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Systematics of the Lobulomycetales, a New Order within the Chytridiomycota

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SYSTEMATICS OF THE LOBULOMYCETALES, A NEW ORDER WITHIN THE CHYTRIDIOMYCOTA

By

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Thesis Advisor: Dr. Joyce E. Longcore

An Abstract of the Thesis Presented
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Based on molecular phylogenetic analyses, the polyphyletic order Chytridiales, one of the four orders in the Chytridiomycota, contains several well-supported clades. One species, *Chytriomyces angularis*, however, does not group within the robust clades of the Chytridiales or any other chytrid order. The light-level morphology and zoospore ultrastructure of this aquatic species also differ from those of the type species of the genus *Chytriomyces*. I amassed nine additional pure culture isolates of soil-inhabiting chytrids with morphologies or rDNA sequences similar to *C. angularis*, including two isolates of *C. poculatus*, and studied the molecular phylogeny of these ten chytrids. Results of the Bayesian and maximum parsimony analyses of nucSSU and partial nucLSU rDNA sequences grouped the nine isolates and the type specimen of *C. angularis* in a monophyletic clade within the Chytridiomycota, exterior to the Chytridiaceae. I examined zoospores of two isolates by transmission electron microscopy, and determined that these isolates had

ultrastructure similar to that of *C. angularis*. The variable morphology of four isolates compared to the two described species, *C. angularis* and *C. poculatus*, in addition to the molecular phylogeny and minor differences in their zoosporic ultrastructure supported the description of two additional species. Genetic, ultrastructural, and light-level morphological data support the establishment of the new order "Lobulomycetales," which contains the family "Lobulomycetaceae." I describe two new monospecific genera, "*Maunachytrium*" and "*Clydaea*," within this family and remove *C. angularis* and *C. poculatus* from the genus *Chytriomyces* and place them in the new genus "*Lobulomyces*."

DEDICATION

I dedicate this work in memory of Clyda Rae Simms Simmons, who believed in me only as a mother could.

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I thank my family and friends, who did not have a clue what I was doing but supported me all the same.

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LITERATURE REVIEW

Systematics of the Chytridiomycota has been and remains a difficult discipline given the few morphological characters of these microscopic, aquatic fungi and the arguments over phylogenetic importance of those characters (Sparrow 1943, Whiffen 1944, Roane & Paterson 1974, Karling 1977, Barr 1980). Genetic sequences are providing a wealth of characters that better illustrate how taxa within the phylum are related, yet some taxa have not been investigated because they are not within well-supported phylogenetic clades (James et al 2000, 2006b). Currently, a major goal of systematists is to increase taxon sampling to better estimate diversity and topology of lineages and, when necessary, to provide revisions to the taxonomy.

Since the advent of molecular phylogenies in fungal systematics, one species, *Chytriomyces angularis*, has fallen outside of the well-supported clades of the Chytridiomycota. I present evidence of the close relationship between *C. angularis* and *C. poculatus* and describe two new species also related to *C. angularis*. The distinct morphological and genetic characters of this group of species warrant the creation of a new order in the Chytridiomycota.

Phylum Chytridiomycota

The Chytridiomycota is one of three phyla within the Fungi whose members reproduce by producing zoospores, which are typically propelled by a single, posterior, smooth flagellum (Hibbett et al in press, James et al 2006b). These fungi,

commonly referred to as chytrids, are ubiquitous, occurring in terrestrial and aquatic environments. Terrestrial species are mostly saprobic on recalcitrant polysaccharides (chitin or cellulose), proteins (keratin), or carbohydrate-rich pollen, and some species are obligate parasites of vascular plants. Aquatic species, including a few marine ones, are saprobic or parasites of algae, protozoa, invertebrates, or other fungi. One species, *Batrachochytrium dendrobatidis*, is a pathogen that targets the skin of amphibians.

The chytrids have several types of development that result in the production of motile, asexual reproductive zoospores bound by a plasma membrane. Once a zoospore reaches a suitable substrate, it retracts its flagellum and the resulting single-celled organism produces a cell wall in a process called encysting. A holocarpic chytrid enlarges such that the entire individual, or thallus, becomes the zoosporangium, which is the zoospore-producing structure of the chytrid. A eucarpic chytrid produces one or more zoosporangia that are attached to the substrate by absorptive, root-like extensions of the thallus called rhizoids, sometimes having a swelling below the zoosporangium at the base of a rhizoidal axis. After encystment, some species develop with the nucleus remaining within the encysted zoospore (endogenous development) and others form a germ tube into which the nucleus migrates (exogenous development) that then develops into the thallus (Barr 1980). The precise location of the migratory nucleus within the zoosporangium in exogenous development varies among taxa (Blackwell et al 2006). When the thallus is mature, protoplasm within the zoosporangium divides into new zoospores that are released into the environment, either through a pore in the zoosporangium, or by the

dehiscence of a cap-like structure called an operculum. Chytrids can also produce resting spores, which are thick-walled sexually or asexually formed structures that persist in unfavorable conditions and can remain dormant for years. Monocentric chytrids produce a single zoosporangium whereas polycentric chytrids produce multiple zoosporangia per individual. The different patterns of thallus development were once used for higher taxonomic placement of chytrid species (Sparrow 1943), yet systematists have since debated the importance of these morphological characters (Whiffen 1944, Roane & Paterson 1974).

