Eumycetozoa = Amoebozoa?: SSUrDNA Phylogeny of Protosteloid Slime Molds and Its Significance for the Amoebozoan Supergroup. PLoS ONE 4(8): e6754. doi:10.1371/journal.pone.0006754

Stalked fruiting body formation was thought to be a synapomorphy of the *Eumycetozoa sensu* Olive 1975, with protosteloid fruiting being the ancestral type of fruiting body and protosteloid amoebae being basal to myxogastrids and dictyostelids (Olive 1975; Spiegel, Lee, and Rusk 1995; Swanson, Spiegel, and Cavender 2002). Previous phylogenies of *Eumycetozoa sensu* Olive 1975 have failed to include enough protosteloid ingroups and nonfruiting amoeboid outgroups to adequately test this hypothesis. Chapter 4 presents SSU rDNA phylogenetic analyses that include 21 protosteloid amoebae and a wide variety of amoeboid protists in the eukaryotic supergroup *Amoebozoa*, and other eukaryotic outgroups. This chapter shows that protosteloid amoebae are widespread throughout the *Amoebozoa,* and occur in at least two lineages, vannellids (revised in Smirnov *et al.* 2007) and acanthamoebids (Sawyer and Griffin 1975), in which fruiting body formation was not previously known. This calls into question the validity of stalked fruiting as a synapomorphy of the *Eumycetozoa sensu* Olive 1975. Because of this, we have suggested that the term protostelid is confusing because it does not refer to a monophyletic or even a paraphyletic group of organisms. We suggest that this type of fruiting should be called protosteloid fruiting and that the organisms that exhibit this type of fruiting should be called protosteloid amoebae. Further, we have shown that complex life cycles are common among amoebozoan taxa and that *Eumycetozoa* might be a more appropriate name for the entire group, especially since it has priority over the name *Amoebozoa.*

Author contributions: Conceived and designed the experiments: LLS FWS JDS. Performed the experiments: LLS JDLS MWB. Analyzed the data: LLS JDLS JDS. Contributed reagents/materials/analysis tools: FWS JDS. Wrote the paper: LLS FWS JDLS JDS

Chapter 5: A New Amoeba with Ballistosporous Protosteloid Fruiting: ***Luapeleamoeba hula*** g. ***ad interim*** sp. ***ad interim*.**

One protosteloid isolate, isolate LHI05, which was included in the phylogeny presented in Chapter 4 is clearly a newly identified species. The fifth chapter of this dissertation is a manuscript being prepared as a description of this new species, with a new genus to accommodate it.

Chapter 6: Overall Conclusions

The final chapter discusses the change in perception of protosteloid amoebae brought about by the research presented in this dissertation. It also summarizes conclusions and discusses the current state of research since the publication of these papers. Further, some interesting biological questions raised by this research are also presented and discussed. Finally, directions for future research including molecular phylogenetics, genomics, and taxonomic revision are also suggested.

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CHAPTER 2

LINDLEY LA, STEPHENSON SL, SPIEGEL FW (2007) PROTOSTELIDS AND MYXOMYCETES ISOLATED FROM AQUATIC HABITATS. MYCOLOGIA 99(4): 504-509.

PROTOSTELIDS AND MYXOMYCETES ISOLATED FROM AQUATIC HABITATS

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Abstract: Protostelids and myxomycetes have been isolated from dead plant parts in many different habitats, including tropical rain forests and deserts. However, underwater habitats have been largely overlooked. The purpose of this study was to determine whether protostelids do occur in aquatic habitats and to survey the myxomycetes associated with these habitats. Protostelids and myxomycetes were isolated from substrates collected from just above and just below the surface of the water. Several species of both groups were present, and their distributions above and below the water were different. It is not surprising that the trophic cells of slime molds occur in ponds because they are known to grow in thin films of water. However, these findings are significant because this is the first study to demonstrate clearly the occurrence of protostelids in underwater environments, and one of the few surveys of myxomycetes from aquatic systems.

Key Words: Fruiting amoebae, *Eumycetozoa*, freshwater, ponds, streams, Mycetozoa

INTRODUCTION

Although the captivating image of the mycetozoan fruiting body is what most people consider, the complete life history also must be remembered. Mycetozoans are found in dry environments including deserts (Stephenson and Stempen 1994b) because their dormant stages, spores and cysts, are resistant. However, their amoeboid trophic cells must live in a film of liquid water even though it may be temporary in arid environments. Since amoebae are, in effect, aquatic, we thought mycetozoans might be present at the other extreme in wetlands, ponds, and lakes. It is possible that these mycetozoans could live for long periods of time in aquatic habitats, resorting to sporulation when the body of water dries or after migrating to substrates at the edge of the water.

To date there have been only a few reports of “aquatic myxomycetes.” One recent paper, (Kappel and Anken 1992) reports that a plasmodium was found growing on the inside of an aquarium. The plasmodium was placed into culture where it formed the fruiting bodies of a long-stalked *Didymium,* which the authors identified as *Didymium nigripes*. Another earlier study describes the “nutrition of some aquatic myxomycetes” identified as *Physarum gyrosum, Physarum nutans,* and *Fuligo cinerea* (Parker 1946). These organisms were reportedly grown completely submerged in water until the fruiting stages. Myxomycetes have been isolated and identified from substrates submerged in swamps (Shearer and Crane 1986), and an early report of an aquatic myxomycete describes the growth of a strain of *Didymium difforme* on the roots of Hyacinths being cultured in nutritive solutions (Ward 1886). This myxomycete repeatedly completed its life-cycle entirely underwater. In addition, *Didymium aquatile*, collected in Brazil is described as living completely submerged as a phaneroplasmodium until the moment of fructification (Gottsberger and Nannenga-Bremekamp 1971). We are not aware of any published reports of protostelids cultured from submerged substrates. However, *Tychosporium acutostipes* and *Schizoplasmodiopsis vulgare* have grown from substrates collected from wetlands (Spiegel, unpub. observation).

The purpose of this study was to determine whether protostelids could be isolated from submerged substrates and to obtain additional data on the myxomycetes present in aquatic environments.

MATERIALS AND METHODS

Substrates.— Samples were collected from just above and just below the surface of the water at the shoreline of ponds or lakes. Samples were designated as being either **Above** (within 5 cm above the water surface) or **Below** (within 5 cm below the water surface). Samples were placed in paper bags and allowed to air dry to inhibit further decomposition. Dried samples were then taken back to the lab and plated for protostelids and myxomycetes. Substrates in these samples included decaying plant matter that had fallen into the water, dead parts of living emergent or submerged plants, pieces of driftwood, leaves, and seeds that were floating or had accumulated along the bank. The different types of substrates were not separated.

Collection Sites.—Five collection sites were chosen in Northwest Arkansas and Northeastern Oklahoma (TABLE 1). Sites 1 (Cincinnati, Arkansas) and 2 (Muskogee, Oklahoma) are farm ponds. Site 3 is near the boat ramp at Carter's Landing on Tenkiller Ferry Lake, OK. Sites 4 and 5 are located in Honor Heights Park in Muskogee, Oklahoma. Site 4 is a small (1 meter x 1 meter x 0.5 m) pond in a rose garden. Site 5 is in the largest pond in Honor Heights Park.

Protostelid Primary Isolation Plates.— Primary isolation plates were set up for protostelids (Spiegel *et al.*2005; Spiegel *et al.* 2004). A small amount of substrate was taken from samples using ethanol flamed forceps and broken into 6-8 mm pieces, soaked in sterile, distilled water for 10 min, and plated on weak malt extract yeast extract agar (WMY). Approximately 8 pieces of substrate were placed on each 100 x15 mm plate, these pieces were evenly spaced around the plate so that between each piece there was approximately 1cm of uninoculated agar between each piece. Protostelids observed on the perimeter of one piece that were one species were assumed to be clones. However protostelids on different pieces of substrate on the same plate were considered to be different clones and therefore separate observations. After approximately 10 days the perimeter of each piece was observed under the 10x objective of a compound microscope. Protostelids were identified by characteristics of their fruiting bodies and amoebae. Higher power objectives were sometimes used to aid in the identification of certain patches of fruiting bodies. If a species determination could not be made, then the organism was isolated into monoeukaryotic culture and deposited in the American Type Culture Collection (ATCC).

Myxomycete Moist Chambers.— The remainder of each sample was used to set up moist chambers to observe myxomycetes (Stephenson and Stempen 1994a). Moist chambers were prepared by placing a 90 mm diameter filter paper inside a sterile 100 x 15mm polystyrene Petri plate. Substrates were placed so that plates were as full as possible without overlapping pieces of substrate. Samples were then moistened with deionized water. The sample pH was recorded approximately 24 hours after samples were hydrated. Plates were then observed under a dissecting microscope every 2 or 3 days for approximately two months. Mature fruiting bodies were observed, collected, identified, placed in small boxes for permanent storage and deposited in the myxomycete herbarium at the University of Arkansas (UARK).

RESULTS

A total of 22 protostelid primary isolation plates containing 176 pieces of substrate were observed. Protostelids were found at every collection site (Table 1). Altogether we made 153 protostelid observations, 67 observations on **Above** plates and 86 observations of **Below** plates (Table 1). Seven species of protostelids were identified on substrates collected just above the surface of the water (9 primary isolation plates, containing 72 pieces of substrate), and nine species were recorded on samples taken from below the water surface (13 primary isolation plates, containing 104 pieces of substrate). The most common species found above the surface was *Soliformovum irregularis* (Fig 1), which was present on 25% of the pieces of substrate (Fig 1). *Tychosporium acutostipes* was present on 11% of the above water pieces and 2% of below water pieces (Fig 1). *Protostelium nocturnum* was found on 10% of above water pieces but not at all on subsurface pieces (Fig 1). *Nematostelium ovatum*, *Protostelium arachisporum,* and *Echinostelium bisporum* were not found on samples taken from above the surface, but these species were present on <7% of pieces collected from below the surface of the water (Fig 1). The most common species (Fig 1) found below the surface was *Schizoplasmodiopsis vulgare*, which was found on 19% of the pieces of substrate (Fig 1). *Schizoplasmodiopsis vulgare, Protostelium mycophaga*, *Schizoplasmodiopsis pseudoendospora*, *Nematostelium ovatum*, *Echinostelium bisporum*, and *Protostelium arachisporum* all occurred in higher percentages on pieces from below the surface of the water. *Nematostelium gracile* was present on 10% of **Above** and 9% of **Below** pieces (Fig 1). One protostelid culture was established from submerged substrates collected from site five. It was identified as *Schizoplasmodiopsis vulgare*, designated as strain HHPBPB3604-1, and deposited in the American Type Culture Collection “ATCC PRA-158”.

The pH range of the substrates in myxomycete moist chambers was 5.69 to 7.71. Myxomycete fruiting bodies, plasmodia, or sclerotia were observed from every collection site except site 5, regardless of pH (Table 1). No samples from site five were plated for myxomycetes. Fourteen species of myxomycetes fruited in the 42 moist chambers (FIG 2). Twenty samples were from above the surface of the water and 22 from below (FIG 2). Twelve myxomycete species were present on samples from above the water line, and four species were present on samples from below. Two species, *Licea biforis* and *Physarum pusillum*, were present both above and below the surface (FIG 2). There were at least six non-fruiting plasmodia, three on the above water plates and three on below water plates (FIG 2). *Comatricha nigra* and *Licea kleistobolus* were present only below the surface (FIG 2). *Arcyria cinerea*, *Perichaena chrysosperma*, *Perichaena* cf. *depressa*, *Didymium anellus*, *Didymium iridis*, *Didymium ochroideum*, *Didymium squamulosum*, *Physarum* cf. *crateriforme*, *Physarum* cf. *cinereum*, and *Licea* sp. all fruited only on substrates from above the surface (FIG 2). A few of the typically sessile *Didymium ochroideum* fruiting bodies have short stalks, an unusual character state in this typically stalkless species (Martin and Alexopoulos 1969). Some of the specimens of *Physarum pusillum* have lime with a faint yellow pigmentation. All myxomycete voucher specimens have been deposited into the myxomycete herbarium at the University of Arkansas (UARK).

DISCUSSION

These results indicate that protostelids and myxomycetes are easy to recover from aquatic habitats. Our data begin to challenge the notion that fruiting amoebae are primarily terrestrial throughout their life history and highlight the need for broader field sampling of many different substrates from a wider variety of habitats, even those that might, *a priori*, be expected to yield few mycetozoans. Habitats that may not have been adequately sampled include freshwater streams, ponds, rivers, and lakes. Salt-water and brackish habitats also have been consistently overlooked, yet naked amoebae are found to be common in the plankton, e.g., (Rogerson and Gwaltney 2000). Some of these amoebae could potentially be trophic cells of mycetozoans.

Naked amoebae (gymnamoebae, amoebae that do not secrete a hard covering or test) (Page 1988) are commonly observed in marine and freshwater habitats. Because it is unlikely that protostelids or myxomycetes would fruit under the conditions that are used to observe and study naked amoebae, it is possible that many mycetozoans and close relatives have been observed in aquatic habitats without being recognized as mycetozoans because of their failure to fruit. Aquatic habitats were found to be both more species rich and biocomplex than terrestrial habitats when surveyed for amoeboid protists (Anderson 2003). Naked amoebae have also been shown to vary in abundance because of localized nutrient enrichment, particle size, earthworm activity, and season (Anderson 2003; Anderson 2000; Bass and Bischoff 2001). Naked amoebae from terrestrial environments have been shown to increase dramatically in numbers in response to moist conditions, which suggests that a truly aquatic environment may be suitable for their growth and reproduction, and some species are reported to be both aquatic and terrestrial (Anderson 2000). This may also be true for some eumycetozoan amoebae.

These results do not show absolutely that trophic cells of protostelids and myxomycetes were actively growing in aquatic habitats. However, several lines of evidence show that these eumycetozoans will grow submerged. As mentioned above, there are a few reports of submerged myxomycete plasmodia (Gottsberger and Nannenga-Bremekamp 1971; Kappel and Anken 1992; Parker 1946; Stephenson and Stempen 1994a; Ward 1886). Also, myxomycetes have been isolated from the air into liquid culture (Miller 1898). In this case fruiting was reliably induced by the addition of partially submerged solid support (sterilized hay) (Miller 1898). Protostelids have regularly been observed to grow submerged in thin films of water, then to penetrate the surface film to fruit in both primary isolation plates and in culture (Olive 1975, Spiegel unpublished observations). Protostelid and myxomycete trophic cells grow readily in liquid culture (Olive 1975, Spiegel 1982, Miller 1898). It could be suggested that the eumycetozoans we observed from submerged substrates may have come from airborne propagules that were floating in the surface film or from aerial contamination as the substrates were brought out of the water. Since it is likely that such propagules would be very dilute in nature, it would be expected that, though present, these mycetozoans would be much less abundant in the plates of substrates collected from below the water. We observed no obviously lower abundance of myxomycetes and protostelids on plates of substrates from below the water when compared with those from above.

Myxomycetes and protostelids may differ in their likelihood to exploit the aquatic environment. Though our sample sizes were small, fruiting bodies of myxomycetes were more common on samples collected from just above the surface of the water, which could indicate that many species do not generally exploit the submerged habitat. They may, however, be present in aquatic environments as populations of amoeboflagellates that do not progress through the life cycle under our culture conditions. In fact, Eumycetozoan trophic cells have been repeatedly isolated from aquatic environments and erroneously described as novel organisms despite the overwhelming ultrastructural and molecular phylogenetic evidence showing that these are myxomycete and/or protostelid amoeboflagellates (Rolf et al. 2003; Walker et al. 2003; Walochnik et al. 2004). Protostelids, on the other hand, do not appear to be depauperate in freshwater habitats, but our data do suggest that the amoebae of some species may exploit this habitat more than others. For instance, the high numbers of *Schizoplasmodiopsis vulgare* from submerged substrates suggest that this species may be well suited to aquatic environments (FIG 1).

In this study, some species (*Licea kleistobolous*, and *Comatricha nigra*) were present only below the water, we attribute this difference to small sample size rather than habitat preference. In addition, some protostelid species (*Protostelium nocturnum, Endostelium sp., Soliformovum expulsum*) are probably systematically underestimated in biogeographic surveys, because they often fruit *en mass*. When grown in monoeukaryotic culture only amoebae are observed during the day, while fruiting bodies can often be observed at night. In this study, all the *Protostelium nocturnum* fruiting bodies were observed early in the morning (on the first few plates checked for the day). It was conspicuously absent from samples checked later in the day.

Based upon SSU rRNA sequences, freshwater gymnamoebae are reported to be phylogenetically divergent from marine amoebae of the same or similar species (Sims et al. 2002). It would be interesting to make intraspecific comparisons in species of myxomycetes and protostelids that occur on both terrestrial and submerged substrates to see if they represent large populations or if the aquatic and terrestrial examples are habitat specific.

While it is not surprising that eumycetozoans do inhabit lakes and ponds, very few researchers have looked for them in these ecological situations, therefore it is likely that some species have been overlooked or underestimated because of a bias toward thinking of mycetozoans as terrestrial.

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FIGS. 1—2 . 1. *Protostelids isolated from aquatic habitats*. 2. *Myxomycetes isolated from aquatic habitats*.

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TABLE I. Collection sites

No.	Description	Location	Lat. (°N)	Long. (°W)	Elev. (M)	Myxomycete samples		Protostelid samples	
						plates[a]	collec.[b]	pieces[c]	obs.[d]
1	Farm pond	Cincinnati, AR	36.0215	94.5059	360	16	5	64	55
2	Farm pond	Muskogee, OK	35.7743	95.4179	168	4	2	48	58
3	Lake	Carter's Landing, Tenkiller Lake, OK	35.7980	94.8914	194	8	7	40	25
4	Rose Garden Pond	Honor Heights Park, Muskogee, OK	35.7707	95.4163	191	14	5	16	9
5	Large Pond	Honor Heights Park, Muskogee, OK	35.7692	95.4193	176	0	0	8	6

[a] Number of myxomycete moist chambers. [b] Number of myxomycete collections deposited into UARK(M). [c] Number of pieces of substrate plated onto protostelid primary isolation plates. [d] Number of protostelid clones observed.

Figure 1: Species of protostelids isolated from aquatic habitats.

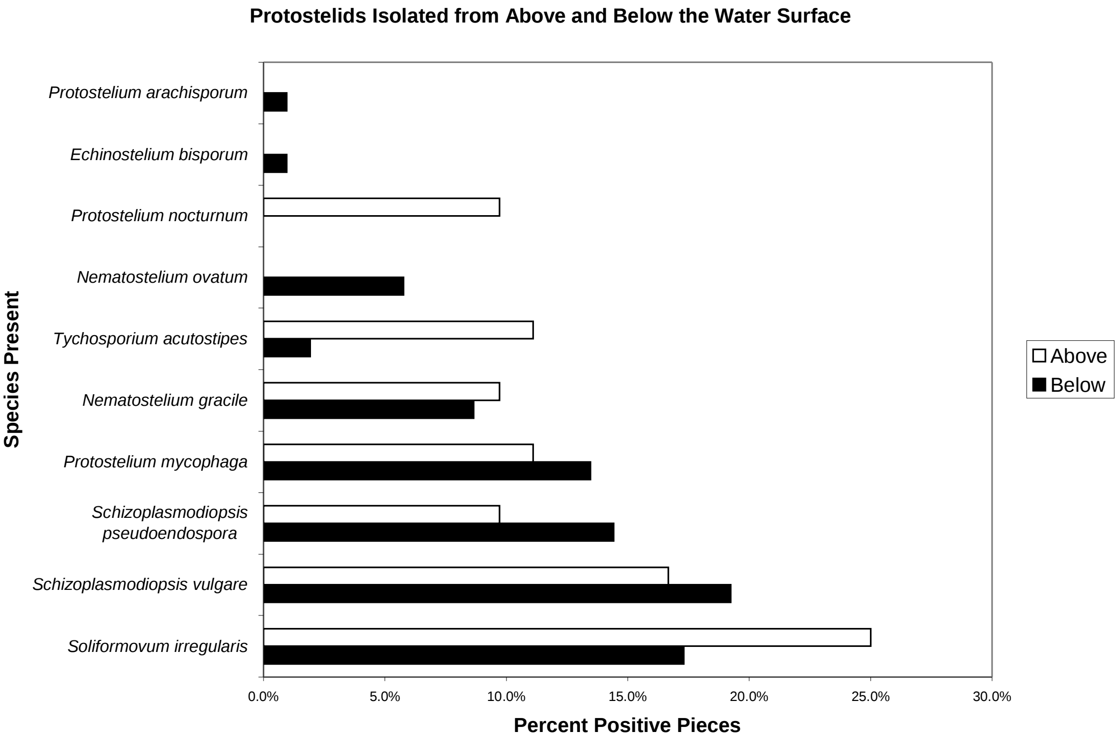
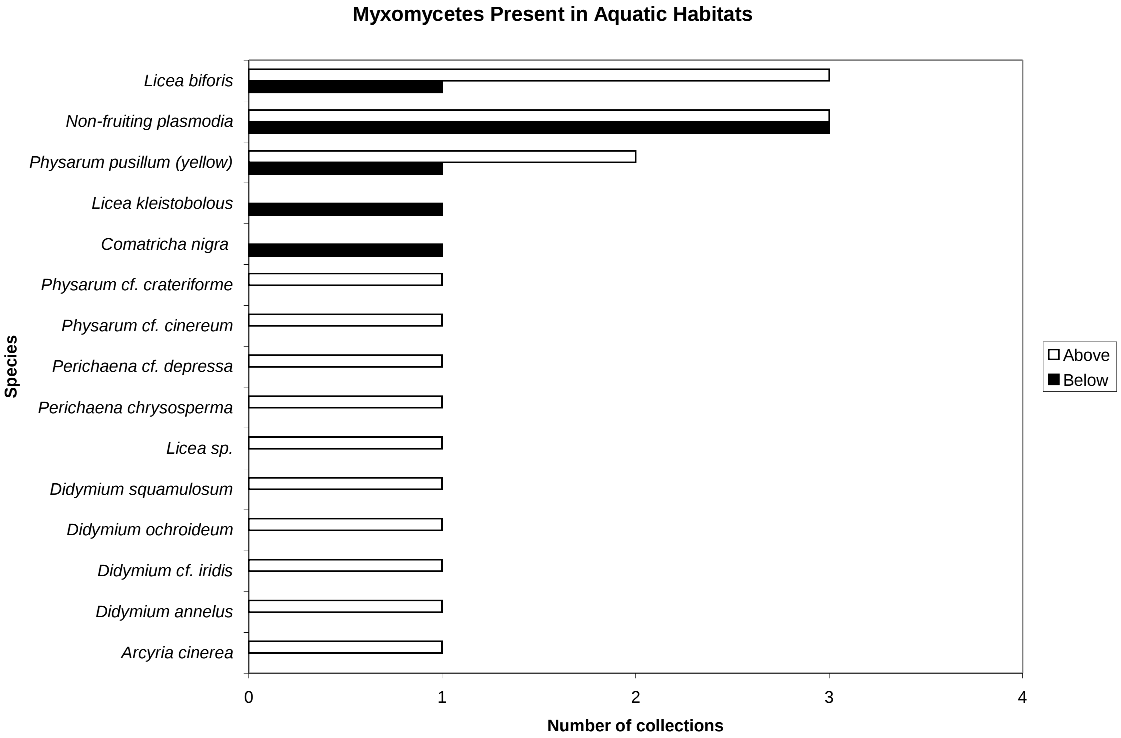


FIGURE 2: Species of myxomycetes isolated from aquatic habitats.



CHAPTER 3

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VARIATIONS IN NUCLEOLAR MORPHOLOGY IN EUMYCETOZOANS

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ABSTRACT

While most biologists are familiar with nuclei that have a single, central, more or less spherical nucleolus, there are many variations on this theme. Several of these permutations are found in the various amoebae of eumycetozoans. These differences in nucleolar morphology may have some phylogenetic significance, but are clearly useful in helping researchers to identify the taxa in which they occur. Using a combination of microscopy techniques, we illustrate the typical nucleoli found in myxomycetes, most protostelids, dictyostelids, and the protostelids *Soliformovum* spp. and *Echinosteliopsis oligospora*. We emphasize the previously unpublished details of *E. oligospora.*

KEY WORDS

Dictyostelid, *Echinosteliopsis*, Microscopy, Myxomycete, Protostelid

VARIACIONES EN LA MORFOLOGIA NUCLEOLAR EN EUMYCETOZOOS

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RESUMEN

A pesar de que la mayoría de biólogos esta familiarizada con el tipo de núcleo celular que posee un solo nucleolo, normalmente en el centro y más o menos de forma esférica, hay muchas variaciones al respecto. Muchas de las permutaciones pueden ser encontradas en las diferentes amebas de los eumycetozoos. Estas diferencias en la morfología nucleolar pueden tener algún significado filogenético y son muy utilizadas por los investigadores para identificar los taxones en los cuales se encuentran. Mediante una combinación de técnicas microscópicas, se ilustran los nucleolos típicos encontrados en mixomicetes, dictiostélidos y los protostélidos *Soliformovum* spp. y *Echinosteliopsis oligospora*.

PALABRAS CLAVE

Dictiostélido, Microscopio, Mixomicete, Protostélido

INTRODUCTION

The slime molds in the taxon *Eumycetozoa* Olive [10, 1] are a remarkable group. Many of their unique qualities, such as the plasmodium of myxomycetes and the cooperative multicellular aggregations of dictyostelids, are well known for their important applications throughout the biological sciences. Less widely appreciated is the array of striking differences within the eumycetozoans. Here we illustrate one such difference: an unusual degree of intraclade variation in nucleolar morphology.

The Mycetozoa (or slime molds) is a polyphyletic group of amoebae that produce fruiting bodies consisting of a stalk and one or more spores [10, 29]. A subset of the Mycetozoa that is hypothesized to be monophyletic is the Eumycetozoa [10].

The taxon Eumycetozoa consists of three groups: protostelids (Protostelia), dicytostelids (Dicytostelia), and myxomycetes (Myxogastria).

Protostelids have a variety of life-cycles that range from simple (amoeba - fruiting body -

amoeba) to more complex (amoeboflagellate - obligate amoeba - fruiting body -amoeboflagellate) [See 20 for illustrations, 27 for definitions]. Dictyostelids have life-cycles that include free-living amoebae that aggregate to form multicellular fruiting bodies with stalks [15]. The myxomycete life-cycle consists of amoeboflagellates, plasmodia (obligate amoebae), and fruiting bodies with spores that germinate as amoeboflagellates [30].

The dictyostelids and myxomycetes are groups that are each clearly monophyletic based on life-cycle characters, amoebal morphology, and molecular systematics [15, 10, 21, 2, 3]. The protostelids are paraphyletic and show greater variation in life-cycle and morphology than the other groups [10, 21, 22, 29]. Spiegel [21, 22, 27] divided the protostelids into 8 groups [Table 1]. Groups that contain species with amoeboflagellates we consider to be eumycetozoans. Among the completely non-flagellated groups of protostelids, Spiegel [21] hypothesized some to be eumycetozoans and others to be non-eumycetozoans [Table 1].

Our interest is in the comparative morphology of eumycetozoan amoebae. In dictyostelids, with the possible exception of size, no major morphological differences have been recorded among the amoebae of the roughly 100 species [15, 10, 29]. The amoeboflagellate state of all the nearly 1,000 described species of myxomycetes is identical and there are only a few variations in plasmodial morphology [6, 10, 20, 21, 26]. Considerable variation is found among the amoebae of protostelids [21, 22, 23, 25, 27, 26, 19, 24].

Nucleolar morphology is a highly variable character within the *Eumycetozoa*, but it is a stable morphological feature of individual taxa (Table 1). For instance, as has already been implied, the nucleoli of all dicytostelids are thought to be identical and distinct from any other eumycetozoan nucleoli [15]. The nucleoli of myxomycetes, also indistinguishable from one another, are the typical round, central nucleoli that we often think of as the “general” eukaryotic nucleolus [7]. Protostelids, however, show a range of nucleolar variation [21, 26, 16]. Within organisms commonly referred to as protostelids there are at least three distinct types of nucleoli. First, the myxomycete-like (Group 5) and the other flagellated protostelids (Groups 1, 2, and 4) contain the single, central, spherical nucleolus identical to those found in myxomycetes [23]. Second, nucleoli of the genus *Soliformovum* (Group 3) which contains two species, *S. irregularis* and *S. expulsum*, are irregularly shaped and diffused throughout the cell [26]. Finally, the protostelid *Echinosteliopsis oligospora*, Eumycetozoa *incertae sedis*, has what appear to be multiple, peripheral nucleoli [16, 17].

Here we illustrate four of the types of nucleoli found within the Eumycetozoa, including the first transmission electron micrographs published for *Echinosteliopsis oligospora.*

MATERIALS AND METHODS

Cultures—The protostelids *Protostelium mycophaga* Type (ATCC PRA-154), *Soliformovum irregularis* Mex 81 (ATCC 26826) and *Echinosteliopsis oligospora* HIO4-33a-3a (ATCC PRA-125) were all cultured on weak malt yeast extract agar (wMY [21]) with *Rhodotorula mucilaginosa*, *Flavobacterium* sp., and *E. coli*, respectively, as their food sources. *Polysphondylium violaceum* (local isolate) was grown on wMY with *E. coli.* All were grown in the laboratory at ambient temperatures (approx. 21-25C).

