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Phylogeny of the Labyrinthulomycota
(Under the Direction of DAVID PORTER)

The labyrinthulids, aplanochytrids, and thraustochytrids comprise a small group of marine saprobes. These organisms cause little economic or environmental impact, but probably play an important role in nutrient cycling and detritus breakdown in marine habitats worldwide. There have been sporadic incidences of labyrinthulid, aplanochytrid, or thraustochytrid species that have become pathogenic and have had major subsequent economic and environmental effects. One is *Labyrinthula zosterae*, the causative agent of wasting disease of the eelgrass *Zostera marina*. This species caused massive decimation of eelgrass populations in the 1930's along the Atlantic coasts of North America and Europe. The dieoff of eelgrass led to the extinction of the limpet *Lottia alveus*, which relied on eelgrass as its habitat. This remains one of only two documented extinctions of a species due to disease. Economic impact has been reported as a result of *Aplanochytrium haliotidis* infestation of abalone mari-culture facilities. These diseases are particularly worrisome because they appear sporadically and with little or no warning. In the interim, the causative organisms probably exist with little harm to the host and only become epidemic when environmental conditions are favorable. Fortunately, at least one isolate of a thraustochytrid also provides a human benefit. *Schizochytrium aggregatum* is being successfully grown in batch conditions for isolation of Ω -3-fatty acids (especially DHA or docohexanoic acid) that the organism produces in abundance. We have undertaken this research to provide an understanding of the phylogeny and taxonomy of these organisms and to increase our basic knowledge about these enigmatic protists. By doing so, we hope to provide a model of evolution that can be referenced by future

researchers who may be mining for new dietary supplements or controlling disease. This work includes four main parts: (1) In the first part of this study, we evaluate the phylogenetic position of these organisms within the Eukaryota. (2) Part two examines the phylogenetic positions of taxa within the group in relation to each other. (3) The third part of this work addresses the taxonomic validity of the aplanochytrids. (4) The final section evaluates species concepts within the aplanochytrids.

INDEX WORDS: Cladistics, *Labyrinthula*, Comparative Morphology, Ectoplasmic network, *Aplanochytrium*, *Thraustochytrium*, *Schizochytrium*, Labyrinthulomycota, Aplanochytriaceae, Evolution, *Ulkenia*, Thraustochytriaceae, *Diplophrys*, *Japonochytrium*, Labyrinthulaceae, Phylogenetics, SSU rDNA

PHYLOGENY OF THE LABYRINTHULOMYCOTA

by

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To Brian, Avory, and Emerson

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The labyrinthulids, aplanochytrids, and thraustochytrids comprise a small group of marine saprobes. As such, these organisms cause little economic or environmental impact, but probably play an important role in nutrient cycling and detritus breakdown in marine habitats worldwide. However, there have been sporadic incidences of labyrinthulid, aplanochytrid, or thraustochytrid species that have become pathogenic and have had major subsequent economic and environmental effects. The most important of these species is *Labyrinthula zosterae*, the causative agent of wasting disease of the eelgrass *Zostera marina* (Muehlstein and Porter, 1991). This species caused massive decimation of eelgrass populations in the 1930's along the Atlantic coasts of North America and Europe (Cottam, 1933, Muehlstein, 1989). The dieoff of eelgrass led to the extinction of the limpet *Lottia alveus*, which relied on eelgrass as its habitat. This remains one of only two documented extinctions of a species due to disease (Carlton et al, 1991, Daszak and Cunningham, 1999). Economic impact has been reported as a result of *Aplanochytrium haliotidis* infestation of abalone mari-culture facilities (Bower, 1986). These diseases are particularly worrisome because they appear sporadically and with little or no warning. In the interim, the causative organisms probably exist with little harm to the host and only become epidemic when environmental conditions are favorable.

Fortunately, at least one isolate of a thraustochytrid also provides a human benefit. *Schizochytrium aggregatum* is being successfully grown in batch conditions for isolation of Ω -3-fatty acids (especially DHA or docohexanoic acid) that the organism produces in abundance (Barclay et al, 1994). Although not yet approved for direct human consumption, the fatty acids are added to animal feeds and consumed by humans indirectly through eggs and dairy products. These fatty acids are known to play an important role in fetal nervous system development and maintenance of cardiovascular health, among other reported benefits (Barclay et al, 1998).

Because the diseases and benefits of these organisms are diverse and sporadic, and we understand that nothing in biology makes sense outside of the framework of evolution, we have undertaken this research to provide an understanding of the phylogeny and taxonomy of these organisms and to increase our basic knowledge about these enigmatic protists. By doing so, we hope to provide a model of evolution that can be referenced by future researchers who may be mining for new dietary supplements or controlling disease. This work includes four main parts: (1) In the first part of this study, we evaluate the phylogenetic position of these organisms within the Eukaryota. (2) Part two examines the phylogenetic positions of taxa within the group in relation to each other. (3) The third part of this work addresses the taxonomic validity of the aplanochytrids. (4) The final section evaluates species concepts within the aplanochytrids. The background for each of these sections is discussed in the following introduction.

In chapter two, the labyrinthulids were first described by Cienkowski in 1867. Cienkowski originally described two species, and seven additional taxonomically valid

descriptions have since been added for a total of nine species. These organisms are marine by nature (with one probable exception) and are characterized by the presence of an membranous, anastomizing ectoplasmic network (EN) that is thought to be extruded via bothrosomes at the cell surface (Porter 1972). The bothrosomes and EN of the labyrinthulids differ from those of the aplanochytrids and thraustochytrids in both structure and function. The bothrosome of labyrinthulas is urn-shaped and the EN enrobes the spindle cells that glide along through the network. The EN itself is immobile except for the advancing network at the colony periphery and occasional bulging where the cells squeeze through. The labyrinthulas were placed in the Rhizopodea (Protozoa) by Calkins in 1934. Bessey (1950) first allied these organisms with fungi by placing them in the Mycetozoa. In 1955, the labyrinthulids were allied with the Chrysophyta in the heterokont algae (Hollande and Enjumet 1955) which was affirmed by Chadeffaud (1956). Affinity was transferred back to the Rhizopodea by Honigberg et al in 1964. Pokorny (1967) once again transferred these organisms to the Mycetozoa. In 1975, Olive recognized similarities between the labyrinthulids and the thraustochytrids and classified the two groups together for the first time in the protistan phylum Labyrinthulomycota. In the late 1980's, ultrastructural data combined with molecular data indicated that the phylum was a natural member of the stramenopile clade of protists (Barr and Allen, 1985, Andersen, 1991, Cavalier-Smith and Chao, 1994, Leipe et al. 1994, 1996).

The thraustochytrids have endured a less turbulent taxonomic history than the labyrinthulids. The thraustochytrids have oval or spherical sporangia anchored to a substrate via unilateral EN. The EN is immobile, but it does not enrobe the cells as in the labyrinthulids. There are six genera with approximately 50 valid species in the group.

They were first described by Sparrow in 1936, who allied them with the chytrid fungi. However, the heterokont nature of the zoospores soon indicated that these organisms were more closely related to the Oomycetes (Sparrow 1943, Dick 1973).

The aplanochytrids are considered as part of the thraustochytrids (as the genus *Labyrinthuloides*). The aplanochytrids superficially resemble the thraustochytrids in that they have a globose sporangium with a unilateral EN. However, the aplanochytrid EN is motile. The cells are able to glide over the substrate surface using the EN in an amoeboid fashion. There are seven species in one genus.

When evaluating a gene to provide an independent phylogeny for the Labyrinthulomycota, we had the following criteria: (1) The candidate gene must be slowly evolving since the Labyrinthulomycota diverge early in the stramenopile lineage and is likely a highly divergent group. (2) The candidate gene must have been used in other stramenopile phylogenies so that appropriate outgroup sequences could be obtained. Based on these criteria, we chose to use the small subunit ribosomal DNA gene for this study to address the following questions: (1) Do the thraustochytrids and labyrinthulids form a monophyletic assemblage? (2) Do the aplanochytrids (or labyrinthuloids) nest within the thraustochytrids? (3) Where in the stramenopilian clade of protists do these organisms show affinity?

In chapter three, we examined species concepts within the labyrinthulids, thraustochytrids, and aplanochytrids using molecular tools. Historically, the labyrinthulids and aplanochytrids are identified to species based on gross colony morphology. The isolate is allowed to grow onto agar medium and is viewed under a

dissecting microscope or by eye. Species identification is based on such characters as colony size, radiating pattern, margin pattern, and agar penetration.

Unlike the other groups, the thraustochytrids are identified on fine characters of thallus morphology. An isolate can only be identified after viewing such developmental characters as number of spores released, presence of a basal rudiment, and dissolution of the cell wall. Identification is often time consuming and tedious, since an isolate needs to be monitored through at least one life cycle to verify characters. A list of valid taxa and their published defining characters for the labyrinthulids, thraustochytrids, and aplanochytrids is given in appendix 1.

We also examined the phylogenetic placement of *Diplophrys*, a genus of uncertain taxonomic affinities. Like the labyrinthulids, aplanochytrids, and thraustochytrids, *Diplophrys* species have ectoplasmic net elements, but they lack associated bothrosomes. This is particularly intriguing since bothrosomes are reported as being responsible for the generation of the ectoplasmic net (Porter 1972).

The following questions were addressed: (1) Is species identification based solely on colony morphology a valid method for the identification of species of aplanochytrids and labyrinthulids? (2) Are the characters used to identify thraustochytrids to genus and species level supported phylogenetically? (3) Do the motile members of the phylum form one lineage? (Did gliding motion evolve once, or more than once?) (4) Were bothrosomes secondarily lost in *Diplophrys*?

In chapter four, the genera *Aplanochytrium* and *Labyrinthuloides* were first described in adjacent issues of the same journal in the early 1970's. In 1973, Perkins described *Labyrinthuloides* as a new genus of labyrinthulid with a different locomotion.

Just months before, Bahnweg and Sparrow (1972) described *Aplanochytrium* as a thraustochytrid that made unique aplanospores. At that time, the thraustochytrid fungi were recognized as having morphological similarities with the Oomycetes. The labyrinthulids, including *Labyrinthuloides*, was placed in the Mycetozoa. Speculation on the relatedness of the labyrinthulids and thraustochytrids was just beginning (Perkins 1972, Darley et al. 1973).

Since the 1970's several researchers have remarked on the similarities between these two genera. The type species, *Aplanochytrium kerguelensis* and *Labyrinthuloides yorkensis* are so similar morphologically that they may be one taxon. Both taxa are described as having globose or spherical sporangia that liberate spherical aplanospores through tears in the sporangial wall. The major difference between the two taxa is in the number of aplanospores released (up to 64 in *L. yorkensis*, versus about 10 in *A. kerguelensis*), and the presence of large conspicuous lipid droplets in the aplanospores of *A. kerguelensis* that are not seen in *L. yorkensis*.

In 1985, Ulken et al. compared cell wall composition, G+C content of DNA, and nitrogen uptake ability for *Aplanochytrium*, *Labyrinthuloides yorkensis*, and *Labyrinthuloides minuta*. Using this data, they concluded “*Aplanochytrium* and *Labyrinthuloides* seem very similar if not identical”. At the same time, Bahnweg and Jackle (1986) examined similarities of thraustochytrid taxa using cell wall analysis, DNA base composition, and DNA/DNA hybridizations. The slight differences they found between *Labyrinthuloides* and *Aplanochytrium* were no more than between two isolates within each genus. They decided that these organisms were “inseparable from one

another on the basis of morphology.” Porter (1989) reaffirmed that *Aplanochytrium* and *Labyrinthuloides* were morphologically very similar.

Using molecular data, morphological data, and historical literature, the following questions were addressed: (1) Are the genera *Labyrinthuloides* and *Aplanochytrium* synonyms? (2) If so, which name should be conserved?

Chapter five constitutes a detailed analysis of the genus *Aplanochytrium* (discussed above). Although only seven species have been described in this genus, we have identified many more morphological variants during several years of collecting. Using electron microscopy, light microscopy, and sequence data, we addressed species concepts in detail including: (1) Does cell shape validly distinguish between species? (2) Does the ectoplasmic net show morphological differences between taxa? (3) Does pattern morphology indicate monophyletic lineages? (4) Is colony size and shape constant per isolate?

Appendix 1. **Valid taxa in the Labyrinthulomycota with the distinguishing characteristics of each.**

Labyrinthulas:

LABYRINTHULA L. Cienkowski, Archiv fur Mikroskopische Anatomie und Entwicklungsmechanik 3: 275 (1867).

The labyrinthulas are recognized by the complete enrobement of the trophic cells by the ectoplasmic net. The trophic cells stream through the ectoplasmic net which grows ahead of the advancing colony.

1. *Labyrinthula vitellina* L. Cienkowski, Archiv fur Mikroskopische Anatomie und Entwicklungsmechanik 3: 275 (1867).

Recognized by having relatively small trophic cells (<18 μm) and a red or yellow colony.

2. *Labyrinthula macrocystis* L. Cienkowski, Archiv fur Mikroskopische Anatomie und Entwicklungsmechanik 3: 275 (1867).

The sori have thick walls surrounding each cells and the colony is pale yellow.

3. *Labyrinthula cienkowskii* W. Zopf Beitrage zur Physiologie und Morphologie niederer Organismnen 2: 61 (1892).

This is the only freshwater species. Cells form sori with no common wall.

4. *Labyrinthula valkanovii* (A. Valkanov) J.S. Karling, American Journal of Botany 31: 50 (1944).

Recognized by very small trophic cells (<8 μm). This taxa is only identified from brown algal hosts.

5. *Labyrinthula algeriensis* A. Hollande and M. Enjumet, Annales des Sciences Naturelles, Zoologie et Biologie Animale, Serie 11 17: 357 (1955).

Unlike other labyrinthulas, this taxon regularly produces zoospores (4 per cell in the sorus).

6. *Labyrinthula roscoffensis* M. Chadeffaud, Comptes Rendus Hebdomadaires de Seances de l'Academie des Sciences, Paris, Serie D 243: 1797 (1956).

Confined to brown algae, but cells are relatively large (15-30 μm) compared to *L. valkanovii*. Sori have a common thin wall.

7. *Labyrinthula coenocystis* H. Schmoller, Archiv fur Mikrobiologie 36: 371 (1960).

The colony is light green in color.

8. *Labyrinthula magnifica* (A. Vlakanov) L.S. Olive, The Mycetozoans: 227 (1975).

This taxon has very large sori (1mm), each with a very thick common envelope.

This taxa is limited to diatom hosts.

9. *Labyrinthula zosterae* D. Porter and L. Muehlstein in L Muehlstein, D. Porter, and F Short, Mycologia 83: 182 (1991).

The cells do not form sori. This taxon is confined to *Zostera marina*.

Aplanochytrids:

APLANOCHYTRIUM (G. Bahnweg and F. Sparrow) C. Leander and D. Porter, Mycotaxon 76: 442 (2000).

The aplanochytrids are recognized by movement across substrate via filose pseudopodia of the ectoplasmic net.

1. *Aplanochytrium kerguelensis* G. Bahnweg and F. Sparrow, Archiv fur Mikrobiologie 81: 46 (1972).

The aplanospores have large, conspicuous oil droplets.

2. *Aplanochytrium yorkensis* (F.O. Perkins) C.A. Leander and D. Porter, Mycotaxon 76: 442 (2000).

The sori develop by one presporangium enlarging, and then dividing by simultaneous nuclear division and cleavage. Colony growth is very slow and the mature colony remains very small.

3. *Aplanochytrium minutum* (S.W. Watson and Raper) C.A. Leander and D. Porter, Mycotaxon 76: 442 (2000).

Sori are absent. This taxon is the most prevalent of our isolates, occurring on almost any substrate including non-living material such as blue-jeans. Colony has very prominent radial lines extending like spokes from the center of the colony.

4. *Aplanochytrium saliens* (J.A. Quick) C.A. Leander and D. Porter, Mycotaxon 76: 442 (2000).

The trophic cells are pear shaped with an anterior pit. This taxon has only been isolated from *Halophila*.

5. *Aplanochytrium haliotidis* (S. Bower) C.A. Leander and D. Porter, Mycotaxon 76: 442 (2000).

This taxon is an obligate pathogen of abalone. Unlike most species, *A. haliotidis* readily makes zoospores in standard culture conditions.

6. *Aplanochytrium thaisii* (B.A. Cox and Mackin) C.A. Leander and D. Porter, Mycotaxon 76: 442 (2000).

Two types of sporangia are produced, thin-walled and thick-walled. Trophic cells are spherical with amoeboid tendencies to become ovoid. Cysts are commonly formed.

7. *Aplanochytrium schizochytrops* (J.A. Quick) C.A. Leander and D. Porter, Mycotaxon 76: 442 (2000).

Trophic cells of this taxon are distinctly round or spherical.

8. *Aplanochytrium sweetingensis* C.A. Leander and D. Porter, Journal of Eukaryotic Microbiology. Submitted.

This taxon is easily recognized by patches of cells on agar and profuse agar penetration.

Thraustochytrids:

THRAUSTOCHYTRIUM F.K. Sparrow, Biological Bulletin of the Marine Biological Laboratory, Woods Hole 70: 259 (1936).

This genus is a catch-all for the group. Any thraustochytrid that does not display the necessary character(s) for the other genera is assigned to *Thraustochytrium*.

1. *Thraustochytrium proliferum* F.K. Sparrow, Biological Bulletin of the Marine Biological Laboratory, Woods Hole 70: 259 (1936).

This taxon has a single basal rudiment. The zoospores become flagellate after discharge from the sporangium, and the sporangial wall is persistent.

2. *Thraustochytrium globosum* Y. Kobayashi and M. Ookubo, Bulletin of the National Science Museum (Tokyo) 33: 60 (1953).

This is a non-proliferous taxon in which the basal part of the sporangial wall persists.

3. *Thraustochytrium pachydermum* E. Scholz, Archiv fur Mikrobiologie 29: 361 (1958).

This taxon is easily recognized by the extremely thick sporangial wall (5 μm).

4. *Thraustochytrium aureum* S. Goldstein, Archiv fur Mikrobiologie 45: 102 (1963).

Presence of a single basal rudiment and a golden colony color distinguishes this taxon.

5. *Thraustochytrium motivum* S. Goldstein, American Journal of Botany 50: 273 (1963).

Forms a single basal rudiment. The wall of the sporangium cracks open to release zoospores.

6. *Thraustochytrium multirudimentale* S. Goldstein. American Journal of Botany 50: 273 (1963).

This taxon is distinguishable by the presence of multiple basal rudiments, each with its own ectoplasmic network.

7. *Thraustochytrium roseum* S. Goldstein. Mycologia 55: 801 (1963).

This is the only taxon that is pink.

8. *Thraustochytrium aggregatum* A. Ulken, Veroffentlichungen des Instituts fur Meeresforschung in Bremerhaven 9: 291 (1965).

This taxon differs from *S. aggregatum* in size and number of zoospores released.

T. aggregatum is small (< 5 μm) and releases < 10 zoospores, while *S. aggregatum* is larger and releases 32-64 zoospores.

9. *Thraustochytrium kinnei* A. Gaertner, Helgolander wissenschaftliche Meeresuntersuchungen 15: 186 (1967).

This is a prolific species. The wall remains after zoospore release. The sporangium cleaves radially via a central vacuole which expands to release zoospores from multiple sites at once.

10. *Thraustochytrium striatum* J. Schneider, Kieler Meeresforschung 23: 16 (1967).

This may be a synonym for *T. kinnei*. The only difference is that this taxon may be slightly larger (sporangium 14-26 μm vs. 14-19 μm).

11. *Thraustochytrium arudimentale* N. Ya. Artemchuk, Veröffentlichungen des Instituts für Meeresforschung in Bremerhaven 13: 233 (1972).

The sporangial wall disintegrates only at the very apex.

12. *Thraustochytrium antarcticum* G. Bahnweg and F.K. Sparrow, American Journal of Botany 61: 760 (1974).

This taxon has a single rudiment, and the zoospores are released as a single flagellated clump.

13. *Thraustochytrium rossii* G. Bahnweg and F.K. Sparrow, American Journal of Botany 61: 760 (1974).

Zoospores are quiescent after release, and many rudiments are present.

14. *Thraustochytrium kerguelense* G. Bahnweg and F.K. Sparrow, American Journal of Botany 61: 762. (as *T. kerguelensis*)

This taxon also releases zoospores in a single flagellated clump, but has 3-10 successively forming rudiments.

15. *Thraustochytrium benthicola* S. Raghu Kumar, Transactions of the British Mycological Society 74: 612 (1980).

This taxon has a single basal rudiment around which the sporangial wall persists. Cleavage planes are random (vs. radial), and the zoospores are motile before discharge.

JAPONOCYTRIUM Y. Kobayashi and M. Ookubo, Bulletin of the National Science Museum (Tokyo) 33: 60 (1953).

This genus is recognized by the presence of a subsoral apophysis.

1. *Japonochytrium marinum* Y. Kobayashi and M. Ookubo, Bulletin of the National Science Museum (Tokyo) 33: 60 (1953).

This monotypic genus is recognized by the presence of a swelling directly beneath the sporangium, the subsoral apophysis.

SCHIZOCYTRIUM S. Goldstein and M. Belsky, American Journal of Botany 51: 72 (1964).

This genus is recognized by clumps of sporangia that are formed via a sporangium undergoing vegetative mitosis.

1. *Schizocytrium aggregatum* S. Goldstein and M. Belsky, American Journal of Botany 51: 72 (1964).

The masses of sori in this species are typically 10 times larger than those of *T. aggregatum*. The sorus releases 32-64 zoospores (vs. <10 in *T. aggregatum*).