At present, the Chytridiomycota is divided into the classes Monoblepharidomycetes and Chytridiomycetes, the latter including the three orders Spizellomycetales, Rhizophydiales, and Chytridiales, 13 families, more than a 100 genera, and approximately 750 species. However, familial affiliations of genera have long been a topic of debate (Sparrow 1943, 1960, Whiffen 1944, Roane & Paterson 1974, Karling 1977), and because of this some genera described since Sparrow's 1960 monograph have never been placed within a family (Longcore 1992, 1993, Longcore et al 1999). Linnaean taxonomy of chytrids based on morphology has not been representative of a likely phylogeny; it has taken recent studies of zoospore ultrastructure (Barr 1980) and genomic DNA sequences (James et al 2000, 2006a, 2006b) to begin to bring more probable reconstructions of phylogenetic relationships in this group of fungi.

Chytrid Systematics

The Use of Light Microscopy

Sparrow (1943) divided the chytrids into two taxonomic series based on the presence or absence of an operculum, concluding that similar types of development in the operculate and the inoperculate series could be explained by parallel evolution. He used the characters of operculation and type of development to organize his treatment of the Chytridiomycota in the influential and only monographs that cover the group (Sparrow 1943, 1960). Whiffen (1944) argued, however, that it was just as probable that type of development (endogenous versus exogenous) was more phylogenetically important than operculation. Karling (1977) concurred that operculation was not the most important character and organized genera and families based on the manner of thallus development. He used this view in his *Chytridiomycetarum Iconographia*, which contains discussions of genera and drawings of selected species. In the 1970s, comparative studies of chytrid light-level morphology showed that patterns of development in a single isolate varied depending on host and substrate (Powell & Koch 1977a, 1977b). These studies showed that the light-level morphological features used for species identification in gross cultures could be lost or changed with development in laboratory media. This added more confusion to chytrid taxonomy, leaving the discipline with an outmoded identification system of species described from gross culture and an undeveloped system of species described from pure culture.

The Use of Zoospore Ultrastructure

In the 1960s and 1970s, ultrastructural characters of zoospores were compiled and compared across taxa to determine their bearing on phylogenetic theories (Fuller 1966, Fuller & Reichle 1967, Lange & Olson 1979). Barr (1980) proposed that zoospore characters associated with the flagellum would be conserved because the flagellum is required for motility. Characters in this region of the zoospore, known as the kinetid (Andersen et al 1991), are 1) the angle and type of fibrillar bridge between the kinetosome and the nonflagellated centriole, 2) the presence and configuration or absence of microtubules (microtubule root) associated with the kinetosome, 3) the association or lack thereof between the nucleus and the kinetosome, 4) the presence or absence of an electron-opaque plug in the base of the flagellum, and 5) any other electron-opaque bodies in the kinetid region.

Sparrow (1960) divided chytrids into two small orders distinguished by sexual traits, the Monoblepharidales and Blastocladiales, and one large and extremely diverse order, the Chytridiales. The Spizellomycetales were the first order to be segregated from the Chytridiales based on ultrastructural characters (Barr 1980). The three synapomorphic ultrastructural characters for the Spizellomycetales are 1) the presence of an association between the nucleus and the kinetosome, 2) the radiation of microtubules from the kinetosome to the anterior of the zoospore, and 3) the lack of association between the mitochondria and microbody-lipid globule complex (Powell 1978). The specific types of microtubule arrangements and nuclear association with the kinetosome were used for the description of five genera in this order. Vavra & Joyon (1966) first discovered the anaerobic chytrids that inhabited the

rumen and hind gut of herbivorous mammals, and erected the genus *Neocallimastix* for these species. The family Neocallimasticaceae that contained these gut fungi was originally classified in the Spizellomycetales, but the family was elevated to the order Neocallimastigales based on morphological and physiological characters (Li et al 1993). Barr (1980) supported the distinguishing of species within a genus by light-level morphology and the use of characters of thallus development in distinguishing families. He proposed, however, that orders and genera be based on ultrastructural characters. He believed light-level morphological traits may also correlate with genus descriptions but maintained that ultrastructural characters be included for new genera and species and that previously described chytrids be examined to better assess phylogenetic placement.