Light microscopy— Agar coated slides [28] were prepared and inoculated with amoebae of each species and allowed to acclimate for approximately one hour in a Petri dish. Amoebae, cysts and spores were then observed on a Zeiss Axioskop 2 Plus under the 40x dry objective using both phase contrast and DIC techniques and photographed using Auto Montage (Syncroscopy).

Transmission Electron Microscopy— A 1cm square piece of agar containing a feeding front of *Echinosteliopis* amoebae was placed amoeba side down into a formvar coated fixation boat containing Karnovsky's fixative and fixed under weak vacuum. After 30 seconds, the sample was rinsed 3X in 0.05 M cacodylate buffer and post fixed in the dark for 30 min in 1% osmium tetroxide, buffered in 0.05M cacodylate buffer. During this time the agar block was floated off the sample and removed. The sample was rinsed 1X in distilled water and prestained overnight in 0.5% uranyl acetate. The uranyl acetate stained sample was dehydrated in a graded ethanol series, 1 min/change (30%, 50%, 70%, 80%, 95%, 3 changes of 100%). Samples were further dehydrated by 2 changes of propylene oxide at 20 min/change, then infiltrated with 50%-50% propylene oxide-Spurr's medium for 1 hour. *Echinosteliopsis* was infiltrated overnight in 100% Spurr in fresh desiccator. After 12 hours a thin layer (> 1 cm) of fresh 100% Spurr's medium was poured over the sample and placed under the vacuum for several hours and then put into a 70C oven overnight. The fixation boat was cut off of the sample and amoebae were identified under the compound microscope. The block was trimmed around the amoebae, sectioned with a diamond knife, placed on copper grids, and post-stained as per standard protocol: rinsed for a few seconds in ddH_20 then stained in uranyl acetate 2% for 4 min. The section-containing-grids were rinsed again and placed in lead citrate for 2 min. then rinsed a final time in water. Grids were observed and photographed in a JOEL 100 CX transmission electron microscope. *Polysphondylium violaceum* amoebae were prepared similarly except that they were fixed in suspension, pelleted by centrifugation between each step, and the pellets embedded in Spurr blocks.

RESULTS

The four variations of nucleoli seen thus far in eumycetozoans are illustrated with light microscopy in Fig 1. The single, central, homogeneous spherical to subspherical type of nucleolus is represented by *P. mycophaga* (Fig 1a). A diffuse, lobed, central nucleolus is typical of the genus *Soliformovum* (Fig 1b). Dictyostelids, represented here by *P. violaceum,* all have a peripheral, reticulate nucleolus that appears as thin straps with enlarged thickenings (Fig 1c). The protostelid *E. oligospora* has one to several nuclei per amoeba and each has numerous peripheral nucleolar bodies and perhaps a small central nucleolus as well (Fig 1d). With through focus examination, the peripheral nucleolar bodies appear to be distinct and not joined into a reticulum.

The ultrastructure of the peripheral nucleoli of *E. oligospora* and the dictyostelids, represented by *P. violaceum*, are distinct from each other (Fig 2). Many electron dense, peripheral bodies were observed in the nuclei of *Echinosteliopsis oligospora* under transmission electron microscopy (Fig 2a,b) These dark nuclear constituents contain a core region of greater electron density and an outer region of less electron density. Sections of the same nucleus indicate that the electron dense core regions are spherical and disjunct from other such regions. The surrounding less electron dense regions are irregular in shape and may or may not be interconnected. The less electron dense portions of the nucleoli are not closely appressed to the nuclear envelope and tend to have half-moon shaped pits filled with nucleoplasm in the areas immediately opposite nuclear pores (Fig 2b). In addition, at least one nucleolar body appears to be located near the center of the nucleus.

Conversely, the nucleoli of *P. violaceum* (Fig 2c) are essentially uniformly electron dense. They are closely appressed to the inside of the nuclear envelope, and there is no indication of a portion of the nucleolus in the center of the nucleus.

DISCUSSION

Single, Central Nucleolus— The typical single, central, round eukaryotic nucleus displayed in the myxomycetes and protostelids (Groups 1, 2, 4, and 5) is not static. In these organisms, as in other "typical" eukaryotes, the nucleolus degenerates when the nucleus divides mitotically [8, 12, 9, 11, 13]. After telophase, multiple nucleoli appear in the reorganizing nucleus of each daughter cell depending on the species [12, 9]. These eventually fuse into a single centrally located nucleolus, but are reported to persist for some time before fusion [9]. This observation contributed to Olive's 1967 hypothesis that the dictyostelids arose from a nonflagellated protostelid ancestor [9]. The recognition that there are a number of distinct types of fragmented nucleoli in the eumycetozoans suggests that this simple hypothesis may be incorrect.

Multiple Peripheral Nucleoli— The prominent dark objects within the *Echinosteliopsis* nuclei are assumed to be nucleoli because previous literature has reported that they have the staining properties of RNA [16, 17]. If these are indeed nucleoli, then they appear to be distinct from the large central nucleoli of Groups 1, 2, 4, and 5 protostelids [10, 21] and the myxogastrids [7], the peripheral lobed nucleoli of the dictyostelids [15], and the diffuse, central, lobed nucleoli of *Soliformovum* [26]. We know of no other organism with a nucleolar morphology that is identical to that found in *Echinosteliopsis oligospora.* The odd nucleolar morphology of *Echinosteliopsis oligospora* does not match that of any eumycetozoan. Further phylogenetic analysis is needed before we can understand the evolution of this nucleolar character.

Multiple Peripheral vs. Peripheral Lobed Nucleoli— There are many striking differences between dictyostelid nucleoli and the nucleoli of *Echinosteliopsis.* For instance, the dictyostelid nucleoli are closely appressed to the nuclear envelope, where *Echinosteliopsis* nucleoli generally maintain a spherical electron dense core with some nucleoplasm remaining between the outer nucleolus and the nuclear envelope. Secondly, one might recall that the dictyostelid nucleus typically contains one nucleolus with many lobes which wrap part way around the nucleus much like a person's fingers wrap around a tennis ball, as has been demonstrated with serial sectioning [5]. Conversely the nucleoli of *Echinosteliopsis* seem to contain individual spheres. Finally, dictyostelid nucleoli also tend to lack distinct granular and fibrillar regions within the nucleolar lobes [5, 15], while these are evident in each *Echinosteliopis* nucleolus. Peripheral lobed nucleoli are found in all dictyostelids examined so far [15, 10].

Central, Diffuse Nucleoli— The central, diffuse *Soliformovum* (Group 3) nucleoli are quite different from the nucleoli of other eumycetozoan amoebae [26]. These nucleoli either have granular and fibrillar regions that are diffuse/interspersed with one another or indistinguishable [26]. A nucleolar morphology similar to *Soliformovum* is possibly found in the feeding amoebae of *Schizoplasmodiopsis amoeboidea*, a protostelid that is dissimilar to other members of the genus *Schizoplasmodiopsis* [4]. An open nucleolar arrangement like this has been hypothesized to be important for cells that are rapidly producing ribosomal precursors [18]. The *Soliformovum* type of nucleolus shows no obvious similarity with that of *E. oligospora* or the dictyostelids.

Taxonomic Implications— Intra-clade nucleolar variation does occur in other groups of Eukaryotes, such as the Hartmannellidae and certain other families within Amoebozoa, and is a character frequently used for taxonomic purposes [14]. It has been suggested that the number and size of nucleoli are strongly correlated with the number and lengths of the chromosomal secondary constrictions (nucleolar organizing regions) [18]. However, many evolutionary and developmental questions about nucleolar morphology remain unanswered.

Nucleolar morphology may be a useful character in taxonomy particularly with respect to eumycetozoans.

Here we have illustrated four nucleolar morphologies present in various eumycetozoan taxa. Of the three major groups of eumycetozoans, the species described as protostelids display the greatest diversity of morphological and life-cycle variations. These morphological variations include three different types of nucleoli within the approximately 36 described species, as compared to one nucleolar morphology for all ca. 100 species of dictyostelids, and one nucleolar morphology for all ca. 1000 species of myxomycetes. Whether a wide array of nucleolar morphologies indicates long evolutionary divergence times, or represents a case of simply controlled morphological variation is still an open question. While nucleolar morphology is a useful character for identification, the biochemical and evolutionary importance of varying nucleolar morphology remains almost completely unknown. To discover the pattern of evolution of nucleolar morphology in the group, a robust phylogeny must be developed.

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Table 1: Nucleolar morphology of Eumycetozoans. Protostelid groupings modified from Spiegel (1990). Dictyostelid taxonomy as in Raper (1984).

		Nucleolar morphology			
Group	Examples	Single, Central, Round	Diffuse	Peripheral Lobed	Multiple Peripheral
Protostelid Group 1	*Protostelium, s.s.*	X			
	Planoprotostelium	X			
Protostelid Group 2	*Ceratiomyxella*	X			
	Nematostelium	X			
	Schizoplasmodium	X			
Protostelid Group 4	*Cavostelium*	X			
	Schizoplasmodiopsis, s.s.	X			
Protostelid Group 5	*Protosporangium*	X			
	Clastostelium	X			
	Ceratiomyxa	X			
Myxomycetes	*Echinostelium*	X			
Protostelid Group 3	*Soliformovum*		X		
Dictyostelids	*Dictyostelium*			X	
	Polysphondylium			X	
	Acytostelium			X	
Eumycetozoa *incertae sedis*	*Protosteliopsis*	X			
	Microglomus	X			
	Echinosteliopsis				X
	Schizoplasmodiopsis amoeboidea		X		
Non-Eumycetozoa *incertae sedis*	*Endostelium*	X			
	Protostelium arachisporum	X			

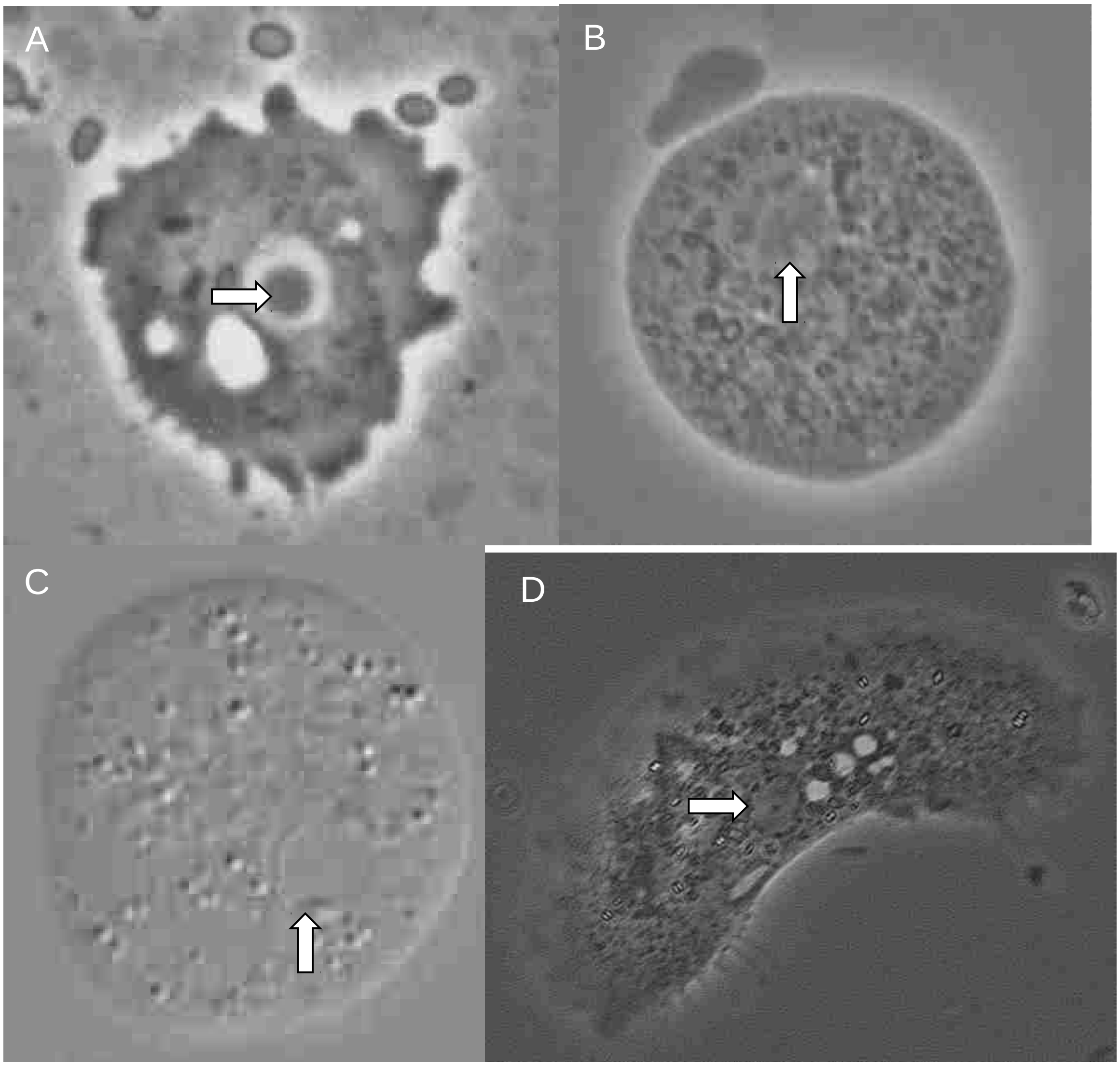

Figure 1: Mycetozoan nucleolar morphologies. Nucleoli indicated by white arrows a) phase contrast *Protostelium mycophaga* amoeba with single, central nucleolus b) phase contrast *Soliformovum irregularis* amoeba with diffuse nucleolus c) differential interference contrast of *Polysphondylium violaceum* amoeba with peripheral lobed nucleolus d) phase contrast *Echinosteliopsis oligospora* amoeba showing multiple peripheral nucleoli.

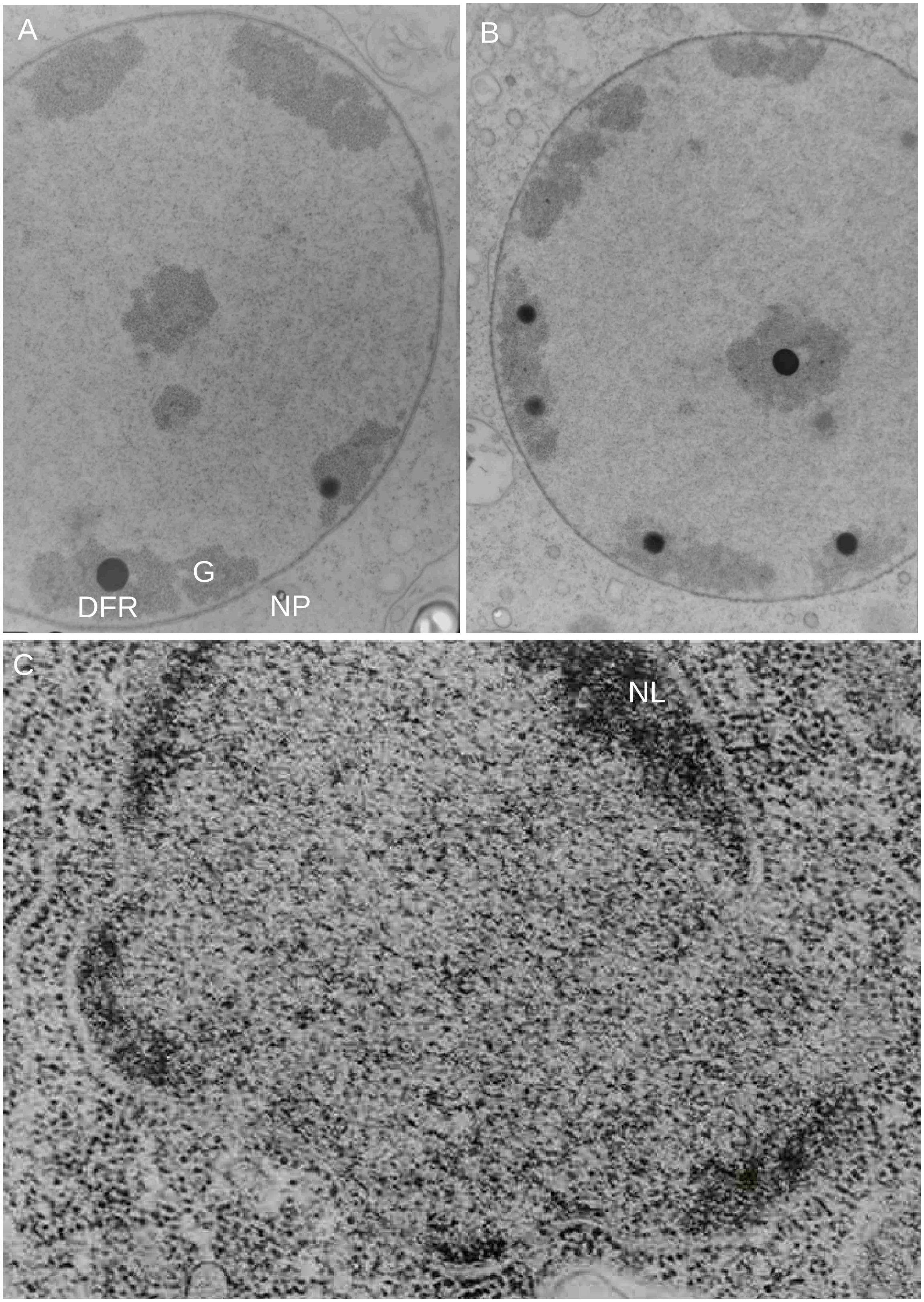
A
G
DFR
NP
B
C
NL

Figure 2: Transmission electron micrographs of different sections through *Echinosteliopsis oligospora* round nucleus with section through a) smaller diameter section b) larger diameter section. Note discrete dense fibrillar region, diffuse granular component, and pits in granular component around nuclear pores. Dense Fibrillar Region (DFR). Granular (G). Nuclear Pore (NP). Compare with section through Dictyostelid nucleus c) *Polysphondylium violaceum* peripheral lobed nucleoli. Nucleolar lobe (NL).

CHAPTER 4

SHADWICK LL, SPIEGEL FW, SHADWICK JDL, BROWN MW, SILBERMAN JD (2009) EUMYCETOZOA = AMOEBOZOA?: SSURDNA PHYLOGENY OF PROTOSTELOID SLIME MOLDS AND ITS SIGNIFICANCE FOR THE AMOEBOZOAN SUPERGROUP. PLOS ONE 4(8): E6754. DOI:10.1371/JOURNAL.PONE.0006754

EUMYCETOZOA = *AMOEBOZOA*?: SSURDNA PHYLOGENY OF PROTOSTELOID SLIME MOLDS AND ITS SIGNIFICANCE FOR THE AMOEBOZOAN SUPERGROUP

running head: Protosteloid Amoebozoans

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ABSTRACT

Amoebae that make fruiting bodies consisting of a stalk and spores and classified as closely related to the myxogastrids have classically been placed in the taxon *Eumycetozoa*. Traditionally, there are three groups comprising *Eumycetozoa*: myxogastrids, dictyostelids, and the so-called protostelids. Dictyostelids and myxogastrids both make multicellular fruiting bodies that may contain hundreds of spores. Protostelids are those amoebae that make simple fruiting bodies consisting of a stalk and one or a few spores. Protostelid-like organisms have been suggested as the progenitors of the myxogastrids and dictyostelids, and they have been used to formulate hypotheses on the evolution of fruiting within the group. Molecular phylogenies have been published for both myxogastrids and dictyostelids, but little molecular phylogenetic work has been done on the protostelids. Here we provide phylogenetic trees based on the small subunit ribosomal RNA gene (SSU) that include 21 protostelids along with publicly available sequences from a wide variety of amoebae and other eukaryotes. SSU trees recover seven well supported clades that contain protostelids but do not appear to be specifically related to one another and are often interspersed among established groups of amoebae that have never been reported to fruit. In fact, we show that at least two taxa unambiguously belong to amoebozoan lineages where fruiting has never been reported. These analyses indicate that we can reject a monophyletic *Eumycetozoa, s.l.* For this reason, we will hereafter refer to those slime molds with simple fruiting as protosteloid amoebae and/or protosteloid slime molds, not as protostelids. These results add to our understanding of amoebozoan biodiversity, and demonstrate that the paradigms for understanding both nonfruiting and sporulating amoebae must be integrated. Finally, we suggest strategies for future research on protosteloid amoebae and nonfruiting amoebae, and discuss the impact of this work for taxonomists and phylogenomicists.

INTRODUCTION

A microscopic drop of water resting upon the tip of a fine hair, this is the search image for organisms historically called protostelids. When researchers see this, they know they might be looking at a protostelid fruiting body. The so-called protostelids are amoebae that make simple fruiting bodies consisting of a delicate stalk that supports one or a few spores (Figure 1) [1-3]. Other fruiting amoebae, the dictyostelids and myxogastrids (also referred to as myxomycetes), make relatively complex fruiting bodies with many cells: the dictyostelids by aggregative fruiting and the myxogastrids by division of large, multinucleate cells into uninucleate spores [1,4,5]. Olive [1, see also 2,6] thought that the simplicity of protostelid fruiting bodies suggested that the ancestors of dictyostelids and myxogastrid amoebae might have made protostelid-like fruiting bodies. Olive called this group the taxon *Eumycetozoa* and envisioned the monophyletic taxa *Myxogastria*, and *Dictyostelia* arising from a paraphyletic taxon *Protostelia* (Figure 251 of [1]) [7]. Different interpretations of morphology by both Olive and Spiegel were used to call this idea into question [1,2,6,8]. However, early molecular phylogenies that included species from all three groups suggested that there might be a clade of eukaryotes that includes myxogastrids, dictyostelids, and protostelids [8-10]. Was this support for the taxon *Eumycetozoa?*

Since 1654 when the first record of a myxogastrid was purported to be a fungus, the insidious perception that fruiting body formation has phylogenetic relevance has perpetuated a divide between those biologists who study amoebae and those biologists who study amoebae that fruit (for reviews see [1,11,12]). Fruiting amoebae are those amoebae that make spores, usually supported by stalks, at some point during their life-cycle, and are typically studied by classically trained mycologists. These amoebae are identified, isolated, and described beginning with their fruiting bodies (for reviews see [1-5,11]). Amoebae that are not known to fruit are typically studied by classically trained protistologists. Such amoebae are identified, isolated, and described by their amoebal morphology and sometimes by their cysts (for review see [13,14]). The reasons for this scientific divide are historical and methodological, not biological.

Until the last decade, when molecular phylogenies began to show otherwise, amoebae were thought of as a polyphyletic assemblage of eukaryotes. Baldauf *et al.* [10] were the first to show that some classical amoebae and some dictyostelids and myxogastrids grouped together. There has been a flush of recent molecular phylogenetic evidence showing that some fruiting and some nonfruiting amoebae belong to the supergroup *Amoebozoa* [10,15-27]. Taxonomic sampling of protostelids has been a major limitation in all of these studies. In fact, those protostelids that have been included in phylogenies do not span the breadth of the purported morphological groups of protostelids [2,8]. In fact, the ribosomal small subunit RNA gene (SSU) sequences from only two very closely related species have been used as exemplars in all the above studies that include any protostelids. We think that the term protostelid has led to confusion in the literature because it implies an evolutionarily cohesive taxonomic unit [2,7], while at the same time, [2,8] the term protostelid is used to describe a morphology [2]. Therefore, to avoid this double meaning we will hereafter refer to these organisms in a descriptive sense as protosteloid amoebae, not as protostelids.

Protosteloid amoebae have simple fruiting structures (Figure 1), and a range of highly diverse amoeboid trophic cells (Figure 2 and [1-3]). Analysis by Spiegel of amoebal morphology as well as fruiting led to five proposed, morphologically identifiable groups of protosteloid amoebae that he thought were good candidates for being closely related to myxogastrids and dictyostelids [2,6]. These five groups include 28 of the 36 species described as protosteloid amoebae. Of the other eight species, one, *Echinostelium bisporum*, is clearly a myxogastrid [28,29], and the rest are of doubtful affinity [2]. Only by including protosteloid amoebae that span this known diversity in analyses with an appropriately broad set of outgroups will it be possible to determine whether there is a clade that corresponds to Olive's [1] hypothesis that there is robust phylogenetic support for the taxon *Eumycetozoa*. If the *Eumycetozoa* hypothesis is correct, then fruiting amoebae should form a monophyletic (or natural) group that includes some protosteloid amoebae, the myxogastrids, and the dictyostelids to the exclusion of nonfruiting amoebae.

To gain insights into the relationships among protosteloid amoebae and where they fit among other amoebae, we have sequenced the SSU of 21 isolates representing 17 species of protosteloid amoebae including multiple representatives of each of the five “eumycetozoan” groups of Spiegel [2] and three other species, *Endostelium zonatum, Protosteliopsis fimicola,* and undescribed protosteloid isolate LHI05, whose morphologies suggest questionable affinity to the other purported eumycetozoans (Figures 1,2). These were included in phylogenetic analyses along with the SSU sequences from a broad range of amoebozoans (for recent reviews of *Amoebozoa* see [19,23,30]), and from a diverse assemblage of outgroup eukaryotes. Several cercozoans and stramenopiles were included, because Spiegel [2,6,8] had suggested members of both groups as possible close relatives to protosteloid amoebae. The SSU gene was chosen because it is the most widely sequenced among amoebozoans and because it has been used to support the phylogenies of a number of clearly monophyletic lineages within *Amoebozoa* [16,17,21,23,26,27,31-36] including dictyostelids [34] and myxogastrids [33]. We included multiple representatives from well supported amoebal lineages in our analysis to (a) look for congruence between our results and other amoebozoan phylogenies and (b) test whether any or all organisms described as protosteloid amoebae fell into a clade of amoebozoans that also included the myxogastrids and dictyostelids, *i.e. Eumycetozoa sensu* Olive [1]. Further, we wanted to know if protosteloid amoebae were indeed a grade of *Eumycetozoa sensu* Olive [1]. We show here that protosteloid amoebae are all members of the supergroup *Amoebozoa* and that there are several discrete lineages that include protosteloid species. There is no evidence for a group that corresponds to *Eumycetozoa sensu* Olive; rather, stalked fruiting is widespread among the supergroup.

RESULTS

The SSU rRNA genes of 21 isolates, representing 17 species of protosteloid amoebae were sequenced to assess their phylogenetic affinities (Table 1). The sequences ranged from 1,786 bp in *Schizoplasmodiopsis pseudoendospora* to 2,493 bp in *Endostelium zonatum* (Table

1). No group 1 introns were observed, and nearly all variation in length was contained within hypervariable regions of the SSU rRNA gene. Those seven isolates with especially short SSU genes <1,850 bp had some truncations in regions that are generally conserved across a diverse array of eukaryotes. Most SSU genes of protosteloid amoebae were AT rich with GC contents ranging from 38% in *Schizoplasmodiopsis amoeboidea* to 50% in isolate LHI05 (Table 1). Within-isolate sequence heterogeneity was detected in nine isolates, and was most extensive in *E. zonatum*, unnamed isolate LHI05, *Protosporangium articulatum*, and all isolates of *Protosteliopsis fimicola* (Table 1).

All of the organisms with protosteloid types of fruiting group within *Amoebozoa* in our maximum likelihood tree (Figure 3). There are several well supported clades that contain protosteloid species. Five of these clades with more than one species include protosteloid amoebae exclusively. We refer to these by the following informal designations: the **protosporangiid clade**, the **protosteliid clade**, the **soliformoviid clade**, the **cavosteliid clade**, and the **schizoplasmodiid clade**. Two species of protosteloid amoebae branch with high support within established, species rich amoebal lineages where no fruiting members have previously been reported. *Protosteliopsis fimicola* is a vannellid (Figures 1K, 2G, 3, 4A), and the undescribed isolate LHI05, is an acanthamoebid (Figures 3, 4B). So far, only one sequenced protosteloid species has no obvious close relatives, *E. zonatum* (Figures 1M, 2I, 3). We had originally included LHI05 in the analysis because its amoeba and mode of fruiting are reminiscent of *E. zonatum* (Figures 1I,M, 2H,I). However, these two taxa do not appear to be specifically related to each other. While all of the protosteloid species branched within a monophyletic *Amoebozoa* in our ML tree, the bootstrap support for a monophyletic *Amoebozoa* is lacking (Figure 3).

The monophyly of myxogastrids and the monophyly of dictyostelids are maintained in our analyses. The myxogastrids form a clade that is divided into the dark spored and light spored lineages [33], and the dictyostelids show the four clades of Schaap *et al.* [34] (Figure 3). Our highest likelihood tree has protosteloid clades as sister to the myxogastrids and dictyostelids. The soliformoviid clade is a poorly supported sister to the myxogastrids and the cavosteliid clade appears as a sister to the dictyostelids, again with weak support (Figure 3).