2. *Schizocytrium minutum* A. Gaertner, Veröffentlichungen des Instituts für Meeresforschung in Bremerhaven 19: 69 (1972).

The sporangia are very small and clump together in tetrads or octads. Each gives rise to just 2 zoospores.

3. *Schizochytrium octosporum* S. Raghu-kumar, Transactions of the British Mycological Society 90: 273 (1988).

The sori are made of >100 sporangia, each releasing 8 zoospores.

4. *Schizochytrium mangrovei* S. Raghu-kumar, Transactions of the British Mycological Society 90: 273 (1988).

The sori are made of just a few sporangia (up to 12). Each sporangium releases a single zoospore.

5. *Schizochytrium limacinum* D. Honda, Mycological Research 102: 439-448 (1998).

This taxon is distinguished by the presence of limaciform amoeboid cells, but zoospores cleaved prior to release (vs. *Ulkenia*).

ULKENIA A. Gaertner, Veröffentlichungen des Instituts für Meeresforschung in Bremerhaven 16: 141 (1977).

This genus is characterized by the zoosporangial protoplasm being released from the sporangium prior to zoospore cleavage.

1. *Ulkenia visurgensis* (A. Ulken) A. Gaertner, Veröffentlichungen des Instituts für Meeresforschung in Bremerhaven 16: 141 (1977).

The sporangial wall dissipates upon spore release. The wall is thick, and the color is hyaline (vs. pink in *T. roseum*).

2. *Ulkenia amoeboidea* (G. Bahnweg and F.K. Sparrow) A. Gaertner, Veröffentlichungen des Instituts für Meeresforschung in Bremerhaven 16: 141 (1977).

Like *Aplanochytrium* species, this taxon is motile on its ectoplasmic network.

However, it produces abundant zoospores which is uncommon in the aplanochytrids.

3. *Ulkenia sarkariana* A. Gaertner, Veröffentlichungen des Instituts für Meeresforschung in Bremerhaven 16: 144 (1977).

The zoosporangial wall is persistent after the protoplasm is released.

4. *Ulkenia profunda* A. Gaertner, Veröffentlichungen des Instituts für Meeresforschung in Bremerhaven 16: 146 (1977).

The thin sporangial wall disperses when the protoplasm is released. The protoplasmic contents are amoeboid and amorphous.

5. *Ulkenia radiata* A. Gaertner, Veröffentlichungen des Instituts für Meeresforschung in Bremerhaven 16: 150 (1977).

This taxon is like *U. profunda* in that a thin wall disperses upon protoplasmic release. However, the protoplasm is star-shaped when released and divides into zoospores by centripetal cleavage.

6. *Ulkenia minuta* S. Raghu-Kumar, Veröffentlichungen des Instituts für Meeresforschung in Bremerhaven 16: 150 (1977).

This taxon develops secondary sporangia before protoplasmic release. The primary sporangium undergoes the typical mitotic cleavage to produce a multinucleate protoplasm. This protoplasm cleaves to produce secondary sporangia, rather than cleaving directly into zoospores. The secondary sporangia then cleave internally to produce the typical amoeboid protoplasm.

ALTHORNIA E.B.G. Jones and D.J. Alderman, Nova Hedwigia 21: 381-399 (1971).

This monotypic genus was isolated as an oyster shell pathogen, but exists free-floating in culture. This organism lacks bothrosomes and ectoplasmic net, but is

probably a true member of the phylum based on other morphological similarities such as the multi-laminar scaly wall.

1. *Althornia crouchii* Jones and D.J. Alderman, Nova Hedwigia 21: 381-399 (1971).

Diplophrids:

DIPLOPHRYS J. Barker, Quarterly Journal of Microscopical Science N.S. 8: 123 (1868).

Diplophrys is probably a natural member of this assemblage, but it lacks one of the two synapomorphies, the bothrosomes. It is otherwise similar to the trophic cells of the aplanochytrids in that it moves via extensions of the ectoplasmic network.

1. *Diplophrys archeri* J. Barker, Quarterly Journal of Microscopical Science N.S. 8: 123 (1868).

This taxon is from freshwater.

2. *Diplophrys marina* M.J. Dykstra and D. Porter, Mycologia 76: 627 (1984).

This taxon is marine.

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CHAPTER 2

THE LABYRINTHULOMYCOTA IS COMPRISED OF THREE DISTINCT LINEAGES.¹

¹Leander, C.A. and D. Porter. 2001. *Mycologia*. 93:459-464.
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ABSTRACT

The labyrinthulids and thraustochytrids, identified by the presence of bothrosomes and the associated ectoplasmic net, are marine saprobes classified as stramenopiles in the kingdom Protista. We have sequenced a partial ssurDNA region for 10 isolates in five genera, including *Diplophrys marina* (a possible labyrinthulid relative which lacks bothrosomes). We also include sequences of two isolates of the northern quahog pathogen, QPX, a thraustochytrid of uncertain taxonomic affinities. Our ssurDNA sequence analysis indicates that members of the Labyrinthulomycota fall into three phylogenetic groups. These groups correspond to the three morphological extremes found within the phylum; the labyrinthulids, the thraustochytrids, and the labyrinthuloids. QPX sequences support its inclusion as a thraustochytrid. *Diplophrys marina* also appears to show phylogenetic affinity with the thraustochytrids.

INTRODUCTION

The labyrinthulids and thraustochytrids comprise a group of common marine protists. In spite of their widespread occurrence as saprobes, they have garnered little scientific attention. Reports of pathogenic species on marine mollusks (Whyte et al 1994, Bower 1987, McLean and Porter 1982) and seagrasses (Muehlstein et al 1988) have periodically increased interest in their biology. Interest also has peaked recently because of the discovery of strains of thraustochytrids that produce abundant ω -3 fatty acids. The docahexanoic acid (DHA) from one strain of thraustochytrid has been commercially marketed as a dietary supplement (Lewis et al 1999, Barclay et al 1994).

Thraustochytrids are also known to produce a carotenoid pigment, canthaxanthin, which may also be useful as a dietary supplement (Valadon 1976).

The taxonomic status of the labyrinthulids and thraustochytrids is in need of phylogenetic investigation. These organisms have been placed among the fungi, slime molds, amoebae, and most recently, the stramenopiles (Cavalier-Smith et al 1994, Patterson 1989). The labyrinthulids and thraustochytrids were taxonomically united by Olive (1975), although their monophyly was questioned by Porter (1974). Porter (1989) later accepted the single phylum, Labyrinthulomycota, based on a suite of shared morphological features including the bothrosome (syn. sagenosome), a discrete organelle that produces the ectoplasmic network (Porter 1972). Other morphological characters such as the presence of tubular mitochondrial cristae and heterokont zoospores first established these organisms as belonging to the stramenopile clade of protists (Patterson 1989). In fact, as early as 1955 Hollande and Enjumet demonstrated the similarity of *Labyrinthula* zoospores to the members of another heterokont group, the Chrysophyceae. Small subunit ribosomal gene sequence analysis on a small number of species has further supported inclusion of the Labyrinthulomycota within the stramenopiles (Leipe et al 1994, Leipe et al 1996, Cavalier-Smith et al 1994).

Traditionally the group has been divided into two families: the Labyrinthulaceae and the Thraustochytriaceae (Olive 1975, Porter 1989). According to Olive and Porter, the Labyrinthulaceae consists of one genus, *Labyrinthula* (Cienkowski 1867). *Labyrinthula*, or marine slime nets, divide to produce a colony of spindle shaped trophic cells that glide through channels of an anastomized ectoplasmic network.

Thraustochytrids also extrude an ectoplasmic network, but their trophic cells are

determinate in growth. Instead, they produce globose, stationary sporangia with scaly walls and a unilateral ectoplasmic network. The Thraustochytriaceae includes seven genera: *Thraustochytrium* (Sparrow 1936), *Japonochytrium* (Kobayasi and Ookubo 1953), *Schizochytrium* (Goldstein and Belsky 1964), *Althornia* (Jones and Alderman 1971), *Ulkenia* (Gaertner 1977) *Aplanochytrium* (Bahnweg and Sparrow 1972), and *Labyrinthuloides* (Perkins 1973). The first five genera listed above readily produce zoospores. In contrast, species of *Labyrinthuloides* and *Aplanochytrium* commonly release non-flagellated aplanospores. Although traditionally recognized as being closely related to the other thraustochytrids, *Labyrinthuloides* and *Aplanochytrium* species also differ from the other five genera in their ability to glide via the ectoplasmic net. This character and their irregular production of zoospores raises questions about their relationship to the other, immobile thraustochytrids. Cell wall composition also suggests that the thraustochytrids and labyrinthuloids may form two distinct groups (Bahnweg and Jackle 1986). The thraustochytrids (including *Thraustochytrium*, *Schizochytrium*, *Japonochytrium*, and *Ulkenia*) contain galactose as the major wall carbohydrate (Darley et al. 1973), while species of *Labyrinthuloides* contain mostly fucose (Bahnweg and Jackle 1986). In contrast, the unity of *Labyrinthuloides* with the other thraustochytrids has been recently supported by Honda et al (1999) who suggest that the Labyrinthulomycota is composed of two phylogenetic groups, a Thraustochytrid Phylogenetic Group (TPG) and a Labyrinthula Phylogenetic Group (LPG).

Our own working hypothesis is the recognition of three distinct morphological types in the Labyrinthulomycota: the labyrinthulids, the thraustochytrids, and the labyrinthuloids. The current study reports ssrDNA sequence data used to examine the

phylogenetic placement of the Labyrinthulomycota within the stramenopile clade, and to establish phylogenetic relationships among members of the three morphological types seen in the group. Also included are sequences of two strains of QPX, an unnamed thraustochytrid parasite of *Mercenaria mercenaria* (quahog) (Maas et al 1999). QPX has bothrosomes, a scaly wall, and produces heterokont zoospores, consistent with its inclusion as a thraustochytrid. In addition we have included *Diplophrys marina*. Dykstra and Porter (1984) suggest its phylogenetic association with the labyrinthulids and thraustochytrids because *Diplophrys* has an ectoplasmic net and thin, scaly walls. Taxonomic inclusion of *Diplophrys* species within the Labyrinthulomycota is uncertain, as they do not have bothrosomes nor are they known to make zoospores.

MATERIALS AND METHODS

Cultures of labyrinthulids and thraustochytrids (including labyrinthuloids) were isolated from various coastal marine environments (Table 2.1), identified using light microscopy, and maintained on 1% SSA or KMV media (Porter 1989). For DNA isolation, organisms were grown in broth media in still petri dishes. DNA was isolated from concentrated cultures using a standard CTAB extraction protocol (Zolan and Pukilla 1986).

Following RNase digestion, genomic DNA was amplified by PCR using primer sets SR1R-NS2 and NS3-NS4 (SR1R from R. Vilgalys at <http://www.botany.duke.edu/fungi/mycolab/primers.htm>, others from White et al. 1990). Resulting PCR products were sequenced using the same primers on a Perkin-Elmer ABI 377 following manufacturers protocols. GenBank accession numbers for new sequences

are listed in Table 2.1. Sequences for QPX and QPXS were obtained from Maas et al (1999). Additional sequences were obtained from GenBank (Figure 2.1).

Sequence analysis and alignment was performed using the programs Lineup, Pileup, and PAUP* (Swofford 1998) available through the Genetics Computing Group, Madison, Wisconsin. Final alignment was done visually (TreeBase Submission ID#: SN595). Of the 1232 base pairs included in our alignment, 707 were unambiguously aligned and subsequently used for parsimony and maximum likelihood phylogenetic analyses. For maximum parsimony analyses (MP) 1000 replicates with random addition of taxa were performed to avoid multiple islands of most parsimonious trees. Starting trees were obtained via stepwise addition with simple addition sequence, and branch swapping was performed with tree-bisection-reconnection (TBR). A bootstrap analysis of 1000 replications was included in the MP search. Maximum likelihood (ML) analyses were also performed with 1000 replicates of random addition of taxa. Analyses were conducted using empirical nucleotide frequencies and two substitution types corresponding with the Hasegawa-Kishino-Yano (HKY) model. A molecular clock was not enforced. Starting branch lengths were obtained using Rogers-Swofford approximation method. Decay indices were generated using AutoDecay (Eriksson 1998). After these initial MP and ML analyses, topological constraints were employed to investigate the apparent polyphyly of the thraustochytrids. Constrained ML and MP trees were compared to unconstrained ML and MP trees using the Kishino-Hasegawa test. Finally, the same phylogenetic searches described above were performed with the exclusion of *Di. marina* from the taxa set to test for increased support of the thraustochytrid lineage. Because this study included broad phylogenetic questions

including placement of the group within the stamenopiles, three possible outgroups were assigned, the dinoflagellate *Pfiesteria* sp., the testate amoebae *Euglypha rotunda*, and the chlorarachniophyte *Chlorarachnion reptans*.

RESULTS

Each phylogenetic analysis (ML and MP) generated just a single tree. The single MPtree including *Diplophrys marina* is shown in Fig. 2.1. The ML tree has nearly identical topology. The only incongruence is the placement of QPX, which is sister to *Di. marina* in our ML analysis and sister to *Th. striatum* in the MP analysis. Because the placement of *Di. marina* is unstable, we also performed phylogenetic analyses excluding *Di. marina*. Exclusion of this taxon greatly increased bootstrap support for the thraustochytrid lineage (Fig. 2.1). When the sequence of *Di. marina* was excluded from the taxon set, ML and MP analyses produce trees with identical topology (Fig. 2.1).

Our work shows that the Labyrinthulomycota is sister to the Oomycetes, *Developayella*, and *Hyphochytrium*. Within the Labyrinthulomycota, the thraustochytrids are most closely related to the labyrinthulids. Together, these form a well supported, derived sister group to the third morphological group, the labyrinthuloids. It is worthy to note that inclusion of *D. marina* lowers bootstrap support for the thraustochytrid lineage. The thraustochytrid group corresponds to the TPG group of Honda et al (1999) (including *Ulkenia profunda*, *Labyrinthuloides haliotidis*, *Thraustochytrium kinnei*, *Th. motivum*, *Th. striatum*, *Di. marina*, and two strains of the thraustochytrid quahog parasite, QPX and QPXS). The thraustochytrids appear to be polyphyletic based on the exclusion of *Schizochytrium aggregatum* from the main clade.

However, when this polyphyly was tested the thraustochytrid constrained MP tree was only 6 steps longer than the unconstrained tree and this length difference is insignificant judged by Kishino-Hasegawa comparisons ($p=0.1337$).

The main clade of thraustochytrids is also paraphyletic due to the unexpected inclusion of *Di. marina* and *Labyrinthuloides haliotidis*. In contrast to the work of Honda et al (1999) we find a labyrinthulid lineage (including two isolates of *Labyrinthula zosterae*, *Labyrinthula* sp. s, and *Labyrinthula* sp. f) (Muehlstein et al 1988), and a distinct labyrinthuloid lineage (including *Labyrinthuloides yorkensis* and *Labyrinthuloides minuta*). The labyrinthulid lineage consists of all *Labyrinthula* species. Likewise, the labyrinthuloid lineage contains only *Labyrinthuloides* species.

DISCUSSION

Previous work has suggested that the nonpigmented stramenopiles group together in a distinct clade (Cavalier-Smith et al 1994). In congruence with these findings, our work shows that the Labyrinthulomycota is sister to the other nonpigmented stramenopiles (the Oomycetes, *Developayella*, and *Hyphochytrium*).

In our analyses, *Di. marina* nests in the thraustochytrid lineage. The taxonomic inclusion of *Di. marina* within the Labyrinthulomycota is supported with this work, although *Di. marina* has a relatively long phylogenetic branch. Inclusion of additional *Thraustochytrium* and *Diplophrys* isolates will add insight into the true nature of this relationship. The unusual result of having a single tree from these analyses gives support to their validity, as does a decay value of 14 for the branch joining *Di. marina* with the thraustochytrids.

Also in the thraustochytrid lineage is *Labyrinthuloides haliotidis*. The sequence submitted to GenBank for *La. haliotidis* was sequenced from nonviable material (L.Goggin pers com) and may need to be reisolated and resequenced to verify any phylogenetic position. It is likely that the sequence may actually be that of a thraustochytrid contaminant. Since this organism is a parasite of abalone in mariculture and there are currently no known outbreaks of disease, *La. haliotidis* is currently unavailable for further study. Alternately, it could be a misidentified thraustochytrid since it readily makes zoospores unlike other *Labyrinthuloides* species. The other taxa in the thraustochytrid lineage are clearly thraustochytrids.

In our analyses, the thraustochytrid *Sc. aggregatum* is positioned basal to the labyrinthulid and thraustochytrid lineages, as is the labyrinthuloid lineage. Although this polyphyletic arrangement of the thraustochytrids is not significant, it is worthy to note that *Schizochytrium* species and *Labyrinthuloides* species are the only organisms normally classified in the Thraustochytriaceae that undergo vegetative mitosis. This phylogenetic relationship may indicate a loss of vegetative mitosis in the more recently derived thraustochytrid group. Recognizing changes in morphological trends will be an important part of our ongoing study since morphological plasticity is well known in the thraustochytrids (Booth and Miller 1968).

Honda et al (1999) report on the presence of possible signature sequences that exist in the ssurDNA of thraustochytrids and labyrinthulids. Our labyrinthulid and labyrinthuloid signature sequences correspond to those of the LPG of Honda et al (1999), as does our sequence of *Sc. aggregatum*. Interestingly, the phylogenetic placement of *Sc. aggregatum* in our study is supported by these sequence similarities. Honda et al (1999),

however, used a different strain of *Sc. aggregatum* (ATCC 28209) with a different signature sequence and found that their strain of *Sc. aggregatum* falls within the main thraustochytrid group (TPG). The correct identification, phylogenetic placement, and species concepts within *Schizochytrium* is clearly in need of further investigation.

Given the consistency of the grouping of the thraustochytrids with the labyrinthulids, it is our contention that the thraustochytrids and labyrinthulids are indeed closely related. Our data show that the *Labyrinthuloides* also form a distinct phylogenetic group. We also suspect that *Diplophrys* is a simple, yet natural member of the phylum that may have lost a synapomorphic character, the bothrosome. Additional ultrastructural data analysis will help us to elucidate the relationships between species within the phylum with an emphasis on mapping morphological trends.

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TABLE 2.1. Source of organisms used for isolation of genomic DNA with GenBank numbers

<i>Thraustochytrium motivum</i>	T91-12	<i>Thalassia testudinim</i> /Florida Bay, FL
AF265337		
<i>Thraustochytrium striatum</i>	T91-6	<i>Spartina altiniflora</i> /Meridian, GA
AF265338		
<i>Labyrinthula zosterae</i> (east coast)	MBL93-2	<i>Zostera marina</i> /Woods Hole, MA
AF265334		
<i>Labyrinthula zosterae</i> (west coast)	Type-154	<i>Zostera marina</i> /San Juan Island, WA
AF265335		
<i>Labyrinthula</i> sp. s	JEL-Sap 93-1	<i>Zostera marina</i> /Adams Pt., NH
AF265332		
<i>Labyrinthula</i> sp. f	Sap F-1	<i>Spartina altiniflora</i> /Sapelo Island, GA
AF265330		
<i>Schizochytrium aggregatum</i>	T91-7	<i>Polysiphonia</i> sp./Meridian, GA
AF265336		
<i>Labyrinthuloides yorkensis</i>	JEL Ly	<i>Zostera marina</i> /Adams Pt., NH
AF265333		
<i>Labyrinthuloides minuta</i>	MMLM	<i>Zostera marina</i> /Middle Marsh, NC
AF265339		
<i>Diplophrys marina</i>	D83	<i>Thalassia testudinim</i> /Florida Bay, FL
AF265331		

FIGURE LEGENDS

Fig. 2.1. The single most parsimonious tree resulting from a heuristic search of all taxa. Bootstrap values (1000 reps) above 50 are shown above branches. Bootstrap values in parentheses indicate values obtained when *Diplophrys marina* is excluded from the analysis. Decay indices are shown below branches. Accession numbers are listed for sequences we obtained from Genbank. Bar = 10 changes. Length= 3,644. CI= 0.45, RI= 0.43. The single most likely tree, which has a corresponding topography, has a Ln likelihood of 9356.