The Use of Molecular Phylogenetic Hypotheses

At the turn of the 21st century, molecular phylogenies define the Chytridiomycota as a polyphyletic fungal group with the majority of its taxa in a monophyletic clade (James et al 2000, 2006a, 2006b). This phylum is basal to the more derived fungal phyla, including the recently elevated Blastocladiomycota (James et al 2006b), the Glomeromycota, Zygomycota, and Dikarya (James et al 2006a, 2006b). Molecular phylogenies in recent studies of chytrid systematics rely on various combinations of sequences from the nuclear ribosomal DNA (rDNA) operon (James et al 2000, 2006b) and conserved transcription factor genes (James et al 2006a). These phylogenies all identified similar monophyletic groups, including the orders Neocallimastigales, Monoblepharidales, and Spizellomycetales. The

Chytridiales, however, are polyphyletic in single-gene (James et al 2000) and six-gene phylogenetic hypotheses (James et al 2006b). The phylogenies produced by James et al (2000) from nuclear small subunit (nucSSU) rDNA sequences divided the Chytridiales into four well-supported clades. These genetically determined clades within the Chytridiales were identical to groupings based on zoospore ultrastructure (Barr 1980, 1990). Several taxa did not fall within any supported clade. *Chytriomyces angularis*, which had already been shown to have different ultrastructural characters from the type species, *Chytriomyces hyalinus*, of the genus *Chytriomyces* (Longcore 1992), was grouped with little support as sister to the order Monoblepharidales, a group with which it had even less in common than with the Chytridiales (James et al 2000).

Correlations among ultrastructural and genetic data have since been used to support taxonomic revisions in the Chytridiales. The monophyletic *Rhizophydium* clade of the Chytridiales (James et al 2000, 2006b) has been elevated to the order Rhizophydiales (Letcher et al 2006), which contains the families Rhizophydiaceae, Terramycetaceae, and Kappamycetaceae. The order and families are based on zoospore ultrastructure and analyses of partial nuclear large subunit (nucLSU) and 5.8S rDNA sequences. The only ultrastructural character state that defines the Rhizophydiales is a negative state, the lack of an electron-opaque flagellar plug, which is a structure that may have been lost. Therefore, without genetic characters, unrelated taxa that have lost a flagellar plug could erroneously be placed within the order. The description of the new order, despite the lack of a synapomorphic zoospore character for the Rhizophydiales, shows the importance placed on molecular

phylogenies by modern chytrid systematists. Molecular phylogenies also have supported the elevation of the former chytridiomycete orders Blastocladiales and Neocallimastigales to the phyla Blastocladiomycota (James et al 2006b) and Neocallimastigomycota (Hibbett et al in press).

The family Chytridiaceae *sensu* Barr, which contains *Chytridium*, the type genus of the order Chytridiales, was emended based on ultrastructural and DNA characters (Letcher et al 2005). The type species of the genus *Chytriomyces*, *Chytriomyces hyalinus*, is within the Chytridiaceae, yet some species currently classified in this genus do not group within this family in any phylogenetic hypothesis (Letcher et al 2005, James et al 2006b). Thus, even organisms considered to be within the same genus by morphological traits have been placed in different lineages within the Chytridiomycota when scrutinized by molecular phylogenetics.

Genus *Chytriomyces*

Karling (1945) described the first two species in the genus *Chytriomyces* as having operculate zoosporangia with subsporangial swellings and producing resting spores that developed exterior to their substrate. He also described the posteriorly flagellated zoospores as being bound in a vesicle after release and before dispersal into the environment. As species were added, the genus concept expanded to allow inclusion of species that lacked one or more of these characters (Letcher & Powell 2002). Letcher & Powell designated *Chytriomyces hyalinus*, one of the first two species in the genus, the lectotype of *Chytriomyces*. Barr (1980) defined groupings of chytrid taxa based on ultrastructural characters of zoospores, and *Chytriomyces*

hyalinus has Barr's Group I type of zoospore (FIG. 1a), which has 1) a nonflagellated centriole parallel to the kinetosome and connected to it by a fibrillar bridge, 2) a microtubule root extending from the kinetosome to the rumposome (fenestrated cisterna), which abuts the lipid globule and the plasma membrane, 3) a Golgi apparatus and electron-opaque plates that surround the microtubule root, 4) a paracrystalline inclusion that lies in the zoospore cytoplasm, and 5) an electron-opaque plug located in the base of the flagellum.

Chytriomyces angularis

Molecular phylogenies (James et al 2000, 2006b, Letcher et al 2005) consistently place *Chytriomyces angularis* Longcore (1992) exterior to species-rich, statistically well-supported clades, including the Chytridiaceae clade in which *Chytriomyces hyalinus* resides. *Chytriomyces poculatus* Willoughby & Townley (1961) and chytrids resembling *C. angularis* had previously been considered part of a species complex (Dogma 1969, Booth 1971a, 1971b), and Longcore (1992) also suggested that *C. angularis* was a close relative of *C. poculatus*. *Chytriomyces poculatus* has a zoosporangium surrounded by cupules of the cell wall, giving it a "ghostly veil" (Willoughby & Townley 1961), but was often seen in gross cultures to be lacking this ornamentation. *Chytriomyces angularis* does not possess these cupules, and may have been the chytrid that previous researchers had noted as a noncupuled variation of *C. poculatus*. Because pure cultures of *C. poculatus* were lacking, neither ultrastructural nor molecular studies have been done. The light-level morphologies of *C. angularis* and *C. poculatus* differ from *C. hyalinus* in two