There is no discrete clade of *Amoebozoa* that exclusively contains all the fruiting species we included in our taxon sample in our highest likelihood tree (Figure 3). That is, we recovered no monophyletic taxon *Eumycetozoa sensu* Olive [1]. There is no clade that exclusively includes the myxogastrids, the dictyostelids, and some subset of the protosteloid species and no nonfruiting amoebae *i.e.* an exclusively fruiting clade that could be consistent with Olive's [1] *Eumycetozoa* hypothesis in a more limited sense.

There is an essentially unsupported clade that occurs in our highest likelihood tree that includes most of the protosteloid species thought to be *Eumycetozoa* by Spiegel [2]. This unsupported clade includes the protosporangiids, protosteliids, soliformoviids, cavosteliids, schizoplasmodiids, myxogastrids and dictyostelids with a number of nonfruiting amoebozoans including archamoebids, *Arachnula*, both *Filamoeba* spp., *Acramoeba*, and the amoebozoan flagellates *Multicilia* and *Phalansterium* (Figure 3).

Three clades that contain both protosteloid amoebae and amoebozoans that have never been reported to fruit were examined in more detail.

Within the poorly supported group that contains the myxogastrids, dictyostelids, many protosteloid amoebae and several nonfruiting amoebozoans, there is an interesting sister group relationship recovered between the schizoplasmodiid clade and the amoebozoan flagellate

Phalansterium solitarium. This group appears with low support in the large tree that is restricted to 1,169 alignable positions (Figure 3). However, *P. solitarium* is alignable with all of the schizoplasmodiids across nearly their entire SSU rRNA genes, including hypervariable regions, such that 1,735 unambiguously aligned positions are amenable to phylogenetic analyses (Figure 4C). More detailed analyses of this region of the tree using *P. solitarium* as an outgroup to the schizoplasmodiids shows that *Nematostelium ovatum* and *Schizoplasmodium cavostelioides* are sister to each other with *Ceratiomyxella tahitiensis* branching basally to them (Figure 4C).

Isolate LHI05 groups with high support within the acanthamoebids with specific and robust affinity to *Protacanthamoeba bohemica* (Figure 4B).

Protosteliopsis is clearly a vannellid with *Vannella placida* as its sister species (Figures 3, 4A). The sister group relationship between *Protosteliopsis fimicola* and *Vannella placida* is upheld when multiple isolates of *P. fimicola* and additional vannellids are included in a fine-scale analysis (Figure 4A).

Hypothesis Testing

Some previous hypotheses about the relationships among the organisms traditionally considered to be eumycetozoans [1] and their relationships with other eukaryotes were not compatible with branching patterns recovered in our maximum likelihood tree. To test these hypotheses we built topologically constrained trees and compared their likelihoods to our maximum likelihood trees and bootstrap trees using the Approximately Unbiased (AU) test. Table 2 lists some important hypotheses of relationships among the purported eumycetozoans and other organisms that have been listed in the literature. Rejection of constrained trees was established at an AU test *p*-value of 0.05.

Brief descriptions of some of the more interesting AU test results follow. A group that exclusively contains all the protosteloid amoebae is rejected (Table 2). *Eumycetozoa* in the strictest sense, *i.e.*, a group that includes all the protosteloid species, myxogastrids, and dictyostelids to the exclusion of other groups is also rejected (Table 2). However, *Eumycetozoa* cannot be rejected if it is defined to include only protosteliids, schizoplasmodiids, soliformoviids, cavosteliids, protosporangiids, dictyostelids, and myxogastrids, where, *E. zonatum, P. fimicola,* isolate LHI05, and all nonfruiting amoebozoans are excluded from the constraint (Table 2).

Within the well supported, species rich clades that contain protosteloid amoebae, *i.e*., the protosteliid clade, the cavosteliid clade, the vannellids, and the acanthamoebids, some hypotheses can be rejected and others cannot. In the protosteliid clade, while *Planoprotostelium aurantium* is nestled within the protosteliid clade in all of our highest likelihood trees, it cannot be rejected as sister to *Protostelium* (Table 2). Likewise in the cavosteliid clade, *Cavostelium apophysatum* cannot be rejected as the sister to all other cavosteliids (Table 2), nor can the protosporangiids be rejected as sister to *Myxogastria* (Table 2). Trees in which LHI05 was excluded from the acanthamoebids were soundly rejected (Table 2). *Protosteliopsis fimicola* branched as the sister taxon to the remaining vannellids when it was excluded from that group, and this was a relationship that could not be rejected (Table 2).

Endostelium zonatum was not rejected as the sister group to any lineage of eukaryotes except *Flamella* sp. (formerly Lobosea sp. “Borok” [26]) and apicomplexans (Table S2).

DISCUSSION

Our findings show that the organisms formerly called protostelids are scattered among *Amoebozoa.* Our trees clearly show: 1) as expected, that protosteloid amoebae are not monophyletic [1,2,6], and 2) contrary to predictions, they are not a grade within *Eumycetozoa*, *sensu* Olive [1,7]. Therefore, our results justify our decision to reject the term protostelids in favor of the strictly descriptive term protosteloid amoebae. While we recognize that the SSU gene presents a problem in resolving deep structure [19,23,27], it is ideal for delimiting well supported groups of clearly related organisms.

When we look past the obvious trait of fruiting, we do find that there are five such groups containing only protosteloid amoebae and that their morphological identity is clear when all other detailed stages of the life-cycle are considered. These correspond to Spiegel's groups I, II, III, IV, and Va (see Figure 3 and Table 2 in [2]). In brief these groups are described below. Monographic treatments with formal taxonomic revisions are being prepared separately.

The Protosteliid Clade - Group I (100% Bootstrap Support)

This group includes the first described protosteloid amoeba, *Protostelium mycophaga* [37]. Its taxa have amoebae with orange pigment and acutely pointed subpseudopodia (Figure 1A, 2A). There are three points of interest within this group: 1) the branch lengths within the species *Protostelium mycophaga* are relatively long, 2) the species which forcibly discharge their spores, *Protostelium nocturnum* [38] and *Protostelium okumukumu* [39], branch basally, and 3) one member of this clade *Planoprotostelium aurantium* makes an amoeboflagellate cell [40]. It was supposed that *Planoprotostelium* was sister to *Protostelium* because of its ability to make flagella and that this ability was lost once, ancestrally to other members of the clade [1,2,38,40-42]. Our optimal trees do not support that hypothesis because the genus *Planoprotostelium* is embedded within the protosteliid clade (Figure 3). However, when *Planoprotostelium* was constrained outside of *Protostelium* it branched as sister to *Protostelium,* and this relationship was not rejected by the AU test (Table 2). While the AU test does not let us reject *P. aurantium* as sister to the rest of the protosteliid clade, the similarity of its fruiting body [40] to that of

Protostelium mycophaga [37] compared to *Protostelium okumukumu* [39] and *Protostelium nocturnum* [38] is quite clear, and we predict that further analysis of a broader taxon sampling of the protosteliid clade will further support its position within the group rather than as a basal lineage.

The Schizoplasmodiid Clade - Group II (100% Bootstrap Support)

The first schizoplasmodiids were described together under the genus name *Schizoplasmodium* [43] based on the plasmodial trophic state that gives rise to the fruiting bodies, and their shared characteristic of a stalk-spore junction with an annular hilum on the spore that articulates with a knob-like apophysis on the stalk (Figure 1B, C, 2B) [2,43-47]. The plasmodial amoeba has both filose and anastomosing subpseudopodia (Figure 2B), and similar “bead on a string” plasmodial mitosis [2,6,43-46,48,49]. Subsequently, schizoplasmodiids were divided into three genera based on variations in fruiting-body stalk length, presence or absence of ballistospores, and presence of an amoeboflagellate in the life-cycle of *Ceratiomyxella tahitiensis* [2,45,50]. While our 129 taxa tree groups all three species, *C. tahitiensis, Nematostelium ovatum,* and *Schizoplasmodium cavostelioides* together with 100% bootstrap support (Figure 3), a more inclusive mask was required in order to recover the branching order among the three species (Figure 4C). The fine-scale analysis resolved that the two non-flagellates, *N. ovatum* and *S. cavostelioides,* are sister to one another with 96% bootstrap support and a Bayesian posterior probability of 0.95 (Figure 4C), which suggests that the flagellate state may have been lost once in this group as previously supposed [46]. However more taxon sampling of the non-flagellate schizoplasmodiid species, including *Schizoplasmodium obovatum, Schizoplasmodium seychellarum,* and *Nematostelium gracile,* will be necessary to resolve this group completely. Given the large number of morphological synapomorphies and the nearly identical SSU rRNA gene sequences, maintaining three genera within the schizoplasmodiid clade may not be well justified. For instance, the separate genus names may well have served to confuse researchers with little firsthand knowledge of protosteloid amoebae, leading them to misclassify members of this clade [51].

The Soliformoviid Clade - Group III (100% Bootstrap Support)

The genus *Soliformovum* includes two species with identical fan-shaped amoebae with acutely pointed subpseudopodia and indistinct, diffuse nucleoli (Figure 2C) [2,52,53]. Both species make a characteristic prespore cell that resembles a “sunny-side-up” fried egg, the character for which the genus is named [2,52].

The Cavosteliid Clade - Group IV (65/69% Bootstrap Support)

This is by far the most morphologically diverse clade of protosteloid amoebae. The cavosteliids all have relatively thin amoebae (Figure 2D,E), with filose subpseudopodia, although flagellates and plasmodia also occur as additional stages within some species of the group (see Figure 3 of [2]). Most have round, centrally located nucleoli, except *Schizoplasmodiopsis amoeboidea,* which has indistinct, diffuse nucleoli similar to those seen in *Soliformovum* [2,52,54]. They all have sculpturing on their spore walls, and the spores are not deciduous [54-57]. Many of these species are common. We were surprised that *Tychosporium acutostipes* branched within this group. Spiegel *et al.* [57] had placed *Tychosporium* as a basal *Protostelium* noting similar prespore cells and some aspects of amoebal morphology, but *Tychosporium* lacked the orange pigment [57]. The cavosteliids are a highly diverse and fascinating group that requires more work. For instance, this clade has the lowest bootstrap support of any of the morphological groups, but support for the group and for nearly every node within the group jumps to nearly 100% if *Cavostelium apophysatum* is removed from the analysis (data not shown). Removal of *Cavostelium apophysatum* from the group was not rejected by the AU test (Table 2). However, we still tentatively accept this clade because of its morphological identity [2].

The Protosporangiid Clade - Group Va in part (100% Bootstrap Support)

The two species of Group Va that we included, *Protosporangium articulatum* and *Clastostelium recurvatum,* have essentially identical life cycles, essentially identical amoeboflagellates (Figure 2F) and non-flagellated amoebae, and fruiting bodies with 2-4 spores (Figure 1I, J) (see [2,6,58-62]). Spiegel's [2] group Va also includes *Ceratiomyxa,* for which we do not have sequence data; it has a similar life-cycle and amoeboflagellates [6,63]. Group Va was thought to be sister to the myxogastrids (Spiegel's group Vb) [2,6,61] on the basis of amoeboflagellate ultrastructure. Although the AU test does not allow us to reject this relationship to myxogastrids, we prefer to be skeptical about this hypothesis until further work either supports or fully rejects it. For the same reason, we also remain skeptical about the sister group relationship with *Protostelium* that we recovered with low support in our highest likelihood tree (Figure 3).

Groups VI & VII

Spiegel suggested that *Endostelium zonatum* and *Protosteliopsis fimicola* might be members of amoeboid groups unrelated to other protosteloid amoebae [2]. Our results support this hypothesis. The placement of *Endostelium zonatum* in the SSU tree is equivocal since it has no strong affinities towards any particular amoebozoan taxon (Figure 3, Table S2) [64-66]. Based on similar amoebal morphology and fruiting body development, we thought that our new protosteloid amoeba, isolate LHI05, and *E. zonatum* might be specifically related. However, *E. zonatum* and isolate LHI05 show no close relationship in our highest likelihood trees, though a possible sister group relationship of LHI05 and *E. zonatum,* embedded within the acanthamoebids was not rejected by the AU test (Table 2). If *E. zonatum* were closely related to LHI05, then it would be an acanthamoebid according to the maximum likelihood constrained tree (data not shown). LHI05 is clearly closely related to *Protacanthamoeba bohemica* among the acanthamoebids (Figures 3, 4B) [32]. In fact constraining LHI05 away from *Protacanthamoeba bohemica* results in a maximum likelihood tree that is strongly rejected by the AU test (Table 1). Two of us, L.L. Shadwick and F.W. Spiegel are currently in the process of describing this new species. *Protosteliopsis fimicola* was originally described as a *Protostelium,* then moved to the monotypic genus *Protosteliopsis* because it was so different (lacking both orange pigment and filose subpseudopodia) from the other species of *Protostelium* [67,68]. *Protosteliopsis fimicola* robustly groups within the vannellids (Figure 3). In fact, *P. fimicola* displaces *Vannella epipetala* as the sister to *Vannella placida* (Figure 4A) [69]. In addition, *P. fimicola* shares similar patterns of SSU sequence microheterogeneity with both *V. placida* and *V. epipetala* [36,69]. These molecular phylogenetic findings are consistent with published light and electron-microscopy images [36,67-72]. For instance, *Protosteliopsis fimicola* has the typically conspicuous contractile vacuole, the anterior hyaline veil, the floating form, and the complete lack of uropodia – all typical characters of vannellids [36,67,70]. It is clear from Olive's drawings that he was sometimes observing an amoebal state intermediate between the locomotive form and the floating form, which partially explains his constant assertion that *Protosteliopsis fimicola* makes filose pseudopodia, which both *P. fimicola* and vannellids lack [36,67,72]. In light of the description of *P. fimicola* and the recent clarification of the genus *Vannella,* it is astounding that no one recognized that *P. fimicola* was a vannellid prior to this study. The misclassification of *P. fimicola* is a testament to both the bias that fruiting induces in the minds of researchers, and the lack of clear, published morphological guidelines for classification of vannellids at the time *P. fimicola* was described [36,71-73].

Eumycetozoa Question

Protosteloid amoebae are at the crux of the *Eumycetozoa* hypothesis *sensu* Olive [1]. Stalked fruiting body formation was thought to be a synapomorphy of *Eumycetozoa* [1,7,8]. Purported eumycetozoans, such as sessile myxogastrids, whose fruiting bodies lacked a stalk were thought to be derived from a stalked ancestor [7]. Other morphological characters were used to support the monophyly of *Eumycetozoa, e.g.*, morphology of stalk-producing cells, amoebal morphology and ultrastructure of amoeboflagellates [6,8,52,61,63,74,75]. These characters were also used to delineate the major groups of protosteloid amoebae as discussed above [2]. However, these characters were not considered in the absence of fruiting for comparison to other morphologically similar nonfruiting amoebae; in fact no amoeboid outgroups were considered at all [6,8,74,75]. These additional characters were used, instead, to support fruiting as a character. Just as morphological characters were used to support stalked fruiting as a character, so were early molecular markers [8-10]. Here, we have shown with the most extensive taxon sampling that we are aware of for both fruiting and nonfruiting amoebae that: 1) protosteloid amoebae are not monophyletic, and 2) amoebozoans with stalked fruiting are not monophyletic. We have recovered the same well supported clades that others have recovered for myxogastrids [33], dictyostelids [34], and most well to moderately supported clades of nonfruiting amoebae found consistently in the literature [16,17,21,23,26,27,31,32,35,36]. However, support for the deeper relationships among these groups is lacking as in all SSU trees of *Amoebozoa* [16,17,19,21-23,25-27,31,35,36,51,76].

Rigor demands that we reject the insidious lure of the fruiting body. Our results show, that stalked fruiting, *s.l.*, by amoebozoans, though taken as significant, has no *a priori* phylogenetic significance [1,7,8]. Rather, it is our view that fruiting has to be taken in context with all the characters of an organism's morphological traits and life history before its significance can be understood.

If we wished to argue for a clade to call *Eumycetozoa* that includes myxogastrids, dictyostelids, and some or all of the protosteliid, schizoplasmodiid, soliformoviid, cavosteliid, and protosporangiid clades, then there are several nodes we could select on the tree as basal to such a group (Figure 3). We think this is unwise for two reasons. First, while it is interesting that our trees show that there may be protosteloid sister groups to both dictyostelids and myxogastrids, the affinities are very poorly supported. Second, it must be recognized that almost all of the groups in the part of the tree that includes most of the fruiting organisms have extremely long branches; thus, some of the deep structure in our tree could simply be a result of long branch attraction [76]. We have attempted to alleviate some long branch effects by using a conservative inclusion set of unambiguously alignable sequence and by including multiple representatives of each lineage where possible, but we cannot confidently rule out long branch attraction. Therefore, we think formal taxonomic revision should be restricted only to well supported clades. Further work, such as comparative genomics, will be necessary to resolve the uncertain deeper relationships. We are inclined to be very conservative when using our results to revise the higher level taxonomy of these organisms. In fact, we are strongly disinclined to even propose informal names for poorly supported groups that happen to occur in our highest likelihood tree unless subsequent research provides more support for them. Taxonomic revisions based on poorly resolved phylogenetic nodes only clutter the literature with names that can lead to confusion. Therefore we think it is best, for now, to relinquish the concept of *Eumycetozoa*.

We would like to make our point as strongly as possible. Our results show that stalked fruiting is widespread among the *Amoebozoa*. Thus, *if* we were presumptuous enough to accept 1) that our tree of this amoeboid supergroup (Figure 3) is true, 2) that stalked fruiting in the supergroup has only one origin, and 3) that fruiting is important as a defining character, then the name *Eumycetozoa* Zopf 1885 would be correct for the whole supergroup since the name has taxonomic priority over the name *Amoebozoa* Lühe, 1913 [1,77-79]. However, at least two issues need to be resolved before we would consider formally renaming *Amoebozoa*. First, the evolutionary and developmental origin of fruiting must be understood. Second, the higher order relationships among fruiting and nonfruiting lineages must be resolved.

MATERIALS AND METHODS

Cultures

At least one protosteloid amoeba from each major morphological group of Spiegel [2] was sampled. Type cultures were used where available, as were multiple isolates. When necessary we isolated organisms from nature into monoeukaryotic or dieukaryotic culture as previously described [2,3,21] and other cultures were acquired from culture collections (Table 3).

Protosteloid amoebae were grown on weak malt yeast extract agar plates (wMY) (0.002g malt extract, 0.002g yeast extract, 0.75g K_2HP0_4, 15.0g Difco Bacto Agar, 1.0L deionized [DI] H_20) with appropriate food organisms (Table 3) in the laboratory at ambient temperatures (approx 21-25°C) [2,3]. *Protosporangium articulatum* could not be established in culture.

All cultures were vouchered by rigorous microscopical examination on a Zeiss Axioskop 2 Plus under the 10x dry differential interference contrast (DIC) and bright field (BF), 40x dry (DIC/Phase contrast (PC)), and 63x oil (DIC) to verify the proper taxon identification of each organism, and to check for possible contaminants. All cultured organisms were observed to form fruiting bodies in culture. All cultures were digitally photographed using AutoMontage (Syncroscopy, Frederick, MD). All cultures, even those obtained from ATCC and CCAP except *Schizoplasmodiopsis pseudoendospora* (which later succumbed to a bacterial contamination) were put into a viable frozen stasis in liquid nitrogen and are stored at the University of Arkansas. Many were also deposited at the ATCC (Table 3).

DNA extraction

DNA from cultures was made available for PCR by using a chelex method that requires little starting material and provides few chances for cross contamination of reagents [80]. Chelex solution was 6% (w/v) chelex100 resin (Bio-Rad Laboratories, Hercules, CA) in double distilled, diethylpyrocarbonate treated H_20. Under a dissecting microscope (Nikon SMZ 1500), organisms were scraped off their media using an ethanol-flamed spear-point needle and placed into 150µl of chelex solution. Negative controls include 1) chelex solution only 2) chelex solution + food organism. All were placed into a thermal cycler for 4 hours at 56°C followed by 30 min. at 98°C, then stored at -20°C until needed. *Protosporangium articulatum* was treated differently because we were not able to grow it in culture. Fruiting bodies on their natural substrate were vouchered through photomicroscopy (Figure 1J), then approximately 30 spores were collected with an insect pin (see [21]). DNA was extracted from the collected spores using the MasterPure Complete DNA and RNA Purification Kit (Epicentre, Madison, WI) according to manufacturer's protocols.

Two additional sequences were included to increase phylogenetic signal from the *Myxogastria*. *Lindbladia tubulina* fruiting bodies were a kind gift from Sergey Karpov, Herzen State Pedagogical University, St. Petersburg, Russia. *Trichia decipiens* fruiting bodies were collected from their natural substrate in Halifax Nova Scotia, Canada during the summer of

2001. DNA from each myxogastrid was isolated from spores or maturing plasmodium using PureGene tissue lysis kit, per manufacturer's recommendations.

PCR and DNA sequencing

The SSU from protosteloid amoebae were amplified with either “universal” eukaryote SSU primer pairs Medlin A, Medlin B [81], 30F, 1492R, 5'SSU17!, or specifically designed biased primers PmycF1, PmycR2, Myxo3' (Table 4). The SSU of *Protosporangium articulatum* was obtained by a nested PCR protocol using Medlin A : B in the first PCR reaction that served as template for a second PCR using 30F : 1492R. For the two myxogastrids, *L. tubulina* and *T. decipiens*, SSU rDNA was amplified with the “universal” eukaryotic primers Medlin A : B [81], cloned and sequenced as previously described [21].

For the SSU from protosteloid species, a stepdown thermal cycling program was used with Platinum Blue RTS PCR Super Mix (Invitrogen, Carlsbad, CA) in 20µl reactions with the following cycling parameters: preheat lid 105°C, initial denaturation 94°C 1min., followed by 5 cycles of 94°C 30 sec., 1 min. for primer annealing at 60°C followed by 3 min. 5 sec. elongation at 72°C. Then 9 cycles were done with denaturation 94°C for 30 sec., with an initial annealing temperature of 59°C that decreased 1°C/cycle to a final 50°C annealing, elongation for 3 min. 5 sec. at 72°C, followed by 20 cycles as above with a 50°C annealing temperature and an elongation time of 2 min. 5 sec. PCR products were either sequenced directly after removal of unincorporated nucleotides and primers using QIAquick Gel-Extraction kit (Qiagen, Valencia, CA), or they were T/A cloned into TOPO vector pCR4 and transformed into TOP 10 *Escherichia coli* cells per manufacturer’s instruction (Invitrogen). For cultures grown on yeast (where no size difference between the SSU of the yeast and amoeba was seen), amplified and cloned SSU inserts were PCR amplified directly from transformed bacterial colonies and screened by TaqI restriction fragment length polymorphism with TaqαI restriction endonuclease (New England Biolabs, Ipswich, MA) to distinguish between yeast SSU clones and protosteloid SSU clones.

DNA sequencing reactions were performed using big-dye chemistry and resolved on an Applied Biosystems 3100 Genetic Analyzer (Foster City, CA). PCR products were sequenced directly, where possible in both orientations. Otherwise, one or two clones and/or a pool of 6-10 clones were sequenced fully in both orientations. Partial sequence was often obtained from additional clones.

For protosteloid amoebae that grew with yeast as food organisms, we partially sequenced one fungal clone (identified by restriction fragment length polymorphism), and fully sequenced all available (1 to 10) protosteloid clones. In several cases, within-isolate SSU sequence microheterogeneity was observed. In these cases pooled clones and/or PCR products were sequenced plus one or two individual clones through regions of heterogeneity (*Protosteliopsis fimicola*, *Endostelium zonatum*, isolate LHI05). In cases of within-isolate microheterogeneity that inhibited sequencing through certain regions of pooled clones/PCR products, all individual cloned SSUs were sequenced and included in preliminary trees. In all cases those partial sequences clustered tightly together with other sequences from the same isolate. An individual clone from each of these isolates was used for subsequent analyses. All new sequences were accessioned in GenBank (Table S1).

Phylogenetic analyses

Protosteloid amoeba SSU sequences were hand aligned into an existing SSU rDNA multiple sequence alignment in MacClade (Sinauer Associates, Sunderland, MA) [82]. Ambiguous regions in the alignment were excluded from phylogenetic analyses. The largest data set consisted of 129 taxa that span the known diversity of *Amoebozoa* plus a wide array of outgroup taxa that included at least two members from most other major eukaryotic lineages.

Multiple representatives from each sequenced amoebozoan lineage were included to deeply sample the molecular diversity available within *Amoebozoa* and to assess congruence with other amoebozoan phylogenies. Phylogenies were inferred using maximum likelihood (ML) as implemented in PAUP* 4.0 [83], RAxML 7.0.4 [84], and GARLI 0.96 [85] using a GTR + Γ + I model of nucleotide substitution (except RAxML which implemented a GTR CAT model for topology search and a GTR + Γ model for tree optimization, both with 20 rate categories one of which essentially corresponds to invariant sites). Specific model parameters were determined using ModelTest 3.7 [86] selected using the Akaike information criterion (AIC) [87] for analyses run in PAUP*, while RAxML and GARLI were allowed to estimate models during their respective analyses. A single optimum ML tree was inferred in PAUP* while the highest likelihood tree was identified from 300 RAxML and 300 GARLI runs each starting from a different parsimony tree. The optimum ML tree was inferred in RAxML with branch lengths optimized in PAUP* is shown in Figure 3. Topological support for branches was assessed from the consensus of 1,000 ML bootstrap trees inferred in RAxML and GARLI. For the 129 taxa data set Bayesian parameters failed to converge even after 30 million generations.

For all other finer-scale phylogenetic analyses, both ML and Bayesian analyses were performed. ML analyses were performed in PAUP* with the nucleotide substitution model and specific parameters selected for each dataset using ModelTest as implemented in PAUP* [86]. For Bayesian analyses we used Mr.Bayes 3.1.2 [88] with 4 Markov chain Monte Carlo (MCMC) chains in each of two independent runs with nst=6 and rates set to invgamma (corresponding to a GTR + Γ +I model of nucleotide substitution). Trees generated prior to convergence of parameters were discarded as ‘burn-in’. Convergence was detected only after the standard deviation of split frequencies dropped below 0.01 and the sump function provided in Mr.Bayes and the program Tracer (part of the BEAST package) [89] all indicated convergence.

Hypotheses testing

Phylogenetic tree topologies conforming to a variety of specific hypotheses were tested in a likelihood framework. Hypothesis testing was done on the 129 taxa tree (Figure 3) using the Approximately Unbiased (AU) test in the program Consel 0.1i [90,91]. Consel compares likelihoods, but the likelihoods calculated by the programs RAxML and GARLI, which were used to generate topologies and estimate models, are not directly comparable [84,85]. Also, each topology generated by RAxML and GARLI has unique model parameters estimated with it. A single set of model parameters was needed for input into PAUP* so that comparable likelihood values could be calculated for every topology no matter how it was generated. RAxML and GARLI each produce a highest likelihood topology (as calculated within the program). We considered the model parameters associated with that highest likelihood topology to be the optimal model parameters produced by that program. Thus PAUP* was used to calculate likelihoods using the optimal model parameters estimated by RAxML for all of the tree topologies generated in RAxML and GARLI. The likelihoods of tree topologies produced by the two methods produced distributions that overlapped almost entirely. PAUP* was used to calculated likelihoods for all topologies using the model estimated by RAxML for all subsequent analyses. Six hundred ten constraint topologies were created by manually constraining specific taxa to branch together followed by reoptimization of the branching among the remaining taxa. Reoptimization was performed by inferring three ML trees in RAxML with a GTR + Γ model (as specified in RAxML) and keeping the highest likelihood of these trees. Thus, 610 constraint tree topologies (hypotheses) were generated. For a list of constraints see table S2. Site likelihoods, required for import into Consel, were calculated from the optimal ML topologies, the 610 specific constraint topologies and a set of plausible topologies consisting of the 1,000 RAxML and 1,000 GARLI bootstrap topologies in PAUP* using the RAxML substitution model. Significant differences in the likelihood of among all trees were tested by the AU, Shimodaira-Hasegawa (SH), and Kishino-Hasegawa (KH) tests as implemented in Consel 0.1i [90,91].

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COMPETING INTERESTS

The authors have no financial, personal, or professional competing interests that could be construed to have influenced this paper.