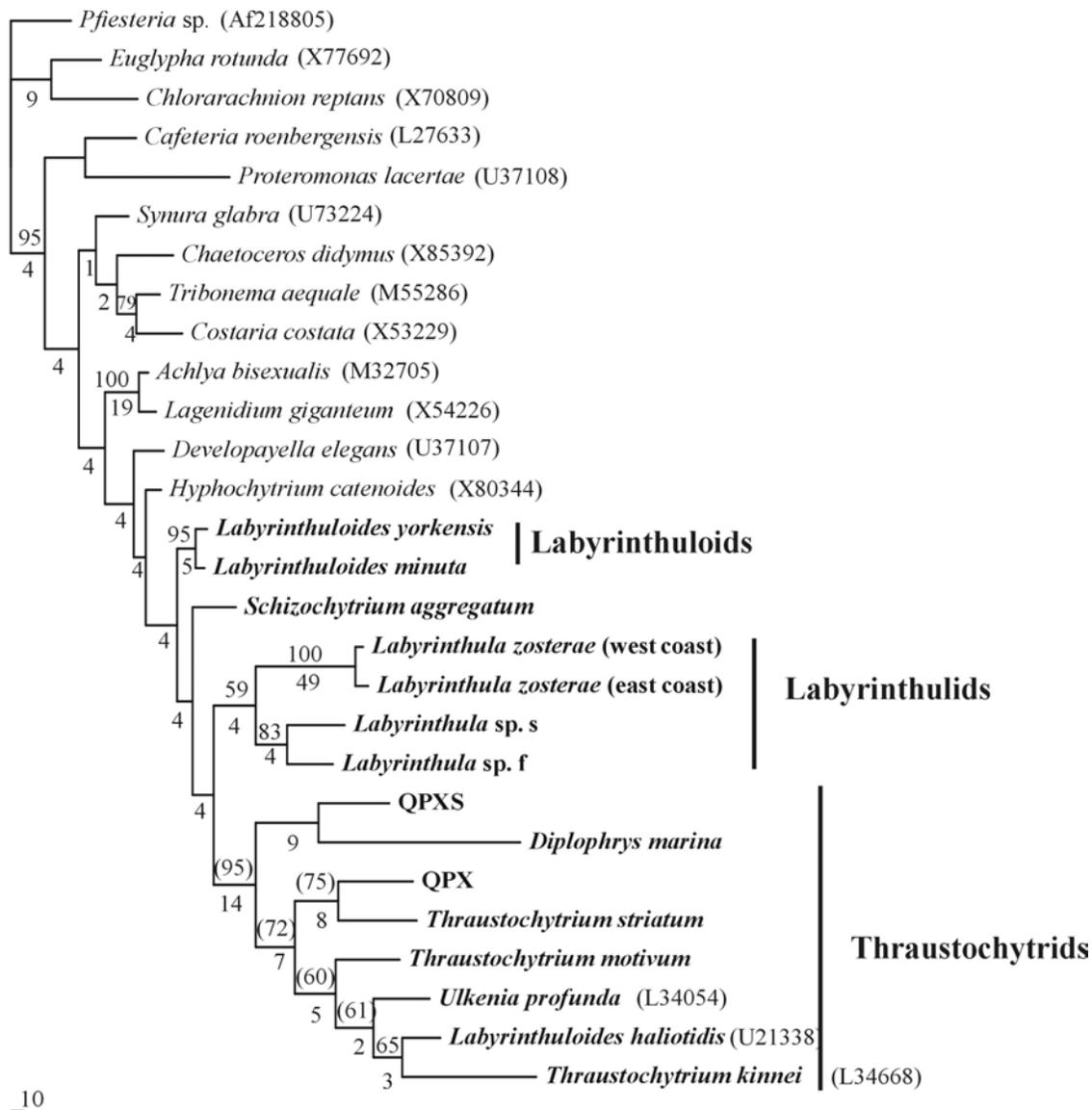


FIGURE 1
Leander and Porter

Figure 2.1.

CHAPTER 3

IDENTIFICATION CONCEPTS WITHIN THE LABYRINTHULOMYCOTA INCLUDING
APLANOCHYTRIUM SWEETINGENSIS N. SP. AND *APLANOCHYTRIACEAE* N. FAM.¹

¹Leander, C.A. and D. Porter. Submitted to Journal of Eukaryotic Microbiology,
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ABSTRACT

The phylum Labyrinthulomycota is comprised of three distinct morphological groups of marine protists; the labyrinthulids (slime nets), the thraustochytrids, and the aplanochytrids (=labyrinthuloids). Although the three groups are easily distinguished based on gross morphological characters, identifying genera and species within each group is challenging. The traditional morphological characters used to separate genera and species range from colony shape (in the aplanochytrids) to sporangial development (in the thraustochytrids). Recent molecular studies have indicated that the traditional characters used to identify genera and species within this phylum may not survive rigorous phylogenetic testing. However, this is the first study to address phylogenetic relationships using a wide range of taxa from each of the three groups found in the Labyrinthulomycota. By comparing molecular phylogenetic reconstructions with those made using traditional morphological characters, we have found that the gross colony characteristics used to separate taxa within the labyrinthulids and the aplanochytrids hold up to phylogenetic testing, while the morphological characters used to identify species and genera within the thraustochytrids do not. We describe one new species, *Aplanochytrium sweetingensis*, and separate Aplanochytriaceae from the Thraustochytriaceae as a third family within the phylum. We also designate PhyloCode definitions for the Labyrinthulomycota, the Aplanochytriaceae, and the Labyrinthulaceae.

INTRODUCTION

The labyrinthulids and the thraustochytrids are clearly distinct morphological groups. In fact, it was not until the mid-1970's that these two groups of organisms were first classified together (Olive 1975). The aplanochytrids (formerly species of *Labyrinthuloides*) show similarities to each of the other two groups but have historically been classified within the thraustochytrids (Darley, Porter, and Fuller 1973; Perkins, 1973; Porter, 1989). The labyrinthulids have spindle-shaped trophic cells that are completely enrobed by the ectoplasmic net. The cells glide through the ectoplasmic net and in some species congregate to form dense sori (Fig. 3.1).

The thraustochytrids are so named because of their morphological similarity to the chytrid fungi. The globose epibiotic sporangia and rhizoid-like unilateral ectoplasmic net system gives them a chytrid-like appearance (Fig. 3.2—3.7). However, this convergent morphological type has been adapted by these two groups due to similar habitat and lifestyle and does not indicate a close evolutionary relationship. Upon closer examination, thraustochytrids can be distinguished from chytrids by the presence of a multi-laminar scaly wall and an ectoplasmic net system that lacks a cell wall and organelles present in chytrid rhizoids.

The aplanochytrids are morphologically similar to the thraustochytrids in that they both possess globose sporangia and scaly walls, but they are motile on their ectoplasmic net (Fig. 3.3). The mode of motility is very different than that of the labyrinthulids, which is one reason why these organisms have been classified with the thraustochytrids rather than the labyrinthulids (Olive 1975). Unlike the labyrinthulids, which glide through the net elements, the aplanochytrids move in a gliding fashion using their

ectoplasmic nets as filose pseudopodia. Like the thraustochytrids, the aplanochytrids have multi-laminar scaly walls and zoospores that are more similar to those of thraustochytrids than to zoospores of labyrinthulids. Recent molecular data has shown that the aplanochytrids in fact form a phylogenetic group distinct from both the labyrinthulids and the thraustochytrids (Leander and Porter 2001).

Because of the distinct morphology exhibited by these three groups, it is no surprise that molecular data supports three corresponding phylogenetic groups (Leander and Porter 2001). However, assignment of genus and species names to isolates proves to be more difficult. Particularly in the thraustochytrids, characters used to assign genera and species are known to be highly variable and dependent on conditions such as nutrient availability and temperature (Booth and Miller 1968, Wethered and Jennings 1985). Even seemingly vital characters such as presence or absence of the ectoplasmic net and sporangial shape are not stable (Raghu-kumar 1988; Raghukumar 1992). Within the labyrinthulids and aplanochytrids, species designations are assigned primarily on gross colony characters (cell size and shape, presence of radial rings, presence of spokes, agar penetrance, colony size, and pattern of ectoplasmic net distribution) (Bahnweg 1973; Bahnweg and Sparrow 1972; Quick 1974a, b; Watson and Raper 1957). It is likely that these characters could also be plastic in nature.

There are five genera of thraustochytrids: *Thraustochytrium*, *Schizochytrium*, *Ulkenia*, *Japonochytrium*, and *Althornia*. *Ulkenia* species are recognized by the release of the sporangial protoplasm prior to cleavage into zoospores (Fig. 3.5) (Gaertner 1977). However, there is at least one species in the genus *Thraustochytrium* (*T. roseum*) that also displays this characteristic (Goldstein 1963). *Japonochytrium* (Fig. 3.4) is a monotypic

genus that is recognized by having a subsporangial apophysis (Kobayasi and Ookubo 1953), a characteristic that we have observed sporadically in several isolates that we would otherwise categorize as belonging to the genera *Schizochytrium* or *Thraustochytrium* (unpublished observations). Sporangia of *Schizochytrium* (Fig. 3.6) divide by vegetative cytokinesis (Goldstein and Belsky 1964), which also is a distinguishing characteristic of taxa such as *T. aggregatum* (Ulken 1965). The genus *Thraustochytrium* is the type for the family Thraustochytriaceae (Sparrow 1943) and serves as a catch-all for those taxa that do not obviously belong in the other three genera. There are 15 species in the genus *Thraustochytrium*, and these are divided into two taxonomic categories based on the proliferous nature of the sporangium. Those that are proliferous in nature produce subsequent sporangia by the growth of one or more basal rudiments that result from incomplete cleavage of the original sporangium (Fig. 3.7). The fifth genus, *Althornia*, is a parasite of oyster (Jones and Alderman 1971). *Althornia* has scaly walls like other thraustochytrids, but it does not have an ectoplasmic net or bothrosomes. *Althornia* is a monotypic genus not available in culture. There is no sequence data available for *Althornia* (Jones, G., pers. commun.) and it is not included in this study.

In the labyrinthulids, species of *Labyrinthula* are distinguished based on substrate affinities, trophic cell size, radiating pattern of the ectoplasmic net, and characteristics of the sori such as color and wall thickness. There are seven species in one genus, although we frequently observe isolates that show characteristics of more than one described species. Many *Labyrinthula* isolates rarely or never form sori or zoospores. Since cell

size overlaps between species, identification is most often based on how the colony grows across the agar surface.

There is also a single genus of aplanochytrid. *Aplanochytrium* (formerly *Labyrinthuloides*) colonies may look superficially like those of *Labyrinthula* species in that they disperse across an agar surface. There is a tendency for *Aplanochytrium* species to form radial spokes or annular rings as they move across the agar. In addition to the presence and shape of these spokes or rays, characteristics such as distinctness of the marginal line are also used to identify species. Cell shape in the aplanochytrids is more important than in identification of the labyrinthulids. Cell shape ranges from round (*A. schizochytrids* and *A. yorkensis*) to ovoid (*A. minuta*) and the cell is often marked with a distinct character such as an anterior pit (*A. saliens*) or a prominent lipid droplet (*A. kerguelensis*). As with the labyrinthulids, many of our isolates of aplanochytrids display overlapping characteristics of two or more described taxa.

One taxon of uncertain phylogenetic affinity, *Diplophrys marina*, is of interest to us because it has an ectoplasmic net but no associated bothrosomes (Dykstra and Porter 1984). Both of these characteristics are unique to the phylum Labyrinthulomycota. The absence of bothrosomes in *D. marina* is particularly intriguing since bothrosomes are implicated in the generation of an ectoplasmic net (Porter 1972). We have previously reported on the phylogenetic placement of one isolate of *D. marina* (Leander and Porter 2001). This relationship was inconclusive and we continue to investigate this proposed relationship with this study.

We are interested in evaluating species concepts within the Labyrinthulomycota by testing the characters described above with a phylogenetic approach to taxonomy. For

taxonomic groups based on characters that appear to be plastic or phylogenetically insignificant, we hope to identify useful characters that can be used as an alternative for identifying phylogenetic groups. Specifically, this paper addresses the following:

1. Do thraustochytrid genera form monophyletic groups based on their defining characteristics (*Schizochytrium*- vegetative cytokinesis, *Japonochytrium*- subsporangial apophysis, *Ulkenia*- amoeboid protoplasmic release, *Thraustochytrium*- 2 groups separated by presence or absence of rudiments)?
2. Can labyrinthulid and aplanochytrid species be identified based solely on colony patterns?
3. Did gliding locomotion evolve just once within the group, or twice? (Did the aplanochytrids, labyrinthulids, and *Diplophrys* arise from within the thraustochytrid group, or from a motile common ancestor?)
4. Were bothrosomes lost in *D. marina* or were bothrosomes a more recently acquired character?

METHODS AND MATERIALS

Culture collections. All new isolates were collected during 1998-1999 from the Bahamas, Puerto Rico, and Sapelo Island, Georgia (Table 3.1). Various substrates were collected from the tidal zone to 120' depth and sealed in sterile bags before bringing them back to shore. The substrate was then divided into small segments and placed on serum/seawater agar (SSA) or a yeast extract media (KMV). Growth was monitored over several days. When colonies of labyrinthulids, aplanochytrids, or thraustochytrids appeared, they were excised to new media and cryopreserved in liquid nitrogen after

several transfers. Thawed cultures were maintained on 1% (SSA) for the duration of this study. Isolates were identified with light microscopy. When we could not positively identify an isolate due to overlapping or ambiguous characters states, we continued to identify it by the isolate identification number rather than a species name.

Sequence generation. Many sequences used in this study have been previously reported (Honda et al. 1999; Leander and Porter 2001). These previous studies did not have adequate labyrinthulid or aplanochytrid sequences to address species validity within these groups. To accurately evaluate the taxonomic characteristics for this paper, we have added sequences from several isolates of *Aplanochytrium*, a second *Diplophrys* sequence, and an additional sequence from *Labyrinthula* sp. F (Table 3.1).

Genomic DNA was isolated with a modified Chelex extraction protocol (Goff and Moon 1993). Cultures were grown for an average of two weeks on 1% SSA media. Small segments of the colony were excised from the agar, added to 200 μ l of chelex extraction buffer, and incubated at 75° C for 30 minutes. The extracts were then boiled for 10 minutes in a water bath before being cooled on ice for 5 min. After centrifugation, the top layer was removed and immediately used as a PCR template.

We used an overlapping combination of two primer sets, NS1-NS4 and NS3-NS8 (White et al. 1990) for PCR amplification on a Perkin-Elmer PCR System 2400. With both primer sets, an annealing temperature of 54° C preceded an extension time of 1 min for 25 cycles. PCR products were sequenced completely in both directions using the above primer sets on an Perkin-Elmer ABI 377 following the manufacturers protocols.

Alignment was performed using the ClustalW package available from the Genetics Computing Group, Madison, WI. Fine alignment was finished by eye. We

included a total of 1,384 unambiguously aligned characters in our phylogenetic analyses. Optimality criterion was set to maximum parsimony for analysis with PAUP* (Swofford 1998). A bootstrap search with 250 replicates, each with 1,000 replicates with random addition of taxa was performed. A second bootstrap analysis was performed with 100 replicates with the optimality criterion set to maximum likelihood.

Morphological data collection. Within the thraustochytrids, we also used morphological data to create an independent phylogenetic hypothesis with which to compare our molecular reconstructions. These morphological characters include: number of basal rudiments, quiescence of zoospores, persistence of the sporangial wall, zoospore release mode, sporangial wall thickness, pigmentation, cleavage patterns, and sporangial size, presence of apophyses, and condition of cytoplasm upon release from the sporangium. The character state matrix used for subsequent phylogenetic analyses is shown in Fig. 3.9. Using PAUP*, the morphological data set was subjected to a branch and bound search set to a criterion of maximum parsimony. The thraustochytrid *ssur*DNA sequences from the molecular analysis (above) were subjected to a separate maximum likelihood search for comparison with the outcome of this morphological analysis.

Microscopy. Compound light images were captured using differential interference contrast (DIC) or bright field. Sections of colonies growing on 1% agar media were excised and placed directly on a standard glass slide with a small drop of seawater. Low magnification images were captured with the use of a reflective glass surface beneath the stage that allowed for maximum light refraction off of the colonies. For SEM, *Aplanochytrium sweetingensis* was grown on Thermanox coated plastic discs

placed on the agar surface near the expanding edge of an established colony. For fixation, the lid of the petri dish was removed and a piece of filter paper was taped to the inside. Approximately 0.5 ml of 4% O_5O_4 was applied to the filter paper. The lid was replaced, and vapor fixation was allowed to proceed for 30 min. Thermanox discs with fixed cells attached were then removed from the agar, dehydrated through a standard ethanol series, and critical point dried with CO_2 . The discs were then mounted on stubs and sputter coated with evaporated chromium. The cells were viewed under a LEO 982 scanning electron microscope.

For TEM, cells were grown on a large cover glass sealed in a chamber designed by Gabridge in 1981. Cells were grown in liquid serum seawater broth (SSB) for 1 week. The media was poured off and cells were pre-fixed for 5 min. with a solution of final concentration 8% gluteraldehyde and 4% O_5O_4 in a 0.1M cacodylate buffer with 1% $CaCl_2$ and 1M NaCl. Fixation followed for 30 min. with an 8% gluteraldehyde solution in the same buffer. Postfixation was for 15 min. with a final concentration of 4% O_5O_4 in the same buffer. All fixations took place at room temperature. Dehydration proceeded through a graded ethanol series. Cells were infiltrated with ethanol-resin mixtures and embedded in pure Epoxy resin (EMS). The chamber was then placed in a 60 °C oven where the resin was allowed to polymerize before being sectioned on a RMC MT-X ultramicrotome, post-stained with uranyl acetate and lead citrate, and viewed under a JEOL 100 CX II transmission electron microscope.

RESULTS

The results from our main molecular phylogenetic analyses are shown in Fig. 3.8. Numbers above branches indicate bootstrap support for the parsimony analysis, while those below branches indicate bootstrap support for the maximum likelihood analysis. One of 22 most parsimonious trees is shown. The representative tree matches the topology generated from maximum likelihood analysis.

Within the labyrinthulids, the two isolates of *Labyrinthula zosterae* (one east coast and the other west coast) form a clade with a bootstrap support of 100. The two isolates of *Labyrinthula* sp. F (Muehlstein, Porter, and Short 1988) also form a clade with bootstrap support of 100. Likewise, *Labyrinthula* sp. S (Muehlstein, Porter, and Short 1988) and the *Labyrinthula* sp. reported by Honda et al. (1999) form a monophyletic group with bootstrap values of 95 and 89. Published photographs of colony morphology of the *Labyrinthula* species reported by Honda (2001) resemble the colony morphology of *Labyrinthula* sp. S.

The inclusion of several isolates of *Aplanochytrium* firmly establishes this group as a highly supported monophyletic assemblage distinct from the thraustochytrids. *Aplanochytrium kerguelensis* and *A. yorkensis* form a weakly supported sister group to the other aplanochytrid isolates (bootstrap = 61). *A. sweetingensis* n. sp. forms a second lineage, while the remainder of the isolates (PR 24-1, *A. minutum*, PR 15-1, PR 1-1, SC 24-1, and PR 12-3) form a highly supported monophyletic group sister to *A. sweetingensis* n. sp. (bootstrap = 94).

The clade comprised of the two *Diplophrys* isolates has a moderate bootstrap value of 65, but this group is consistently placed within the same thraustochytrid clade.

The branches of both *Diplophrys* isolates are significantly long, so the relationship could be due to long-branch attraction. The thraustochyrids form two well-supported clades. The first is a basal lineage and includes *Thraustochytrium multirudimentale*, one isolate of *Schizochytrium minutum*, and one isolate of *S. aggregatum*. The second thraustochyrid clade is a recent lineage that is sister to the labyrinthulids. This clade includes the remaining 16 thraustochyrid isolates included in this study and the *Diplophrys* lineage. Many relationships within this clade are well supported with high bootstrap values. These include *T. striatum* and *S. limacinum* (bootstrap 100) with QPX (bootstrap 100 and 98), *Ulkenia radiata* and *U. profunda* (bootstrap 100), *T. motivum* and *T. striatum* (bootstrap 100), and *U. visergensis* and *Japonochytrium sp.* (bootstrap 100) with a second isolate of *U. profunda* (bootstrap 100).

The phylogenetic hypothesis resulting from our morphological search is shown in Fig. 3.10. *Thraustochytrium* species, except *T. aggregatum*, form a monophyletic clade with the inclusion of *Japonochytrium*. *Ulkenia* species form a paraphyletic group to the *Schizochytrium* species and *T. aggregatum*. *Schizochytrium* species form a monophyletic group with the inclusion of *T. aggregatum*. Figure 3.11 shows the unrooted result of the maximum likelihood analysis of our molecular data for only the thraustochyrid taxa. *Thraustochytrium* taxa are polyphyletic with six independent lineages. *Schizochytrium* taxa are also polyphyletic with three lineages. Likewise, *Ulkenia* species form two polyphyletic lineages, one of which includes *Japonochytrium*.

In order to correlate our phylogeny with previous taxonomies based on morphology, we evaluated the thraustochyrid taxa used in this study for 11 morphological characters. The results of the morphological investigation are presented in Fig. 3.9.

Using the resulting data matrix, the morphological phylogeny is composed of a lineage of all *Thraustochytrium* species with the inclusion of *Japonochytrium*. A lineage of *Schizochytrium* species also appears, with the inclusion of *Thraustochytrium aggregatum*. The three taxa of *Ulkenia* are likewise closely related with regards to morphology (Fig. 3.10). By comparison, evaluation of an unrooted molecular phylogeny of the thraustochytrid isolates indicates four independent *Thraustochytrium* lineages, three *Schizochytrium* lineages, and two *Ulkenia* lineages, one of which includes *Japonochytrium*.

DISCUSSION

Phylogenetics and identification. In general, the gross colony identification used within the labyrinthulids and the aplanochytrids is supported with monophyletic, well-supported clades from molecular phylogenetic analyses of the *ssurDNA* region. However, none of the 11 morphological characters used to assess thraustochytrid identification successfully merged with the relationships suggested from these molecular phylogenetic trees. Details within each of the three groups are discussed below.