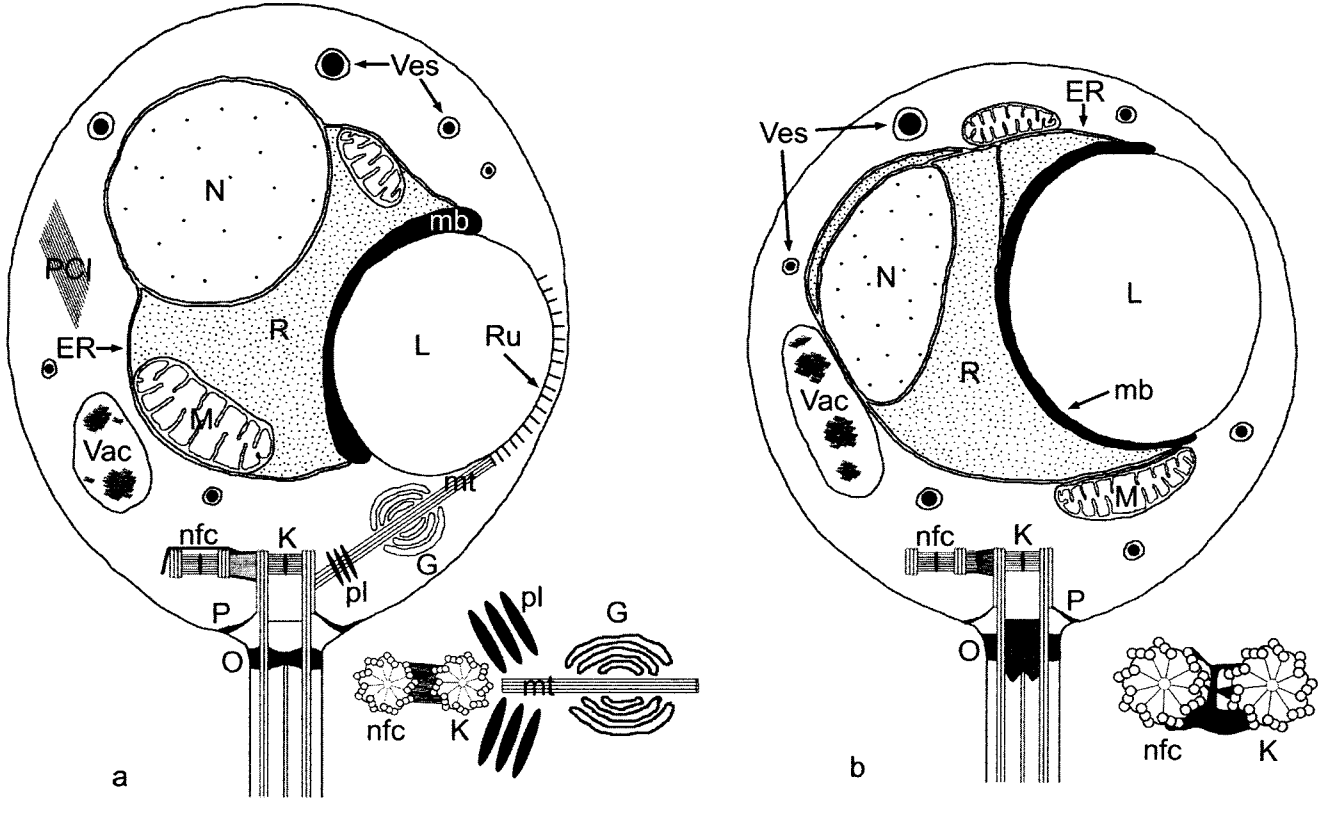

FIGURE 1. Zoospore diagrams of *Chytriomyces hyalinus* and *C. angularis* (= "*Lobulomyces angularis*"). a. Ultrastructure of *C. hyalinus* zoospore, adapted from Letcher et al (2005). b. Ultrastructure of *L. angularis* zoospore, adapted from Longcore (1992). KEY TO FIGS. 1, 5, & 7: ER, endoplasmic reticulum; G, Golgi apparatus; K, kinetosome; L, lipid globule; M, mitochondrion; mb, microbody; mt, microtubule root, N, nucleus; nfc, nonflagellated centriole; O, electron-opaque plug; P, props; PCI, paracrystalline inclusion; pl, electron-opaque plates; R, clustered ribosomes; Ru, rumposome; Vac, vacuole; Ves, vesicles.

respects. Neither *C. angularis* nor *C. poculatus* possess a swelling in the rhizoidal axis directly below the zoosporangium (FIG. 2), which is present in the type species of *Chytriomyces*. Also, zoospores of *C. hyalinus* are bound in a vesicle after their emergence from the zoosporangium; *C. angularis* and *C. poculatus* do not release zoospores enclosed in a vesicle.

The ultrastructure of *Chytriomyces angularis* (= "*Lobulomyces angularis*") (FIG. 1b) is unique among previously examined chytrids in the possession of anterior and posterior electron-opaque extensions of the flagellar plug and in the pattern of the dense fibrillar bridge connecting four microtubule triplets of the kinetosome and three triplets of the nonflagellated centriole. *Chytriomyces angularis* lacks several ultrastructural features that are found in *C. hyalinus*, including the Golgi apparatus, microtubules and electron-opaque plates associated with the kinetosome, a rumposome on the lipid globule, and a paracrystalline inclusion in the cytoplasm. These negative characters were not useful in determining the relatedness of *C. angularis* to other species in the genus. Based on its unique zoospore ultrastructure, however, and its position outside of other clades in molecular phylogenetic hypotheses, *C. angularis*, and possibly the morphologically similar *C. poculatus*, may represent a new genus.