ABBREVIATIONS

AU test: approximately unbiased test, MCMC: Markov chain Monte Carlo, ML: maximum likelihood, SSU: small subunit ribosomal RNA gene

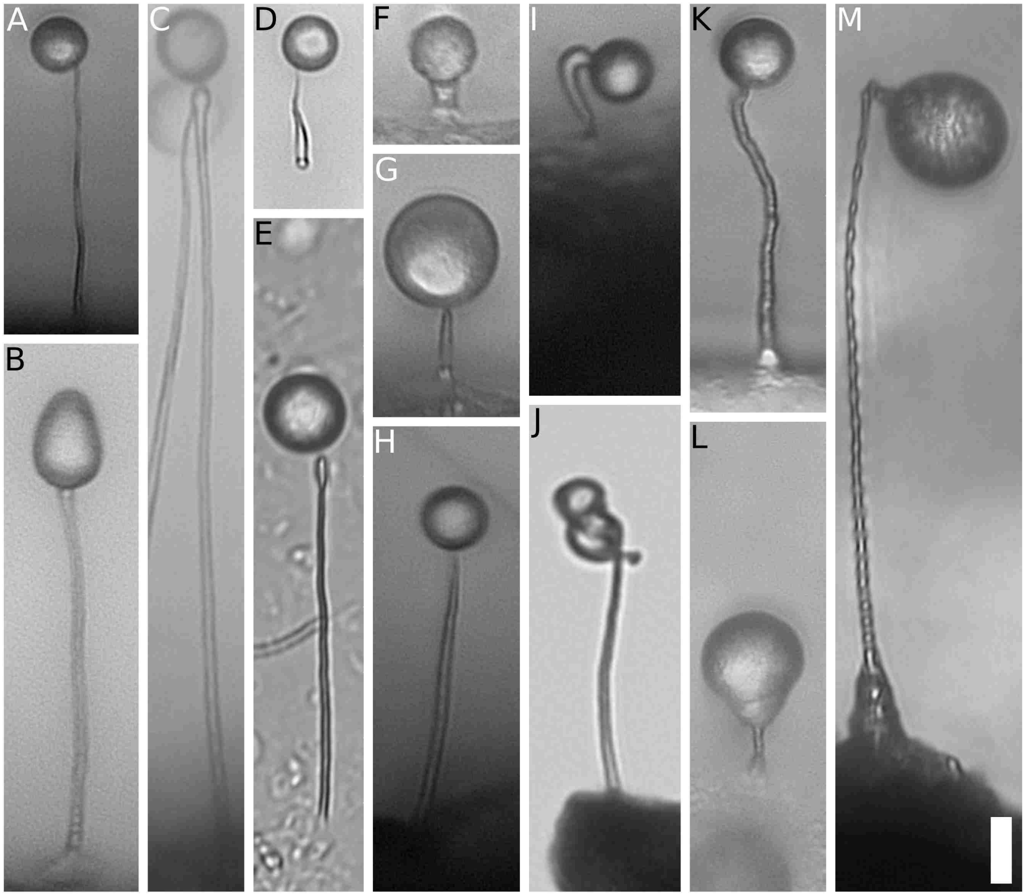

Figure 1. Protosteloid Fruiting Bodies.

Bright field light micrographs of standing protosteloid fruiting bodies on culture or natural substrates (*P. articulatum*). A) *Protostelium mycophaga* brightfield microscopy, B) *Nematostelium ovatum*, C) *Ceratiomyxella tahitiensis*, D) *Soliformovum expulsum*, E) *Soliformovum irregularis*, F) *Cavostelium apophysatum*, G) *Schizoplasmodiopsis amoeboidea*, H) *Tychosporium acutostipes*, I) *Clastostelium recurvatum*, J) *Protosporangium articulatum*, K) *Protosteliopsis fimicola*, L) isolate LHI05, M) *Endostelium zonatum.* Scale bar is 10µm.

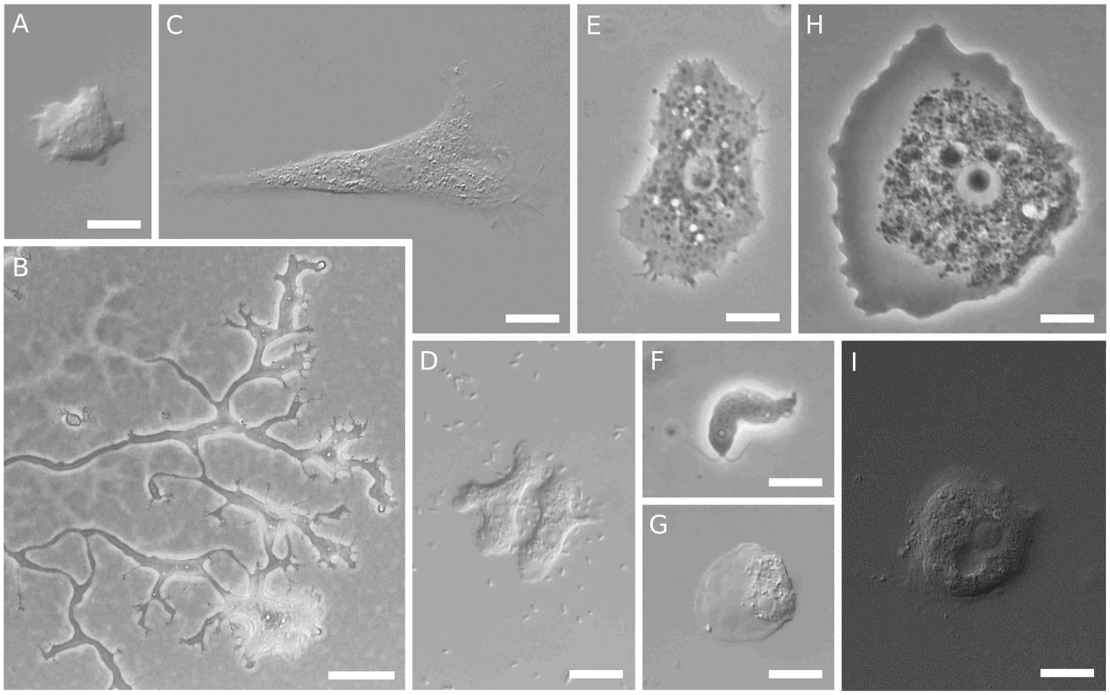

Figure 2. Protosteloid Amoebae.

Light micrographs of protosteloid amoebae. A) *Protostelium mycophaga* differential interference contrast microscopy (DIC), B) *Nematostelium ovatum* (PC), C) *Soliformovum expulsum* (DIC), D) *Cavostelium apophysatum* (DIC), E) *Schizoplasmodiopsis amoeboidea* (PC), F) *Protosporangium articulatum* (PC), G) *Protosteliopsis fimicola* (DIC), H) isolate LHI05 (PC), I) *Endostelium zonatum* (DIC). Scale bars are 10µm, except B which is 50µm.

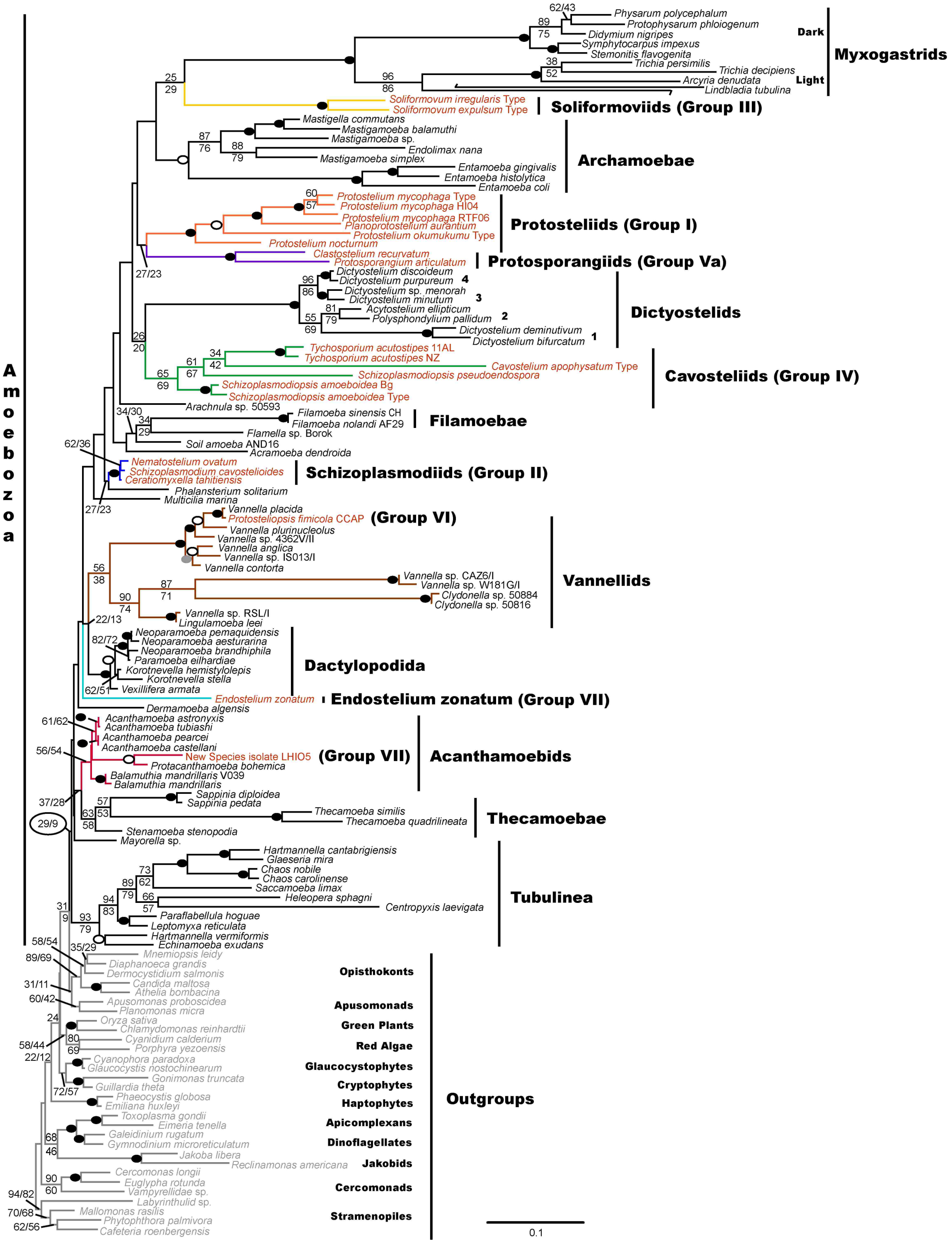
Amoebozoa
Myxogastrids
Dark
Light
62/43
89
75
96
86
38
52
25
29
Physarum polycephalum
Protophysarum phloiogenum
Didymium nigripes
Symphytocarpus impexus
Stemonitis flavogenita
Trichia persimilis
Trichia decipiens
Arcyria denudata
Lindbladia tubulina
Soliformovum irregularis Type
Soliformovum expulsum Type
Soliformoviids (Group III)
Mastigella commutans
Mastigamoeba balamuthi
Mastigamoeba sp.
Endolimax nana
Mastigamoeba simplex
Entamoeba gingivalis
Entamoeba histolytica
Entamoeba coli
87
76
88
79
Archamoebae
60
57
Protostelium mycophaga Type
Protostelium mycophaga HI04
Protostelium mycophaga RTF06
Planoprotostelium aurantium
Protostelium okumukumu Type
Protostelium nocturnum
Protosteliids (Group I)
Clastostelium recurvatum
Protosporangium articulatum
Protosporangiids (Group Va)
27/23
96
86
Dictyostelium discoideum
Dictyostelium purpureum
4
Dictyostelium sp. menorah
Dictyostelium minutum
3
81
79
55
69
Acytostelium ellipticum
Polysphondylium pallidum
2
Dictyostelium deminutivum
Dictyostelium bifurcatum
1
Dictyostelids
26
20
34
42
61
67
65
69
Tychosporium acutostipes 11AL
Tychosporium acutostipes NZ
Cavostelium apophysatum Type
Schizoplasmodiopsis pseudoendospora
Schizoplasmodiopsis amoeboidea Bg
Schizoplasmodiopsis amoeboidea Type
Cavosteliids (Group IV)
Arachnula sp. 50593
34/30
34
29
Filamoeba sinensis CH
Filamoeba nolandi AF29
Flamella sp. Borok
Soil amoeba AND16
Acramoeba dendroida
Filamoebae
62/36
Nematostelium ovatum
Schizoplasmodium cavostelioides
Ceratiomyxella tahitiensis
Schizoplasmodiids (Group II)
Phalansterium solitarium
Multicilia marina
27/23
Vannella placida
Protosteliopsis fimicola CCAP
(Group VI)
Vannella plurinucleolus
Vannella sp. 4362V/II
Vannella anglica
Vannella sp. IS013/I
Vannella contorta
56
38
87
71
90
74
Vannella sp. CAZ6/I
Vannella sp. W181G/I
Clydonella sp. 50884
Clydonella sp. 50816
Vannella sp. RSL/I
Lingulamoeba leei
Vannellids
22/13
Neoparamoeba pemaquidensis
Neoparamoeba aesturarina
Neoparamoeba brandhiphila
Paramoeba eilhardiae
Korotnevella hemistylolepis
Korotnevella stella
Vexillifera armata
82/72
62/51
Dactylopodida
Endostelium zonatum
Endostelium zonatum (Group VII)
Dermamoeba algensis
61/62
Acanthamoeba astronyxis
Acanthamoeba tubiashi
Acanthamoeba pearcei
Acanthamoeba castellani
56/54
New Species isolate LHIO5
Protacanthamoeba bohemica
(Group VII)
Balamuthia mandrillaris V039
Balamuthia mandrillaris
Acanthamoebids
37/28
29/9
57
63
53
58
Sappinia diploidea
Sappinia pedata
Thecamoeba similis
Thecamoeba quadrilineata
Stenamoeba stenopodia
Mayorella sp.
Thecamoebae
Hartmannella cantabrigiensis
Glaeseria mira
Chaos nobile
Chaos carolinense
Saccamoeba limax
Heleopera sphagni
Centropyxis laevigata
73
62
89
79
66
57
94
83
Paraflabellula hoguae
Leptomyxa reticulata
Hartmannella vermiformis
Echinamoeba exudans
31
9
93
79
Tubulinea
58/54
35/29
89/69
31/11
60/42
Mnemiopsis leidy
Diaphanoeca grandis
Dermocystidium salmonis
Candida maltosa
Athelia bombacina
Apusomonas proboscidea
Planomonas micra
Opisthokonts
Apusomonads
24
Oryza sativa
Chlamydomonas reinhardtii
58/44
80
69
Cyanidium calderium
Porphyra yezoensis
Green Plants
Red Algae
22/12
Cyanophora paradoxa
Glaucocystis nostochinearum
Gonimonas truncata
Guillardia theta
72/57
Phaeocystis globosa
Emiliana huxleyi
Glaucocystophytes
Cryptophytes
Haptophytes
Toxoplasma gondii
Eimeria tenella
68
46
Galeidinium rugatum
Gymnodinium microreticulatum
Jakoba libera
Reclinamonas americana
Apicomplexans
Dinoflagellates
Jakobids
Outgroups
90
60
Cercomonas longii
Euglypha rotunda
Vampyrellidae sp.
Cercomonads
94/82
70/68
62/56
Labyrinthulid sp.
Mallomonas rasilis
Phytophthora palmivora
Cafeteria roenbergensis
Stramenopiles
0.1

Figure 3. 129 Taxa SSU Maximum Likelihood Tree of Protosteloid Amoebae, Other Amoebozoans and Eukaryotes as Outgroups. Colored branches indicate lineages in which protosteloid fruiting occurs. Black branches highlight amoebozoan lineages, and gray branches show other eukaryotes used as outgroups. Red, black and gray fonts indicate species of amoebae with protosteloid fruiting, nonfruiting amoebozoans, and other eukaryotes used as outgroups, respectively. To allow the figure to fit legibly on a single page, and to conserve the long branch length, the long branch leading to *Lindbladia* has been broken and shifted above and left. One hundred twenty nine taxa and 1,169 aligned positions were used to infer the optimal maximum likelihood (ML) tree in RAxML 7.0.4 using the following model (GTR + Γ, α= 0.513834, 20 discrete rate categories). ML bootstrap values from analyses of 1,000 RAxML datasets and 1,000 GARLI 0.96 datasets are shown above and below the node respectively. ML bootstrap values: ● 90-100, ○ 80-90, ◍ 70-80, unmarked < 20. Black circle highlights the support values for monophyly of *Amoebozoa*. For the GARLI analyses, the following model was used (GTR + Γ + I, α= 0.71950104, 4 discrete rate categories). The scale bar represents evolutionary distance in changes per site.

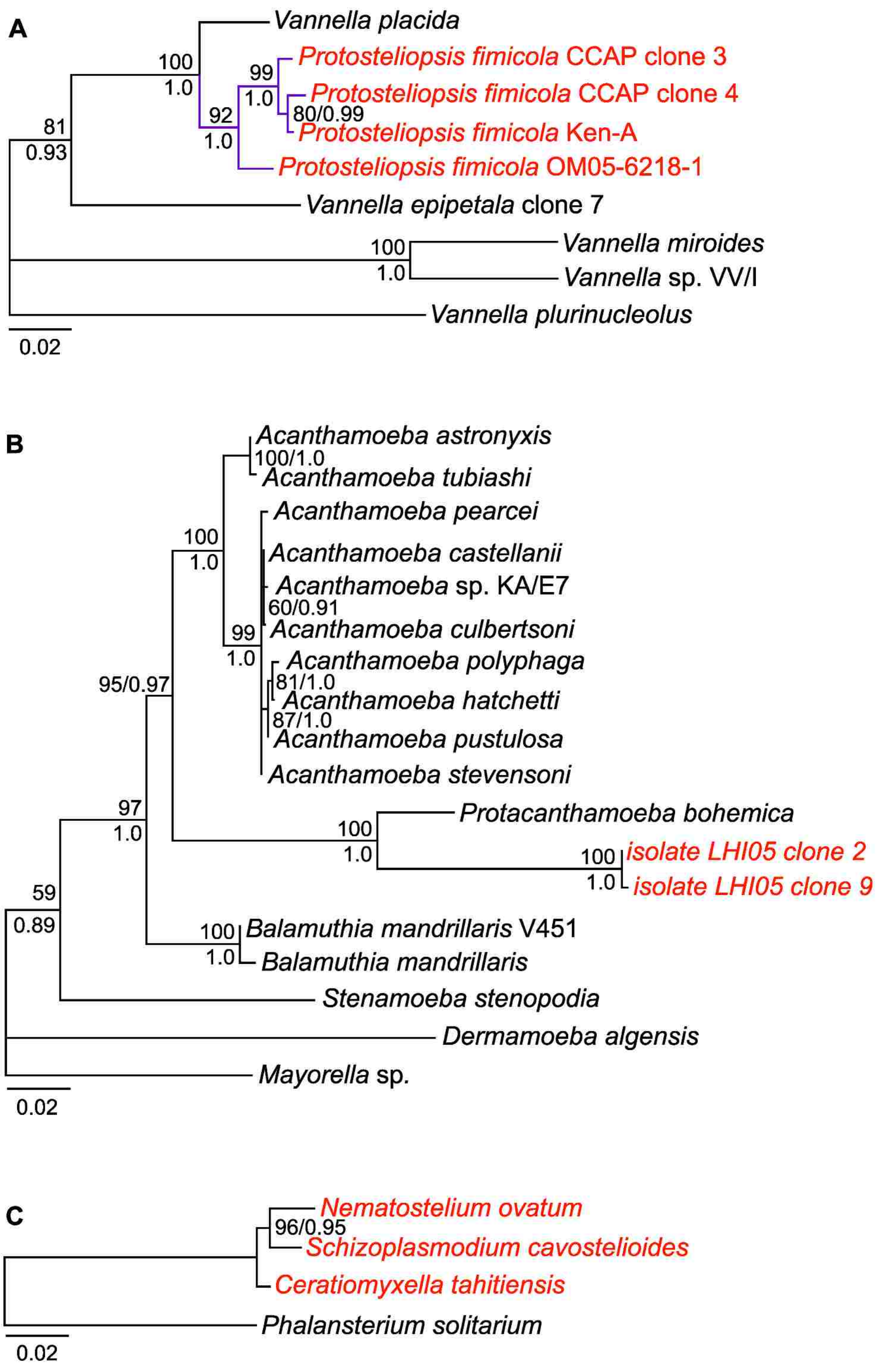
A
Vannella placida
Protosteliopsis fimicola CCAP clone 3
Protosteliopsis fimicola CCAP clone 4
Protosteliopsis fimicola Ken-A
Protosteliopsis fimicola OM05-6218-1
Vannella epipetala clone 7
Vannella miroides
Vannella sp. VV/I
Vannella plurinucleolus
100
1.0
99
1.0
80/0.99
92
1.0
81
0.93
100
1.0
0.02
B
Acanthamoeba astronyxis
100/1.0
Acanthamoeba tubiashi
Acanthamoeba pearcei
Acanthamoeba castellanii
Acanthamoeba sp. KA/E7
60/0.91
Acanthamoeba culbertsoni
Acanthamoeba polyphaga
81/1.0
Acanthamoeba hatchetti
87/1.0
Acanthamoeba pustulosa
Acanthamoeba stevensoni
100
1.0
99
1.0
95/0.97
Protacanthamoeba bohemica
isolate LHI05 clone 2
isolate LHI05 clone 9
100
1.0
100
1.0
97
1.0
59
0.89
Balamuthia mandrillaris V451
Balamuthia mandrillaris
100
1.0
Stenamoeba stenopodia
Dermamoeba algensis
Mayorella sp.
0.02
C
Nematostelium ovatum
96/0.95
Schizoplasmodium cavostelioides
Ceratiomyxella tahitiensis
Phalansterium solitarium
0.02

Figure 4. SSU Maximum Likelihood Trees Assessing Placement of Protosteloid Amoebae within Selected Clades. For all trees, the scale bars represent evolutionary distance in changes per site. Red font indicates protosteloid amoebae. ML bootstrap values from analyses of 1,000 datasets and Bayesian posterior probabilities are shown above and below the nodes respectively. A) Placement of *Protosteliopsis fimicola* among vannellids. ML tree of 9 SSU genes and 1,837 aligned positions inferred with a GTR + Γ + I (α= 0.5042, 4 discrete rate categories, and I=0.3421) model of nucleotide substitution. For the Bayesian analyses two runs, each consisting of 4 MCMC chains, were run for 2,000,000 generations, sampling every 100th tree. The first 100 trees were discarded as burnin after assessing for convergence of parameters. Purple branches highlight *Protosteliopsis fimicola* clade. B) Placement of protosteloid Isolate LH105 among acanthamoebids. ML tree of 18 ssu genes and 1,476 aligned positions inferred with a TrN + Γ, α=0.2228, 4 discrete rate categories model of nucleotide substitution. For the Bayesian analyses two runs each consisting of 4 MCMC chains were run for 2,000,000 generations, sampling every 100th tree. The first 4,000 trees were discarded as burnin after assessing for convergence of parameters. C) Branching order of Schizoplasmodiids rooted with *Phalansterium*. Four taxa and 1,735 aligned positions were used to infer the optimal ML tree with a TrN + Γ, α=0.3693, 4 discrete rate categories model. For the Bayesian analyses two runs each consisting of 4 MCMC chains were run for 5,000,000 generations, sampling every 100th tree. The first 5,000 trees were discarded as burnin after assessing for convergence of parameters.

Table 1. Characteristics of Protosteloid Amoeba SSU rRNA Gene Sequences.

Group	Organism	Clone / PCR	bp	%GC	heterogeneity
I	*Protostelium mycophaga* type	PCR	1809	41.8	none
I	*Protostelium mycophaga* HI04	PCR	1819	41.7	none
I (I)	*Protostelium okumukumu* type	PCR	1813	45.2	none
I	*Protostelium nocturnum*	PCR	1800	45.9	1s
II	*Schizoplasmodium cavostelioides*	2 clones	1937	44.6	not detected
II	*Nematostelium ovatum*	1 clone	1918	43.8	not detected
II	*Ceratiomyxella tahitiensis*	1 clone	1883	44.3	not detected
III	*Soliformovum expulsum* type	10 clones	1894	45.3	1y
III	*Soliformovum irregularis* type	1 clone	1898	45.8	not detected
IV	*Cavostelium apophysatum* type	2 clones	1794	47.9	not detected
IV	*Schizoplasmodiopsis pseudoendospora*	1 clone	1786	47.9	not detected
IV(I)	*Tychosporium acutostipes* NZ	1 clone	1835	46.4	not detected
IV(I)	*Tychosporium acutostipes* KE	1 clone	1856	46.3	not detected
IV	*Schizoplasmodiopsis amoeboidea* bg	10 clones	1876	38.4	none
IV	*Schizoplasmodiopsis amoeboidea* type	10 clones	1927	38.6	1r,1y
Va	*Protosporangium articulatum*	5 clones	2312	40.3	4r,5y,1k
Va	*Clastostelium recurvatum*	1 clone	2119	38.6	not detected

Table 1 (continued). Characteristics of Protosteloid Amoeba SSU rRNA Gene Sequences.

Group	Organism	Clone / PCR	bp	%GC	heterogeneity
VI	*Protosteliopsis fimicola* OM05	PCR	1945	40.5	1y,1d
VI	*Protosteliopsis fimicola* Ken-A	PCR	1970	40.7	4r,6y,1k,1w,2s
VI	*Protosteliopsis fimicola* CCAP	Clone 4 / PCR	1945	40.5	in PCR prod.
VI	*Protosteliopsis fimicola* CCAP	Clone 3 / PCR	1945	40.1	in PCR prod.
VII	*Endostelium zonatum*	Clone 1 / PCR	2493	43.4	in PCR prod.
(VII)	Unnamed LHI05	Clone 9 / PCR	2254	50.1	in PCR prod.
(VII)	Unnamed LHI05	Clone 2 / PCR	2253	50.2	in PCR prod.

SSU sequence length in base pairs (bp), %GC content, and within isolate sequence micro-heterogeneity. Organized by protostelid groups I-VII of Spiegel [2], parenthetical groups proposed in later papers [39,57], or expected based on morphology. For sequence heterogeneity, 'none' = PCR product sequenced and no heterogeneity found, 'not detected' = 1-3 clones sequenced and no sequence heterogeneity detected, type and number of sites exhibiting heterogeneity explicitly noted by standard IUPAC code (s = C or G, y = C or T, r = A or G, k = G or T, w = A or T, d = A or G or T), 'in PCR prod.' = heterogeneity noted in PCR product, sequencing failed through regions of heterogeneity and multiple clones were sequenced individually.

Table 2. *P*-Values for the AU Tests of Selected Hypotheses.

Hypothesis Tested	Constraint Tested	*p*-value	R/NR
Monophyletic protosteloid amoebae (607)	((all protosteloid amoebae) . . .);	0.037	R
Eumycetozoa sensu lato (606)	((all fruiting amoebozoans) . . .);	0.017	R
Eumycetozoa sensu strictu (608)	((I,II,III,IV,Va,D,M) . . .);	0.368	NR
Pla excluded from *Protostelium s.l.* (603)	((*Protostelium s.l.*)*Pla* . . .);	0.127	NR
Ca excluded from cavosteliids (610)	((cavosteliids)*Ca* . . .);	0.476	NR
protosporangiids sister to *Myxogastria* (436)	((Vb,M) . . .);	0.508	NR
LHI05 sister to *Ez* (605)	((LHI05,*Ez*) . . .);	0.077	NR
LHI05 excluded from acanthamoebids (604)	((acanthamoebids)LHI05 . . .);	1x10-5	R
Pf excluded from vannellids (602)	((vannellids)*Pf* . . .);	0.142	NR
AU test control (all taxa together) (601)	(. . .);	0.714	NR

Hypotheses are rejected at a *P*-value of <0.05. R/NR = Rejected or Not Rejected respectively. *P*-values of rejected hypotheses are shown in red. Constraints = ((constrained taxa) excluded taxa, all remaining taxa indicated by . . .);. D = dictyostelids, M = myxogastrids, I = protosteliids, II = schizoplasmodiids, III = soliformoviids, IV = cavosteliids, Va = protosporangiids, LHI05 = Isolate LHI05, *Ez* = *Endostelium zonatum*, *Pf* = *Protosteliopsis fimicola*, *Pla* = *Planoprotostelium aurantium*, *Ca* = *Cavostelium apophysatum*. See Table S2 for a comprehensive list of all 610 hypotheses tested, exact constraints used, likelihood scores, and *p*-values obtained.

Table 3. Protosteloid Amoeba Cultures Used.