The labyrinthulids form a monophyletic group with very strong bootstrap support when *Diplophrys* sequences are excluded (Leander and Porter 2001). Within the labyrinthulids, each of the pairs of species that we identified (*Labyrinthula* sp. F, and *Labyrinthula zosterae* east and west coast isolates) formed monophyletic groups (Fig. 3.9). In addition, our undescribed *Labyrinthula* sp. S and the unidentified *Labyrinthula* sp. isolated by Honda et al. formed a clade with bootstrap support of 95. We suspect that this pair forms a third species complex. This information suggests that characteristics

used to identify species of *Labyrinthula* are simple and adequate. These taxa can be identified visually using the colony characteristics described above with a dissecting microscope. The easiest characteristic to evaluate is the fanning morphology of the colony as it grows across a standard 1% SSA media (Fig. 3.12-3.14). *Labyrinthula* sp. S expands rapidly across the agar surface, with only slight masses of cells forming at the margins (Fig. 12) (Muehlstein, Porter, and Short 1988). This taxon often grows into the agar surface. Cells stream out in nearly linear masses with fine reticulations forming a continuous, uniform margin. In contrast, *Labyrinthula* sp. F has highly digitate margins. Masses of cells occur throughout the colony, but this species does not grow into agar (Fig. 3.13) (Muehlstein, Porter, and Short 1988). *Labyrinthula zosterae* has a very irregular expanding edge with formation of large masses of cells along the margins (Fig. 3.14).

Likewise, the aplanochytrids form species complex clades that can be identified with gross colony characteristics (Fig. 3.15-3.16). Aplanochytrid colonies tend to have either rays originating from the center of the colony and extending outwards as in *A. minutum* (Fig. 3.15), or patches of cell clusters as found in *A. yorkensis* (Fig. 3.16) (Quick 1974b). *Aplanochytrium yorkensis* and *A. kerguelensis* are recognized as distinct but very similar species (Leander and Porter 2000). This is supported by the bootstrap result of 61 for the least inclusive clade comprised of these two taxa on our molecular tree. A second clade can be found with the association of *A. minutum* and PR 24-1, an *A. minutum*-like isolate. Sister to the *A. minutum* clade is a monophyletic group of four similar, but non- identical isolates (PR 15-1, PR1-1, SC24-1, and PR12-3). The cells of PR1-1, PR15-1, and PR12-3 are oblong, while those of SC24-1 are round. The surface

pattern of the rays varies from thick and highly branched rays to thin rarely branching rays. While these cultures are distinct morphologically, there is little bootstrap support within this clade. This suggests that the sequence data is also very similar. This clade (bootstrap 96) collectively forms an *A. minutum* species complex. A third lineage consists of *A. sweetingensis* n. sp. This isolate differs morphologically from all described taxa and forms a branch that is basal to the *A. minutum* group. Herein we describe this isolate as a new species based on the morphological uniqueness and sequence distance of this taxon.

Within the thraustochytrids, there is little correlation between morphology and phylogeny. Not one of the 11 morphological characters assessed in this study corresponds to our *ssurDNA* phylogeny (Fig. 3.10, 3.11). Bootstrap values are high for several relationships that do not share traditional morphological traits. Among these are clades containing two genera such as *Ulkenia visergensis* and *Japonochytrium* sp., and *Thraustochytrium striatum* and *Schizochytrium limnacium*. It is also disconcerting that two isolates of the same taxon are found in completely different parts of the *ssurDNA* phylogeny as is the case with both isolates of *Schizochytrium aggregatum*, *Ulkenia profunda*, and *Thraustochytrium striatum* (Fig. 3.8). Finally, the thraustochytrids do not form a monophyletic assemblage, as do the labyrinthulids and aplanochytrids. Instead, we find a well-supported main group of isolates, and then a group of 3 isolates that appear to be a basal lineage to the rest of the phylum. Assuming that *ssurDNA* sequences adequately reflect a true phylogeny, we must conclude that not only do we lack a phylogenetic systematic treatment of the thraustochytrids, but also that

identification to genus and species level even with these phylogenetically invalid morphological characters is highly subjective due to character plasticity.

There is molecular evidence supporting that gliding motility is an ancestral trait in the Labyrinthulomycota that has been lost within some thraustochytrid lineages. The motile organisms (the aplanochytrids, the labyrinthulids, and *Diplophrys* taxa) are ancestral to a main thraustochytrid group, however a smaller thraustochytrid lineage (including *T. multirudimentale*, *S. minutum*, and *S. aggregatum*) is sister to this entire labyrinthulid/aplanochytrid/thraustochytrid lineage with a bootstrap support of 85. This suggests that gliding may have evolved from a thraustochytrid-like descendent, and has been secondarily lost within the main group of thraustochytrids. *Diplophrys* taxa form a well-supported least inclusive clade (bootstrap 100) ancestral to this derived thraustochytrid lineage, suggesting that bothrosomes may have been secondarily lost as well.

TAXONOMY

In recent years, the Linnaean system of taxonomy has come under considerable scrutiny. The Linnaean system is especially challenging for workers dealing with protists and other organisms whose taxonomy is in continual flux beyond the genus and species levels. One solution has been the development of the PhyloCode: Phylogenetic Code for Biological Nomenclature (<http://www.ohiou.edu/phylocode/index.html>), which has been established as an alternative to a ranked system. For the following discussion, we designate Linnaean names with (L) and PhyloCode names with (P).

Based on the morphological and molecular data discussed above, we separate the genus *Aplanochytrium* from the Thraustochytriaceae(L) and designate a third family within the phylum, the Aplanochytriaceae(L) following Article 41 of the Botanical Rules of Nomenclature (Greuter et al. 2000).

Aplanochytriaceae (n. fam.) C. Leander and D. Porter. Includes those genera possessing an ectoplasmic net system that does not enrobe the vegetative cells, yet serves as a locomotor device. Type genus: *Aplanochytrium* (G. Bahnweg and F. Sparrow) Leander and Porter 2000.

Aplanochytriaceae (n. fam.) C. Leander and D. Porter. Comprehendo generis gestum retia ectoplasmica ratio qui non complexo crescens loculi, etiam munus cum lapsus fabrica. Type genus: *Aplanochytrium* (G. Bahnweg and F. Sparrow) Leander and Porter 2000.

Under the PhyloCode, clades are designated with a uninominal, and may be named using node-based, stem-based, or apomorphy-based definitions. We chose to use apomorphy-based definitions for the following three clades. We are not transferring the Thraustochytriaceae(L) at this time because there is not strong evidence that this family is comprised of a monophyletic assemblage.

Aplanochytriaceae(P) (new clade name) as the clade stemming from the first species to possesses an ectoplasmic net system that is used as a locomotor device, yet whose system does not enrobe the vegetative cell, synampomorphic with these features in

Aplanochytrium kerguelensis. *A. kerguelensis* G. Bahnweg and F.K. Sparrow, the type species of *Aplanochytrium*, will serve as the type species for this definition.

Labyrinthulaceae(P) (converted clade name, E. Haeckel 1868, *Jenaische Zeitschrift für Medicin und Naturwissenschaft* 4, 127) as the clade stemming from the first species to possess an ectoplasmic net enrobing the vegetative cells, synapomorphic with these features in *Labyrinthula vitellina* . *Labyrinthula vitellina* L. Cienkowski, the type species of *Labyrinthula*, will serve as the type species for this definition.

Labyrinthulomycota(P) (converted clade name, R.H. Whittaker 1969, *Science* 163, 150-163) as the clade stemming from the first species to posses bothrosomes and/or an ectoplasmic net, synapomorphic with these features in *Labyrinthula vitellina*.

Labyrinthula vitellina, the type species of *Labyrinthulaceae* will serve as the type species for this definition.

Because the PhyloCode does not currently cover species level names, the following description of *Aplanochytrium sweetingensis* corresponds to the traditional rules established by the Botanical Rules of Nomenclature (Greuter et al. 2000). When

rules for naming species have been established for the PhyloCode, corresponding names will be submitted for species within the *Aplanochytriaceae*(P).

Aplanochytrium sweetingensis (n. sp.)

Isolate SCL 1-1 is morphologically and molecularly distinct warranting a new species description (Fig. 3.8, 3.17-3.20). We describe *Aplanochytrium sweetingensis* as designated by the holotype culture SCL 1-1 maintained in cryopreservation at the University of Georgia, Department of Botany culture collection of fungi and algae.

This isolate has never been observed to form zoospores. Its cells divide rapidly into two or four cells. Although cells are irregularly shaped (Fig. 3.19), no amoeboid stages have been observed. Cells have many small refractile granules (Fig. 3.20). *A. sweetingensis* n. sp. colonies spread relatively slowly in a continuous gliding fashion and readily penetrate a 1% agar surface. The only other aplanochytrid taxa that penetrate agar include *A. schizochytrops*, in which penetration is irregular and restricted to the center of the colony, and *A. saliens*, which has ovoid cells and is host restricted to *Halophila englemanni*.

Aplanochytrium sweetingensis n. sp. C. Leander and D. Porter. Uninucleate motile cells spheroid (2.5 μm diameter) or irregularly spheroid with obvious lipid droplets; glide slowly and continuously by means of ectoplasmic nets. Form visible clumps on agar by binary or quaternary divisions. Colonies are dispersed and penetrate readily into 1% agar media.

Aplanochytrium sweetingensis n. sp. C. Leander and D. Porter. Uninucleatae cellulae motiles spheroidae (2.5 µm diam.) vel incompositae spheroidae cum obvio labiato guttulae; lente prolabentes et protinus per retia ectoplasmica. Formo promptus acervus in agarum per fissionem quaternarium et binarium. Coloniae dispersae et prompte penetrae in 1% agarum medium.

PhyloCode names will be submitted to the PhyloCode registration bank when such registration becomes available. Taxonomic structure of the phylum under both systems is outlined in Fig. 3.21.

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FIGURE LEGENDS

Fig. **3.1—3.3.** Morphological groups within the Labyrinthulomycota. **1.** Diagram of *Labyrinthula* sp. with spindle-shaped vegetative cells enrobed by the ectoplasmic net. **2.** A non-proliferous *Thraustochytrium* sp. with unilateral non-motile ectoplasmic net. **3.** An *Aplanochytrium* sp. gliding via ectoplasmic net.

Fig. **3.4—3.7.** Morphological characteristics used to identify thraustochytrid genera. **4.** *Japonochytrium* with subsporangial apophysis (arrow). **5.** *Ulkenia* with release of uncleaved, amoeboid protoplast. **6.** *Schizochytrium* with clump of sporangia as a result of vegetative cytokinesis. **7.** A proliferous *Thraustochytrium* species with a single basal rudiment (arrow).

Fig. **3.8.** Characters and character-state data matrix used for the generation of morphological topology shown in Figure 10.

Fig. **3.9.** One of 22 most parsimonious trees corresponding to the topology generated from a maximum likelihood search of *ssur*DNA sequence data. Numbers above branches indicate bootstrap support with a parsimony analysis, 250 replicates, each with 1000 random addition of taxa. Length = 2839, CI = 0.52, RI = 0.63. Numbers beneath branches indicate bootstrap support from a maximum likelihood analysis with 100 replicates. Likelihood score = 7684. To distinguish between duplicate taxa, isolates for which we have contributed sequences are in bold.

Fig. **3.10.** Single tree generated from a branch-and-bound algorithm of thraustochytrid taxa using morphological characters.

Fig. **3.11.** Maximum likelihood result from an analysis of thraustochytrid taxa using *ssur*DNA sequence data.

Fig. **3.12—3.14**. Colony morphology in the labyrinthulids. Bar = 1 mm **12**.

Labyrinthula sp. S with thin, fanning colony morphology and even margin. **13**.

Labyrinthula sp. F with robust, meandering colony morphology and digitate margin. **14**.

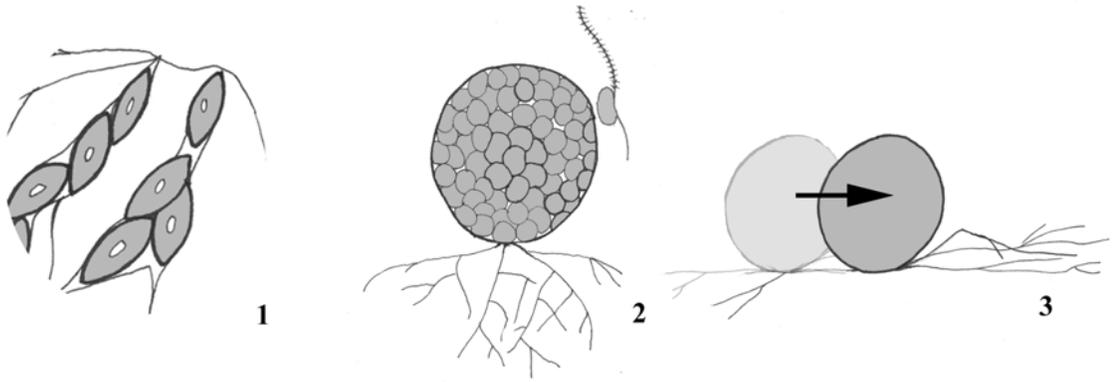
Labyrinthula zostera with thick massed cells.

Fig **3.15—3.16**. Colony morphology in the aplanochytrids. Bar = 4 mm **15**.

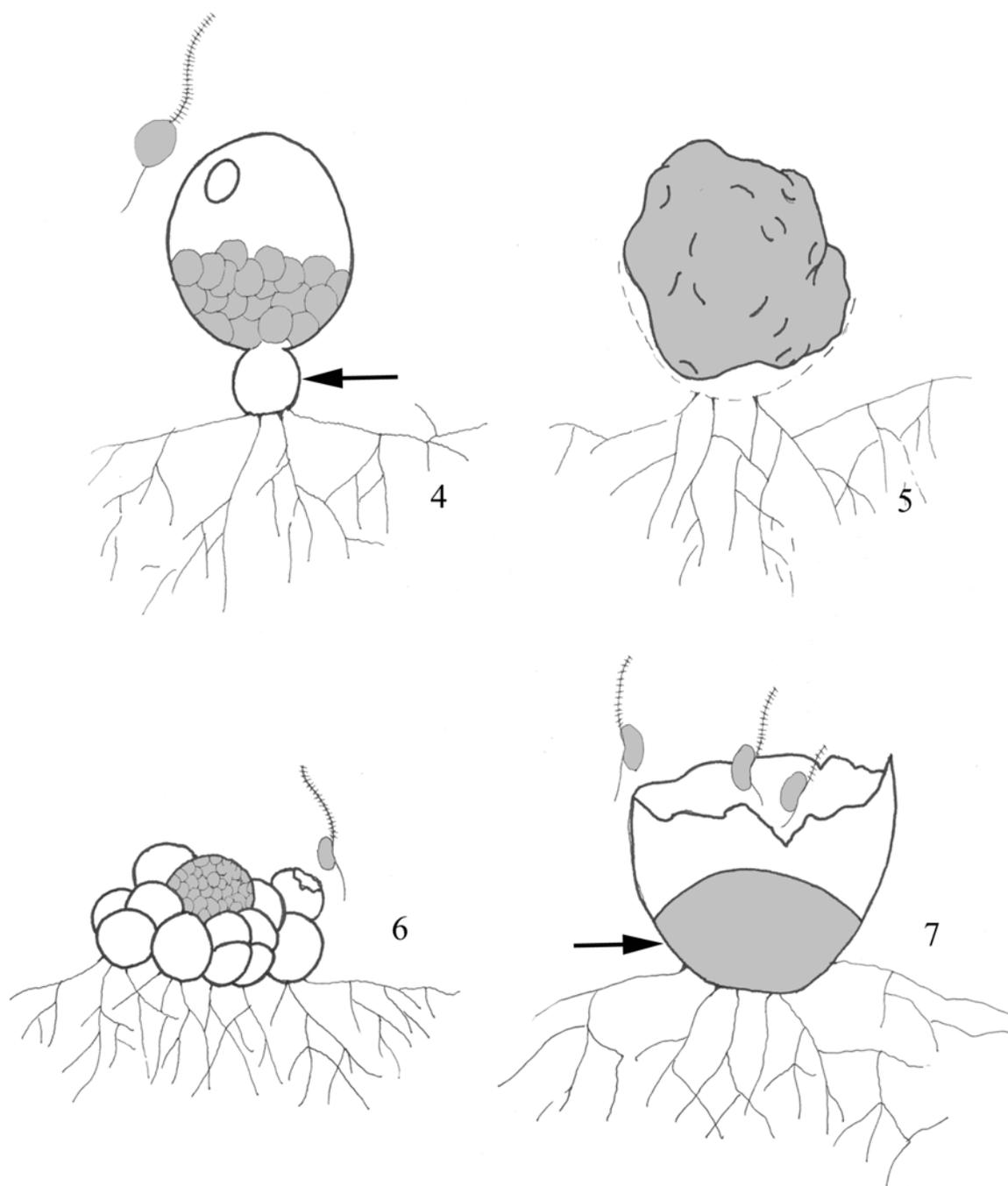
Aplanochytrium minutum with characteristic rays. **16**. *Aplanochytrium yorkensis* showing colony with patches of cells on agar.

Fig. **3.17—3.20**. *Aplanochytrium sweetingensis* n. sp. **17**. Colony morphology as seen with dissecting microscope showing patches of cells. Stellate light pattern at the center of the colony is due to profuse agar penetration. Bar = 4 mm **18**. Cells at margin of colony with ectoplasmic net extensions. DIC. Bar = 4 μ m. **19**. Cluster of cells showing thin ectoplasmic net elements and irregular shape. EN = ectoplasmic net. SEM. Bar = 2 μ m. **20**. Cell ultrastructure with lipid droplet and multivesicular body. CW = cell wall, G = Golgi body, L = lipid droplet, M = mitochondria, MVB = multivesicular body, N = nucleus. TEM. Bar = 0.5 μ m.

Fig. **3.21**. Linnean taxonomic treatment of the Labyrinthulomycota with corresponding phylocode equivalents.



Figures 3.1-3.3



Figures 3.4-3.7.

Morphological characters:

1. Zoospores with a quiescent phase (+ or -)
2. Maximum number of basal rudiments (0-5)
3. Maximum sporangium size (s = <12 μm , m = 13-24 μm , l = >24 μm)
4. Persistent sporangial wall (+ or -)
5. Mode of zoospore release (r = rupture, c = crack, f = fissure, wd = wall dissolution, p = pore)
6. Zoospore shape (vl = very long and thin, l = long and thin, o = slightly oval to nearly round, r = round)
7. Wall thickness (t = thick, n = thin)
8. Pigment (+ or -)
9. Vegetative cytokinesis (+ or -)
10. Amoeboid release of cytoplasm (+ or -)
11. Sporangial apophysis (+ or -)

Data matrix:

	1	2	3	4	5	6	7	8	9	10	11
<i>T. multirudimentale</i>	-	4	l	+	r	vl	n	-	-	-	-
<i>T. striatum</i>	-	0	l	+	r	l	t	-	-	-	-
<i>T. aureum</i>	-	1	m	+	p	o	n	+	-	-	-
<i>T. kinnei</i>	+	1	m	+	r	vl	t	-	-	-	-
<i>T. motivum</i>	-	1	l	+	c	o	n	-	-	-	-
<i>T. pachydermum</i>	+	0	l	-	wd	r	t	-	-	-	-
<i>T. aggregatum</i>	-	0	s	-	wd	o	n	-	+	-	-
<i>S. minutum</i>	-	0	s	-	wd	vl	n	-	+	-	-
<i>S. aggregatum</i>	-	0	s	-	f	o	n	+	+	-	-
<i>S. limacinum</i>	-	0	m	-	wd	o	n	-	+	+	-
<i>U. radiata</i>	-	0	m	-	wd	l	n	-	-	+	-
<i>U. profunda</i>	-	5	m	-	wd	o	n	-	-	+	-
<i>U. visergensis</i>	-	0	m	-	wd	o	t	-	-	+	-
<i>Japonochytrium sp.</i>	-	0	l	+	p	r	t	-	-	-	+

Figure 3.8.

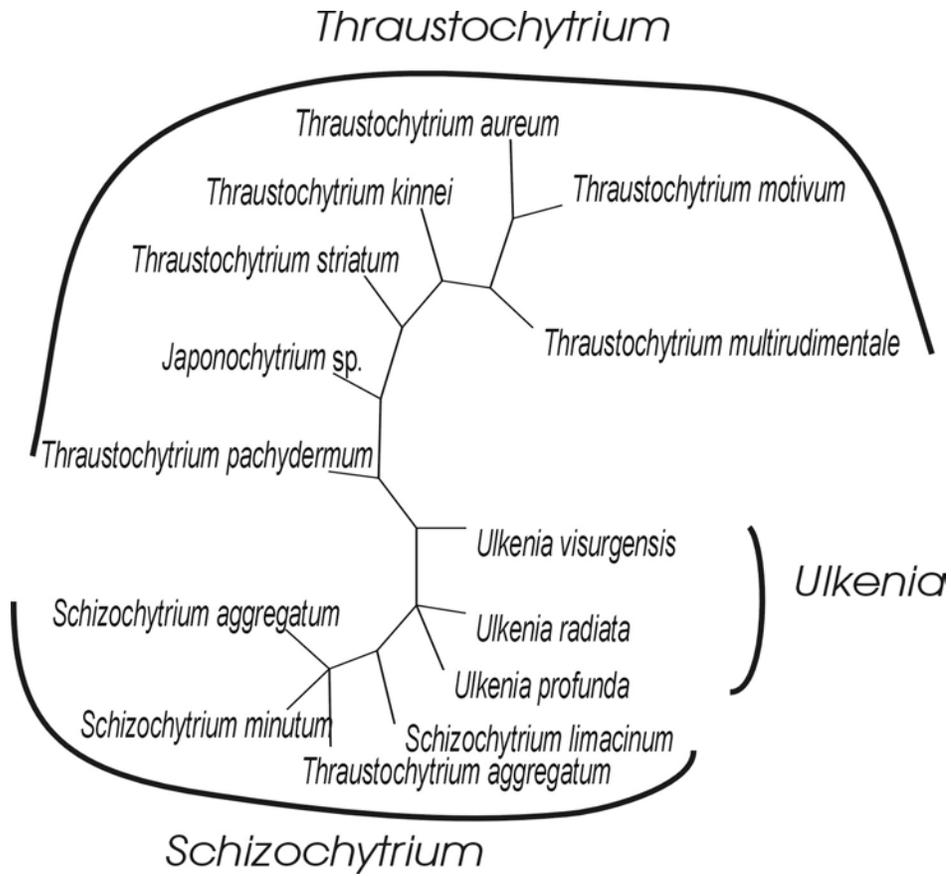


Figure 3.10.