Objective

Early work on chytrid systematics (Sparrow 1943, 1960) based on morphological characters underestimated the diversity we now know is present in these basal fungi. Although morphology and development were the most accessible

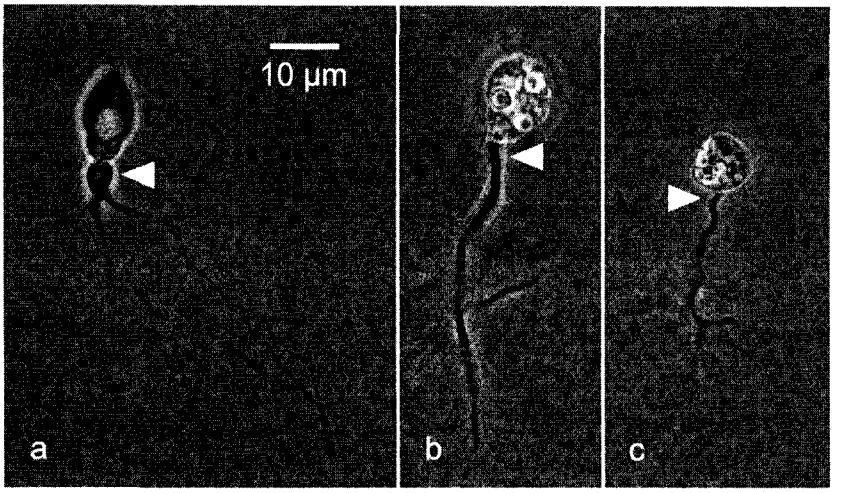

FIGURE 2. Comparison of rhizoids between Chytridiaceae and *Chytriomyces angularis* clade organisms. a. JEL161 *Podochytrium* sp. (18 h), with subsporangial swelling at the base of the rhizoidal axis (white arrowhead) and rhizoids tapering at ends. b. JEL45 *C. angularis* (= "*Lobulomyces angularis*") (18 h), lacking subsporangial swelling (white arrowhead) and with isodiametric rhizoids. c. JEL374 *C. poculatus* ("*Lobulomyces poculatus*") (24 h), rhizoidal system as in *C. angularis*.

traits upon which to base taxonomic decisions at the time, they do not reflect our current understanding of chytrid phylogenetics. Analyses of ultrastructural and genetic characters support some taxonomic groups and have exposed undescribed lineages. It is incumbent upon systematists to apply these new characters to the taxonomy of novel and described species.

My objective was to describe the group contained within the *Chytriomyces angularis* clade. To support taxonomic revisions of this scale, I have 1) increased the sample size of specimens in the *C. angularis* clade by accumulating taxa from culture collections and new isolations, 2) sequenced and analyzed nuclear small subunit ribosomal DNA (nucSSU rDNA) and partial nuclear large subunit ribosomal DNA (nucLSU rDNA) of the collected isolates, and 3) compared ultrastructural zoospore characters of selected isolates to the type isolate of *Chytriomyces angularis*.

Chapter 2

LOBULOMYCETALES, A NEW ORDER IN THE CHYTRIDIOMYCOTA

Introduction

Some species of chytrids have morphologies that make them easily recognizable. Two such species are *Chytriomyces poculatus* Willoughby and Townley and *C. angularis* Longcore. *Chytriomyces poculatus* is an operculate, elongate chytrid, which is unique in that the zoosporangium is surrounded by a "ghostly veil," or cupules (Willoughby & Townley 1961). Researchers noted noncupuled forms of *C. poculatus* (Dogma 1969, Booth 1971a, 1971b), but it was not until the naming of *C. angularis* that these two species were recognized as being distinct. The light-level morphology of *C. angularis* is similar to *C. poculatus*, with the exception of the cupules on the zoosporangium. Because of its similarity to *C. poculatus*, Longcore (1992) also placed *C. angularis* in the genus *Chytriomyces*, but found that the zoospore ultrastructure of *C. angularis* differed from *C. hyalinus* Karling, the type of the genus (Letcher et al 2002) and was unique within the Chytridiomycota. Longcore (1992) hypothesized that *C. angularis* might represent a new genus but waited to describe such a genus until more chytrids were isolated that had light-level and ultrastructural characters in common with the species.

Since that time, molecular phylogenies (James et al 2000, 2006b, Letcher et al 2005) have consistently placed *C. angularis* exterior to well-supported groups, which coincided with groups based on ultrastructural data (Barr 1980, 1990). Analyses of molecular and ultrastructural data have supported major taxonomic changes in the

Chytridiomycota, including the segregation of the Rhizophydiales from the Chytridiales (Letcher et al 2006) and the amendment of the Chytridiaceae to exclude taxa not related to *C. hyalinus* (Letcher et al 2005).