Name	Collection #	Source	Culture Collection	Food	Group	Genbank
Cavostelium apophysatum [t]	G-17	supplied	ATCC 38567	Fla	IV	FJ766476
Ceratiomyxella tahitiensis	HIO4-93L-1	collected	UA	M,*Cl*	II	FJ544419
Clastostelium recurvatum	NZ05-10a-4	collected	ATCC PRA-189, UA	*Kp*	Va	FJ766474
Endostelium zonatum	LHIO5M6a-1	collected	ATCC PRA-191, UA	F	VII	FJ766469
Nematostelium ovatum	JDS 6241	collected	UA	M,K	II	FJ544420
Protosporangium articulatum	1-Bg3-9-1	N/A	N/A	U	Va	FJ792705
Protostelium mycophaga [t]	Type	collected	ATCC PRA-154, UA	*Rm*	I	FJ766484
Protostelium mycophaga	HI04 85a-1b	collected	UA	*Rm*	I	FJ766483
Protostelium nocturnum	LHI05M6a-1a	collected	ATCC PRA-194, UA	Fla	I	FJ766481
Protostelium okumukumu [t]	HIO4-37a-1a	collected	ATCC PRA-156, UA	*Rm*	I	FJ766482
Protosteliopsis fimicola	H76-34	purchased	CCAP 1569/I,	F	VI	FJ766470
Protosteliopsis fimicola	H76-34	purchased	CCAP 1569/I,	F	VI	FJ766471
Protosteliopsis fimicola	Ken-A '20DE'	collected	UA	F	VI	FJ766472
Protosteliopsis fimicola	OM05-6218-1	collected	UA	F	VI	FJ766473
Schizoplasmodiopsis pseudoendospora	PBR-G5-1	collected	ATCC PRA-195	Fla	IV	FJ766475
Schizoplasmodiopsis amoeboidea [t]	RA81-20	supplied	ATCC 46943	Fla	IV	FJ766477
Schizoplasmodiopsis amoeboidea	BG7A-12B	collected	UA	Fla	IV	FJ766478
Schizoplasmodium cavostelioides	NZ05-24L-2	collected	ATCC PRA-197, UA	M,K	II	FJ544418
Soliformovum expulsum [t]	YAP 76-9	supplied	ATCC 48083	M	III	FJ766479
Soliformovum irregularis [t]	Mex 61-81	supplied	ATCC 26826	*Ec*	III	FJ766480

Table 3 (Continued). Protosteloid Amoeba Cultures Used.

Name	Collection #	Source	Culture Collection	Food	Group	Genbank
Tychosporium acutostipes	NZ05-15a-2	collected	ATCC PRA-196, UA	Fla	IV	FJ792704
Tychosporium acutostipes	KEA-11A-L	collected	UA	Fla	IV	FJ792703
Unnamed	LHI05M5g-1	collected	ATCC PRA-198, UA	F	none	FJ792702
Unnamed	LHI05M5g-1	collected	ATCC PRA-198, UA	F	none	FJ794612

Name abbreviations: ^{t} = Type cultures. Collection number = collection number of the source material used to isolate the culture/collection. Source abbreviations: supplied = supplied by, collected = isolated from substrates collected in the field, purchased = Purchased from culture collection. Culture Collection abbreviations: ATCC = American Type Culture Collection (Eumycetozoan Special Collection), UA = University of Arkansas (cryopreserved), CCAP = Culture Collection of Algae and Protozoa. Food organism abbreviations: Fla20 = *Serratia liquefaciens* strain Florida 20 of Olive ATCC BAA-1466, M = *Dyadobacter sp*. Strain Malaya (MAL 82 of Olive) ATCC BAA-1468, *K* = *Tilletiopsis* sp. strain Kitani of Olive, *Ec* = *Escherichia coli* ATCC 23432, *Cl* = *Cryptococcus laurentii* (kindly provided by E.F. Haskins), *Rm* = *Rhodotorula mucilaginosa* of Olive ATCC 14023, *Kp* = *Klebsiella pneumoniae* ATCC 23432, F = *Sphingomonas* sp. Strain FLAVO ATCC BAA-1467, U = uncultured. Genbank = genbank accession number.

Table 4. SSU rDNA primers for protosteloid amoebae.

Medlin A : B	Medlin A : PmycR2	PmycF1 : PmycR2	30F : 1492R	5’SSU17! : Myxo3'
S. irregularis	*T. acutostipes* NZ	*P. mycophaga*	*P. articulatum* 2°	*S. pseudoendospora*
C. recurvatum		*P. nocturnum*		*P. fimicola*
E. zonatum		*S. amoeboidea*		
S. cavosteliodes		*N. ovatum*		
C. apophysatum		*C. tahitiensis*		
T. acutostipes KE		*S. expulsum*		
P. articulatum 1°		isolate LHI05		

PmycF1: 5' TCC TGC CAG TAG TCA TAT GCT 3', PmycR2: 5' GCA GGT TCA CCT AGG GAG 3', Medlin A: 5' CCG AAT TCG TCG ACA ACC TGG TTG ATC CTG CCA GT 3', Medlin B: 5' CCC GGG ATC CAA GCT TGA TCC TTC TGC AGG TTC ACC 3' [81], 30F: 5' AAA GAT TAA GCC ATG CAT G 3', 1492R: 5' ACC TTG TTA CGA CTT 3', 5'SSU17!: 5' CTG GTT GAT CCT GCC AG 3', and Myxo 3': 5' TAA TGA TCC AAA GGC AGG TTC ACC TAC 3'.

SUPPORTING INFORMATION (on disk)

Table S1. Genbank Accession Numbers for Additional Sequences Used in Phylogenetic Analyses. Bold font highlights protosteloid species. * = organisms sequenced in this study.

Table S2. Constrained Taxa Abbreviations and Constraints Used for Statistical Tests. Each well supported amoebozoan lineage is constrained with all other amoebozoan and outgroup lineages. The first two columns refer to the taxon and its abbreviation in the constraint. The taxa constrained to branch together are within the parentheses. For more complex constraints, where some taxa are constrained away from a group the notation used is ((taxa constrained together) taxa constrained away);. All other taxa (from 129 taxa dataset see figure 3) that are not constrained in or out of a group are omitted from this table for ease of reading. Constrained trees (not shown) were built in RAxML and likelihood scores (-lnL) were estimated in PAUP* using the constraints shown. Significant differences in the likelihood among all trees were tested by the AU, SH, KH tests as implemented in Consel [90].

CHAPTER 5

RUNNING TITLE---*LUAPELEAMOEBA HULA ad interim* DESCRIPTION

A NEW AMOEBA WITH BALLISTOSPOROUS PROTOSTELOID FRUITING: *LUAPELEAMOEBA HULA* G. *AD INTERIM* SP. *AD INTERIM*

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ABSTRACT. We describe a new ballistosporous protosteloid amoeba *Luapeleamoeba hula* g. *ad interim* sp. *ad interim.* Protosteloid amoebae, sometimes called protostelids, are those amoebae that make fruiting bodies that consist of a stalk and one to a few spores. Ballistospores are those spores that are forcefully propelled away from their stalks. This new species was cultured from dead leaves of mamaki (*Pipturus albidus*) from the Manuka Natural Area Reserve, Hawaii, USA. Light microscopic examination showed that this amoeba has a rigid stalk with a small apophysis and a spore that changes shape continuously until it discharges from the stalk. In addition, this amoeba was observed to maintain a strict diurnal rhythm in which fruiting body formation occurred only in the late afternoon. This new species is unique in both its amoebal and fruiting body morphology. In addition, it appears to use a method of ballistospore discharge that is unique among protosteloid amoebae. Spore discharge appears to be a result of shape changes in the spore itself. This is the eighth species of protosteloid amoeba described to forcibly discharge its spore, and the fourth with a clearly described diurnal rhythm. In addition, recent molecular phylogenetic analyses showed that this new species has SSU rRNA gene sequences which clearly separate it from other protosteloid amoebae and place it as sister to *Protacanthamoeba bohemica* among the acanthamoebids. Because this new species does not fit into any described genus, we have proposed an *ad interim* genus—*Luapeleamoeba.*

***Key words*:** ballistospore, free-living amoeba, protostelid, acanthamoebid, stalk, taxonomy, terrestrial, Hawaii

Protosteloid amoebae, often called protostelids, are those amoebae that make simple fruiting structures comprised of a stalk supporting one to a few spores (Olive 1975; Spiegel 1990). They consume organisms that decompose plants and are observed in the laboratory by microscopically examining the edges of dead plant matter (Spiegel et al. 2004; Spiegel et al. 2007). In an ongoing survey of Hawaiian protosteloid amoebae, we found a distinctive ballistosporous species that fruited heavily only in the late afternoon. At other times of the day, no fruiting bodies of this species were found. Because this new species fruited in abundance, it was easily brought into monoeukaryotic culture. Phylogenetic analyses of small subunit ribosomal RNA gene sequences from one isolate (LHI05) of this new species have shown that it branches as sister to *Protacanthamoeba bohemica* among the acanthamoebids, a clade in which fruiting body formation had not previously been described (Shadwick et al. 2009). Light micrographs presented here show that this organism is morphologically distinct from species in the genus *Protacanthamoeba*. In addition, members of the genus *Protacanthamoeba* make distinctive cysts in culture which this new species does not. Here this organism is described as new genus and species on the bases of its unique molecular phylogenetic, morphological, and life cycle characters.

MATERIALS AND METHODS

Collections. Collecting trips were made by LL Shadwick, JDL Shadwick, and FW Spiege in late July and early August, 2005, to the island of Hawaii. At each collecting site a GPS reading was recordedand samples of dead standing vegetation and ground litter were collected and placed in paper bags. The samples were air dried, then sent to the University of Arkansas. The new species was recorded only from the Manuka Natural Area Reserve, Hawaii, USA. GPS: 19.110217° N, 155.825600° W.

Primary isolation plates. Subsets of the vegetation samples were plated out with standard techniques (Spiegel et al. 2004). Briefly, eight pieces of substrate were placed on a primary isolation plate (PIP) of wMY agar (0.002g malt extract, 0.002 g yeast extract, 0.75g K2HPO4 and 15g agar / L distilled ddH20) then saturated with sterile distilled water. After three to five days of culture, the edges of substrates in PIP were scanned for protosteloid fruiting bodies using the 10x objective of a compound microscope.

Monoeukaryotic culture. The new species was isolated into mono-eukaryotic culture by picking spores from fruiting bodies observed in a PIP with a sterile glass needle and placing them onto a streak of the bacterial food organism *Sphingomonas* sp. Strain FLAVO ATCC BAA-1467 on wMY agar plates, or by cutting an agar block full of fruiting bodies from the primary isolation plate and allowing spores to drop onto a WMY plate containing the food organism. Cultures were passed to fresh media every month to two months.

Strains examined. We have isolated and examined two independent cultures, LHI05-M5g-1 and LHI0-M5g-2, of this species from dead leaves of mamaki (*Pipturus albidus* [Hook & Arn.] A. Gray ex. H. Mann), and from ground litter samples, collected by Lora L. Shadwick, John D. L. Shadwick, and Frederick W. Spiegel from the Manuka Natural Area Reserve on August 26, 2005.

Light microscopy. The new species was observed repeatedly in culture under a compound light microscope using 10x brightfield (BF), 20x BF, 40x dry differential interference contrast (DIC) and phase-contrast (PC), and 63x DIC optics, and digitally photographed using Auto-Montage software (Syncroscopy, Frederick, Maryland, USA), which allows for in-focus images of three-dimensional objects by combining a series of through-focus images.

Fixation. Some amoebae were gently fixed on a glass slide to maintain their locomotive form when photographed under the light microscope (Fig. 7, 9, 10). An agar block containing as many amoebae as possible was cut from the culture with a flamed spear-point needle and gently inverted onto a clean glass slide. Liquid wMY was added drop wise to the edge of the block so that it could wick under the block. This was allowed to nearly dry for approximately 10 minutes so that the amoebae would settle onto the glass. A cocktail of 1% OsO_4 in wMY was added, and was followed immediately with enough glutaraldehyde to reach a final concentration of roughly 1%. Additional wMY was wicked under the agar block to rinse the cells and to float the agar block off of the cells so that the block could be replaced with a clean cover slip. Amoebae were then viewed under the compound microscope, and photographed as described above (Fig. 7, 9, 10).

RESULTS

The unique protosteloid amoeba, isolate LHI05-M5g, which we propose to call *Luapeleamoeba hula* g. *ad interim* sp. *ad interim*., was found fruiting in abundance between the hours of 3 pm and 6 pm on native substrates in a primary isolation plate (PIP). Mature fruiting bodies, also called sporocarps, consisted of a stalk and a single spore that when viewed from the side were distinct from all described protosteloid fruiting bodies in that the spores continuously changed shape and could be viewed as obovate, round, ovate, or slightly elongated (Fig. 1, 4, 11-14). Amoebae on an agar surface were also distinctive in that they were often somewhat triangular in outline with a conspicuous contractile vacuole, broad lamellipodium and blunted subpseuodopodia (Fig. 2). Because the area around the contractile vacuole is often the thickest part of the cell which tapers gently toward the lamelipodium which is thinnest, these amoebae often had ashape reminiscent of a shield volcano (Fig. 2).

Fruiting bodies viewed from the side range in size from 14.1 µm to 28.8 µm tall averaging 23.7 µm (n = 7) when measured from the base of the stalk to the top of the spore (Fig.

1, 4, 11-14). Maturing fruiting bodies have a portion of the stalk embedded within the spore (Fig. 3, viewed from top), while fully mature spores are more rounded so that the stalk is not clearly visible through the spore (Fig. 3, 4). Spores, when viewed and measured from the top, are generally round with an average diameter of 14.75 μm that ranges from a minimum of 11.3 μm to a maximum of 18.11 μm (n = 83). Spores viewed from the side are generally more irregular in shape and are often taller than they are wide (Fig. 1, 4, 11). Spore length measured from the side ranges from 11.5 μm to 18.4 μm with an average of 14.4 μm, maximum width measured from the side ranges from 8.9 μm to 17 μm with an average of 13.4 μm, (n = 3). An apical apophysis of the stalk is embedded within the spore when the spore is attached (Fig. 12-14), thus the apophysis cannot be seen until the spore has been discharged (Fig. 5). Stalks remain standing after ballistosporous ejection of the spores. Stalks are rigid and do not change shape or bend (Fig. 5). Total stalk length, including the apophysis, without the spore present, ranges from 5.9 μm to 9.8 μm averaging 7.7 μm (n = 17). The length of the apophysis ranged between 1.0 μm and 2.71 μm with an average of 1.92 μm (n = 10).

Amoebae of the new species are usually uninucleate with a single, central, round nucleolus (Fig. 6, 7, 9, 10). Amoebae in their locomotive form in monoeukaryotic culture on an agar surface are usually somewhat triangular (Fig. 2, 7, 10) however, they vary greatly in size (Fig. 10) and shape especially when changing directions (bottom left amoeba in Fig. 10). The amoebae have a broad, hyaline lamellipodium at the leading edge and a large granular cytoplasmic region that contains the nucleus and a conspicuous contractile vacuole (Fig. 7, 10). The length of the locomotive form averages 42.9 μm, but ranges from 31.0 μm to 54.0 μm (n = 10). The breadth of locomotive form averages 38.4 μm, but ranges from 32.9 μm to 51.1 μm (n = 10), with an average length/breadth ratio of 1.15 ranging from 0.61 to 1.64 (n = 10). The lamellipodium has an average length of 7.3 µm ranging from 4.4 µm to 9.5 µm, so that the average ratio of the length of the lamellipodium versus the length of the amoeba is 0.17, ranging from 0.08 to 0.24 (n = 10). The locomoting amoebae quickly round up when exposed to bright light (Fig. 6, 8). Migrating amoebae typically have blunt, sometimes triangular, subpseudopodial extensions which are most visible towards the leading edge (Fig. 9). The large contractile vacuole (Fig. 2, 6, 7) continuously builds in size averaging 5.5 µm (n = 11) with a maximum of 7.1 µm until its contents are expelled (Fig. 9), then smaller contractile vacuoles migrate together and coalesce to form a new conspicuous contractile vacuole—the contents of which will eventually be expelled (Fig. 9). The contractile vacuole is usually found just behind the nucleus (Fig. 7, 10). The nucleus (Fig. 6, 7) is often slightly eliptical and ranges in length from 5.1 µm to 8.1 µm with an average of 6 µm (n = 12), and breadth averaging 5.5 µm and ranging from 4.5 µm to 6.5 µm, containing a single, central, round nucleolus with a diameter ranging from 2.02 µm to 2.82 µm, average 2.4 µm (n = 12). The three-dimensional shape of the amoeba was determined by adjusting the focal plane of the microscope (data not shown). This shape is hinted at in Fig. 2 where some of the light shining through imperfections in the plastic petri plate and agar surface hits the amoeba from an oblique angle causing the portions of the amoeba that stick up the highest or at the highest angle relative to the agar surface to be white which fades into darker and darker shades of gray as the amoeba slopes down toward the edges. Notice that some of the subpseudopodia stick up from the agar surface as evidenced by their white color. The amoeba appears to be thickest around the rim of the contractile vacuole and decreases thickness sharply towards the posterior with a more gentle slope towards the anterior (Fig. 2). The edge of the amoeba is slightly ruffled in appearance (data not shown). The amoebae were seen digesting a variety of bacteria and fungal spores on primary isolation plates (Fig. 8, 15-17) but grew and sporulated well in monoeukaryotic culture on *Sphingomonas* sp.

Time-series images, approximately 30 seconds apart, of the new species on primary isolation plates (PIP) revealed that spores changed shape atop the fully formed stalk (Fig. 11--14).Amoebae were also documented undergoing cytokinesis (Fig. 18--20).

The floating form is disc-shaped in three dimensions (data not shown) with no distinct anterior and posterior regions. The amoeba in figure 6 shows some of the characteristics of the floating form since it is an optical section of a living amoeba somewhat flattened between a slide and a cover slip in liquid media. For instance, the nucleus and contractile vacuole are no longer posterior (Fig. 6). A thin hyaline region surrounds the edge of the floating amoebae with granular cytoplasm in the interior (Fig. 6).

Cysts (data not shown) differ from spores in that they are never stalked. Cysts have been observed only rarely, even though we have continuously maintained the cultures for more than 5 years. Cysts, when they appear, are spherical and extremely thin-walled.

DISCUSSION

Isolate LHIO5, which we propose to call *Luapeleamoeba* g. *ad interim*, is an amoebae with a simple fruiting body consisting of a single stalk and a single spore. When amoebae form this type of a fruiting body they are referred to as protosteloid amoebae (Shadwick et al. 2009). Most protosteloid amoebae have been placed into morphological groups in which the amoebae and prespore cells share strong similarities, but the fruiting bodies are unique and thus often delineate the species (Spiegel 1990). This new species does not fit into any previously described genus of fruiting or nonfruiting amoebae. The morphology of its fruiting bodies distinguish it from all other protosteloid amoebae. Its amoebal stage is distinctive from most other protosteloid amoebae in that it has never been observed to make filose sub-pseudopodia (Olive 1975, Spiegel

1990), a characteristic that also separates it from a few nonfruiting amoebae. Finally, molecular phylogenetic analyses of its SSU rDNA gene sequences indicate that isolate LHI05 represents a distinct lineage that is closely related to the acanthamoebid *Protacanthamoeba bohemica* (Shadwick et al. 2009).

Luapeleamoeba hula g. *ad interim* is a unique amoeba with a distinctive ballistosporous protosteloid fruiting body. There are seven previously described species of ballistosporous protosteloid amoebae (see discussion portion of Spiegel et al. 2006 for a recent review). These ballistosporous species include *Schizoplasmodium cavostelioides*, *Schizoplasmodium obovatum*, and *Schizoplasmodium seychellarum* (Olive and Stoianovitch 1966; Olive and Stoianovitch 1976). These are species that use a Buller's drop-like spore dispersal mechanism (Pringle et al. 2005; van Niel, Garner, and Cohen 1972). The other species use various mechanisms that involve changes in the shape and sometimes bursting of portions of the stalk; examples include *Clastostelium recurvatum* (Olive and Stoianovitch 1977), *Soliformovum expulsum* (Olive and Stoianovitch 1981), *Protostelium nocturnum* (Spiegel 1984), and *Protostelium okumukumu* (Spiegel el al.2006). These ballistosporous species are are not phylogenetic sisters in molecular analyses (Shadwick *et al.* 2009), and occurr in at least three of Spiegel's (1990) morphological groups of protosteloid amoebae. All of these species have relatively short fruiting bodies (*i.e.*, less than 25 µm tall).

Luapeleamoeba hula g. *ad interim* s. *ad interim* uses a unique mode of ballistosporous spore dispersal. There are three ballistosporous protosteloid amoebae (*e.g.*, *Protostelium nocturnum*, *Clastostelium recurvatum*, and *Soliformovum expulsum*) where nearly the whole fruiting body disappears (Olive and Stoianovitch 1977, Olive and Stoianovitch 1981; Spiegel 1984). All of these protosteloid amoebae appear to use their stalks (which change shape) for ejecting their spores (which do not change shape). In contrast, *Luapeleamoeba hula* g. *ad interim* sp. *ad interim* appears to use its spore (which changes shape) to launch itself from the top of the stalk (which does not change shape). The stalk then persists for up to an hour. The protosteloid amoeba, *Protostelium okumukumu*, ejects its spore using changes in the stalk shape, but part of its stalk remains after spore ejection so that a field of partial stalks, reminiscent of beard-stubble is often observed (Spiegel et al. 2006). Although the stalks of *Luapeleamoeba hula* g. *ad interim* sp. *ad interim* do persist for some time, we do not find fields of stalks remaining for many hours or days after a large fruiting event.

Like *L. hula* s. *ad interim,* both *Protostelium nocturnum* and *Clastostelium recurvatum* maintain a strong diurnal rhythm, in which all fruiting is limited to certain parts of the day (Olive and Stoianovitch 1977; Spiegel 1984). However, both *Protostelium nocturnum* and *Clastostelium recurvatum* generally fruit heavily late at night and early in the morning (Olive and Stoianovitch 1977; Spiegel 1984). Unlike these other protosteloid amoebae, *L. hula* s. *ad interim* fruits only in the mid to late afternoon (between 3 and 6 pm).

To our knowledge, this amoeba has been recorded only twice, and both records are from the same site on the Big Island of Hawaii. The first record is a drawing of two fruiting bodies and an amoeba made by F.W. Spiegel from substrates collected at Manuka Natural Area Reserve in 1998, which he labeled ‘new species T’ (personal observation). For the second record, both L.L. Shadwick and F.W. Spiegel established monoeukaryotic cultures of this new species from substrates they collected together at the same site in 2005 (this study).

Phylogenetic analyses of small subunit ribosomal DNA have recently placed this new species within the *Acanthamoebidae* (Shadwick et al. 2009), most closely related to *Protacanthamoeba bohemica* (Dykova et al. 2005) (Volkonsky 1931; Page 1967; Sawyer and

Griffin 1975; for review of *Acanthamoebidae* see also Adl et al. 2005). However, the new species has several morphological characters that set it apart from most other acanthamoebids. The amoebae are generally larger than other acanthamoebids. It rarely makes cysts, and the cysts it does make are extremely thin walled (data not shown). Its subpseudopodia are much more blunted than acanthopodia. No other acanthamoebids have been reported to make fruiting bodies. Although this may not be a distinguishing character because it is possible that other acanthamoebids do form fruiting bodies and that these have been overlooked. The new species has a SSU rDNA gene sequence that easily distinguishes it from other acanthamoebids. Thus, this new amoeba is described as a new genus and species within the eukaryotic super-group *Amoebozoa* Lühe 1913, emend. Cavalier-Smith, 1998, within the *Acanthamoebidae* Sawyer and Griffin 1975.

Eukaryota Chatton, 1925, *Amoebozoa* Lühe, 1913, emend. Cavalier-Smith, 1998,
Acanthamoebidae Sawyer and Griffin, 1975.

Generic Diagnosis. *Luapeleamoeba* g. *ad interim*, during locomotion, these flabellate, uninucleate amoebae have a single broad, hyaline lamellipodium with blunted triangular subpseudopodia at the leading edge. Following the lamellipodium is a thick granuloplasmic region containing a single nucleus and a conspicuous contractile vacuole that is usually posterior to the nucleus. The amoebae generally lack uropodia. In addition, the amoeba is thickest near the contractile vacuole and nucleus, tapering gradually toward the edges, thus the amoeba has the overall appearance of a minute shield volcano. No flagellate form is known. The cysts are rare and thin-walled. Aerial fruiting bodies have been observed in the type species but may not be characteristic of all members of the genus.

Etymology. Derived from the Hawaiian word *luapele,* which means volcanic crater, for the conspicuous contractile vacuole in the amoeba (Fig. 2, 6, 7).

Type species. *L. hula* sp. *ad interim*

The diagnosis of the type species of the genus *Luapeleamoeba* g. *ad interim* follows.

Eukaryota Chatton, 1925, *Amoebozoa* Lühe, 1913, emend. Cavalier-Smith, 1998,

Acanthamoebidae Sawyer and Griffin, 1975, *Luapeleamoeba* g. *ad interim*

Species Diagnosis. *L. hula* sp. *ad interim,* Sporocarps average 23.7 μm tall. Stalk 8.97 μm in total length; with an apophysis at the tip of the stalk, embedded within the spore. Stalk is inflexible along its whole length including the articulation below the apophysis. There is one spore per stalk. The spore is uninucleate, with continuously variable shape, but often in the shape of an upside-down pear when viewed from the side and round when viewed from the top. Spores germinate as uninucleate, nonflagellate amoebae that are characteristic of the genus.

Amoebae often flabellate on agar surface, with broad, hyaline, anterior lamellipodium with short rounded triangular subpseudopodia.The total length of the locomotive form averages 42 μm, breadth of locomotive form averages 38 μm with an average length/breadth ratio of 1.15 μm. The body of the amoeba contains granular cytoplasm, a conspicuous contractile vacuole, and nucleus containing a single, central, round nucleolus. The lamellipodium extends an average of 7.3 μm beyond the granular cytoplasm.The amoeba appears to be thickest around the rim of the contractile vacuole and decreases thickness sharply toward posterior with a more gentle slope toward anterior. The edge of the amoeba is ruffled in appearance.

Floating form is disc-shaped with no distinct anterior and posterior regions, and a hyaline region surrounds amoeba with granular cytoplasm in the interior.

Cysts are rare and thin-walled.

Etymology. The specific epithet is from the Hawai'ian *hula* which means dance, for the spore of this species continuously changes shape as if it were dancing.

Type locality. Manuka Natural Area Reserve, Hawaii USA. GPS: 19.110217° N, 155.825600° W, a native upland dry/mesic forest, elevation 547 meters above sea level.

Prevalence. This species has been recorded twice at the type locality on the big island of Hawai'i. We know of no other records of this species.

Ecology/Habitat. This species has been isolated in association with decaying plant matter. It has been shown to eat both fungi and bacteria associated with decaying plants.

Specimens examined. We have isolated and examined two cultures of this species from dead leaves of mamaki (*Pipturus albidus* [Hook & Arn.] A. Gray ex. H. Mann) and ground litter samples from the type locality the Manuka Natural Area Reserve on the Big Island of Hawai'i in the state of Hawaii, USA. The type specimen, LHI05-M5g-1, from mamaki ground litter was isolated by FW Spiegel. The isotype, LHI05-M5g-2, was isolated from the same collection by L L Shadwick, Collection Date August 26, 2005. This species had previously been observed and recorded as 'new species T' from a Manuka Natural Area Reserve collection HI98-81a on October 14, 1998, from standing dead fronds of *Nephrolepus* sp.

Deposition of Type Specimens. The type culture LHIO5M5g-1 has been submitted to the American Type Culture Collection Accession # ATCC PRA-198 to be included in the Eumycetozoan Project Special Collection.