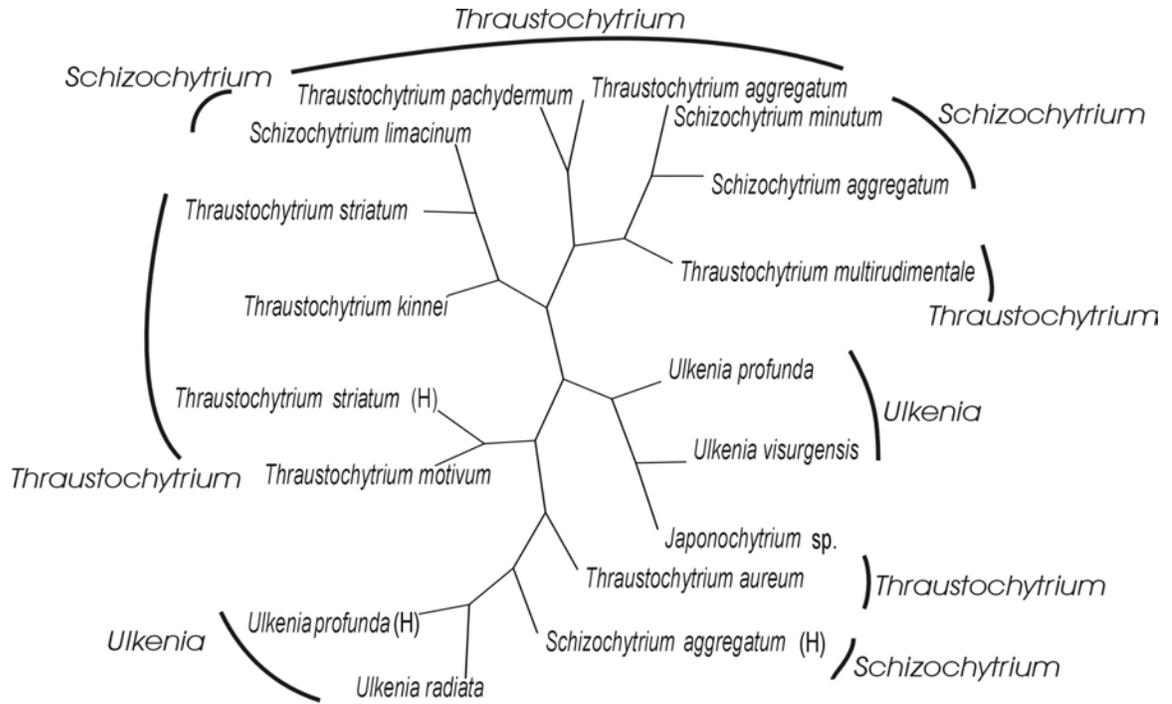
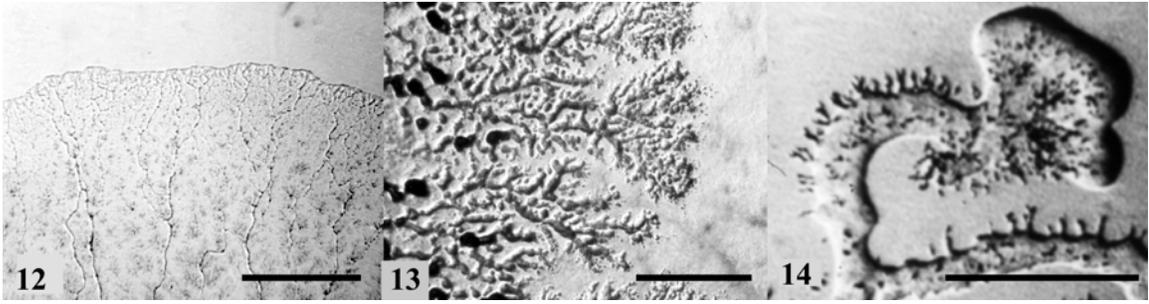


Figure 3.11.



Figures 3.12-3.14.

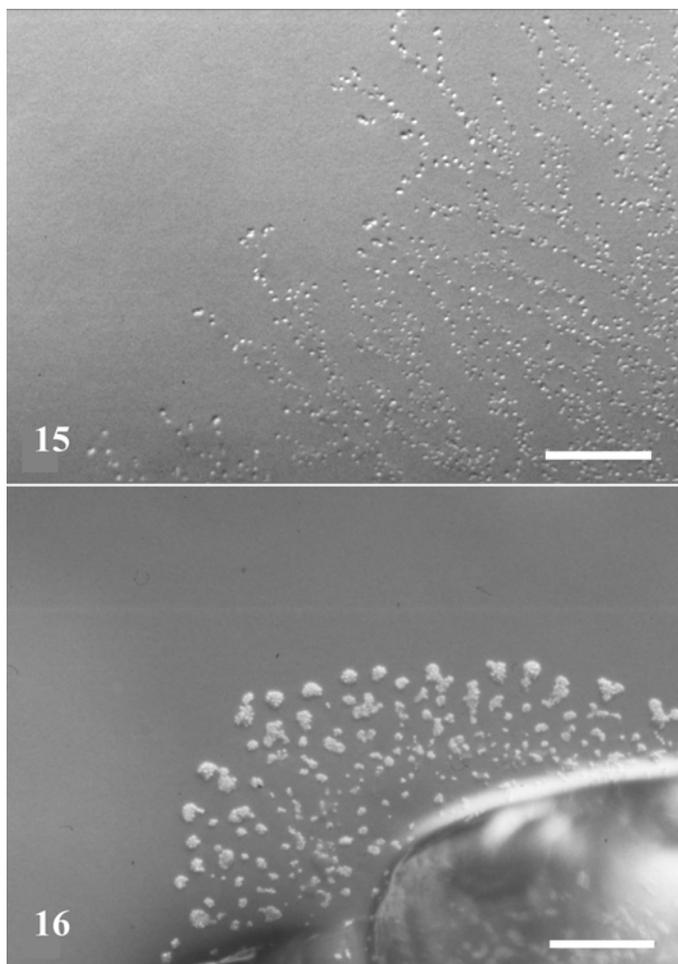
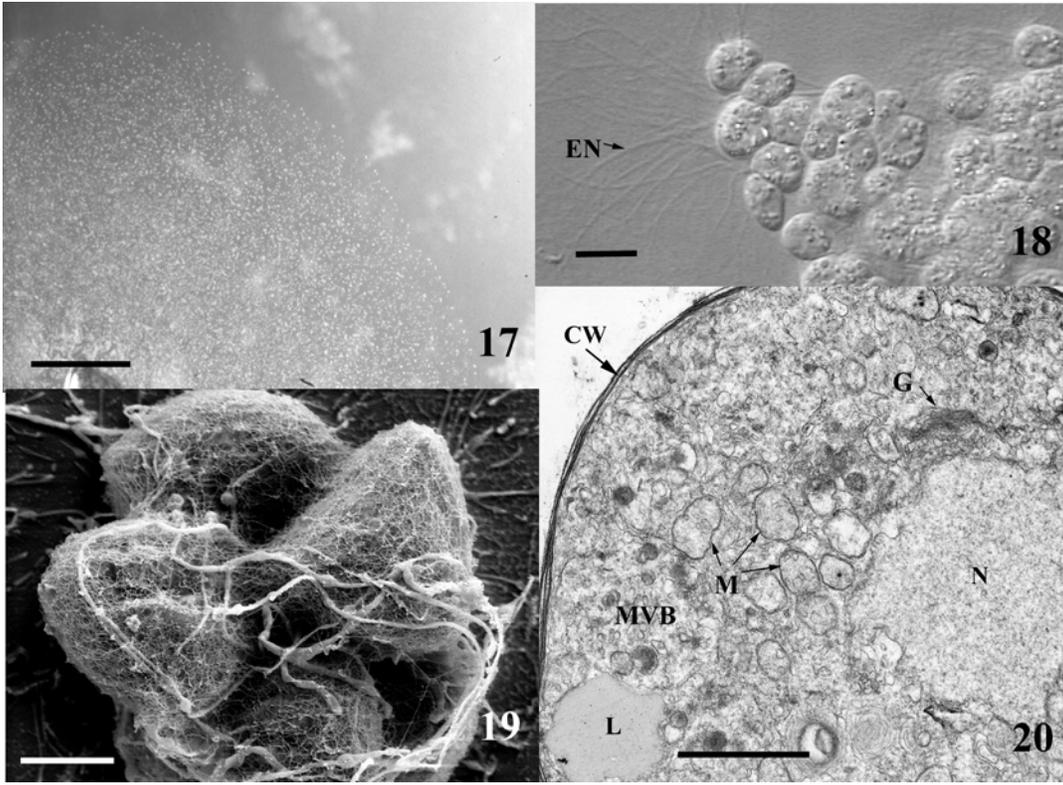


Figure 3.15-3.16.



Figures 3.17-3.20.

*Linnaean taxonomy**PhyloCode equivalent*

Phylum Labyrinthulomycota	□	<i>Labyrinthulomycota</i>
Class Labyrinthulomycetes		
Order Labyrinthulales		
Family Thraustochytriaceae		<i>Thraustochytriaceae</i>
Labyrinthulaceae		<i>Labyrinthulaceae</i>
Aplanochytriaceae		<i>Aplanochytriaceae</i>

Figure 3.21.

CHAPTER 4

REDEFINING THE GENUS *APLANOCHYTRIUM* (PHYLUM LABYRINTHULOMYCOTA).¹

¹Leander, C.A. and D. Porter. 2000. *Mycotaxon*. 76:439-444.
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ABSTRACT

In the early 1970's, two similar genera, *Labyrinthuloides* (Perkins, 1973) and *Aplanochytrium* (Bahnweg and Sparrow, 1972), were described within months of one another. Ironically, these two genera were originally associated with entirely different groups of fungi. *Labyrinthuloides* was recognized as having morphological similarities to the labyrinthulids (or marine slime nets), and *Aplanochytrium* was associated with the thraustochytrid fungi. However, contemporary researchers were beginning to recognize that the thraustochytrids and labyrinthulids were closely related (Perkins, 1972). By the mid 1980's, biochemical and morphological evidence linking *Labyrinthuloides* and *Aplanochytrium* was mounting (Ulken et al., 1985), and suggestions were made that these genera were identical (Ulken et al., 1985, Porter, 1989). In 1999, Honda et al. published a molecular phylogeny of the Labyrinthulomycota, including ssurDNA sequence data from *Aplanochytrium kerguelensis*. Leander and Porter (submitted) also reported a molecular phylogeny of the group, including *Labyrinthuloides yorkensis* and *Labyrinthuloides minuta*. We have compared this sequence data from *A. kerguelensis*, *L. minuta*, and *L. yorkensis*, and together with the morphological similarities, we conclude that *Aplanochytrium* and *Labyrinthuloides* are synonymous. Following the IBC rules of nomenclature, *Aplanochytrium* is recognized as the correct name for this genus by means of precedence. Additionally, the five species of *Labyrinthuloides* are transferred to *Aplanochytrium*. We also transfer one species of *Labyrinthula*, *L. thaisi*, to *Aplanochytrium* based on morphological similarities.

INTRODUCTION

In 1973, Perkins described a new genus of labyrinthulid. The genus *Labyrinthuloides* was erected based on a way of locomotion that differed dramatically from other labyrinthulids. Rather than being enrobed by the ectoplasmic net and gliding through it as in *Labyrinthula*, *Labyrinthuloides* consists of cells that can glide over a substrate by being pushed or pulled by the ectoplasmic net. Since the erection of this genus, four additional species have been described or moved to *Labyrinthuloides*. However, just months before the erection of *Labyrinthuloides*, Bahnweg and Sparrow (1972) had described a very similar genus, *Aplanochytrium*.

Aplanochytrium was recognized as being similar to the thraustochytrids in its thallus morphology, but was elevated to a separate genus because it makes aplanospores rather than the typical biflagellate zoospores. The aplanospores are described as “drifting” away from the parent sporangium (Ulken et al, 1985). *Aplanochytrium* has remained monotypic. Since the establishment of these two genera, there has been repeated speculation that they are indeed very closely related.

Evidence linking *Labyrinthuloides* and *Aplanochytrium* has been available for nearly 20 years. Morphologically, *Aplanochytrium kerguelensis* is most similar to (if not identical to) *Labyrinthuloides yorkensis*. These taxa are both described as having globose or spherical colorless sporangia that liberate spherical aplanospores through tears in the sporangial wall. *Labyrinthuloides yorkensis* also make zoospores, while the original generic description of *Aplanochytrium* states that only aplanospores are produced. The major morphological difference between the two taxa is in the number of aplanospores released (up to 64 in *L. yorkensis*, versus about ten in *A. kerguelensis*), and the presence

of large conspicuous lipid vacuoles in the aplanospores of *A. kerguelensis* that are not seen in *L. yorkensis*.

In 1985, Ulken et al compared cell wall composition, G+C content of DNA, and nitrogen uptake ability for *Aplanochytrium*, *Labyrinthuloides yorkensis*, and *Labyrinthuloides minuta*. Results were so similar that they concluded, “*Aplanochytrium* and *Labyrinthuloides* seem to be very similar if not identical”. Bahnweg and Jäckle (1986) examined similarities of thraustochytrid taxa using cell wall analysis, DNA base composition, and DNA/DNA hybridizations. Although they found slight differences between species of *Labyrinthuloides* and *Aplanochytrium*, the differences were usually no more than between two isolates within each genera. They concluded that these differences “indicate the presence in the oceans of a diverse flora of such organisms practically inseparable from one another on the basis of morphology.” In 1989, Porter reaffirmed that *Aplanochytrium* and *Labyrinthuloides* are morphologically very similar.

In 1999, Honda et al. published a molecular phylogeny of the Labyrinthulomycota, including *Aplanochytrium kerguelensis*. Recently, Leander and Porter (submitted) also reported a molecular phylogeny of the labyrinthulids, including two species of *Labyrinthuloides*, *L. minuta* and *L. yorkensis*. We have compared the ssurDNA sequence data generated from these two studies in an attempt to analyze the validity of uniting *Aplanochytrium* and *Labyrinthuloides*.

Based on morphological descriptions from the original paper of *Labyrinthula thaisi*, it is obvious that this species is an aplanochytrid. This species was described before the genus *Labyrinthuloides* was established, but has never been officially transferred to *Labyrinthuloides*, as were several other taxa. Unlike other species of

Labyrinthula, *L. thaisi* cells are not enrobed in an ectoplasmic net, and they glide on the ectoplasmic filaments, as do species of *Labyrinthuloides* and *Aplanochytrium*. *L. thaisi* is described as making spore products that glide away from the sporangium on net elements. As this is the distinguishing character of *Aplanochytrium*, we transfer *L. thaisi* to *Aplanochytrium*.

METHODS AND MATERIALS

Small subunit rDNA sequence data for *Aplanochytrium kerguelensis* was obtained from Genbank. Other sequence data (for *L. minuta*, *L. yorkensis*, *Labyrinthula zosterae*, *Thraustochytrium motivum*, and *Thraustochytrium striatum*) was retained from use in a previous study (Leander and Porter, submitted). Sequences were aligned using Lineup and Pileup, available from the Genetics Computing Group, Madison, WI. Our aligned sequences (1155 bases) were analyzed by eye for base pair differences. We counted a substitution or an indel as one base difference. Distance relationships were analyzed in PAUP* (Swofford, 1998) using a Neighbor Joining algorithm.

RESULTS

When comparing the alignment by eye, *L. minuta* and *L. yorkensis* differed by 19 bases. *Aplanochytrium kerguelensis* differed from *L. minuta* by 17 bases, and from *L. yorkensis* by 11 bases. Thus, the sequence data from *Aplanochytrium* is more similar to each of the *Labyrinthuloides* species than the *Labyrinthuloides* species are to each other (alignment available from the authors upon request). In comparison, two isolates of *Labyrinthula zosterae*, one collected from the west coast and one from the east coast of

the United States, differ by 16 bases (including indels) (data not shown). Thus, the sequence similarities between *A. kerguelensis* and *L. yorkensis* are remarkable.

Our distance analysis indicates that *Aplanochytrium* nests in a monophyletic clade with the *Labyrinthuloides* species. *Aplanochytrium* is more similar in sequence to *Labyrinthula zosterae* than it is to species of *Thraustochytrium* (figure 4.1).

DISCUSSION

Keeping in mind the similarities between *Aplanochytrium* and *Labyrinthuloides* that have been previously described (Ulken, 1985, Bahnweg and Jäkle, 1986), as well as the sequence similarities discussed above, it is our contention that *Labyrinthuloides* is a synonym for *Aplanochytrium*. In the original description of *Labyrinthuloides* the nomenclatural type was designated by the citation of isolate 15-6-2 and the type locality was set forth. The original description of *Aplanochytrium* also includes a type slide designation of isolate 17-4-I and type locality. Thus, *Labyrinthuloides* and *Aplanochytrium* must each be considered to have been validly published. Because *Aplanochytrium* was validly published first, the name takes priority over *Labyrinthuloides*. The name *Aplanochytrium* is the correct name following the International Code of Botanical Nomenclature (Tokyo Code), article 11.5.

Sequence similarity between the ssurDNA region of *L. yorkensis* and *A. kerguelensis* suggest that these organisms may be the same species. However, we feel that the morphological differences warrant retention of individual, yet closely related, taxa. The six recognized species of *Labyrinthuloides* are transferred to *Aplanochytrium*, as is *Labyrinthula thaisi*, based on morphological criteria discussed above.

TAXONOMY

Aplanochytrium Bahnweg and Sparrow, Arch. Mikrobiol. 81. p. 46. 1972. emend.

Leander and Porter. T: *A. kerguelensis* Bahnweg and Sparrow, Arch. Mikrobiol. 81. p. 46. 1972.

(=) *Labyrinthuloides* Perkins, Arch. Mikrobiol. 90. p. 5. 1973.

Sporangia are globose or subglobose, sessile or free. Walls are made of scales.

Bothrosomes are present. Ectoplasmic net system is endobiotic or exobiotic, and does not enrobe the cells. Cells move independently, and may reverse direction.

Applanospores always formed. Zoospores may be formed. Plasmodia and amoeboid stages may be formed.

1. **Aplanochytrium kerguelensis** Bahnweg and Sparrow. Arch. Mikrobiol. 81: 45-49, 1972. T: isolate 17-4-I. Botany Department and University of Michigan Herbarium, Ann Arbor, Michigan, *Bahnweg*.

2. **Aplanochytrium yorkensis** (Perkins) Leander and Porter, comb. nov.

Labyrinthuloides yorkensis Perkins, Arch. Mikrobiol. 90: 1-17. 1973. T: Perkins, Arch. Mikrobiol. 90, p. 5. 1973.

3. **Aplanochytrium minuta** (Watson and Raper) Leander and Porter, comb. nov.

Labyrinthula minuta Watson and Raper, J. gen. Mikrobiol. 17: 368-377. 1957. emend. Perkins, Mycologia, 4: 697-702. 1974. LT: Watson and Raper, J. gen. Microbiol. 17. p. 373, 1957.

4. **Aplanochytrium saliens** (Quick) Leander and Porter, comb. nov. *Labyrinthula*

saliens Quick, Trans. Amer. Micros. Soc., 93: 52-61. 1974. emend. Quick, Trans. Amer. Micros. Soc., 93: 344-365. 1974. T: prepared slide P10-ML-3H89 (1-16-69), Marine

Research Laboratory Herbarium, Florida Department of Natural Resources, St.

Petersburg, Florida, *Quick*.

5. **Aplanochytrium haliotidis** (Bower) Leander and Porter, comb. nov.

Labyrinthuloides haliotidis Bower, Can. J. Zool., 65: 1996-2007. 1987. T: specimens Nos. 1986-0893 to 0897, National Museum of Natural Science, Ottawa, Ontario, Canada, , *Bower*.

6. **Aplanochytrium thaisii** (Cox and Mackin) Leander and Porter, comb. nov.

Labyrinthula thaisii Cox and Mackin, Trans. Micros. Soc. 93: 62-70. 1974. T: holotype #BP171816, slide material, National Fungus Collection, United States Dept. of Agriculture, Agricultural Research Service, Beltsville, Maryland 20702.

7. **Aplanochytrium schizochytops** (Quick) Leander and Porter, comb. nov.

Labyrinthuloides schizochytops Quick, Trans. Amer. Micros. Soc., 93: 344-365. 1974. T: prepared slide L9a-RM 10-21-69, Marine Research Laboratory Herbarium, Florida Department of Natural Resources, St. Petersburg, Florida, *J.A. Quick*.

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with spheroid spindle cells. *Trans. Am. Microsc. Soc.* 93: 344-365.

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Watson, S. and K. Raper. 1957. *Labyrinthula minuta* sp. nov. *J. Gen. Microbiol.* 17: 368-377.

FIGURE LEGENDS

Fig. 4.1. Neighbor Joining tree. Length = 618. CI = 0.94, RI = 0.77.