To further study the placement of *C. angularis* and its relationship to *C. poculatus* with a larger taxon sampling, I acquired the type culture of *C. angularis*, two isolates of *C. poculatus*, and seven other morphologically or genetically similar isolates. I compared their light-level morphology and zoospore ultrastructure, and determined the placement of these chytrids within the Chytridiomycota based on analyses of their nucSSU and partial nucLSU regions of the rDNA operon. *Chytriomyces angularis* and *C. poculatus* are related to each other, and I placed them in a new genus. These organisms still do not group within any other clade in the Chytridiomycota, and I describe the *C. angularis* clade as a new order, the "Lobulomycetales," which contains one family and three genera.

Materials and Methods

Chytrid Isolates

Isolates of *Chytriomyces angularis*, *C. poculatus*, and morphologically similar chytrids (TABLE 1) were provided by Dr. Joyce Longcore of the University of Maine. Isolate PL70 was provided by Dr. Peter Letcher of the University of Alabama. Alpine fungi (AF) isolates were baited from soil samples provided by Dr. Allen Meyer of the University of Colorado at Boulder.

TABLE 1. Culture designations, identities, origins, and habitats of "Lobulomycetales" isolates.

Culture no.	New Taxon Identification	Previous Identification	Origin	Habitat
AF007	-[c]	*Rhizophydium* sp.	Peru	Alpine barren soil
AF011	-	*Rhizophydium* sp.	Peru	Alpine barren soil
AF017	-	*Rhizophydium* sp.	Peru	Alpine barren soil
AF021[ab]	"*Maunachytrium keaensis*"	*Rhizophydium* sp.	Hawaii, USA	Alpine barren soil
JEL45	"*Lobulomyces angularis*"	*Chytriomyces angularis* Longcore	Maine, USA	Sphagnum from acidic lake
JEL178[a]	"*Lobulomyces* sp."	*Chytriomyces angularis*	Maine, USA	Spring under mixed hard woods
JEL343	"*Lobulomyces poculatus*"	*Chytriomyces poculatus* Willoughby & Townley	Maine, USA	Soil under Pine trees
JEL369[ab]	"*Clydaea vesicula*"	*Chytriomyces angularis*	California, USA	Soil under *Eucalyptus* trees
JEL374	"*Lobulomyces poculatus*"	*Chytriomyces poculatus*	New Zealand	Tree canopy detritus
PL70	"*Clydaea vesicula*"	*Chytriomyces angularis*	Utah, USA	Creek bank

[a] taxa examined by light microscopy
[b] taxa examined by transmission electron microscopy
[c] "-" indicates no description

Novel isolates were baited with spruce pollen and sterilized snakeskin from gross cultures containing ~5 g of soil and ~50 mL of sterilized, distilled water in a 90 X 25 mm deep-dish plastic Petri dish. Baits with chytrid growth were removed from the gross culture and placed on PmTG agar medium (0.5 g peptonized milk, 0.5 g tryptone, and 2.5 g glucose in 500 mL distilled water; Barr 1986) containing penicillin (200 mg/L) and streptomycin sulfate (~350 mg/L). I tumbled the baits across the surface of a plate of the PmTG agar with a sterile needle in an effort to remove bacteria from the chytrids and their substrate before placing the cleaned baits in another sterile plate. Once growth was sufficient, the chytrid isolate was transferred to a plate of PmTG agar medium without antibiotics to verify that it was free of bacteria. Gross cultures and subsequent isolation plates were incubated at 10 C in the dark to simulate montane environmental conditions. I transferred pure cultures into slant tubes of PmTG agar medium and stored them at 4 C.

Medium Optimization and Temperature Maxima. I modified the recipe of the medium PmTG by changing the carbon source in an attempt to optimize growth of the isolates and encourage zoospore release. The five grams of glucose was removed for one treatment, PmT, or replaced with 5 g of cellobiose (C) or keratin (K). Isolates JEL45, JEL178, JEL343, JEL369, JEL374, PL70, and AF021 were incubated in the four liquid medium treatments PmTC, PmTK, PmTG [+ control], and PmT at 17 C for 2 weeks in the dark. Growth was visually determined and a sample of each flask was observed by light microscopy to determine the isolate's viability. Unexpectedly, the PmT medium produced the most robust growth in all isolates but *C. angularis*

(data not shown). I used the new medium for all subsequent growth of *Chytriomyces angularis* clade isolates except for *C. angularis*, which only grew in PmTG. Isolates were grown in duplicates in 75 mL liquid medium in 125 mL flasks at 17, 20, 25, 30, and 35 C for 2 weeks in the dark to determine maximum temperatures for isolate growth.

Growth Records. I grew isolates JEL178, JEL369, and AF021 for light-level morphological comparison of representative developmental stages. These isolates were chosen because of the differences in their thallus morphology that distinguished them from *Chytriomyces angularis* and *C. poculatus*. Once zoospores of an isolate were observed on a plate of agar medium, the plate was flooded with ~2 mL sterile distilled water and placed at room temperature for 30 minutes. The zoospore solution was aspirated from the plate and placed onto a fresh plate of PmT agar medium, which then was allowed to dry in a laminar flow cabinet. Plates were sealed with Parafilm, and placed at 17 C in the dark. During development, thin slices of agar with thalli were removed aseptically and examined with a Nikon E400 microscope equipped with phase optics and a Spot RT digital camera.