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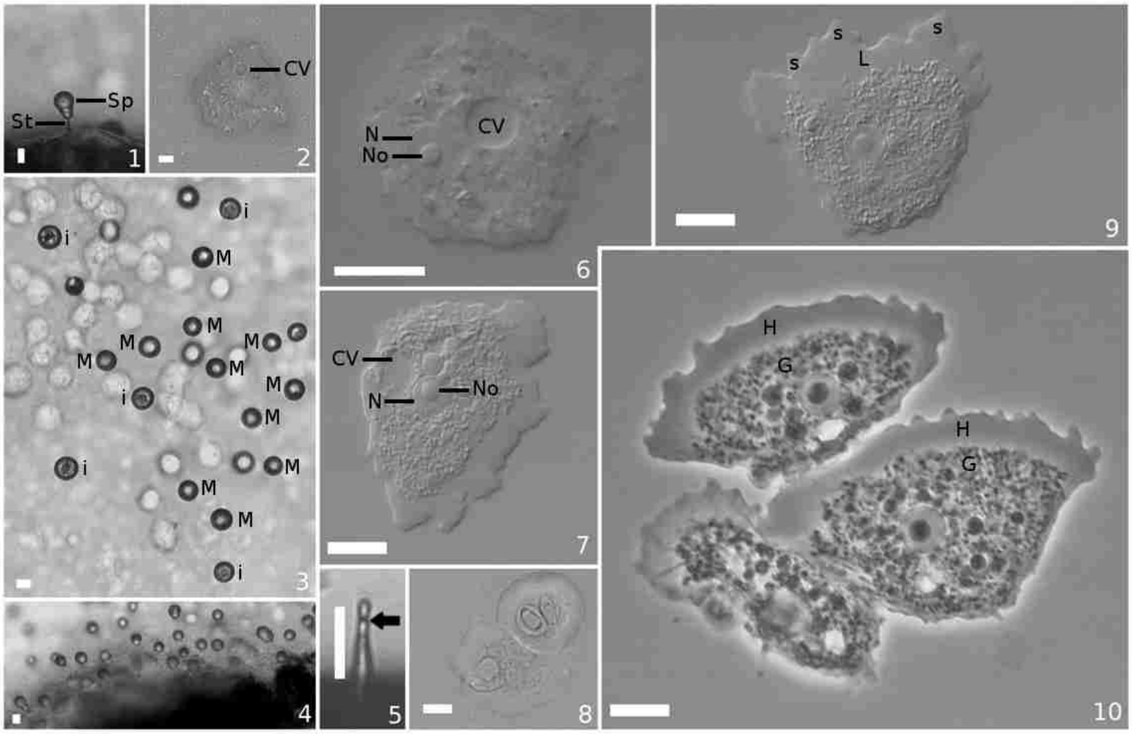

Fig. **1--10**. Light micrographs of *Luapeleamoeba hula* g. *ad interim* s. *ad interim* strain LHI05M-5a-1. 1. Side view of fruiting body on native substrate in primary isolation plate (PIP). 2. Living amoeba on agar surface in monoeukaryotic culture. 3. Fruiting bodies in various stages of development on agar surface viewed from top in PIP. 4. Fruiting bodies in various stages of development on native substrate in PIP. 5. Stalk with apophysis viewed from side after spore has discharged. 6. Living amoeba in liquid media flattened with cover slip under 63x oil differential interference contrast microscopy (DIC). 7. Amoeba gently fixed on slide to maintain locomotive form, 40x dry DIC. 8. Two living amoebae in PIP digesting fungal spores. 9. Amoeba gently fixed on slide to maintain locomotive form showing blunted triangular subpseudopodia extending from broad hyaline lamellipodium, 40x dry DIC. 10. Three amoebae gently fixed on slide to maintain locomotive form 40x dry phase contrast. Scale bar 10 µm throughout. A black arrow points to the apophysis. Abbreviations are as follows: stalk (St), spore (Sp), contractile vacuole (CV), immature fruiting body (i), mature fruiting body (M), nucleus (N), nucleolus (No), subpseudopodia (s), lamellipodium (L), hyaloplasm (H), granuloplasm (G).

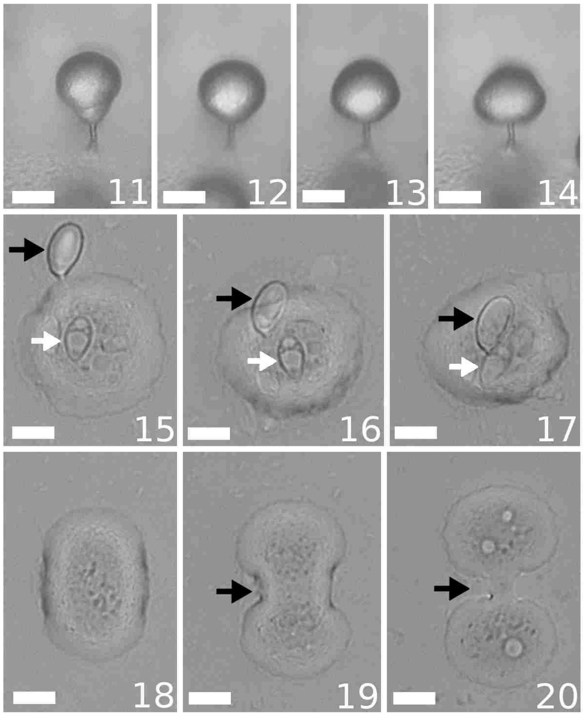

Fig. **11--20**. Time series of events in *Luapeleamoeba hula* g. *ad interim* sp. *ad interim* life cycle.11--14. Changes in spore shape. 15--17. Ingestion of basidiospore. 18--20 Cytokinesis. All images taken with 20x dry lens bright field microscopy on agar surface in primary isolation plate (PIP). Approximately 30 seconds elapsed between images. Scale bar 10µm throughout. Figure 11 is cropped from the same original image as Figure 1L (Shadwick *et al*. 2009). The larger black and smaller white arrows in figures 15--17 follow two separate fungal spores in the process of being ingested and digested by the amoeba. The black arrows in figures 19 and 20 point to the growing cleavage furrow.

CHAPTER 6:

OVERALL CONCLUSION

This chapter summarizes the findings presented in this dissertation and their implications. It begins by summarizing the conclusions reached in chapters 2-5. This is followed by a discussion of the change in perception of protosteloid amoebae that this research has precipitated. Next, the current state of research following publication of chapters 2-4 is summarized. Then, suggestions for future work are presented. The discussion of future work is divided into two parts. The first part suggests future work in this direct line of research. The second part is a discussion of biological questions that are raised by the work presented herein. Finally, a few concluding remarks are presented.

RECAPITULATION OF CONCLUSIONS

Conclusion 1: Protosteloid and myxogastrian amoebae are common in freshwater environments.

Conclusion 2: Three distinct nucleolar morphologies occur among protosteloid amoebae.

Conclusion 3: *Echinosteliopsis oligospora* has a unique nucleolar ultrastructure compared to other eukaryotes.

Conclusion 4: Amoebae with a protosteloid life cycle are common within the supergroup *Amoebozoa.*

Conclusion 5: Protosteloid amoebae are found in eight amoebozoan clades:

a) Four clades appear to be exclusively protosteloid.

b) Three clades contain protosteloid species with robust phylogenetic affinities to nonfruiting amoebozoans.

c) One protosteloid species lacks clear phylogenetic affinities to any other eukaryote with publicly available gene sequence data.

Conclusion 6: The term protostelid is confusing and the assemblage of organisms for which it has been used should be referred to as protosteloid amoebae instead.

Conclusion 7: The taxon *Amoebozoa* (Lühe 1913 emend. Cavalier-Smith 1998) and the taxon *Eumycetozoa* (Zopf 1885 emend. Olive 1975) could be interpreted as identical. In this case, *Eumycetozoa* is the older name and has nomenclatural priority.

Conclusion 8: *Protosteliopsis fimicola* is a vannellid amoeba with protosteloid fruiting. This is the first report of fruiting among vannellids.

Conclusion 9: Protosteloid isolate LHI05 (*Luapeleamoeba hula* g. *ad interim* sp. *ad interim*) from Hawaii represents a new genus and species within the taxon *Acanthamoebidae.*

CHANGE IN PERCEPTION: FROM PROTOSTELIDS TO PROTOSTELOID AMOEBAE

This body of work has changed our perception of the protosteloid amoebae (previously called protostelids) such that we now think of these organisms as amoebae that sometimes happen to fruit rather than thinking of them as a homogenous group of fruiting organisms (like fungi) that also happen to sometimes exist as amoebae. This is important because it pushes us to carefully consider and describe amoebal stages of protosteloid life cycles and to recognize the importance of collaborating with protistologists who study amoebae. This collection of papers also highlights the need in the protistological community to consider that amoebae may have more to their entire life cycles than just the amoeboid states and/or cysts and to consider that complex life cycles are not only common among amoebae but might be an ancestral character of the amoebozoan lineage.

CURRENT STATE OF RESEARCH

Chapter 2: Since the publication of the second chapter of this dissertation (Lindley *et al.* 2007), which was the first paper to report on protosteloid amoebae from aquatic habitats and also one of small handful of papers to report on aquatic myxogastrian amoebae, researchers from as far away as the Philippines, Paraguay, and Japan have expressed an interest in studying aquatic slime molds. A second paper has been published on aquatic protosteloid amoebae from northern Germany (Tesmer and Schnittler 2009) in which the authors carried out extensive sampling and found 93 records covering 10 taxa of protosteloid amoebae from submerged substrates. They also suggested that the actively discharged spores of some protosteloid amoebae are a trait that is useful only in terrestrial environments (Tesmer and Schnittler 2009). It is interesting to note that in both studies, the majority of species found in aquatic habitats are nondeciduous and nonballistosporous. For instance, like our study, the German study found that *Schizoplasmodiopsis vulgare* was more common on submerged substrates than on nearby terrestrial substrates (Lindley *et al.* 2007; Tesmer and Schnittler 2009). While I think it is unlikely that many species of protosteloid amoebae thrive in aquatic habitats and/or frequently complete their entire life cycles in underwater environments; it is quite possible that innundation may reduce competition in favor of those species that have the ability to tolerate submersion. It is also possible that inundation might aid in dispersal of amoeboid floating forms and fruiting bodies that easily float because of their hydrophobic basal discs. If these hypotheses were true, they could explain why some species are common on substrates that are sometimes submerged. In any case, it is clear that protosteloid species are easy to find in these undersampled habitats. It is possible, perhaps even likely, that new species of protosteloid amoebae await discovery and description in these undersampled habitats.Thus, future ecological surveys and carefully designed experiments should include habitually undersampled habitats for protosteloid amoebae such as wetlands and aquatic environments.

Chapter 3: Since the publication of the third chapter of this dissertation (Lindley *et al.* 2006), a molecular phylogeny of protosteloid amoebae has been published (see Chapter 4, Shadwick *et al*. 2009). The variety of nucleolar arrangements found among protosteloid amoebae is congruent with the assertion by Shadwick *et al.* (2009) that protosteloid amoebae comprise a diverse array of amoebozoan lineages. Surprisingly, molecular phylogenetic analyses (Shadwick *et al.* 2009) showed that taxa, both *Soliformovum* species and *Schizoplasmodiopsis amoeboidea*, with diffuse nucleoli are not limited to the soliformoviid clade, as they also occur in the cavosteliid clade. Unfortunately, complete ssu gene sequences are not available for *Echinosteliopsis oligospora*, so it could not be included in the phylogenetic analyses. I have generated partial beta tubulin and elongation factor 1 alpha (EF1-α) gene sequences from *Echinosteliopsis oligospora* HI04-33a-3a (ATCC PRA-125) [Appendix], which suggest that it branches as sister to myxogastrids. Because this finding is somewhat incongruent with the morphology of the amoebal stages of the life cycle, it is unclear whether this result is trustworthy or if it is an artifact of PCR (*e.g.* contamination of a reagent), or an artifact of the phylogenetic analyses.

Chapter 4: At the time chapter 4 was submitted for publication, there were no publically available gene sequences of protosteloid amoebae except for *Planoprotostelium aurantium* and *Protostelium mycophaga* (Spiegel, Lee, and Rusk 1995, Baldauf and Doolittle 1997, Fiore-Donno *et al.* 2005, Schaap *et al.* 2006, and Brown *et al.* 2007). Now in addition to the gene sequences presented in this dissertation, Fiore-Donno *et al*. (2010) have independently sequenced SSU rRNA genes of three species, *Cavostelium apophysatum*, *Schizoplasmodiopsis amoeboidea*, and *Soliformovum irregularis*, of protosteloid amoebae with microscopic fruiting bodies which share 99% sequence identity to those sequences presented in this dissertation

(Shadwick *et al.* 2009) (Table 1).

Table 1. Comparison of SSU rRNA gene sequences published in Fiore-Donno *et al.* (2010) and those presented in this dissertation (Shadwick *et al*. 2009 and Appendix).

	This dissertation			**Fiore-Donno *et al.* 2010**	
Species	**Genbank #**	**bp**	**% ID**	**bp**	**Genbank #**
Soliformovum irregularis	FJ766480.1	1881	99%	1848	EF513181.1
Cavostelium apophysatum	FJ766476.1	1777	99%	1732	EF513172.1
Schizoplasmodiopsis amoeboidea	FJ766477.1	1927	99%	1909	EF513179.1
Schizoplasmodiopsis vulgare	N/A (Sv 5pr)	444	63%	1812	EF513180.1

Number of base pairs (bp). Percent nucleotide identity for aligned sequence (%ID). Unpublished partial gene sequence of SSU rRNA that I generated from *Schizoplasmodiopsis vulgare* strain HHPBPB3604-1 (Sv 5pr) [Appendix]. Sequences of approximately the same length are aligned for nearly their entire length.

Additionally, Fiore-Donno *et al.* (2010) have published a SSU rRNA gene sequence from *Schizoplasmodiopsis vulgare*. I have aligned this recently published sequence (EF513180.1) to the partial (444 bp) SSU rRNA gene sequence which I have generated for *Schizoplasmodiopsis vulgare* (Appendix), and these two sequences share only 63% identity across 442 bp of alignable sequence. While the source material for the partial SSU rRNA gene sequence that I generated is carefully vouchered (*Schizoplasmodiopsis vulgare*, designated as strain HHPBPB3604-1 and deposited in the American Type Culture Collection ATCC PRA-158) [See Lindley *et al.* 2007; Appendix], the source material for the sequence (EF513180.1) presented by Fiore-Donno *et al.* (2010) is not vouchered. This makes it impossible to determine whether Fiore-Donno *et al.*'s (2010) putative SSU rRNA gene sequence from *Schizoplasmodiopsis vulgare* is from an organism that is morphologically similar to the *S. vulgare* isolate HPBPB3604-1. However, it is possible that the differences between the SSU rRNA gene sequence for *Schizoplasmodiopsis* *vulgare* published by Fiore-Donno *et al.* (2010) and the partial gene sequence that I have generated (Appendix) reflect genuine molecular diversity among organisms correctly identified as *Schizoplasmodiopsis vulgare*. If it is indeed the case that both putative *S. vulgare* SSU rRNA sequences (Fiore-Donno *et al.* 2010, Appendix of this dissertation) are representative of *S. vulgare* SSU rRNA gene sequences, then it might indicate that specimens currently identified as *Schizoplasmodiopsis vulgare* represent a species complex or a group of distantly related organisms with similar sporocarps. In fact recent molecular phylogenetic work has shown that another common species of protosteloid amoeba (*Protostelium mycophaga*) is a diverse species complex (Shadwick 2010).

One of the limitations of the phylogenetic analyses presented in chapter 4 of this dissertation is that they are based on a single gene, SSU rRNA, and fail to resolve the deepest nodes of the tree (*i.e.*, the branching order between lineages). For instance the *Eumycetozoa* hypothesis could not be adequately addressed because the branching order among clades of fruiting amoebae and nonfruiting amoebozoans was unresolved. In addition, one protosteloid amoeba, *Endostelium zonatum*, could not be unambiguously asigned to the amoebozoan supergroup (Shadwick *et al.* 2009). In this study (Shadwick *et al.* 2009), no protosteloid clade or lineage was unambiguously identified as the sistergroup to myxogastrids or dictyostelids. It is hoped that the addition of multiple gene sequences to phylogenetic analyses of protosteloid amoebae might help to resolve the branching order between major amoebozoan lineages and provide some bootstrap support for those groupings as in other lineages/studies. In fact multigene phylogenies and phylogenomic studies have resolved many problematic regions of the tree of life including placement of floating taxa (*e.g.* Tekle *et al.* 2008, Minge *et al.* 2009 and Parfrey *et al.* 2010), recovery of morphologically defined groups that generally fail to be recovered in molecular phylogenetic analyses (Hampl *et al.* 2009), and increasing resolution in the deep nodes in the tree of life (Burki *et al.* 2008, Parfrey *et al.* 2010).

Additional gene sequences (elongation factor 1-alpha) have recently been published for a few protosteloid amoebae—*Cavostelium apophysatum*, *Schizoplasmodiopsis amoeboidea, Soliformovum irregularis*, and *Schizoplasmodiopsis vulgare*, and *Ceratiomyxa fruticulosa* (Fiore-Donno *et al.* 2010).

Published phylogenetic analyses using a combination of SSU rRNA and EF1-α gene sequences (Fiore-Donno *et al.* 2010) include too few protosteloid amoebal sequences and too few nonfruiting amoeboid outgroup taxa to convincingly demonstrate relationships among microscopic protosteloid amoebae. These analyses (Fiore-Donno *et al.* 2010) do show a well supported (95% and 99% ML bootstrap, 1.0 Bayesian posterior probability) relationship between *Ceratiomyxa,* myxogastrian amoebae, and dictyostelid amoebae. However, gene sequences of the likely sister group to *Ceratiomyxa,* the protosteloid amoebae *Protosporangium* and *Clastostelium* were not included in the analyses published by Fiore-Donno *et al.* (2010). Bakker (2008) did recover a clade in which *Ceratiomyxa* grouped as sister to *Clasotstelium recurvatum* with 66% bootstrap support and this *Ceratiomyxa/Clastostelium* clade was sister to *Cochliopodium* with no statistical support). Preliminary attempts to determine whether a clade including *Protosporangium, Clastostelium* and *Ceratiomyxa* (Spiegel 1990) is supported with phylogenetic analyses of SSU rDNA gene sequences have generally grouped *Clastostelium* and *Protosporangium* as sister to *Ceratiomyxa* (preliminary analyses not shown). In my preliminary analyses, the *Clastostelium, Protosporangium, Ceratiomyxa* clade did not group as sister to myxgastrids and dictyostelids. It would be interesting to see rigorous phylogenetic analyses that included the published gene sequence from additional isolatates of *Ceratiomyxa* (Fiore-Donno *et* *al.* 2010) to see if these produce trees with higher support values for a grouping that includes *Ceratiomyxa, Protosporangium,* and *Clastostelium.* If this group is recovered will it be sister to myxogastrids as suggested by Spiegel (1990) or as sister to myxogastrids and dictyostelids as suggested by Fiore-Donno *et al.* (2010)?

I have generated some additional gene sequences (beta tubulin, alpha tubulin, actin, elongation factor 1α, and heat shock proteins 70 and 90) from a few species of protosteloid amoebae (Appendix, Table A1) with the goal of producing a multigene phylogeny of protosteloid amoebae.

These sequenes have yet to be included in published molecular phylogenetic analyses primarily because different genes have been successfully sequenced from different species (see Appendix Table 1), a problem that makes broad-scale phylogenetic comparison difficult if not impossible. I have, however, compared the elongation factor 1 alpha gene sequence from *Cavostelium apophysatum* which I generated (Appendix) to that published by Fiore-Donno *et al.* (2010), and these are nearly identical (Table 2). Some EF-1 alpha analyses and combined SSU rDNA and EF-1 alpha analyses presented in Fiore-Donno *et al.* (2010), recover a clade that includes; *Cavostelium apophysatum, Schizoplasmodiopsis vulgare,* and *Schizoplasmodiopsis amoeboidea*. This clade corresponds to the cavosteliid clade, Spiegel's group IV, and has support values ranging from bootstrap support of 56% to 67% (Fiore-Donno *et al.* 2010). In addition this clade was supported with 1.0 Bayesian posterior probability with the caveat that the Bayesian analyses had not converged and therefore bayesian support values for this analysis might be misleading (Huelsenbeck *et al.* 2002, Erixon *et al.* 2003, Beiko *et al.* 2006, Nylander *et al.* 2008). Preliminary analyses of an elongation factor 1 alpha gene sequence which I generated from *Protostelium mycophaga* also provides some additional support for the grouping of

Planoprotostelium aurantium and *Protostelium mycphaga* 100% bootstrap support (tree not shown).

Table 2. Comparison of elongation factor 1 alpha gene sequence for *Cavostelium apophysatum* published in Fiore-Donno *et al.* (2010) and that presented in this dissertation (Appendix).

	This dissertation			**Fiore-Donno *et al.* 2010**	
Species	**Culture #**	**bp**	**% ID**	**bp**	**Genbank #**
Cavostelium apophysatum	Type	1276	99.30%	1286	EF513185.1

Number of base pairs (bp). Percent nucleotide identity for aligned sequence (%ID). Sequences are of approximately the same length and are aligned for nearly their entire length.

In addition, a beta tubulin gene duplication in the most recent common ancestor of the two *Soliformovum* species provides additional support for the monophyly of the genus *Soliformovum* (Appendix Figure A4). Preliminary beta tubulin analyses also recover a clade that includes members of the cavosteliid clade (Spiegel's group IV), *Schizoplasmodiopsis vulgare* and *Cavostelium apophysatum*, with weak bootstrap support (61%) [Appendix, Figure A4]

Finally, I had generated SSU rRNA and partial Actin gene sequences from *Protostelium arachisporum* HI05-3a that I isolated from dead attached fern leaves collected at Onomea Bay on the Big Island of Hawai'i (Appendix). These gene sequences clearly indicate that it is closely related to *Luapeleamoeba hula* g. *ad interim* sp. *ad interim* and should be transferred to the genus *Luapeleamoeba ad interim.* In fact the partial actin gene sequences from the two species are intermingled in molecular phylogenetic analyses (data not shown). However, light microscopy of the amoebal and fruiting stages of these species clearly show that *Luapeleamoeba hula* g. *ad interim sp. ad interim* is morpohologically distinct from *Protostelium arachisporum* (Figure 1). For instance, *L. hula* g. *ad interim* sp. *ad interim* has a proportionally shorter stalk than *P. arachisporum*. *L. hula* g. *ad interim* sp. *ad interim* also typically has larger amoebae that lack acanthopodia, whereas *P. arachisporum* has generally smaller amoebae which sometimes exhibit fine subpseudopodia that are reminiscent of acanthopodia (Appendix Figure A1).

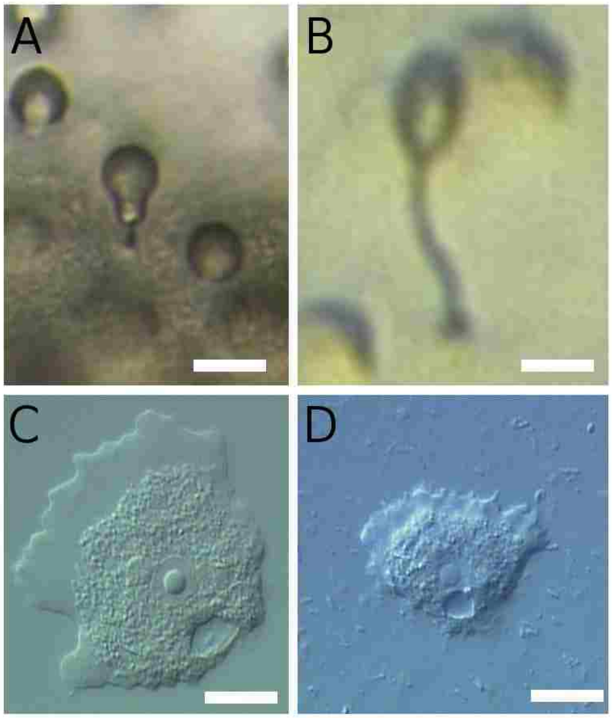

Figure 1: Morphological comparison of the fruiting body and amoebal stages present in the life cycles of *L. hula* g. *ad interim* sp. *ad interim* and *P. arachisporum* isolate HI05-3a using light microscopy. (A) *L. hula* sp. *ad interim* fruiting body photo taken by F.W. Spiegel, (B) *P. arachisporum* fruiting body photo taken by L.L. Shadwick through the lid of the petri plate as a voucher before extracting DNA, (C) *L. hula* sp. *ad interim* amoeba differential interference contrast microscopy (DIC), (D) *P. arachisporum* amoeba (DIC). Scale bar is 10µm.

Further phylogenetic analyses of the SSU rRNA gene clearly indicate that *Protostelium arachisporum* and *Luapeleamoeba hula* g. *ad interim* sp. *ad interim* group together and are the sister group to *Protacanthamoeba bohemica* (analyses not shown). Anna-Maria Fiore-Donno generated SSU rRNA and EF1α gene sequences from an independent isolate of *Protostelium arachisporum* and similarly found that *Protostelium arachisporum* groups with *Protacanthamoeba bohemica* (personal communication). Thus, we have agreed to publish these findings together in a separate paper on which we are currently working.

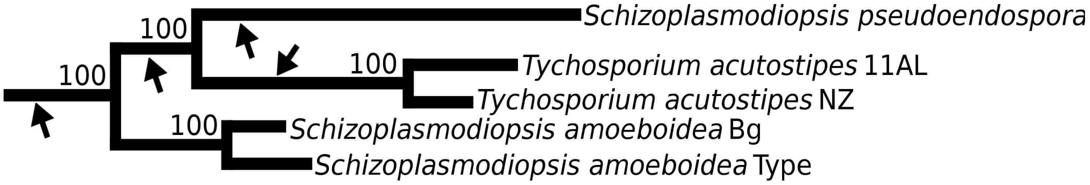

Figure 2. Removal of *Cavostelium apophysatum* from ssu-based phylogenetic analyses results in maximum bootstrap support for the cavosteliid clade. Black arrows indicate the various positions where *Cavostelium apophysatum* frequently branched in bootstrap trees generated for Shadwick *et al.* (2009). Cartoon tree drawn from analyses done as part of Aberer (2011) and from analyses done on a 128 taxon dataset prior to publication of Shadwick *et al.* (2009).

Finally, improvements in phylogenetic methods and subsequent reanalysis of existing data can increase bootstrap support for particular phylogenetic groupings (Aberer 2011). The rogue taxon analysis (Aberer 2011) done on the 141 taxon SSU rRNA gene based dataset assembled for Shadwick *et al.* (2009) shows increased bootstrap support for several clades that were recovered, but poorly supported in Shadwick *et al.* (2009). For instance, bootstrap support for *Amoebozoa* increased to 75% from 52% when rogue taxa were removed from the analysis (trees not shown). In addition analyses done by Aberer (2011) shows (as was mentioned in Shadwick *et al.* 2009) that bootstrap support for the cavosteliid clade increases to 100%, and every remaining node within the cavosteliid clade increased to 100% when *Cavostelium apophysatum* is removed from the analysis (Fig. 2). This is highly consistent with the visual inspection of the boostrap trees associated with the 129 taxon dataset that was done prior to publication of (Shadwick *et al.* 2009). This visual inspection revealed that *Cavostelium apophysatum* branched in various positions within the cavosteliid clade or as sister to the cavosteliid clade in bootstrap trees (see black arrows Fig. 2). Additionally, at least one bootstrap tree showed *Cavostelium apophysatum* branching as sister to dictyostelids, and another bootstrap tree showed it branching among dictyostelids (data not shown). Finally, the AU test failed to reject trees in which *Cavostelium apophysatum* was excluded from the cavosteliid clade (Table S2 in Shadwick *et al.* 2009). Taken together these results suggest that (1) the position of *Cavostelium apophysatum* in ssu based phylogenetic analyses is unresolved (2) the other putative members of the cavosteliid clade (*S. amoeboidea*, *T. acutostipes*, and *S. pseudoendospora*) do form a well resolved clade, and (3) *Cavostelium apophysatum* may or may not branch within the cavosteliid clade.

FUTURE WORK

The following suggestions for further work are centered on the goal of continuing to develop protosteloid amoebae as useful organisms for further biological inquiry.

Taxonomic Revisions: The work presented in this dissertation directly suggests that some taxonomic revisions are necessary. At the generic level, I have three recommendations:

(1) *Protosteliopsis fimicola* should be moved into the genus *Vannella.* This recommendation is based on both strong molecular phylogenetic evidence (Shadwick *et al.* 2009) and undeniable morphological identity of the amoebae (Smirnov *et al*. 2007).

(2) *Schizoplasmodiopsis amoeboidea* should be assigned to a new genus. Bortnick (1993) also reached this conclusion after a careful study of morphology and ultrastructure. However, before this dissertation, it was unclear whether *S. amoeboidea* should be moved to the genus *Soliformovum* because of the similar nucleolar ultrastructure, or given a new genus name entirely. Phylogenetic analyses based on SSU rRNA gene sequence (Shadwick *et al.* 2009) recovers *S. amoeboidea* branching sister to *Tychosporium*, *Cavostelium*, and

Schizoplasmodiopsis pseudoendospora with low support, but none the less, quite separate from the the placement of *Soliformovum.* Visual inspection of bootstrap trees showed no evidence that *S. amoeboidea* branched with *Soliformovum* (data not shown). Because there is no molecular phylogenetic evidence that *Schizoplasmodiopsis amoeboidea* branches with the genus *Soliformovum* I do not think it should be transferred to the genus *Soliformovum.* Molecular phylogenetic analyses indicate with 100% bootstrap support that the genus *Schizoplasmodiopsis* is paraphyletic with respect to the genus *Tychosporium* (Fig. 2). These analyses (Shadwick *et al.* 2009) also suggest that it is possible that the genus *Schizoplasmodiopsis* might also be paraphyletic with respect to the genus *Cavostelium* (see black arrows in Fig. 2); but these analyses are inconclusive on this matter because *Cavostelium apophysatum* is a rogue taxon (Aberer 2011) and was not rejected as branching sister to the cavosteliid clade by the Approximately Unbiased test (Table S2 in Shadwick *et al.* 2009). Thus, based on the information described above, I recommend that *S. amoeboidea* should be given an entirely new genus name.

(3) Species in the genera *Nematostelium* and *Ceratiomyxella* should be transferred to the genus *Schizoplasmodium* for three reasons. First, their extremely close evolutionary relationship to one another (Shadwick *et al.* 2009, Spiegel 1990) casts doubt on the utility of three separate genera. Second, species in the genus *Nematostelium* were originally described as members of the genus *Schizoplasmodium* (Olive and Stoianovitch 1966b) and only later transferred to the genus *Nematostelium* (Olive 1970) because they had fruiting bodies with long stalks. I do not consider stalk length by itself to be a strong enough character for erecting a new genus in the absence of other distinguishing data. Third, *Schizoplasmodium* is the older name (Olive and Stoianovitch 1966a, Olive and Stoianovitch 1966b, Olive 1970, Olive and Stoianovitch 1971, Olive and Stoianovitch 1976).