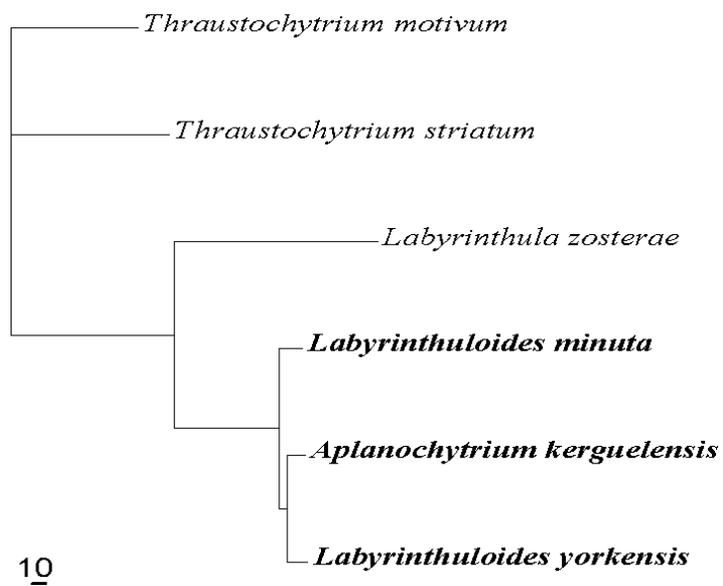


Figure 1. Neighbor Joining tree.
Tree length = 618.
CI = 0.94. RI = 0.77.

FIG. 4.1

CHAPTER 5

COMPARATIVE MORPHOLOGY AND TAXONOMY OF THE APLANOCHYTRIDS

(LABYRINTHULOMYCOTA).¹

¹Leander, C.A. and D. Porter. 2001. To be submitted to European Journal of Protistology.

ABSTRACT

The aplanochytrids comprise one of three families in the small phylum of marine saprobes, the Labyrinthulomycota. The family Aplanochytriaceae has one genus with eight described species. During our observations of collections of aplanochytrids, we have discovered that most isolates are difficult to identify to species level due to character plasticity or ambiguity. For this study, we have selected ten isolates for morphological comparison. Of these isolates, we could positively identify three, which we have designated as typical specimen for *Aplanochytrium yorkensis*, *Aplanochytrium sweetingensis*, and *Aplanochytrium minutum*. We evaluated colony size, shape, pattern, and agar penetration; cell shape, size, and inclusion characteristics; and ectoplasmic net morphology using light and scanning electron microscopy. We also report on a phylogenetic analysis using ssurDNA sequence data from these ten isolates, with the addition of *Aplanochytrium kerguelensis* sequence data obtained from Genbank. By comparing the resulting phylogenetic hypothesis with the morphological characters described above, we have identified valuable taxonomic characters that can be used to identify species specific clades within the aplanochytrids.

INTRODUCTION

The aplanochytrids are a small group of marine heterokont protists that are often associated with dead and decaying plant material or sediments. One species (*Aplanochytrium haliotidis*) is known to be pathogenic to juvenile abalone (Bower, 1987). The remaining taxa are primarily saprobic with varying degrees of host specificity. The vegetative cells of all aplanochytrids are capable of movement with

filose pseudopodia. This characteristic distinguishes them from the other two groups within the phylum, the labyrinthulids (which are enrobed by and glide through the ectoplasmic net) and the thraustochytrids (which are immobile, except for the zoospore stage).

The aplanochytrids are classified within their own family, the Aplanochytriaceae, within the Labyrinthulomycota. Members of this phylum either have an ectoplasmic net and associated bothrosomes, or are derived from an ancestor with these traits which have been secondarily lost. Within the Aplanochytriaceae, all known members retain both bothrosomes and the ectoplasmic net. Some taxa rarely make zoospores, and rely on non-flagellated aplanospores for dispersal (*A. yorkensis*). Within 20 minutes of sporangial liberation, aplanospores form ectoplasmic net elements and glide away (Bahnweg and Sparrow, 1972). Other taxa readily make zoospores, but rarely or never make aplanospores (*A. haliotidis*). The family contains one genus, *Aplanochytrium*, which was first described by Bahnweg and Sparrow in 1972. Although *Aplanochytrium* remained monotypic until recently, the transfer of five species from the genus *Labyrinthuloides* and one species from *Labyrinthula* to *Aplanochytrium* resulted in a total of seven species (Leander and Porter, 2000). One new species, *A. sweetingensis*, has since been added (Leander and Porter, submitted) for a total of eight recognized species in the genus.

Identification within the thraustochytrids and aplanochytrids can be challenging due to the plastic nature of fundamental morphological features. Important identification characters can change depending on growth conditions such as medium, temperature, and salinity (Booth and Miller 1968, Wethered and Jennings 1985). The original descriptions

of the species within *Aplanochytrium* are from observations of isolates under varying cultural conditions. Thus, the problem of taxonomic validity arises. The original culture conditions and identifying characters of the eight described taxa are outlined below.

The type species of *Aplanochytrium* is *A. kerguelensis* (Bahnweg and Sparrow 1972). *A. kerguelensis* was originally isolated from subantarctic waters and was grown in culture on pine pollen. The isolate was classified as a new genus because of the exclusive formation of aplanospores, but the genus definition has since been modified to allow the inclusion of taxa that make zoospores in addition to aplanospores (Leander and Porter 2000). The aplanospores of *A. kerguelensis* contain a large eccentric vacuole, which is a distinguishing character of the species, and a granular cytoplasm. The vacuole is a conspicuous part of the developing sporangium and disappears just before cleavage into aplanospores. A few to 50 spores are released through rupture in the sporangial wall, or the aplanospores germinate within the old sporangial wall to make clusters of sporangia. The ectoplasmic net is formed from several places on the spore body and extends in all directions.

The next year, Perkins described *Labyrinthuloides yorkensis* (Perkins 1973), which is now recognized as a species of *Aplanochytrium* (Leander and Porter 2000). *A. yorkensis* was isolated from oyster mantle, water samples, sediment, sand, and detritus and was maintained in axenic culture on a glucose/gelatin hydrolysate medium (MV) for the original species description (Perkins 1972). Unlike *A. kerguelensis*, *A. yorkensis* does make zoospores as well as amoeboid cells. Perkins also describes various membrane-bound inclusions within the cytoplasm, which may be the same structures as those causing the granular appearance in *A. kerguelensis*. *A. yorkensis* forms stellate, cream-

colored colonies without rays. Mature sporangia are rarely motile. The ectoplasmic net has two major radiating filaments that subsequently branch into finer threads. Movement is slow and continuous.

A. minutum (previously *Labyrinthula minuta*, then *Labyrinthuloides minuta*) was originally isolated from a green algae, *Ulva* sp. collected from the undersurface of a boat (Watson and Raper, 1957). We have also isolated *A. minutum* from many substrates including various green and red alga, *Zostera marina*, sediments, and sand. The cells are more elongate than those of *A. yorkensis* and *A. kerguelensis* and divide into tetrads. Zoospores are not described in the original description, but have been reported by Watson (1957). *A. minutum* tends to spread in distinct rays as a monolayer over agar surfaces. Cells remain motile for all of the life-cycle except for during the formation of four daughter cells, and have very fine ectoplasmic net elements without apophyses (Watson and Raper, 1957). Movement is by alternately reversing directions.

In 1974(a), Quick described the fourth species of aplanochytrid, *Aplanochytrium saliens* (as *Labyrinthula saliens*) from the marine grass *Halophila englemannii* and was originally grown on a modified blood serum agar (Quick, 1974a). *A. saliens* is relatively rare (found in two of 12 host plants), and has not been reported since the original description. The sporangia are spherical, but may be compartmentalized making the sporangium appear to have a rough texture. Four to 20 aplanospores are released via fissures in the wall of the sporangium and are characterized by the presence of an anterior pit. The shape of the cells is distinct, with a pointed posterior and a rounded, inflated anterior. Colonies of *A. saliens* alternate between those rich in vegetative cells and those rich in sporangia. The colonies rich in vegetative cells embed the agar and appear milky

with concentric layers. Sporangial rich colonies form white flecks (clusters of sporangia) that do not penetrate the agar surface. After about five days in culture, the sporangial rich colonies become vegetative. The ectoplasmic net is described as being subdichotomously branched, entangled, and without vesicular apophyses, but with a gently curved thickened trunk that diverges into several thinner branches. Anastomosis is common. Movement is sporadic with intermittent rapid advances.

Aplanochytrium schizochytrrops was also described by Quick in 1974(b). *A. schizochytrrops* was isolated from the seagrass *Halodule wrightii* and grown on bovine serum agar. Although *A. schizochytrrops* is reported to be present on 50% of the sampled host plants, it has not been reported since its original description. Vegetative cells are spherical to ovoid and enlarge to produce sporangia that are spherical when single, but clump to form irregular masses. Vegetative cells have obvious large vacuoles. As an alternative to sporangial formation, vegetative cells can produce unwallied plasmodia, which fragment internally to produce sporangia or fragment completely into vegetative cells or new plasmodia. Like *A. saliens*, the sporangia consist of separate compartments and produce ectoplasmic networks that are straight and tapering without apophyses. The ectoplasmic net of *A. schizochytrrops* rarely anastomizes. Also like *A. saliens*, *A. schizochytrrops* is characterized by vegetative cell rich strains, and sporangial rich strains. Movement is a slow continual gliding.

A. haliotidis is a pathogen of abalone, but grows well in Eagle's minimal essential media (MEM). It was described in 1987 by Bower. Sporangia and vegetative cells are spherical and the cytoplasm contains few vesicles. Division is only by binary fission. Swellings along the ectoplasmic net are typical. Unlike most aplanochytrids,

aplanospores are not formed, but zoosporangia are readily made with the addition of seawater. Three to 10 zoospores are released through a tear in the wall. Movement is by slow gliding.

A. thaisii was described by Cox and Mackin in 1974 as a labyrinthulid. *A. thaisii* was isolated from the marine gastropod *Thais haemastoma floridana*, and was grown on beef serum agar. Like *A. saliens* and *A. schizochytrops*, this taxon is characterized by sporangia-rich colonies alternating with vegetative-rich colonies. Both types of colonies occur in monolayers. The vegetative colonies seem to be determinate since they never return to a sporangial stage. Vegetative cells divide by binary or quaternary divisions, producing tetrads that are enveloped by a mucilaginous sheath. Cysts are also reported. Aplano-sporangia are immobile with small reflective drops. Vegetative cells are spherical. Zoospores are readily produced in the host tissue, but not on agar. Motile plasmodia are reported to pinch off new vegetative cells, or fragment into many vegetative cells.

A. sweetingensis is a recently described taxon (Leander and Porter, 2001) collected from fallen mangrove leaves and maintained on 1% serum seawater agar (SSA). Vegetative cells are irregular to spherical and move by a slow gliding motion. The colony appears as patches, which are clumps of vegetative cells. Division is binary or quaternary. Sporangial formation has never been observed. Agar penetration is profuse throughout the entire colony. The ectoplasmic net appears as long, thin tapering branches without swellings.

During our collection of aplanochytrids, we have noticed many isolates with overlapping characters that fit more than one taxon description. The purpose of this

study was to examine various isolates for taxonomically valid characters. We used a combination of scanning electron microscopy, light microscopy, and sequence analysis to address the phylogeny and taxonomy of the aplanochytrids.

METHODS AND MATERIALS

Identification. Because only one type specimen for aplanochytrids is available, we have designated typical strains of *A. minutum* (PR6-2), *A. sweetingensis* (SC1-1, type), and *A. yorkensis* (D255). Most isolates used in this study were collected during 1998-99 from Miami, Florida, Puerto Rico, and the Bahamas. The exception is our typical *Aplanochytrium yorkensis* isolate, which was collected from New Hampshire in 1991 (Table 5.1). All isolates were stored in liquid nitrogen and subsequently thawed for this study. During documentation, the aplanochytrids were maintained on serum seawater agar (SSA) and transferred biweekly.

Microscopy. Colony measurements and light microscopy were performed when colonies were eight days of age. Measurements were taken from 10 separate transfers of each isolate. To make corresponding transfers, .5cm blocks were excised from the edge of the advancing colony and placed in the center of a new petri dish with .5cm thick 1% SSA. Images were taken at magnifications from 10x- 60x with a dissecting microscope for analysis. We used these images to evaluate colony shape, pattern, and agar penetrance. Compound light microscopic images were also taken at this stage of development. Compound light microscopy was used to evaluate characteristics including presence or absence of amoeboid cells, presence or absence of dense lipid drops and refractile granules, and cell size and shape.

For scanning electron microscopy (SEM), isolates were grown for several days on 13 mm diameter Thermanox Plastic Coverslips (NUNC products) that were bare or thinly coated with SSA. A 30-minute osmium tetroxide vapor fixation preceded dehydration through an ethanol series as described previously (Leander and Porter submitted). Samples were then critical point dried using CO₂ and thinly coated with chromium. Scanning images were taken on a LEO 982 field emission SEM. SEM was used primarily to evaluate characteristics of the ectoplasmic network. For surface pattern comparisons, one isolate of *Schizochytrium aggregatum* (PR10-1) and one isolate of *Labyrinthula* sp. were prepared and viewed in an identical fashion. Several isolates were prepared in an identical fashion, but coated with gold to test for surface pattern consistency. The non-pigmented stramenopiles are closely related to the chrysophyte algae, which have surface scales made of silica. Surface pattern in the aplanochytrid isolate PR15-1 and in *Aplanochytrium sweetingensis* was evaluated with EDS on an Oxford EDS system with a light element detector for the presence of silica.

For transmission electron microscopy (TEM), the type culture of *Aplanochytrium sweetingensis* was fixed, dehydrated, and embedded in Epon Embed 812 as described previously (Leander and Porter, submitted). Sections were viewed on a JEOL 100CX.

Sequence data. Molecular analyses were performed using ssurDNA sequences that were aligned for a previous study (Leander and Porter, submitted), with the addition of three new isolates (Table 5.1). Sequence data from *A. kerguelensis* was obtained from Genbank. A branch-and-bound search using parsimony criterion was performed on PAUP*. Although a branch-and-bound search is guaranteed to give the most parsimonious tree, a bootstrap analysis with 1000 replicates was performed to provide

support for individual lineages. A maximum likelihood analysis was also conducted. Morphological characters generated from microscopical analyses were compared to the resulting phylogenetic hypothesis.

RESULTS

Figure 5.1 shows the colony pattern differences between isolates in this study. These images show two general trends in colony shape. Colonies either have distinct spokes radiating from the center outward (Fig. 5.1a,b), or colonies form clumps of cells on the agar without a radial pattern (Fig. 5.1c). PR24-1, *A. minutum*, PR15-1, and SC24-1 have fine to medium, straight to slightly curved rays extending from the center outwards as in Fig. 5.1a. Isolates M8-6, PR1-1, and PR12-3 also have distinct spokes, but the spokes are thicker and meandering (Fig. 5.1b). *A. yorkensis* has no spokes, but large clumps of cells appear as white flakes on the agar (Fig. 5.1c). *A. sweetingensis* also has no spokes, but clumps of cells on the agar are much smaller than those of *A. yorkensis*. At the other extreme is isolate M4-2, which has no spokes and very dense layers of cells (Fig. 5.1d). *A. sweetingensis* and isolate M4-2 readily penetrate the agar throughout the entire colony. This characteristic is not seen in any of the other isolates or taxa.

Paired 2 sample t-test analysis indicated that colony size is very stable within an isolate. The colony size of each isolate was significantly different from that of all other isolates with an alpha value set at 0.05 (Fig.5.2).

Selected compound microscopic images are shown in Fig. 5.3. Cell shape varied from spherical (*A. yorkensis*, and isolates M4-2, PR1-1, and SC24-1) to oblong (*A. minutum* and isolates M8-6, PR12-3, PR24-1, and PR15-1) (Fig. 5.3a,b). Some cells

from isolates PR1-1, PR24-1, and SC24-1 were subspherical in shape, and those of *A. sweetingensis* were very subspherical (Fig. 5.3c). Isolates M8-6, PR24-1, and PR12-3 had few cells that were weakly amoeboid. Some isolates (M8-6, PR24-1, PR15-1, and PR12-3) had occasional cells that were much larger than the regular vegetative cells. Many isolates also had refractive granules and inclusions. M4-2 had the most conspicuous inclusions in all cells, while SC24-1 also had refractile inclusions, but much less than those of isolate M4-2 (Fig. 5.4a,b). PR15-1 and PR12-3 had inclusions that were quite obvious in the larger cells, but absent from smaller vegetative cells. Cell length ranged from an average of 2.4 μm in SC24-1 to 5.5 μm in isolate PR24-1 (Fig. 5.2). Cells that were oblong in shape were much larger than round cells, while the subspherical cells of *A. sweetingensis* were mid-sized.

SEM micrographs are shown in Fig. 5.5 and Fig. 5.6. In addition to ectoplasmic net characteristics, we discovered a hexagonal/pentagonal array over the surface of all aplanochytrid isolates (Fig. 5.6). This pattern is not seen in SEMs of the thraustochytrid *Schizochytrium aggregatum* or of *Labyrinthula* sp., and is obscured or very faintly visible when aplanochytrids are coated with gold rather than chromium (Fig. 5.6e). Cells were examined for the presence of silica but no detectable amounts could be found using EDS X-ray microanalysis. Examination of the cell wall in thin section showed no evidence of the hexagonal pattern, but it was discovered that the wall is made of monolayered sheets many micrometers in length with interspersed sections of overlapping small scales similar to those found in thraustochytrids (Figure 5.7).

The ectoplasmic network of isolates M4-2 and M8-6 consists of fine filamentous extensions with numerous bead-like attachments that are not seen in other isolates (Fig.

5.5a). The taxa and isolates with round cells (*A. yorkensis*, *A. sweetingensis*, and PR1-1) are covered with layers of very fine ectoplasmic extensions resembling a spider's web (Fig. 5.5b, 5.6c). Cells of isolate SC24-1 are also covered with ectoplasmic net, but the network of this isolate is distinct from all others in that it consists of broad extensions, rather than fine filaments (Fig. 5.5c). The hexagonal/pentagonal array mentioned above is clearly evident on cells of these three isolates when the ectoplasmic covering has been dislodged (Fig. 5.6c). The ectoplasmic nets of *A. minutum*, PR24-1, PR15-1, and PR12-3 are very similar. These nets consist primarily of fine filaments with occasional flattened or broad sections (Fig. 5.5d). A summary of morphological traits for each isolate is in Table 5.2.

Results from ssurDNA sequence analysis are shown in Fig. 5.8. The phylogram presented is one of four most parsimonious trees and is rooted arbitrarily with *A. yorkensis*. Three main relationships occur. The first is a sister relationship between *A. yorkensis* and *A. kerguelensis*, the second is a least inclusive clade of M4-2 and *A. sweetingensis* (bootstrap of 94), and the third is a larger lineage consisting of the other seven isolates (bootstrap of 99). Morphological data generated from microscopical analyses were compared to this resulting phylogenetic tree and are discussed below.

DISCUSSION

The most obvious character that we examined with phylogenetic significance is the presence of rays in colony shape. The radiating pattern typical of *Aplanochytrium minutum* occurs in all members of the largest lineage (bootstrap 99). Furthermore, the least inclusive clade containing isolates PR1-1 and PR12-3 have rays that are much

broader than those seen in the other isolates. Thus, as we have found in the labyrinthulids (Leander and Porter, submitted), colony shape seems to be a fundamental identifying character within the aplanochytrids. We refer to this clade as an *A. minutum* lineage. Colonies in this lineage also grow to the largest size of the aplanochytrid isolates examined in this study.

The clade containing isolates *A. sweetingensis* and M4-2 possess spherical cell-shape (round to round-irregular), small cell size, and ectoplasmic net covering the cells. However, these character states are seen in other isolates (SC24-1 and *A. yorkensis*) and are probably the result of convergent evolution. One character that is synapomorphic to this clade is agar penetration. These are the only two isolates that display profuse agar penetration throughout the entire colony on 1% SSA. We suggest that M4-2 is an isolate of *A. sweetingensis* that has a small colony size and grows in very dense layers.

A. kerguelensis and *A. yorkensis* have long been recognized as being very similar morphologically (Ulken et al. 1985; Bahnweg and Jackle 1986; Leander and Porter 2001). These taxa both have round cells and are similar in size and colony morphology. However, unlike *A. yorkensis*, *A. kerguelensis* does not make zoospores (Bahnweg and Sparrow 1972; Perkins 1973). The number of aplanospores released by each is also different (about 10 in *A. kerguelensis* and up to 64 in *A. yorkensis*). Finally, *A. kerguelensis* has large conspicuous vacuoles that are absent from *A. yorkensis*. *A. kerguelensis* is not available in culture so although they form a sister relationship and are very similar morphologically, we hesitate to combine the two without additional morphological evidence uniting these taxa.

In addition to the phylogenetically significant characters mentioned above, there are also several correlations worth mentioning. All round cells are covered with the fine ectoplasmic net elements, while all oblong cells are naked (Fig. 5.5). In addition, round cells are much smaller than oblong cells (Fig. 5.2). Oblong cells all have an ectoplasmic net with flat, wide areas, and grow in a pattern characterized by rays (Fig. 5.1,5.5).

The hexagonal array observed on the surface of aplanochytrids is curious. One possible explanation is that this pattern is from the wall scales. The multi-layered cell wall of the thraustochytrids is made of overlapping circular polysaccharide plates. These plates appear to be randomly distributed and sluff off as the sporangium ages (Harrison and Jones 1974). In the labyrinthulids the wall is thought to be made of a single layer of plates that are connected to one another with an unknown adhesive (Moss 1985). If these adhered plates form such a hexagonal array in the labyrinthulids, it has not been observed because the labyrinthulids are enrobed by the ectoplasmic net that obscures the cell wall. The hexagonal pattern seen on the surface of the aplanochytrids may be adhesive material, but we have not observed this pattern in cross-section TEM analysis as would be expected. We have identified that the aplanochytrid wall is composed primarily of large layers several micrometers long, rather than small (less than 0.5 μm) overlapping scales as in the thraustochytrids. The aplanochytrid wall is more like a multilayered labyrinthulid wall than the wall of the thraustochytrids.