Transmission Electron Microscopy (TEM) Techniques

The methods of TEM followed the procedures of Barr (1981). Of the isolates showing light-level morphologies that differed from *Chytriomyces angularis* and *C. poculatus*, JEL369 and AF021 produced sufficient numbers of zoospores for ultrastructure analysis. I grew isolates on 20 plates of PmT agar medium for ~5 days

at 17 C in the dark, flooded the plates with ~2 mL of sterile distilled water for 30 min, harvested the zoospore solution, and fixed the zoospores with an equal volume of 3 % glutaraldehyde in 0.1 M *s*-collidine buffer, pH 7.4. I left the zoospores in this solution at room temperature for 2 h after which they were refrigerated for 24 h. After pelleting and washing the zoospores in 0.5 M *s*-collidine buffer, I post-fixed the zoospores in 1 % osmium tetroxide in 0.05 M *s*-collidine for 30 min. After sequential 0.05 M s-collidine buffer and distilled water washes, I embedded the zoospores in 2 % agarose. The agar was then cut into ~1 mm pieces, washed in 10 % acetone and *en bloc* stained overnight with 2 % aqueous uranyl acetate at 4 C. I dehydrated the agar pieces in an acetone series and embedded the pieces in Epon-Araldite. Blocks were serial sectioned with a diamond knife onto carbon-coated, Pioloform-covered nickel slot grids, and ultrathin sections were stained with 0.05 % aqueous lead citrate for 4-6 min. Sections were examined on the Philips CM 10 at 80kV and photographed with a Gatan Bioscan Camera (Gatan, Inc.).

Molecular Techniques

DNA Extractions. I grew isolates in liquid medium in 50 mL centrifuge tubes for 1 week in the dark at 17 C. Isolates were centrifuged and pelleted, and all but ~1 mL of the liquid was poured off. The suspended pellet was pipetted into a 1.5 mL Eppendorf tube, which was centrifuged at 8,000 RPM for 4 min. The supernatant was aspirated and the pellet dehydrated in a speed-vac concentrator for 1 h. A portion of the dried material was placed into a new 1.5 mL Eppendorf tube along with 500 µL 1X CTAB buffer (1 % (w/v) CTAB, 50 mM Tris (pH 8.0), 10 mM Na_2EDTA, 0.7 M NaCl),

sand, and approximately 50 μL of 1 mm zirconium silica beads. The tube was vortexed for 3 min and then placed in a 65 C water bath for 1 hour, with inversions every 20 min. A 500 μL volume of 24:1 chloroform: isoamyl alcohol (C:IA) solution was added, and the tube was inverted thrice with 1 min intervals between inversions. The tube was centrifuged at 12,500 RPM for 12 min, after which the top aqueous layer was placed into a new 1.5 mL Eppendorf tube along with 250 μL of 24:1 C:IA. The new tube was centrifuged at 12,500 RPM for 8 min, and approximately 300 μL of the top aqueous layer of each tube was placed into another 1.5 mL Eppendorf tube containing 200 μL of 80 % ethanol. The tube was inverted several times and then stored at 20 C overnight to allow precipitation of DNA. The tube was centrifuged at 13,000 RPM for 7 min, after which the supernatant was discarded and 1 mL of 80 % ethanol was added. The tube was centrifuged at 13,000 RPM for 2 min, ethanol discarded, and the pellet dehydrated in a speed-vac concentrator for 10 min or a chemical hood for 1 h. The dried DNA pellet was then suspended in 50 μL of sterile distilled water. DNA product was visualized on 1X TAE (Tris-Acetate-EDTA) or 1X TBE (Tris-Borate-EDTA) gels stained with ethidium bromide or GelStar (Cambrex).

Amplification and Sequencing. DNA was diluted into new solutions, each with a final volume of 200 μL. I followed the polymerase chain reaction (PCR) protocol as described by Vilgalys & Hester (1990). A PCR solution for each isolate consisted of 5.875 μL water, 2.5 μL 10X PCR Buffer (15 mM $MgCl_2$), 4 μL of a 1.25 mM dNTP solution, 1.25 μL of a 10 μM solution of each primer, 0.125 μL AmpliTaq DNA polymerase (Applied Biosystems), and 10 μL of the DNA template (0.1-1 ng/μL). I

used primers SR1R (5'-TACCTGGTTGATQCTGCCAGT-3') and SR6.1 (5'-TGTTACGACTTTTACTT-3') for amplification of the nucSSU rDNA region (Vilgalys & Hester 1990, James et al 2000). I used primers SR1R, SR6.1, SR1.5 (5'-AAGGCAGCAGGCGCGCAAATTAC-3'), and NS6 (5'-GCATCACAGACCTGTTATTGCCTC-3') for the sequencing of the nucSSU rDNA region (James et al 2000, White et al 1990). I used primers LR0R (5'-ACCCGCTGAACTTAAGC3') and LR5 (5'-TCCTGAGGGAAACTTCG-3') for amplification and sequencing of the nucLSU rDNA region (White et al 1990).