For convenience, protosteloid amoebae with microscopic fruiting structures have been classified in two families—those with flagellate cells in their life cycle (the *Cavosteliidae* [Olive 1964a]), and those without flagellate cells in their life cycle (the *Protosteliidae* [Olive and Stoianovitch 1966b]). However, molecular phylogenetic analyses presented in this dissertation (Shadwick *et al.* 2009) show that protosteloid amoebae with and without flagella are widely interspersed and that some clades containing protosteloid amoebae include both flagellated and nonflagellated species as predicted by Spiegel (1990, 1991, Spiegel *et al*. 1995). Thus, I recommend that the families *Cavosteliidae* (Olive 1964a) and *Protosteliidae* (Olive and Stoianovitch 1966b) be abandoned.

It is clear from the work presented here that the taxa *Eumycetozoa* (Zopf 1885 emend. Olive 1975) and *Amoebozoa* (Lühe 1913 emend. Cavalier-Smith 1998) are in need of closer examination. However, recircumscription of these taxa without resolution of the branching order among amoebozoan lineages will clutter the literature with useless names and cause confusion. However, I think it is possible, perhaps likely, that evidence showing that *Eumycetozoa* and *Amoebozoa* are synonomous will continue to accumulate. If this happens then *Eumycetozoa* is the older name and should be preserved as previously discussed (Shadwick *et al.* 2009).

Finally, although there is no strong evidence for a particular branching order among fruiting amoebozoans, there is strong evidence for several clades of protosteloid amoebae (Spiegel 1990, Bakker 2008, Shadwick *et al.* 2009, Shadwick 2010). Under the classification scheme proposed by Adl *et al.* (2005) all the protosteloid amoebae were lumped into a group called Protostelia (Olive 1975). Protostelia were placed in a group called the Eumycetozoa (Zopf 1884, emend. Olive 1975) along with Myxogastria and Dictyostelia. Since this classification scheme is not well justified by our current knowledge of these organisms, I suggest that the

Eumycetozoa should be temporarily abandoned and that *Myxogastria*, *Dictyostelia*, and the major clades of protosteloid amoebae should all be considered as clades within *Amoebozoa* without intervening ranks until future research establishes what those should be. Potential clades that might, in the future, be considered *Eumycetozoa* are discussed further under the heading Biological Questions. I therefore suggest the following higher level taxonomic revisions. The protosteliid clade, the soliformoviid clade, the schizoplasmodiid clade, the cavosteliid clade, and the protosporangiid clade (as depicted in Shadwick *et al.* 2009, Figure 3 chapter 4) should be formally named as amoebozoan taxa. These taxa would consist of the following species/genera (summarized in Table 3), but will likely include more taxa as new taxa are described and phylogenetically placed.

Taxon 1 (protosteliid clade): This taxon would consist of the species *Protostelium nocturnum*, *Protostelium okumukumu*, *Protostelium mycophaga*, *Planoprotostelium aurantium*, and a few species that are currently being described (Spiegel 1984, Spiegel 1990, Spiegel *et al.* 1994, L. Shadwick *et al.* 2009, J. Shadwick 2010).

Taxon 2 (schizoplasmodiid clade): This taxon would include the genera *Schizoplasmodium*, *Nematostelium,* and *Ceratiomyxella* (Olive and Stoianovitch 1966 and 1976, Olive 1970, Spiegel 1990). I do not envision including *Phalansterium solitarium* within the Schizoplasmodiid clade even though there is some indication from molecular phylogenetic analeyses (Shadwick *et al.* 2009) that it is the sister group to the Schizoplasmodiid clade because, in contrast with members of the schizoplasmodiid clade, *Phalansterium solitarium* does not have a plasmodial stage in its life cycle nor has it been reported to form fruiting bodies. Additionally, there is no evidence to indicate that *Phalansterium solitarium* might group within the schizoplasmodiid clade.

Taxon 3 (soliformoviid clade): This taxon would consist of the genus *Soliformovum* (Spiegel 1990, Spiegel *et al.* 1994, L. Shadwick *et al.* 2009).

Taxon 4 (cavosteliid clade): At present, this taxon could be anchored around *Schizoplasmdiopsis pseudoendospora* (the type species of the genus *Schizoplasmodiopsis*). The clade should include species and genera that branch with *S. pseudoendospora* in molecular phylogenetic analyses and also species that bear morphological similarity to known members of the clade (Spiegel 1990, Bortnick 1993, Bakker 2008, Shadwick *et al.* 2009, Fiore-Donno *et al.* 2010). Thus the clade should include *Schizoplasmodiopsis* sp. and *Tychosporium acutostipes*. *Cavostelium apophysatum* could be included as an *incertae sedis* member of this clade based on the morphological similarities to members of the group and weak/ambiguous molecular phylogenetic signal (Spiegel 1980, Bakker 2008, Shadwick *et al.* 2009, Fiore-Donno *et al.* 2010, Aberer *et al.* 2011). If the branching order in Figure 3 of Chapter 4 were eventually shown to be correct, the cavosteliid clade would include the genera *Schizoplasmodiopsis, Tychosporium, Cavostelium*, and a *"Schizoplasmodiopsis" amoeboidea* that was reassigned to a new genus. Additionally, *Schizoplasmodiopsis micropunctata* would be transferred to the genus *Tychosporium* as phylogenetic analyses of SSU rRNA gene sequences from *Tychosporium acutostipes* and *Schizoplasmodiopsis micropunctata* have shown these two species are synonomous (Bakker 2008). The remaining species of *Schizoplasmodiopsis* are expected to be distributed among the genera *Schizoplasmodiopsis, Tychosporium,* and *Cavostelium* as future research dictates such that only species that form a monophyletic group with *S. pseudoendospora* are included in the genus *Schizoplasmodiopsis.* Finally, if *Cavostelium apophysatum* becomes unambiguously placed inside the clade before the taxon is formally named, then it should be used as the type species for the taxon instead of *Schizoplasmodiopsis* *pseudoendospora*.

Taxon 5 (protosporangiid clade): This taxon would include the genera *Protosporangium* and *Clastostelium* (Spiegel and Feldman 1988, Spiegel 1990). While some morphological characters indicate that the protosporangiid clade and species in the genus *Ceratiomyxa* might be sister groups there is, thus far, no indication that one clade is nested within the other. I think there is sufficient difference between the microscopic fruiting stage of protosporangiids and the macroscopic fruiting stages of the ceratiomyxas to warrant separate taxonomic groupings. However, if future research were to show that *Clastostelium recurvatum* is nested among *Protosporangium* species then it should be subsumed into the genus *Protosporangium.* Likewise if future research shows that the protosporangiid clade is nested among *Ceratiomyxa* species, then perhaps all of these species should be subsumed into the genus *Ceratiomyxa.* However, I do not think the latter revisions (subsuming *Clasostelium* into *Protosporangium* or *Protosporangium* into *Ceratiomyxa*) are warrented at this time.

While the dictyostelids and myxogastrids are much more speciose groups than any of these proposed protosteloid clades, it is my opinion that the several protosteloid lineages are sufficiently disctinct based on morphological, ultrastructural, and molecular phylogenetic characteristics to warrant equal taxonomic rank with these more speciose groups. There are more described species of myxogastrids and dictyostelids, but this could easily be because these species produce large multicellular fruiting bodies and are thus more often noticed by researchers. The relatively large fruiting bodies of myxogastrids and dictyostelids have also provided researchers with more morphological characters that have been used to delineate species. It is my opinion that there may be many species of protosteloid ameobae within each protosteloid clade that have thus far eluded description because the morphological differences among protosteloid amoebae are often subtle (Shadwick 2010). In this way, I think the myxogastrids could easily be compared to the highly speciose Testacealobosia (testate amoebae), which can generally be identified by their tests even long after they are dead, whereas the major protosteloid lineages could be compared to any of a number of less speciose more ephemeral amoebozoan lineages of equal taxonomic rank such as the Leptomyxida or the Tubulinida (Adl *et al.* 2005).

Table 3: Clades of protosteloid amoebae.

Clade	Spiegel Group	Species included	References
Protosteliid	I	*Protostelium mycphaga*, *Planoprotostelium aurantium* (*Protostelium aurantium* comb. a*d interim*), *Protostelium nocturnum*, *Protostelium okumukumu*, *Protostelium apiculatum ad interim*, *Protostelium rodmani ad interim* (Shadwick 2010)	Spiegel 1984, Spiegel 1990, Spiegel *et al.* 1994, Shadwick *et al.* 2009, Shadwick 2010
Schizoplasmodiid	II	*Schizoplasmodium* sp., *Nematostelium* sp., and *Ceratiomyxella* sp.	Olive and Stoianovitch 1966 and 1976, Olive 1970, Spiegel 1990, Shadwick *et al.* 2009
Soliformoviid	III	*Soliformovum irregularis, Soliformovum expulsum*	Spiegel 1990, Spiegel *et al.* 1994, Shadwick *et al.* 2009
Cavosteliid	IV	*Schizoplasmodiopsis sp. Tychosporium acutostipes, Insertae sedis*: *Cavostelium apophysatum*	Spiegel 1990, Bortnick 1993, Bakker 2008, Shadwick *et al.* 2009, Fiore-Donno *et al.* 2010
Protosporangiid	Va	*Clastostelium recurvatum* and *Protosporangium* sp.	Olive and Stoianovitch 1977, Olive and Stoianovitch 1972, Spiegel and Feldman 1988, Spiegel 1990, Shadwick *et al.* 2009

Expanding Molecular Phylogenies: A robust phylogeny of protosteloid, myxogastrian, dictyostelid, and nonfruiting amoebozoans could serve as a backbone for inferring evolutionary history of characters (character-state mapping) and as a basis for future taxonomic revision. Future efforts to strengthen our understanding of the evolutionary history of protosteloid amoebae should include adding more taxa and more genes to molecular phylogenetic trees.

More taxa: Finding a sister group for some species in the molecular phylogenetic analyses presented in this dissertation could be as simple as obtaining gene sequence from a known or presumed closely related organism. In fact, Matt Brown has obtained SSU rDNA sequence from *Endostelium amerosporum,* and it was shown to be sister to *Endostelium zonatum* in preliminary phylogenetic analyses (Personal communication). This is a strategy that has worked in other groups, *e.g.* the recent expansion in the number of dictyostelid species and clades uncovered in Romeralo *et al.* (2011).

Obtaining gene sequence from all species within a group is necessary for elucidating the branching order among these species, and might also help to alleviate long and/or short branch attraction artifacts that cause these groups to be placed erroneously in broader phylogenetic studies. Thus, it might be useful to obtain SSU rDNA sequence from the additional *Protosporangium* species: *Protosporangium bisporum, P. conicum, P. fragile;* additional *Ceratiomyxa* species: *Ceratiomyxa hemisphaerica, C. morchella, C. sphaerosperma*; the remaining *Schizoplasmodium* and *Nematostelium* species: *Schizoplasmodium obovatum, S. seychellarum, Nematostelium gracile;* and the remaining *Schizoplasmodiopsis* species: *Schizoplasmodiopsis reticulata*.

In some cases it may be useful to sequence multiple isolates of a species espcially if preliminary results or morphological comparison lead to suspicion of a species complex as in

Protostelium mycophaga (Shadwick 2010), *Schizoplasmodiopsis amoeboidea* (note that SSU rDNA gene sequences are not identical for the two isolates included in Shadwick *et al.* 2009), and *Protostelium arachisporum* (preliminary comparison of sequences presented in the appendix with that generated by A.M. Fiore-Donno, analyses not shown).

Obtaining gene sequence from multiple isolates of the same species is also useful where it is thought that two species might be synonymous. This has been demonstrated with *Schizoplasmodiopsis micropunctata* and *Tychosporium acutostipes* (Bakker 2008). Synonomy is also suspected between *Nematostelium gracile* and *Ceratiomyxella tahitiensis* (F. W. Spiegel and George Ndiritu personal communication).

There is at least one case in which morphology of a protosteloid amoeba suggests that it might belong in the genus *Acanthamoeba* (F.W. Spiegel Personal Communication). Especially note that several characteristics of *Protostelium pyriformis* (shown n Bennett 1969, Olive and Stoianovitch 1969) are consistent with descriptions of acanthamoebae (Volkonsky 1931, Pussard 1966, Page 1967, Bowers and Korn 1968, Sawyer and Griffin 1975) including (1) overall amoebal morphology, (2) filose subpseudopodia (acanthopodia), and (3) double walled cysts including the acanthamoeba-like operculum from which the amoeba emerges. These features are, in my opinion, enough evidence to unambiguously place *Protostelium pyriformis* within the family *Acanthamoebidae sensu* Sawyer and Griffin 1975, and the genus *Acanthamoeba sensu* Volkonsky 1931 emend. Page 1967. In this case gene sequence is expected to add additional support to this morphology-based hypothesis. Perhaps gene sequence of multiple isolates might show whether *P. pyriformis* is synonymous with a single known *Acanthamoeba* species, whether the taxon *Protostelium pyriformis* includes multiple *Acanthamoeba* species that happen to fruit, or whether *P. pyriformis* is indeed a distinctive species among the *Acanthamoebidae.*

There are two cases where no sister group for a species is known or suspected based on morphology; *Echinosteliopsis oligospora* and *Microglomus paxillus.* In these cases it would be reassuring to sequence multiple isolates of a single species as was done with *Protosteliopsis fimicola* (Shadwick *et al.* 2009). For instance partial EF1-α gene sequence from *Echinosteliopsis oligospora* (Appendix) groups among myxogastrian gene sequences in molecular phylogenetic analyses (analyses not shown), but this result is suspect because this is incongruent with morphology of the amoebae and thus highly unexpected. In this case molecular sequence data from another isolate, or multiple isolates—all of which agreed, would be quite convincing.

More genes: In addition to SSU based phylogenetic inferences, protein coding gene sequences could also help in resolving the molecular phylogeny of protosteloid amoebae. Some efforts have been made in this direction. However, while, many protein coding genes have been sequenced and used for phylogenetic analyses of amoebozoans, currently different genes are sequenced in different labs for different groups of amoebozoans. Examples include actin (Baldauf *et al.* 2000, Fahrni *et al.* 2003; Nikolaev *et al.* 2004; Brown *et al.* 2007, Tekle *et al.* 2008, Yoon *et al.* 2008), *β*-tubulin (Baldauf *et al.* 2000, Tekle *et al.* 2008, Yoon *et al.* 2008), α-tubulin (Baldauf *et al.* 2000, Schaap *et al.* 2007, Tekle *et al.* 2008, Yoon *et al.* 2008), and elongation factor 1-α (Baldauf and Doolittle 1997, Baldauf *et al.* 2000, Fiore-Donno *et al.* 2005, Fiore-Donno *et al.* 2009). While this approach can be useful for solving phylogenies within lineages, it hampers broader comparisons among lineages.

Some of the reasons that different genes are used for phylogenetic analyses among different lineages are because there is variation among different lineages in different protein coding genes in (1) degree of difficulty to obtain sequences (2) number of gene duplications (*i.e.*, establishing orthology vs paralogy), and (3) the quality of phylogenetic signal. These issues were evident from my own efforts to isolate and sequence protein coding genes from protosteloid amoebae (see Appendix). For instance, actin is so conserved that it fails to delineate *Protostelium arachisporum* and *Luapeleamoeba hula ad interim* as separate species (preliminary analyses not shown). Whereas both morphology and SSU rRNA gene sequences clearly delineate these two species (Figure 1, Appendix Table A1). In addition, beta tubulin is so conserved in some species that *Ceratiomyxella tahitiensis,* a member of the schizoplasmodiid clade, branches among species in the protosteliid clade (Appendix Figure A4). This is despite the fact that beta tubulin is divergent in other species such that beta tubulin sequence from *Protosteliopsis fimicola* is by far the longest branch in any analyses in which it is included (analyses not shown). In addition, I have noted several gene duplications in terminal taxa (species/genera). In most cases these consist of some relatively conserved copies of the gene and others that are more divergent. In the case of *Protosteliopsis fimicola,* where the beta tubulin gene sequence is divergent, it is not clear whether the PCR product that I have obtained is indeed orthologous to the other beta tublin genes in my dataset.

The generation of multigene datasets from genomes and expressed sequence tag (EST) projects may help to alleviate this last problem if EST libraries from key taxa are deeply sequenced. This is because many if not all copies of any given gene are usually sequenced during the course of such a project, and the sequence for a broad variety of genes becomes available for phylogenetic analysis. Finally, any molecular phylogenetic or phylogenomic analyses on these organisms should include sequences from a wide variety of amoeboid organisms. Nonfruiting species that have been suggested, in phylogenetic analyses presented in chapter 4 of this dissertation (Shadwick *et al.* 2009), might be closely related to protosteloid amoebae such as *Flamella, Arachnula, Filamoeba, Acramoeba, Phalansterium, Multicilia, Protacanthamoeba* *bohemica*, and *Vannella placida* are especially important. It is also necessary to include species from other amoebozoan taxa that represent the known diversity, not just those that are thought to be specifically related to protosteloid taxa, for two reasons. First, broad taxon sampling has been shown to help resolve previously unresolved portions of the phylogenetic trees (*e.g.* Dunthorn *et al.* 2008, Yoon *et al.* 2008, Pick *et al.* 2010, Parfrey *et al.* 2010). For instance, in the phylogenetic analyses presented in chapter four of this dissertation multiple phylogenetically diverse SSUrRNA gene sequences from several species/lineages of vannellids, acanthamoebids, tubulinids, and a wide variety of other amoebozoans were necessary in order to recover the major amoebozoan lineages as clades. Second, broad taxon sampling helps to increase the chance that an unexpected, but valid evolutionary relationship will be found such as the relationships uncovered between *Protosteliopsis fimicola* and *Vannella placida* and between Isolate LHIO5 and *Protacanthamoeba bohemica* (Fig. 3 chapter 4 Shadwick *et al.* 2009).

BIOLOGICAL QUESTIONS

Protosteloid amoebae are potentially useful organisms for shedding light on a variety of lines of biological inquiry. These organisms have already been used to broaden our understanding of the biogeography of microscopic eukaryotes (recent papers include Shadwick *et al.* 2009; Ndiritu *et al.* 2009; Tesmer, and Schnittler 2009; Aguilar *et al.* 2007). However, many of these organisms could eventually be developed into model organisms that could help us understand the evolution of several additional biological phenomena (1) complex life cycles, (2) flagella/flagellate cells, (3) fruiting body formation, (4) spatial organization of the nucleus, (5) multicellularity, and (6) amoebal morphology. A few questions raised by the work presented in this dissertation on our understanding of the evolution of fruiting bodies, flagella, and complex life cycles among protosteloid amoebae are considered.

A) Evolution of Fruiting Bodies

1. How many times have stalked fruiting bodies evolved within the supergroup *Amoebozoa*? As discussed in Chapter 4 (Shadwick *et al.* 2009), fruiting amoebozoans are not exclusively a monophyletic group. Even if one were to designate a subset of amoebozoans to be a monohyletic taxon *Eumycetozoa*, it seems unlikely that all fruiting amoebozoans could be covered without reducing *Amoebozoa* to synonomy with *Eumycetozoa*. Conversely, there is no evidence that one could delimit any group of amoebozoans to be an exclusively fruiting monophyletic group unless one limits or splits *Eumycetozoa* to synonomy with *Myxogastria*. Each solution evades rather than illuminates the question of the origin of fruiting. Stalked fruiting occurs within at least 10 of the major amoebozoan lineages (Fig. 3, chapter 4), and specifically protosteloid fruiting occurs within at least nine of these lineages (if the myxogastrian *Echinostelium bisporum* is considered to have protosteloid fruiting). However, because detailed studies of fruiting body formation (George 1968; Reinhardt 1968; Spiegel *et al.* 1979; Whitney 1984; Spiegel and Feldman 1989), and ultrastructure (Dykstra 1978; Furtado and Olive 1971; Olive, Bennett, and Deasey 1984; Reinhardt 1966; Spiegel and Feldman 1989; Whitney 1984; Spiegel and Feldman 1993) are known for only a few species, we do not know how many origins of fruiting, much less how many origins of protosteloid fruiting, are represented within the eukaryotic supergroup *Amoebozoa*. The following discussion considers the implications of three possible scenarios for the number of times fruiting body formation might have evolved within the amoebozoan supergroup.

a) Single origin of fruiting among amoebozoans: If there were a single origin of fruiting within *Amoebozoa*, then it would imply that fruiting body formation is a pleasiomorphic state for known amoebozoans and has thus been either lost or overlooked in all amoebozoan species for which it has not been reported. A combined approach of 1) improving phylogenetic support at deep nodes within the amoebozoa, 2) genomic comparison of fruiting body forming and putative non-fruiting body forming amoebozoans, and 3) functional analyses of genes suspected to be involved in fruiting body formation would likely shed light on this issue.

b) Eleven or more origins of fruiting among amoebozoans: It is possible that future work might show that there were 11 or more origins of fruiting among amoebozoans; including (1) *Myxogastria,* (2) *Dictyostelia,* (3) the protosteliid clade, (4) the soliformoviid clade, (5) the protosporangiid clade, (6) the cavosteliid clade, (7) the schizoplasmodiid clade, (8) the vannellids, (9) *Endostelium,* and (10) the acanthamoebids, (11) copromyxids (Shadwick *et al.* 2009, Cavalier-Smith *et al.* 2004, Brown 2010). This would imply that formation of a fruiting body either is relatively simple for amoeboid organisms to evolve, imparts strong selective advantage for fruiting amoebae in nature, that *Amoebozoa* may be a very old lineage and with even a slight selective advantage, multiple innovations of fruiting evolved, or finally some combination of the above factors have produced the array of fruiting amoebas which currently exist.

c) Five origins of fruiting among amoebozoans: More moderately, and considering a plausible interpretation of the ssu phylogentic tree (Fig. 3 chapter 4) one could envision that there are only five origins of fruiting among amoebozoans: (1) acanthamoebids, (2) vannellids, (3) *Endostelium*, (4) copromyxids, and (5) a modified *Eumycetozoa* which might include myxogastrian amoebae, soliformoviids, protosteliids, protosporangiids, dictyostelids, cavosteliids, schizoplasmodiids, filamoebae, archamoebae, *Phalansterium, Multicilia, Acramoeba*, *Arachnula,* and *Flamella* (Shadwick *et al.* 2009). If this were eventually found to be true; using a combination of phylogenomic, cell biological, and evolution/developmental biology approaches; then it would suggest that fruiting might have been lost or overlooked in the nonfruiting "eumycetozoan" taxa (a subset of the taxa listed under number 5 above: filamoebae, archamoebae, *Phalansterium, Multicilia, Acramoebae, Arachnula,* and *Flamella*). One would also expect that fruiting body formation would be not only genetically, biochemically, and mechanistically similar among these putative eumycetozoan organisms, but also dissimilar with the noneumyceotozoan organisms.

2. How many times have protosteloid amoebae evolved active spore discharge?

When one compares what is known about the ballistosporous protosteloid species (reviewed in chapter 5 of this dissertation) with the phylogeny presented in (Shadwick *et al.* 2009), it seems possible that ballistosporous fruiting bodies might have evolved several times among protosteloid amoebae (Figure 2). This notion is based on the observations that the ballistosporous species are widespread among protosteloid amoebae (Spiegel *et al.* 2006) and also that each of the ballistosporous species indicated in Figure 2 appears to use a distinct mechanism of ballistosporous dispersal (Spiegel *et al.* 2006). I expect that high speed videomicroscopy (as in Pringle *et al.* 2005, Yafetto *et al.* 2008) could shed light on the mechanisms of ballistosporous dispersal among protosteloid amoebae as it has among fungi.

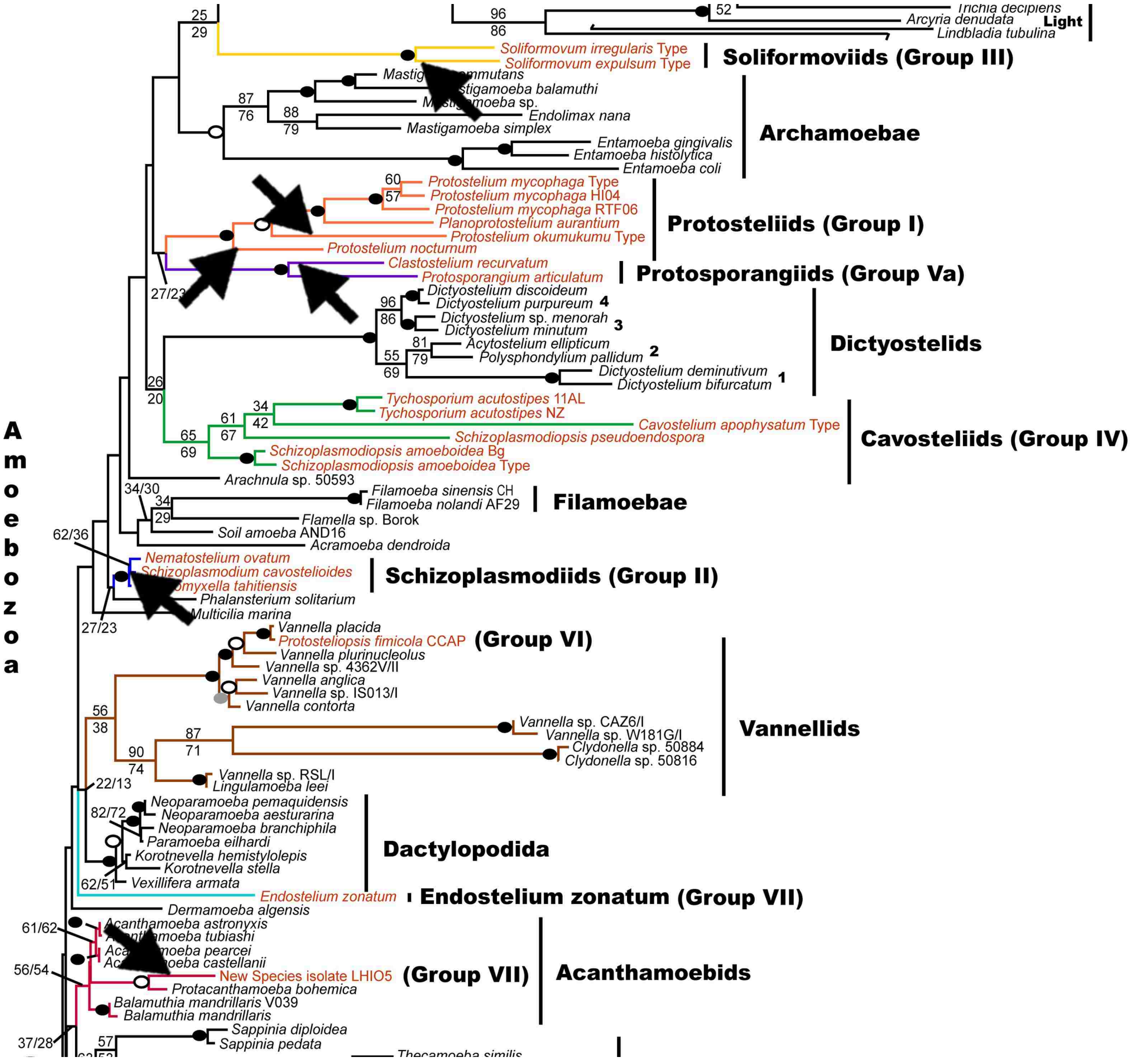

Figure 2: Portion of Maximum Likelihood phylogenetic tree based on ssu rRNA gene sequences excerpted from Figure 3 of Shadwick *et al.* 2009. Large black slanted arrows indicate ballistosporous species. Colored branches indicate protosteloid lineages. ML bootstrap values from analyses of 1,000 RAxML datasets and 1,000 GARLI 0.96 datasets are shown above and below the node respectively. ML bootstrap values: black oval = 90–100, white oval with black outline = 80–90. See chapter 4 of this dissertation (e.g., Shadwick *et al.* 2009) for additional details of this molecular phylogenetic analysis.

3. What biological questions are raised by examining the distribution of spore ornamentation among clades of protosteloid amoebae? It has been noted that the majority of short fruiting bodied protosteloid amoebae (those less than 25 microns tall (Spiegel *et al.* 2006) are ballistosporous. I have further noticed that the few short fruiting bodied protosteloid amoebae that are not ballistosporous have ornamented spores. No tall (> 25 microns) species of protosteloid amoebae have been reported to make ballistosporous fruiting bodies. Spore ornamentation was used as a defining character for the genus *Schizoplasmodiopsis sensu lato* and *Cavostelium apophysatum,* therefore by implication the Cavosteliids (Olive and Stoianovitch 1975, Dykstra 19778, Spiegel 1990), and is a nearly universal trait among myxogastrians, including the protosteloid myxogastrian *Echinostelium bisporum* (Spiegel and Feldman 1989). Among non-myxogastrian protosteloid amoebae, spore ornamentation is found only in the genera *Schizoplasmodiopsis, Cavostelium,* and *Tychosporium.* These genera group together weakly (65/69% bootstrap support) to form the cavosteliid clade (in Shadwick *et al.* 2009). Cavosteliids are all essentially nondeciduous. The ultrastructure of the spore and cyst ornamentation among these species with ornamented spores suggests that ornamentation may not always be homologous (Dykstra 1978, Whitney 1984). These observations lead me to the question of whether or not spore ornamentation aids in dispersal of protosteloid amoebae? This is a question that cannot be answered at this time, but it might be an interesting topic of future research.