The surface hexagonal array is interspersed randomly with pentagons such that the entire surface pattern resembles that of naturally occurring carbon Fullerene molecules (or Bucky Balls) (Kroto et al. 1985). These molecules follow the net closing formula postulated by the mathematician Euler and form balls that typically have 20

hexagonal to 12 pentagonal rings. The hexagonal array seen on the surface of the aplanocytrids is made of an average of 30 hexagonal to 7 pentagonal rings (a significantly lower ratio of pentagons to hexagons). Nevertheless, it appears that this array may be a naturally occurring least energetic way to form a sphere from semi-rigid structures.

In summary, the most commonly encountered species of aplanochytrids can be identified with macroscopic colony characteristics when isolates are grown on a standard medium such as SSA. All isolates that display a ray pattern belong to the *A. minuntum* clade, regardless of cell shape. Isolates with patches on the agar surface in which cells penetrate the agar throughout the entire colony belong to the *A. sweetingensis* group. Finally, isolates that form patches on the agar but do not readily penetrate the surface can be assigned to the *A. yorkensis/A. kerguelensis* group.

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Table 5.1. Taxon and isolate sequence and isolation information.

	GenBank Accession Number	Substrate	Location
A. minutum	L27634	Zostera marina	Middle Marsh, North Carolina
A. kerguelensis ^a	AB022103		
<i>A. sweetingensis</i>	AF348520	Rhizophora mangle	Sweetings Cay, Bahamas
<i>A. yorkensis</i>	AF265333	Zostera marina	Adams Point, New Hampshire
M4-2	(new)	Syringodium filiforme	Miami Harbor, Florida
M8-6	(new)	<i>Cladophora</i> sp.	Miami Harbor, Florida
PR1-1	AF348516	Dictyota cervicornis	San Juan, Puerto Rico
PR12-3	AF348517	<i>Chaetomorpha</i> sp.	San Juan, Puerto Rico
PR15-1	AF348518	Thalassia testudinum	San Juan, Puerto Rico
PR24-1	AF348519	Syringodium filiforme	San Juan, Puerto Rico
SC24-1	AF348521	Thalassia testudinum	Sweetings Cay, Bahamas

^a Reference Honda et al. 1999

Table 5.2. Morphological characters of the aplanochytrids.

	Average Colony Diameter	Colony Margin Shape	Colony Pattern	Agar Penetration	Cell Shape	Average Cell Length	Inclusion	Ectoplasmic Network
<i>A. minutum</i> (PR6-2)	4.1 cm	Even, smooth	Straight, mid-sized rays	Superficial at center of older colonies	Oblong	4.6 μ m	Small, few	Fine, with broad, flat areas
PR15-1	3.6 cm	Even, smooth	Straight, narrow rays	None	Oblong	5.3 μ m	In larger cells	Fine, with broad, flat areas
SC24-1	2.8 cm	Even, smooth	Straight, narrow rays	None	Spherical	2.4 μ m	Small, many	Flat, broad extension covering cells
PR12-3	3.3 cm	Slightly uneven	Curving, wide rays	Superficial at center of older colonies	Oblong	5.0 μ m	In larger cells	Fine, with broad, flat areas
PR1-1	3.2 cm	Uneven, rough	Curving, wide rays	Superficial at center of older colonies	Spherical	3.0 μ m	Some in all cells	Fine filaments, and web-like covering cells
M8-6	2.6 cm	Even, smooth	Slightly curving, narrow rays	Superficial at center of older colonies	Oblong	4.1 μ m	Small, few	Fine, with beads
PR24-1	2.8 cm	Even, smooth	Curving, narrow rays	Superficial at center of older colonies	Oblong	5.5 μ m	Small, few	Fine, with broad, flat areas
<i>A. sweetingensis</i> (SC1-1)	1.7 cm	Even, smooth	Small clumps	Profuse	Sub-spherical	4.4 μ m	Small, many	Fine filaments, and web-like covering cells
M4-2	0.9 cm	Uneven, rough	Dense sheet	Profuse	Spherical	2.7 μ m	Large, many	Fine with beads, and web-like covering cells
<i>A. yorkensis</i> (D256)	0.6 cm	Even, less smooth	Large clumps	Limited to center of older colonies	Spherical	4.3 μ m	Large, many	Fine filaments, and web-like covering cells

FIGURE LEGENDS

Fig. 5.1. Variation in colony morphology of the aplanochytrids when grown on standard media (SSA). 60x. A. *A. minutum* with straight narrow rays and an even margin. B. PR1-1 with broad rays and an uneven margin. C. *A. sweetingensis* with patches of cells and an even margin. D. M4-2 with a dense sheet of cells and an uneven margin.

Fig. 5.2. Colony diameter and cell length averages of isolates grown on SSA for 8 days. A. Colony diameter averages in cm (n=10). B. Cell length averages in μm (n=10).

Fig. 5.3. Variation in cell shape of the aplanochytrids when grown on standard media (SSA). 1,000X. A. SC24-1 with spherical cells. B. *A. minutum* with oblong cells. C. *A. sweetingensis* with subspherical cells.

Fig. 5.4. Inclusions within cells of the aplanochytrids. 1,000X A. M4-2 with many large refractive inclusions. B. SC24-1 with fewer and smaller refractive inclusions.

Fig. 5.5. Ectoplasmic net variations within the aplanochytrids. Bar = 2 μm . A. M4-2 with fine filaments and bead-like extensions and web-like covering over cells. B. *A. sweetingensis* with very fine, web-like covering over cells. C. SC24-1 with broad, dense ectoplasmic net covering cells and extending to the left. D. *A. minutum* with fine filaments and broad, flat areas.

Fig. 5.6. Hexagonal array over the surface of aplanochytrid isolates. Bar = 1 μm . A. PR24-1 B. PR15-1 C. *A. sweetingensis* with hexagons showing through the web-like covering. D. *A. minutum* E. M4-2 coated in gold (versus chromium). Arrows indicate hexagons barely visible with gold coating. F. PR24-1.

Fig. 5.7. TEM of the cell wall of *Aplanochytrium sweetingensis*. Arrows indicate area of several overlapping small scales. Stars indicate ends of large monolayered sections. Bar = 0.5 μm .

Fig. 5.8. One of four most parsimonious trees generated from a branch-and-bound search rooted arbitrarily with *A. yorkensis*. Topology matches that found from a maximum likelihood search. Bootstrap values were generated with a 1000 replicate analysis using maximum parsimony. Length = 174. CI = 0.90, RI = 0.72.

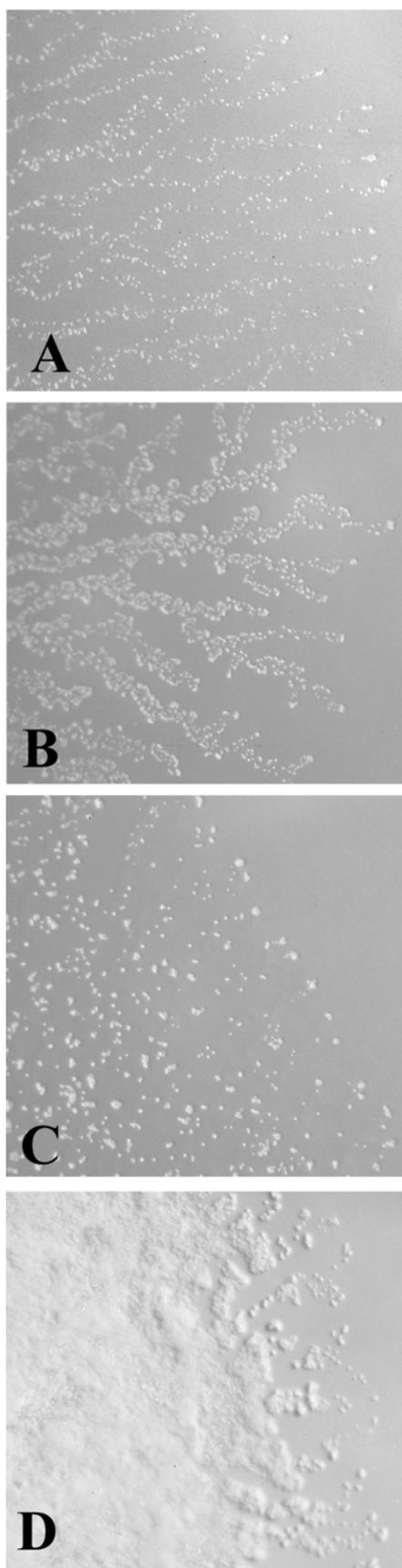
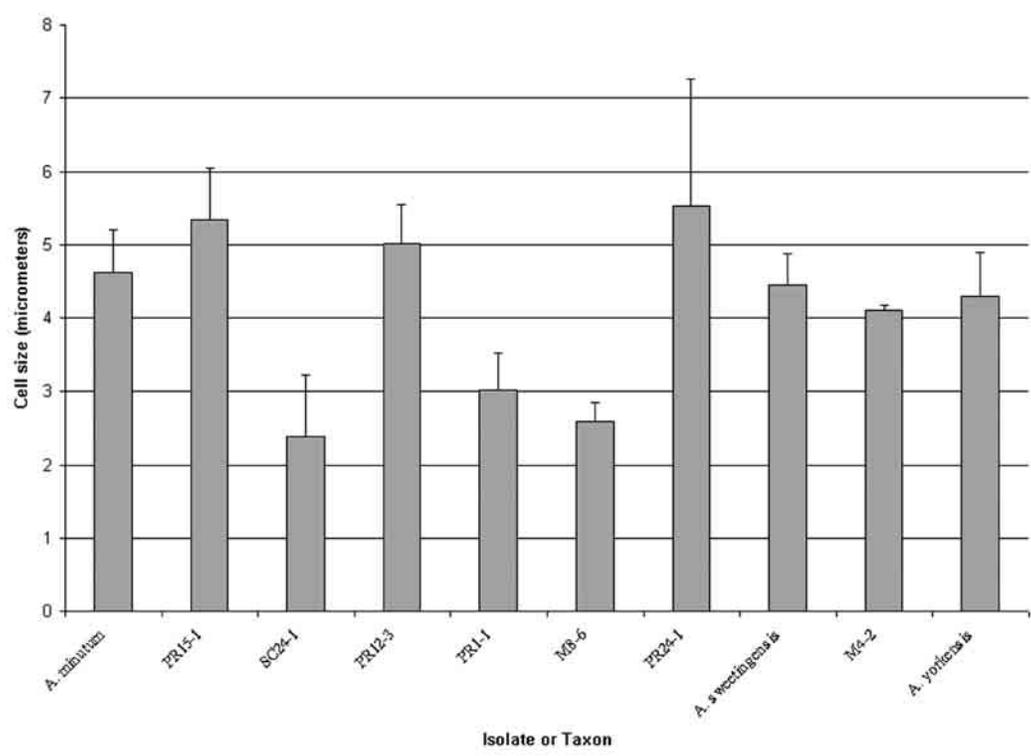
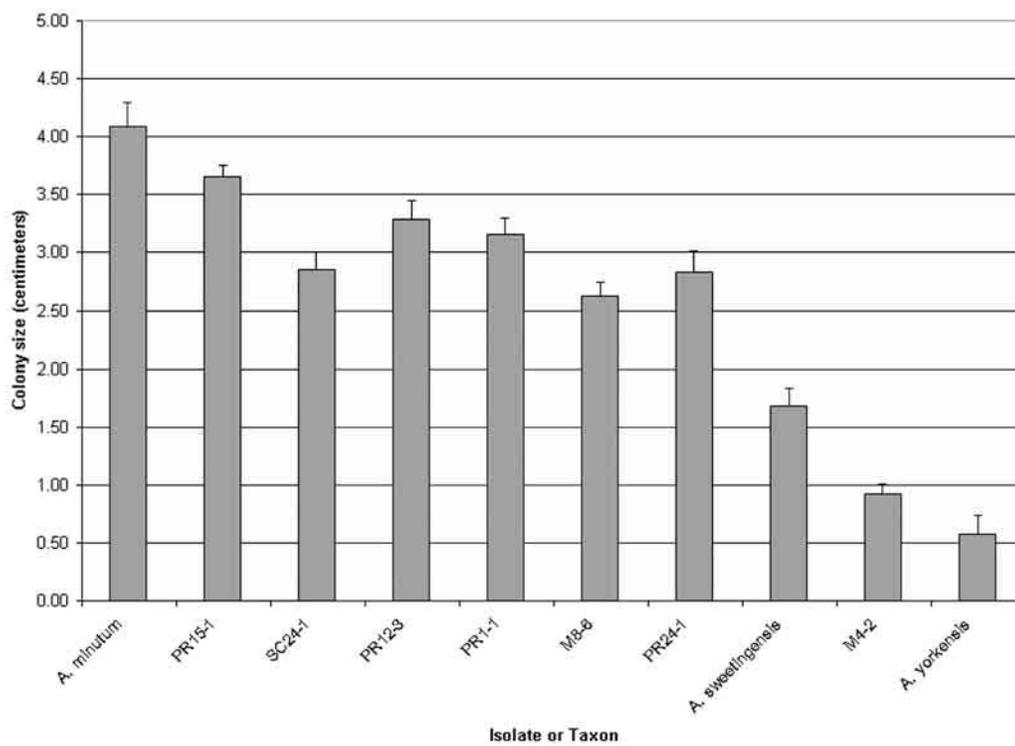


Figure 5.1.



A.



B. Figure 5.2.

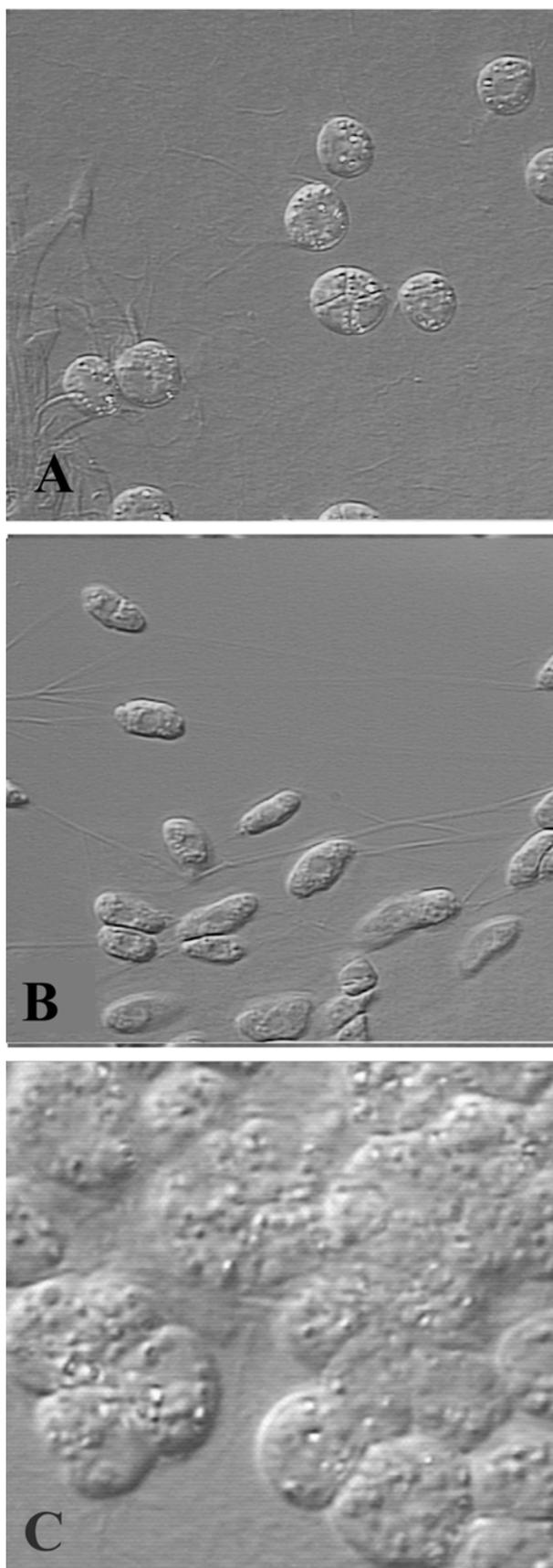


Figure 5.3.

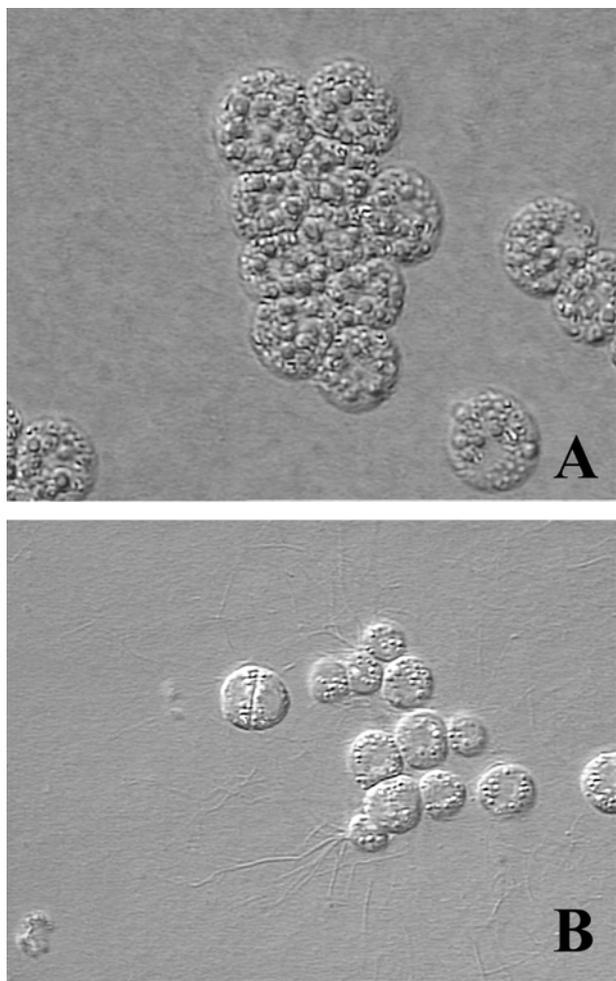


Figure 5.4.

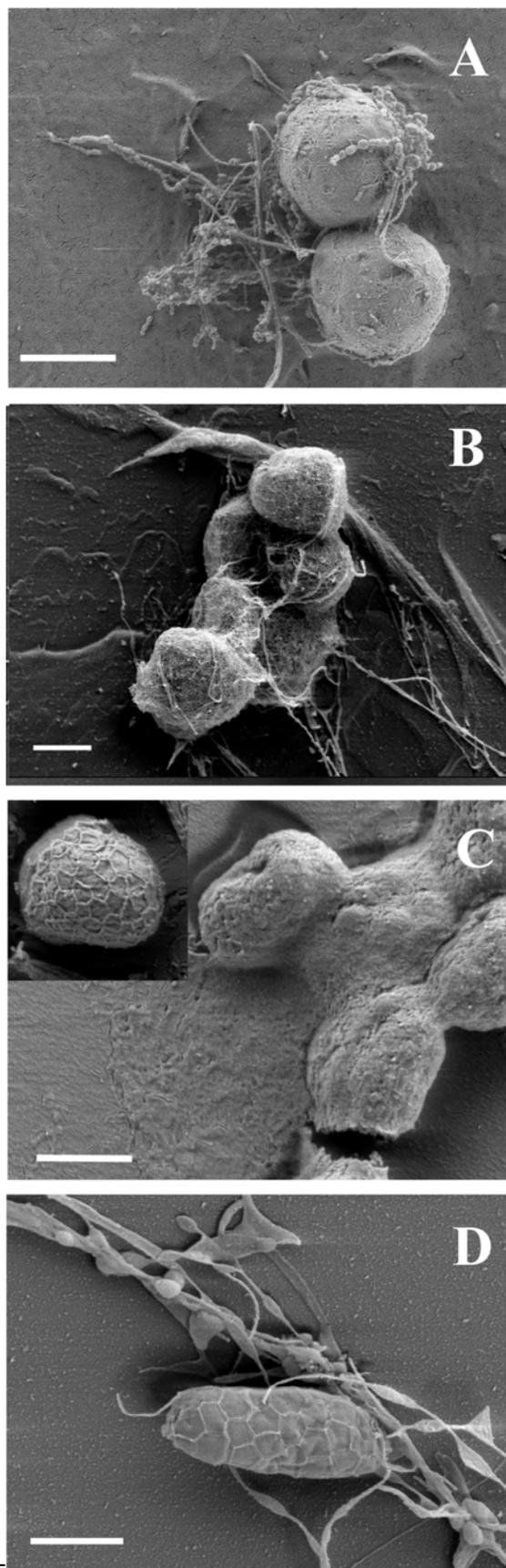


Figure 5.5.