B) Evolution of Flagellates

1. What can the distribution of flagellate species within protosteloid lineages tell us about the evolution of flagellates within those lineages? While the majority of protosteloid amoebae do not produce flagella, a few species are capable of making flagella. It is unlikely that flagella evolved independently in these taxa, since it is likely the last common ancestor of amoebozoans was a flagellate (Minge *et al.* 2009) and furthermore—that all extant eukaryotes are likely derived from ancestral flagellates (for a recent review see Simpson and Roger 2009). The distribution of flagellate protosteloid species across the molecular phylogeny presented in this dissertation (Shadwick *et al.* 2009) suggests that flagella/flagellate stages of the life cycle might have been lost more than once among protosteloid amoebae. For example, molecular phylogenetic analyses presented in this dissertation suggest that an amoeboflagellate-producing species of protosteloid amoeba, *Planoprotostelium aurantium*, is probably embedded within the protosteliid clade (100% ML Bootstrap support) [Figure 3] where more basal branching species do not produce flagella [Shadwick *et al.* 2009].

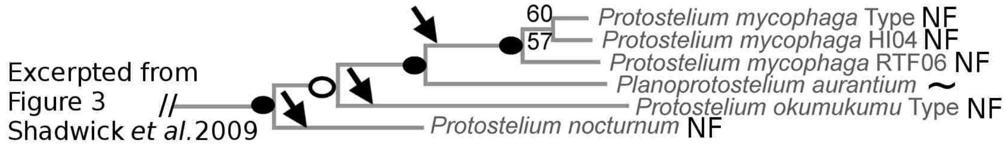

Figure 3: Molecular phylogenetic analyses based on ssu rRNA gene sequences presented in this dissertation (Chapter 4) suggest that the flagellate (~) member of the protosteliid clade is embedded among nonflagellate (NF) species. Arrows indicate lineages in which flagella are inferred to have been lost. ML bootstrap values from analyses of 1,000 RAxML datasets and 1,000 GARLI 0.96 datasets are shown above and below the node respectively. ML bootstrap values: closed oval = 90–100, open oval with black outline = 80–90. See chapter 4 of this dissertation (e.g., Shadwick *et al.* 2009) for additional details of this molecular phylogenetic analysis.

This suggests that flagella have probably been lost multiple times within the protosteliid clade (black arrows in Figure 3), rather than the more parsimonious condition of being lost only once so that a flagellate species, *Planoprotostelium aurantium,* is sister to all of the nonflagellate species (*Protostelium mycophaga, Protostelium nocturnum,* and *Protostelium okumukumu*) in the protosteliid clade as previously supposed (Spiegel 1990, Olive 1975). It must be noted that in ssu phylogenetic analyses,, *Planoprotostelium aurantium* was not absolutely excluded as a possible sister group to the other protosteliid species according to the AU test (Shadwick *et al.* 2009). However, phylogenetic analyses of the SSU rRNA gene of additional isolates of the protosteliid clade showed that not only was the flagellate producing *Planoprotostelium aurantium* embedded among nonflagellate producing species, it was also completely intermingled (0 branchlengths) with the nonflagellate producing *Protostelium mycophaga* var. *crassipes* (Shadwick 2010). This intermingling of flagellate- and nonflagellate-producing isolates in phylogenetic trees strongly suggests that these amoebae may frequently lose the ability to make a flagellum.

In addition, there are two clades of protosteloid amoebae, the schizoplasmodiid clade and the cavosteliid clade, in which multiple losses of flagella and/or flagellate stages of the life cycle cannot be ruled out (Shadwick *et al.* 2009).

(1) Within the schizoplasmodiid clade (100% ML bootstrap support), we cannot be certain of the number of times flagella/flagellate stages of the life cycle have been lost. While the flagellate cell producing species, *Ceratiomyxella tahitiensis,* is well supported (ML bootstrap is 96% and posterior probability is 0.95) as the deepest branching species in the analyses presented in chapter 4 [e.g.Shadwick *et al.* 2009], several nonflagellate species, that are expected to be part of the schizoplasmodiid clade (based on morphologic data), were not included in these analyses (Shadwick *et al.* 2009). Therefore it is possible that future researchers will find that a nonflagellate species such as *Schizoplasmodium seychellarum*, *S. obovatum* or *Nematostelium gracile* is the deepest branching member of the schizoplasmodiid clade. Notably, one of these species, *Nematostelium gracile,* differs from *Ceratiomyxella tahitiensis* only in that it lacks a flagellate stage of its life cycle. Thus, it is possible that *Nematostelium gracile* might be found to represent, not a distinct species, but a variant of *Ceratiomyxella tahitiensis* that failed to produce the flagellate stage of the life cycle under the culture conditions used by Olive and Stoianovitch (1966b). Thus the possibilities that (1) a nonflagellate member of the schizoplasmodiid clade that has not yet been included in molecular phylogenetic analyses is the deepest branching species in the schizoplasmodiid clade or (2) *Ceratiomyxella tahitiensis* and *Nematostelium gracile* form a species complex, present issues that must be addressed before we can know whether

flagella/flagellate stages of the life cycle have been lost once or more than once among species in the schizoplasmodiid lineage.

(2) The cavosteliid clade may contain one species with a flagellate stage in its lifecyle: *Cavostelium apophysatum* (Shadwick *et al.* 2009). Unfortunately *Cavostelium apophysatum* seems to be a rogue taxon (Aberer 2011) in ssu based molecular phylogenetic analyses and is therefore especially difficult to place phylogenetically (Shadwick *et al.* 2009, Fiore-Donno *et al.* 2010). If future work does show that *Cavostelium apophysatum* does indeed belong in the cavosteliid clade, then the branching order of *Cavostelium apophysatum* with respect to the other species must be determined before we can know how many times flagella/flagellate stages of the life cycle have been lost within this group.

2. What is the evolutionary significance of the flagellar apparatus?

Ultrastructural characteristics of the flagellar apparatus have been used for phylogenetic inference among protosteloid amoebae (see Spiegel 1990, 1991). Some strong groupings such as the grouping of *Clastostelium recurvatum* with *Protosporangium articulatum* are inferred from analyses of both morphologic and molecular data (Spiegel 1991, Shadwick *et al.* 2009) and are interpreted as real evolutionary relationships.

However, there are some discrepancies between the two types of analyses. Most flagellate protosteloid amoebae, *Protosporangium, Clastostelium, Ceratiomyxa, Ceratiomyxella*, have two basal bodies comprising their flagellar apparatus, but a few, *Planoprotostelium aurantium* and *Cavostelium apophysatum*, only have one. Phylogenetic analyses using characters of the flagellar apparatus (Spiegel 1991) group those protosteloid species, *Planoprotostelium aurantium* plus *Cavostelium apophysatum,* with a single basal body per flagellar apparatus while phylogenetic analyses of the SSUrDNA gene sequences (Shadwick *et al.* 2009) do not recover these taxa as sister groups. The failure of these species to group together in phylogenetic analyses of SSUrDNA gene sequences is concordant with Spiegel's (1982) assertion that these two species have probably both lost the second basal body independently and that the character state of having a single basal body per flagellar apparatus is convergent. However, the possibility that *Cavostelium apophysatum* might group with *Planoprotostelium aurantium* is not explicitly excluded, based on the phylogenetic analyses in this dissertation (Shadwick *et al.* 2009).

Finally, it is important to remember that characters of the flagellar apparatus, including those of protosteloid amoebae (reviewed in Spiegel 1980), are strikingly similar across all eukaryotes and may be misleading when evolutionary distances are great and taxon selection is lean because at least some of the characters of the flagellar apparatus are likely to be symplesiomorphic and thus uninformative in determining evolutionary relatedness (as in Spiegel 1981a,Spiegel 1981 b, Spiegel 1991, Spiegel and Feldman 1988). In addition, some characters like a cone of microtubules connecting the basal bodies to the nucleus may impart a structural advantage under some circumstances, and may have arisen multiple times (Spiegel *et al.* 1986), and therefore it might not be wise to assume that this character implies evolutionary relatedness (as in Cavalier-Smith *et al.* 2004).

C. Complex Life Cycles

1. Why do many species of protosteloid amoebae appear to have much more complex life cycles than other amoebozoans? It is important to remember that people draw life cycles. They draw the parts of the life cycle that interest them, the parts of the life cycle they look for, and the parts of the life cycle they have either observed or that they expect to see. Mycologists who study slime molds generally begin their life cycle observations with spores from mature fruiting bodies (Figure 4). Protistologists who study amoebae rarely draw life cycles. Thus, the life cycles of most amoebozoans are assumed to be relatively simple or virtually unknown (Figure 5). Complex life cycle is operationally defined here as a life cycle that consists of one amoeboid state that develops into another where the second morphologically distinctive amoebal state cannot revert to the first by a simple physiological change [*e.g.* the flagellate/nonflagellate transition when water is added or removed]. Please note that under this definition a complex life cycle may or may not include, fruiting body formation and/or meiosis. While we know that the last common ancestor of amoebozoa must have been capable of meiosis, we do not know whether it had a complex life cycle as defined herein.

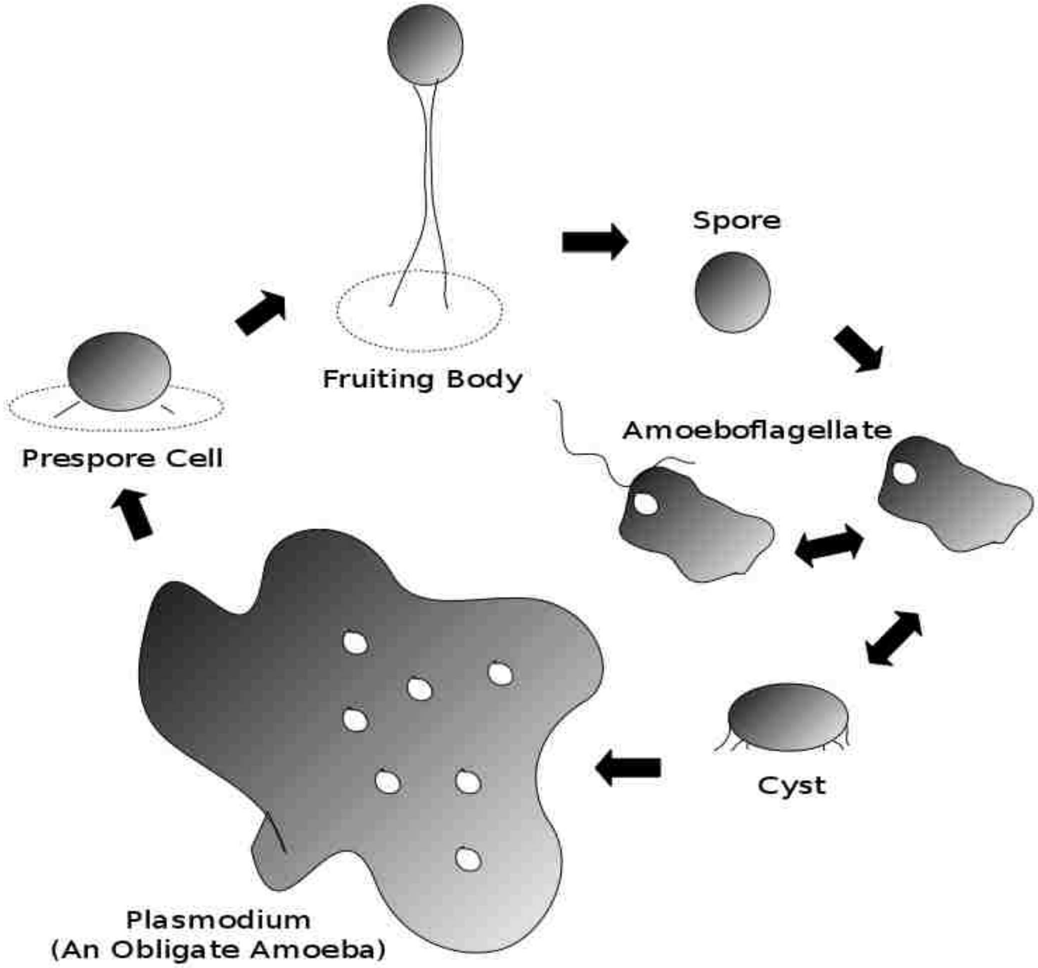

Figure 4: Generalized life cycle stages of protosteloid amoebae.

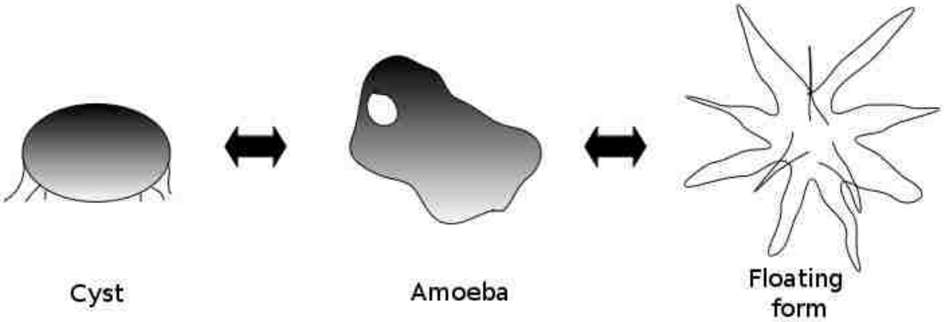

Figure 5: Generalization of known life cycle stages for most amoebozoans.

Molecular phylogenetic analyses presented in this dissertation (Shadwick *et al.* 2009) show that fruiting and nonfruiting amoebozoans (at least those amoebozoans with no described fruiting stage in their life cycle) are interspersed with one another. This should lead us to think more critically of life cycles involving amoeboid organisms. For instance, it causes us to observe amoebal and floating forms carefully in protosteloid amoebae and to begin to look for fruiting body formation in amoebae that were previously assumed not to fruit. The complex life cycles of protosteloid amoebae which are scattered among amoebozoans suggests that either complex life cycles have evolved many times among amoebozoans (Spiegel *et al*. 1995) or a complex life cycle was a character of the most recent common ancestor of the group. If complex life cycles were an ancestral character among amoebozoans, then one might conjecture that the most recent common amoebozoan ancestor possessed many of the life cycle stages present in modern amoebozoans (Figure 6).

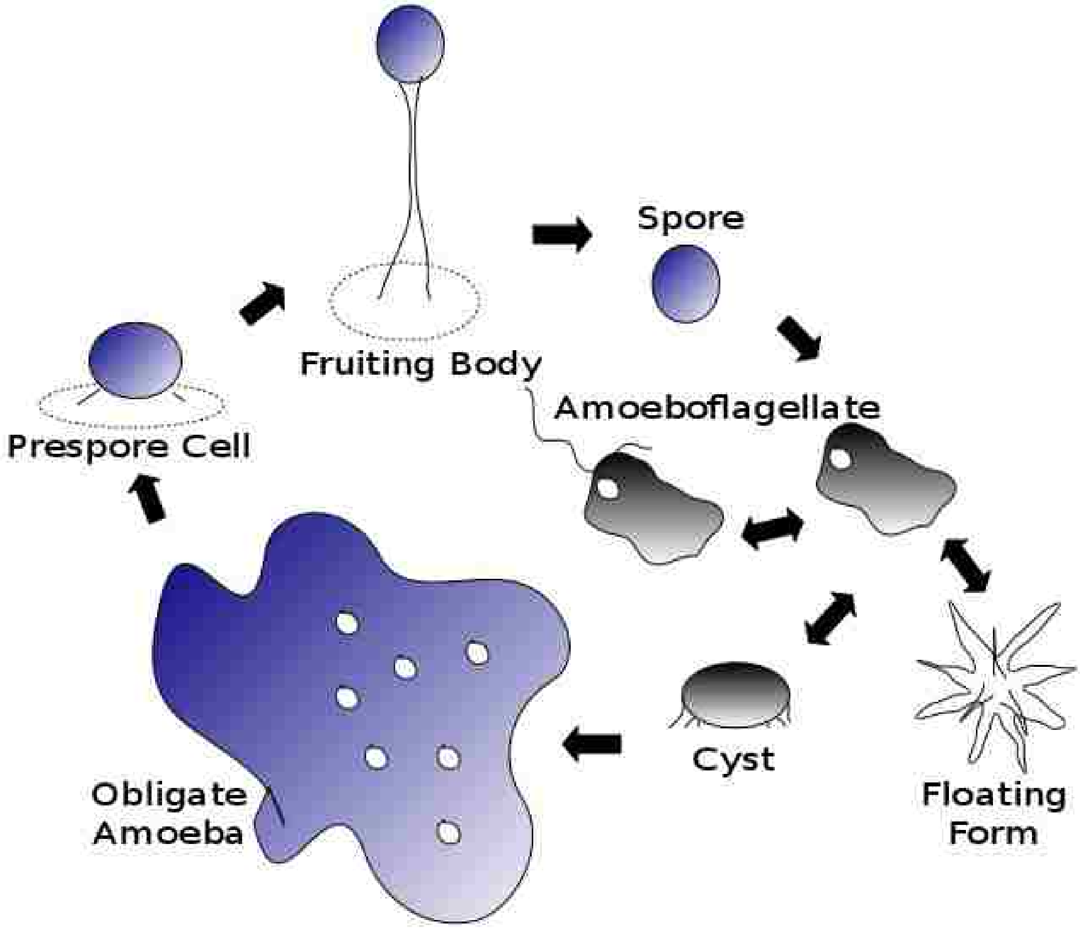

Figure 6: Summary of common life cycle stages that have been described among amoebozoans. Stages in blue are those primarily attributed to organisms that have previously been called slime molds, while stages in black / gray have been the primary focus of protistologists who study non-fruiting amoebae (*i.e.* those amoebozoans that have never been considered slime molds).

2. How do concepts about the evolution of nonflagellate stages of the life cycle of protosteloid amoebae affect ideas about the evolution of amoebozoans?

Nonflagellate amoeboid feeding cells in the life cycles of protosteloid amoebae have been placed in two groups (1) nonflagellated amoeboflagellates and (2) obligate amoebae. The obligate amoeba is generally the amoeboid stage that gives rise to the fruiting bodies, while the amoeboflagellate, if present, usually develops from spore germination (Spiegel and Feldman 1985).

Nonflagellated Amoeboflagellates: It has been suggested that the nonflagellate amoebal stages of *Protostelium mycophaga* (Spiegel *et al.* 1995a), *Protostelium nocturnum* (Spiegel 1984), *Protostelium okumukumu* (Spiegel *et al.* 2006), and *Tychosporium acutostipes* (Spiegel *et al.* 1995b) are simply the result of the loss of the ability of a flagellate cell to make a flagellum (Figure 7). This type of nonflagellate is thought to be homologous to an amoeboflagellate (Spiegel *et al.* 1995a). If the Spiegel, Lee, and Rusk's (1995) assumptions are correct, then molecular phylogenetic analyses presented in chapter four of this dissertation suggest that this process—the process of an amoeboflagellate losing its flagellum—probably happened more than once within the protosteliid clade as discussed above (Figure 3). Further, if *Tychosporium acutostipes* were eventually found to belong to any clade other than the protosteliid clade, as is suggested by molecular phylogenetic analyses (65-69% ML bootstrap support which jumped to 100% when *Cavostelium apophysatum* was removed from the analysis [Figure 3 in Shadwick *et al.* 2009]), then nonflagellate amoeboflagellates would be found to occur in more than one clade of protosteloid amoebae.

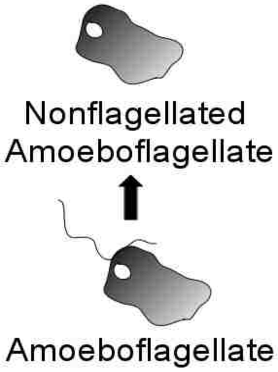

Figure 7: An amoeboflagellate cell loses its flagellum. The resulting nonflagellate is referred to as a nonflagellated amoeboflagellate.

Obligate Amoebae: The obligate amoebae of protosteloid amoebae that belong to the same clade (*e.g.*, the schizoplasmodiid clade) are generally indistinguishable from one another. However, the obligate amoebae of a given clade (*e.g.*, the schizoplasmodiid clade) do not bear clear morphological and ultrastructural similarities with the obligate amoebae found in other clades (*e.g.*, the protosporangiid clade) (Spiegel 1990, 1991; Spiegel and Feldman 1985, 1991; Spiegel *et al.*1995a). It was generally assumed that most amoeboflagellates and many aspects of their flagellar apparatus were homologous to one another (Spiegel 1991; Spiegel *et al.* 1995a; Olive 1975; Cavalier-Smith *et al.* 2004), and that flagellates were a synapomorphic characteristic for protosteloid amoebae (Spiegel1995a, Olive 1975). As discussed above, the homologies among flagellates and characteristics of the flagellar apparatus may be difficult to assign and/or possibly be symplesiomorphic. However, Spiegel and Feldman (1985) and Spiegel *et al.* (1995a) suggested that obligate amoebae might not all be homologous to one another. In Spiegel's view, this meant that there were multiple origins of obligate amoebae within the *Eumycetozoa sensu* Olive (1975) where several lineages with obligate amoebal life cycle stages evolved independently from amoeboflagellates (Figure 8).

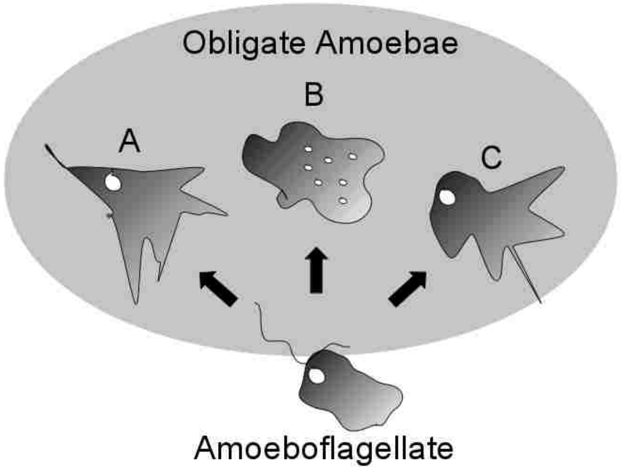

Figure 8: An ancestral amoeboflagellate gives rise to three independent lineages that include obligate amoebae in their life cycle labeled A, B, and C. One of those lineages, lineage B, has a plasmodial obligate amoeba.

Working under the *Eumycetozoa* hypothesis Olive (1975) and Spiegel *et al.* (1995a) assumed that the most recent common ancestor of the protosteloid amoebae had both an amoeboflagellate cell and a fruiting body in its life cycle (Figure 9.1), that obligate amoebae were independently gained in several major protosteloid lineages (Figures 9.2 and 9.3), that the amoebal state in *Protostelium mycophaga* is an amoeboflagellate that has lost the ability to produce a flagellum (Figure 9.4), and finally that the amoeboflagellate state can be lost after evolution of an obligate amoebal state (Figures 9.5 and 9.6). It was also possible, according to their figure (Spiegel *et al.* 1995a) that the original eumycetozoan *sensu* Olive (1975) could have had a full, complex life cycle and that obligate amoebal stages could have been lost and gained again independently (Notice that arrows between 9.1 and 9.2 and 9.1 and 9.3 could go both ways [Figure 9]).

Thus, theoretically, obligate amoebae come from two sources. First they could evolve independently from amoeboflagellates (as an additional life cycle stage), or they could evolve from existing obligate amoebae (summarized in Figure 9). These two scenarios can be thought of as similar to paralogy and orthology, where all of these amoebae are at some point in history the descendents of a common amoeboflagellate. Thus, amoeboflagellates and obligate amoebae (within a species) could be thought of as “paralogous” to one another, and obligate amoebae (among species, within a clade) could be thought of as “orthologous” to one another. While obligate amoebae (of different clades) are analogous to one another.

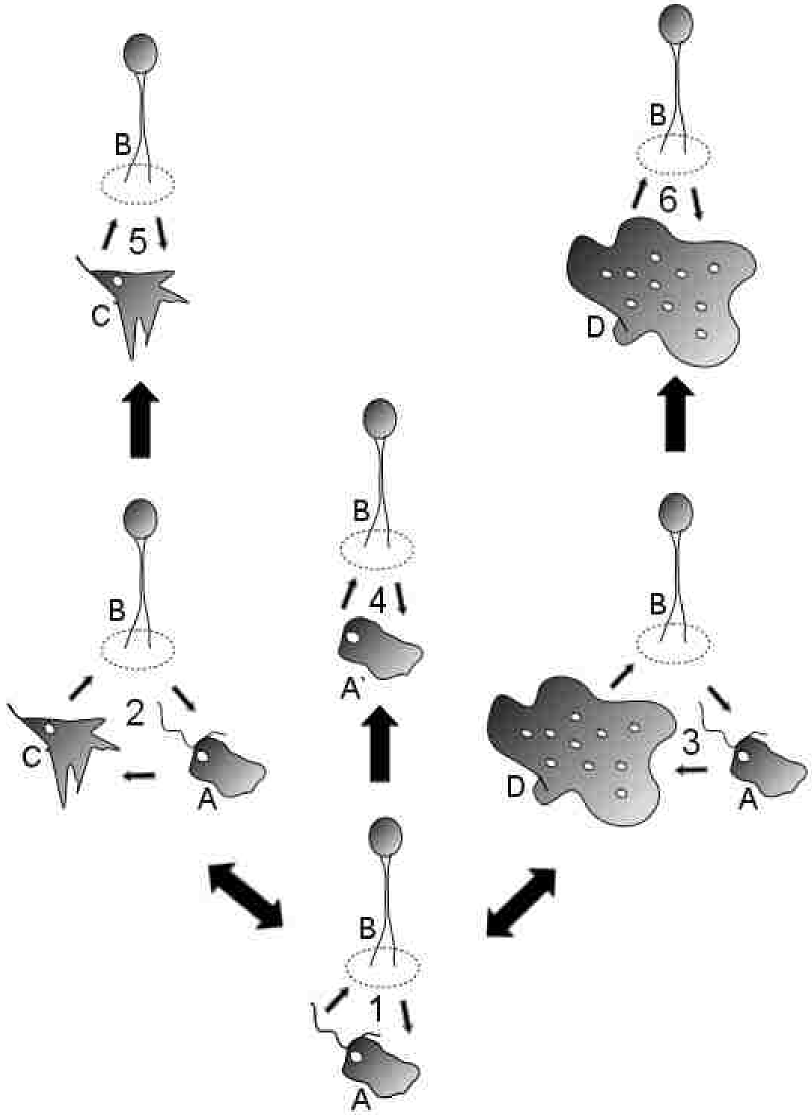

Figure 9: Life cycle evolution among protosteloid amoebae. Cell types with the same shape share synapomorphic morphological characters. (A) amoeboflagellate, (A`) nonflagellated amoeboflagellate, (B) fruiting body, (C) and (D) nonhomologous obligate amoebae. Redrawn from Spiegel *et al.* 1995a.

The molecular phylogenetic analyses presented in chapter four of this dissertation alter this scheme by: (1) forcing us to add nonfruiting amoebae and nonamoeboid flagellates, as well as (2) equating the ancestral eumycetozoan *sensu* Olive (1975) with the ancestral amoebozoan *sensu* Cavalier-Smith (1998).

In summary, it is possible that an ancestral organism with a complex life cycle could have given rise to the diversity that we currently observe in *Amoebozoa* (Lühe 1913 emend. Cavalier-Smith 1998) by subsequent loss and/or derivation of the obligate amoebal, flagellate, and fruiting stages of the life cycle. If this were the case, then this might cause us to view some nonfruiting amoebozoans *sensu* Cavalier-Smith (1998) as nonfruiting obligate amoebae, some as nonflagellate amoeboflagellates, and others as nonfruiting flagellates that have lost some ancestral life cycle stages (Figures 6 and 9).

CONCLUDING REMARKS

This seemingly disparate collection of papers on protosteloid amoebae is anchored by the notion that these organisms are first and foremost amoebae. While they exhibit special characteristics that are not known in other groups of amoebae (*i.e.*, protosteloid fruiting body formation), these characteristics should not be counted disproportionately compared to other key characters in the study of these amoebae. Further, research on both fruiting and nonfruiting amoebae would benefit from increased communication and collaboration between those who study fruiting amoebae and those who study nonfruiting amoebae, because all these amoebae share many, many more commonalities than differences.

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