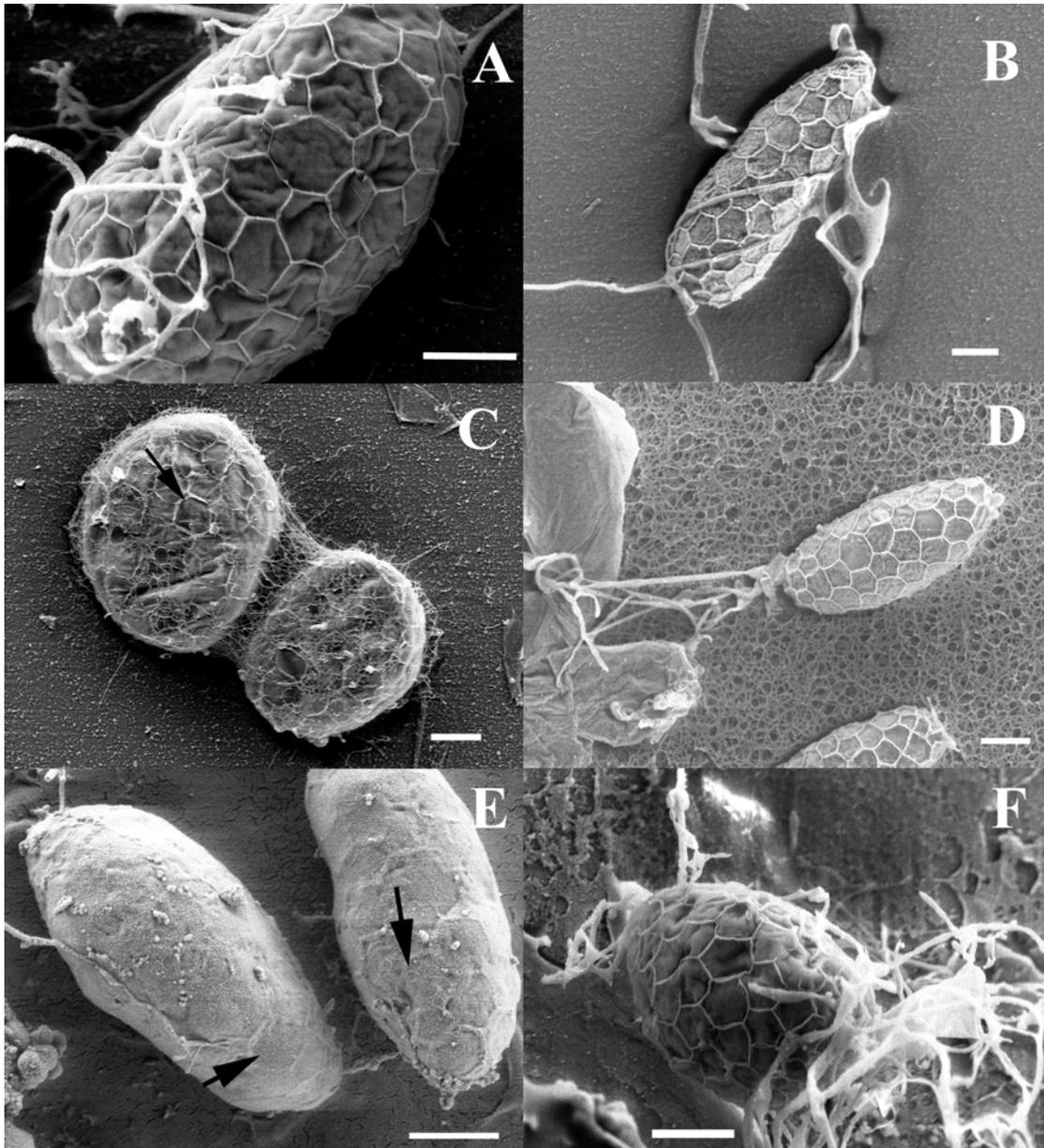


Figure 5.6.

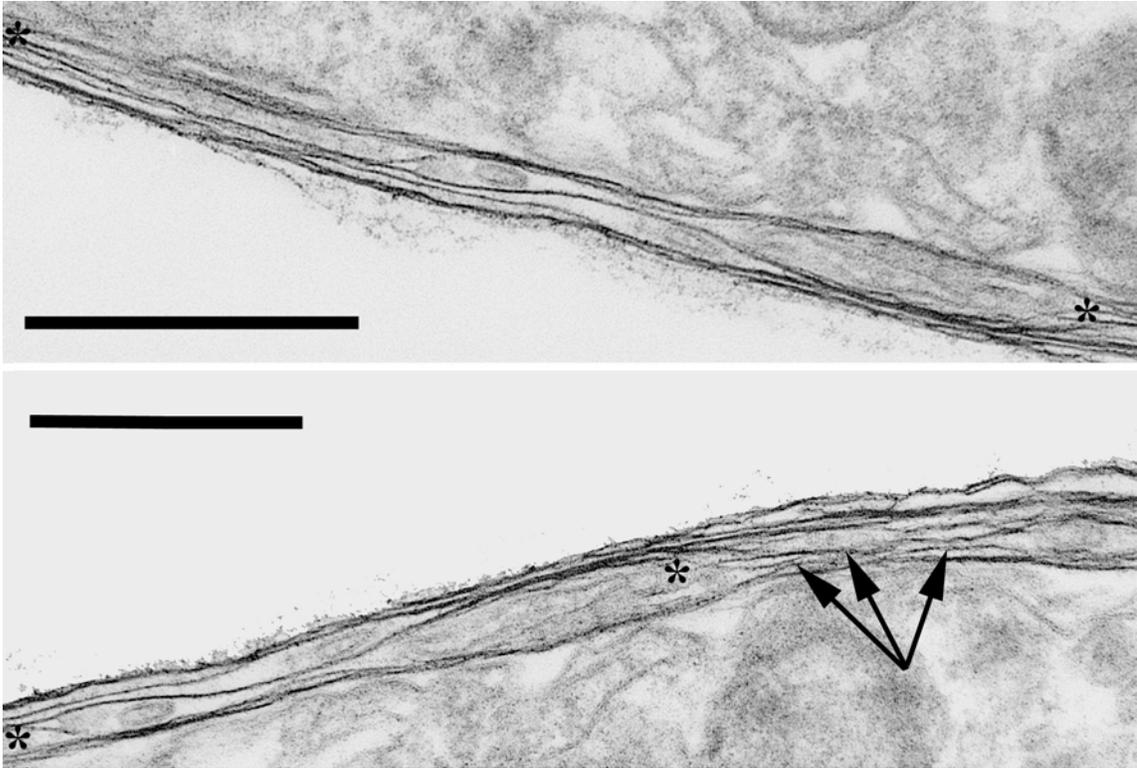


Figure 5.7.

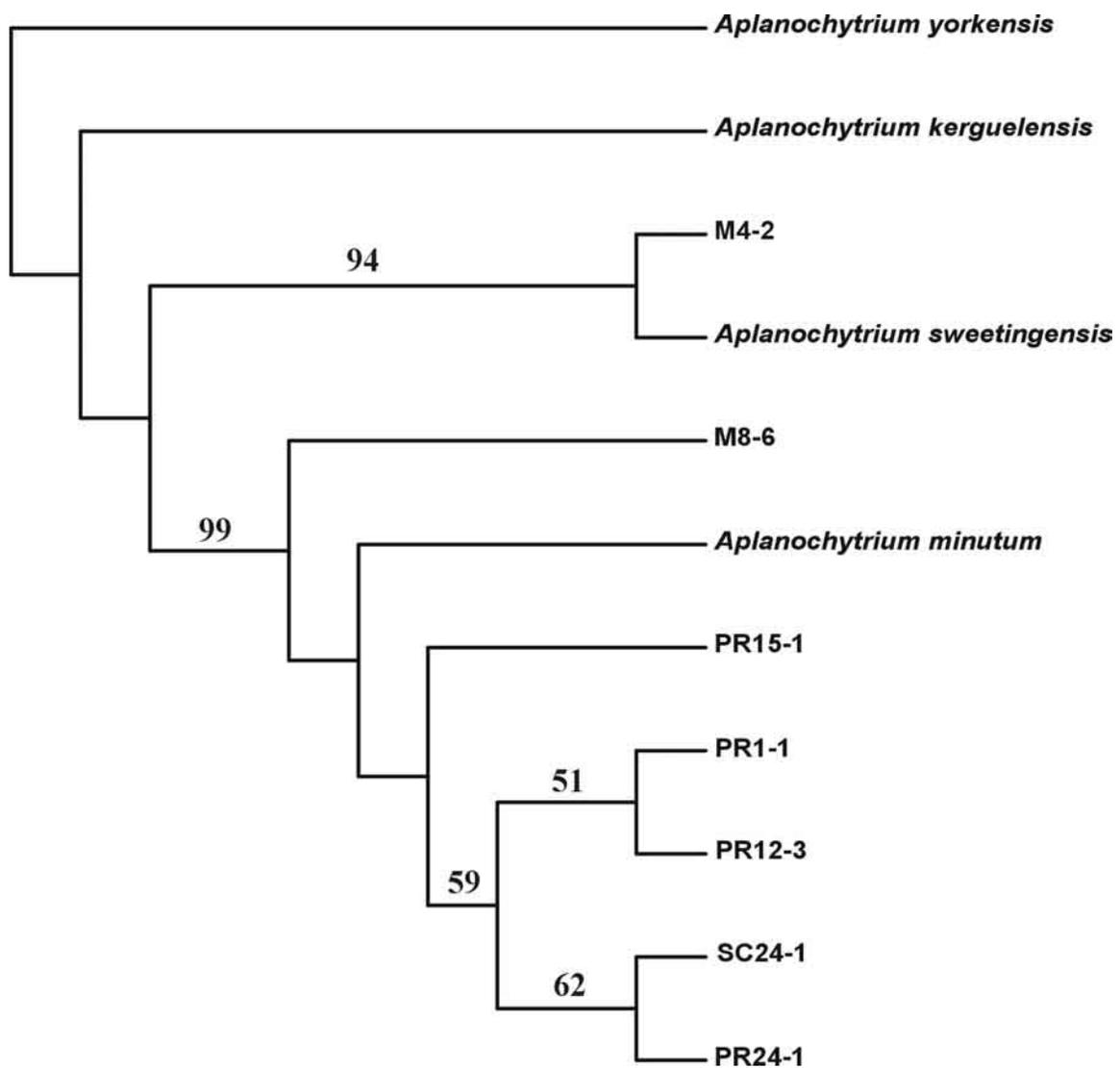


Figure 5.8

CHAPTER 6

CONCLUSION

During the course of this study, we examined the phylogeny of the Labyrinthulomycota by comparing trees generated with ssurDNA sequence data to those based on morphological characteristics. We also examined the traditional characters used to identify taxa within the Labyrinthulomycota for phylogenetic consistency. Ideally, sequence phylogenies will consistently mesh with data obtained from morphological examination, and a robust combined hypothesis can be put forth. This was the case with two of the lineages within the Labyrinthulomycota, the Labyrinthulaceae and the Aplanochytriaceae. Within the Thraustochytriaceae however, no obvious morphological characters support the phylogeny obtained with ssurDNA sequence analysis. This result reflects a problem with species and genus level taxonomy within the thraustochytrids, which is documented as being based on unstable characters (Booth and Miller 1968, Wethered and Jennings 1985). Taxonomically, we have expanded the phylum to include three families, redefined the genus *Aplanochytrium* to include species of the former genus *Labyrinthuloides*, and described one new species within *Aplanochytrium*.

In the second chapter of this study, we performed a preliminary phylogenetic analysis using ssurDNA sequence data. We concluded that the Labyrinthulomycota is a study leads us to suspect that *Labyrinthuloides* (now *Aplanochytrium*) is not a natural member of the Thraustochytriaceae, but may be more closely related to the weakly

supported monophyletic assemblage within the stramenopiles, sister to the other non-pigmented lineages (the Oomycota and the Hyphochytridiomycota) (Fig. 6.1).

To further investigate these relationships, we decided to expand the data set for the labyrinthulids and aplanochytrids in part two of this study.

In chapter three, species concepts within the Labyrinthulomycota were in need of validation. The labyrinthulids and aplanochytrids are identified on colony characteristics such as growth pattern and margin morphology (Muehlstein, Porter, and Short 1988). In contrast, the thraustochytrids are identified by developmental characters that are known to be highly plastic in nature (Booth and Miller 1968, Wethered and Jennings 1985). We began to question the inclusion of *Diplophrys marina* within the Labyrinthulomycota because *D. marina* lacks bothrosomes (Dykstra and Porter 1984). While investigating the taxonomic placement of the aplanochytrids (described above), we began to search for phylogenetically valid characters for taxon identification.

We found that the colony characteristics used for species identification within the labyrinthulids and aplanochytrids were supported with ssrDNA sequence data. Since these organisms are easily grown on a standard media such as SSA, colony recognition becomes a simple way for identification even in the field (Table 6.1). Within the thraustochytrids developmental characters used for identification are not supported by our sequence data at the species or genus level.

The expanded data set presented in this study confirmed that the aplanochytrids are ancestral to the labyrinthulids and thraustochytrids suggesting that gliding motility has been secondarily lost within the main thraustochytrid lineage. A second, smaller thraustochytrid clade consistently appears as basal to the rest of the group. Although this

placement is statistically insignificant, the possible polyphyletic nature of the Thraustochytriaceae cannot be dismissed. We found that *Diplophrys marina* may be a natural member of the phylum, but results were inconclusive due to an inconsistent placement of *D. marina* within the phylogeny. We also established the Aplanochytriaceae as a family independent from the Thraustochytriaceae. Finally, we described one new species of *Aplanochytrium* based on morphological and sequence data.

In chapter four, given the morphological similarities between *Labyrinthuloides* and *Aplanochytrium*, we questioned the integrity of these two genera (Bahnweg and Sparrow 1972, Perkins 1973). After evaluating historical documentation, morphological similarities, and sequence data, we concluded that these two genera are in fact synonyms. Because *Aplanochytrium* takes priority over *Labyrinthuloides*, the five species of *Labyrinthuloides* were transferred to *Aplanochytrium*. We also transferred one species from *Labyrinthula* to *Aplanochytrium* based on morphological criteria.

In chapter five, during our observations of aplanochytrids, we observed many isolates that we could not identify to species. Using ssurDNA sequence data, we compared a molecular phylogeny with a suite of nine morphological characters from 11 taxa/isolates. We discovered that agar penetration, colony diameter, and the rayed colony pattern of some aplanochytrids were phylogenetically significant characters. Colony margin shape, cell shape, cell size, inclusion characteristics, and ectoplasmic net morphology were not phylogenetically significant in comparison to ssurDNA phylogenies.

In conclusion, we have found that the Labyrinthulomycota forms a monophyletic assemblage at the base of the stramenopile clade of protists. The nearest sister groups are

the other non-pigmented stramenopiles, the oomycetes and the hyphochytrids. At least three family-level taxonomic units exist, the Labyrinthulaceae, the Aplanochytriaceae n. fam., and one or more lineages of the Thraustochytriaceae.

We discovered that the genus *Labyrinthuloides* is a synonym for *Aplanochytrium*. Following a redefinition of *Aplanochytrium*, *Labyrinthuloides* is no longer a valid genus within the Labyrinthulomycota. The genus *Aplanochytrium*, which was formerly monotypic, now has eight valid species including *A. sweetingensis* n. sp..

We found that the use of colony morphological characters used to identify species within the Labyrinthulaceae and the Aplanochytriaceae lead to the identification of phylogenetically valid taxa, while the developmental characteristics used to identify genera within the Thraustochytriaceae do not. Finally, we conclude that *Diplophrys marina* is a natural member of the Labyrinthulomycota that has secondarily lost bothrosomes. *D. marina* is inconsistently placed as basal to the main clade of the thraustochytrids, or as basal to the labyrinthulids.

Future work is needed to elucidate phylogenetically valid identifying characters within the thraustochytriaceae. Other zoosporic fungi including the chytrids and the oomycetes can be identified to major groups based on ultrastructural characteristics of the zoospore (Barr 1978, Alexopoulos, Mims, and Blackwell 1996, James et al 1999,). Zoospores of the thraustochytriaceae vary in shape, but have never been evaluated at an ultrastructural level. We would not be surprised to find valid identifying characters if ultrastructure of the zoospores were investigated. The monophyletic nature of the Labyrinthulaceae and the Aplanochytriaceae has been well established with this work.

However, the Thraustochytriaceae appears to be polyphyletic. Sequencing of additional isolates would provide insight into the true phylogenetic nature of this family.

The hexagonal array seen on the surface of the aplanochytrids remains a puzzle. Although we assume a manifestation of some aspect of the cell wall scales, the exact identity of this pattern is unknown. An investigation into the nature of this pattern would provide additional support for the morphological unity of the Aplanochytriaceae. It would be particularly interesting to investigate the presence or absence of such an array on the surface of *Labyrinthula* cells, which had been stripped of the overlying ectoplasmic net. The presence of such an array on naked *Labyrinthulid* cells would be evidence of an adhesive material holding wall scales in place in both the aplanochytrids and the labyrinthulids.

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Table 6.1. Identifying morphological characteristics for colonies of *Labyrinthula* and *Aplanochytrium* taxa.

	Margin Morphology	Stream or ray morphology	Spindle cell stream thickness	Agar Penetration
<i>Labyrinthula</i> sp. s	Smooth	Straight	Narrow	In older colonies
<i>Labyrinthula</i> sp. f	Rough	Curved	Thick	Rarely in older colonies
<i>Labyrinthula zosterae</i>	Very rough	Very curved	Very thick	None
<i>Aplanochytrium minutum</i> group	Smooth or rough	Straight or curved	Thin or thick	In older colonies
<i>Aplanochytrium sweetingensis</i>	Smooth or rough	None	N/A	Profuse
<i>Aplanochytrium yorkensis</i>	Smooth	None	N/A	In older colonies

FIGURE LEGENDS

Fig. 6.1. The Labyrinthulomycota in relation to other stramenopile lineages.

Fig. 6.2. Morphological characters with molecular phylogenetic support within
Aplanochytrium.

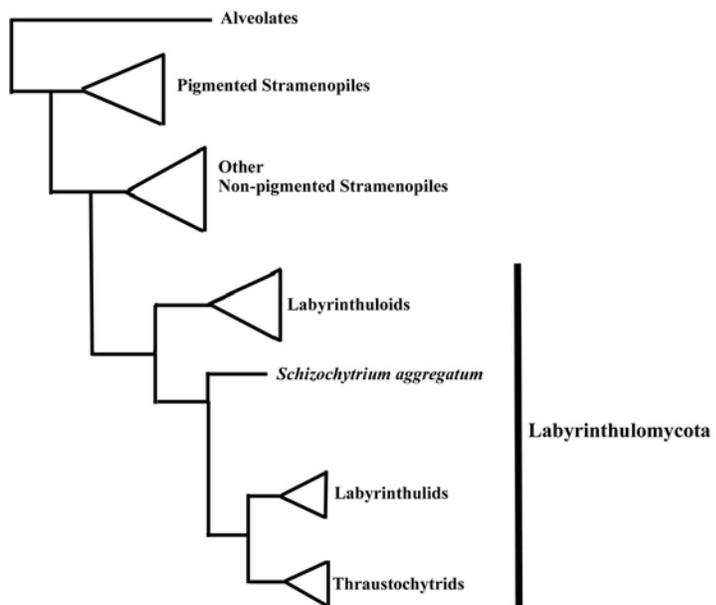


Figure 6.1.

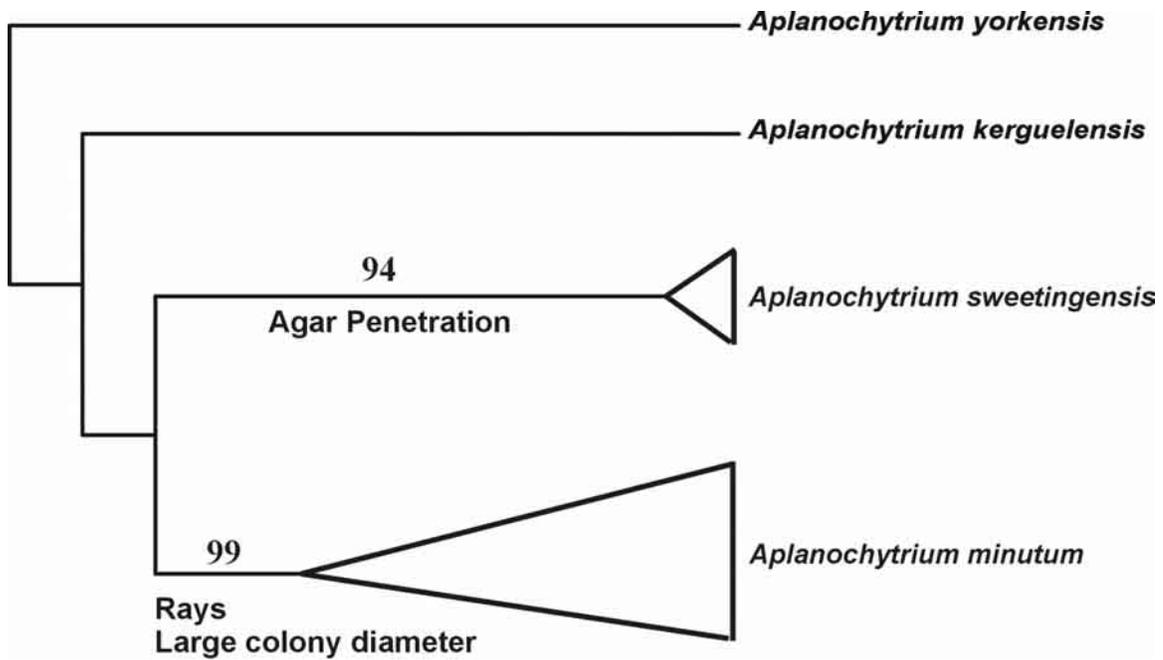


Figure 6.2.