Purification of PCR Product: nucSSU rDNA. The PCR product of the nucSSU rDNA region was purified from 25 μL of each PCR product using the QIAquick PCR Purification Kit (QIAGEN Inc.) following the manufacturer's protocol. At the final step, a 1.5mL Eppendorf tube was used to collect the PCR product and, judging from the intensity of the bands on the 1XTAE gels, 30-130 μL of water was used to rinse the column. For each isolate, 4 μL of the amplified product was mixed with 4 μL dye terminator sequencing chemistries and 2 μL of the 2 μM primer. Sequences were produced with the use of automated sequencers in the Biology Department at Duke University, Durham, North Carolina.

Purification of PCR Product: nucLSU rDNA. The PCR products of the nucLSU rDNA amplifications were run in a 1X TAE gel to separate the products from smaller fragments. Bands were excised from the gel, placed into fresh 1.7 mL Eppendorf

tubes, and cleaned following the QIAquick Gel Extraction protocol (QIAGEN, Inc.). At the last step, 30 μL of distilled MilliQ water was used to elute the PCR product, which was then stored at 20 C. The purified nucLSU rDNA products were sequenced at the University of Maine Sequencing Lab.

Molecular Phylogenetic Analysis

I analyzed chromatograms of the sequences of the *Chytriomyces angularis* clade using ChromasPro 1.32 (Technelysium Pty. Ltd.). I formed contigs of the sequences from each isolate and aligned them to sequences of other selected taxa of the Chytridiomycota and Neocallimastigomycota (TABLE 2) with ClustalX 1.8 (Thompson et al 1997). Using GeneDoc 2.6.002 (Nicholas & Nicholas 1997), I deleted nucleotides at sites that did not have a homologous site in the outgroup *Rozella* isolates or that I determined were ambiguously aligned across the data set, and I produced similarity scores between isolates from TABLE 1. Maximum parsimony (MP) analysis and the computation of Bayesian posterior probabilities were conducted in PAUP* 4.0b10 (Swofford 2003). Support for MP branches was estimated with 1000 bootstrap replicates to form a 50 % majority rule consensus tree. Modeltest 3.4 (Posada & Crandall 1998) calculated the most appropriate model of DNA substitution to be the general time-reversible model with invariant sites and rates of substitution among sites approximated by a gamma distribution (GTR+I+G). The parameters of this model were set into the analysis produced by MrBayes v3.1.2. (Ronquist & Huelsenbeck 2003). Bayesian tree inference with Markov chain Monte Carlo sampling used two simultaneous Markov chains running 10 million

TABLE 2. Taxa of the Chytridiomycota and Neocallimastigomycota used for phylogenetic analysis.

Species	Isolate ID	Order: Family/Clade	GenBank Accession No.	
			nucSSU	nucLSU
Asterophlyctis sarcoptoides	JEL186	Chytridiales: Chytridiaceae	AH009060	AY439070
Chytridium sp.	JEL341	Chytridiales: Chytridiaceae	DQ536482	DQ273831
Chytriomyces hyalinus	MP4	Chytridiales: Chytridiaceae	DQ536487	DQ273836
Chytriomyces spinosus	JEL59	Chytridiales: Chytridiaceae	AH009063	DQ273839
Entophlyctis luteolus	JEL129	Chytridiales: Chytridiaceae	AH009064	AY349094
Obelidium mucronatum	JEL57	Chytridiales: Chytridiaceae	AH009056	AY349070
Phlyctochytrium planicorne	JEL47	Chytridiales: Chytridiaceae	DQ536473	DQ273813
Podochytrium dentatum	JEL30	Chytridiales: Chytridiaceae	AH009055	DQ273838
Podochytrium sp.	JEL161	Chytridiales: Chytridiaceae	AH009054	AY988517
Rhizoclosmatium globosum	JEL06	Chytridiales: Chytridiaceae	AH009057	AY349063
Rhizoclosmatium sp.	JEL347h	Chytridiales: Chytridiaceae	AY601709	DQ273769
Rhizophydium sp.	JEL354	Chytridiales: Chytridiaceae	AY635827	DQ273785
Unknown chytrid	JEL187	Chytridiales: Chytridiaceae	AY635825	DQ273783
Cladochytrium replicatum	JEL180	Chytridiales: *Cladochytrium* clade	AY546683	AY546688
Endochytrium sp.	JEL324	Chytridiales: *Cladochytrium* clade	AY635844	DQ273816
Nowakowskiella sp.	JEL127	Chytridiales: *Cladochytrium* clade	AY635835	DQ273798
Karlingiomyces sp.	JEL93	Chytridiales: *Polychytrium* clade	AF164278	DQ273814
Polychytrium aggregatum	JEL109	Chytridiales: *Polychytrium* clade	AY601711	AY546686
Synchytrium decipiens	AFTOL634	Chytridiales: *Synchytrium* clade	DQ536475	DQ273819
Synchytrium macrosporum	AFTOL635	Chytridiales: *Synchytrium* clade	DQ322623	DQ273820
"*Lobulomyces angularis*"	JEL45	"Lobulomycetales": "Lobulomycetaceae"	AF164253	DQ273815
"*Lobulomyces poculatus*"	JEL343	"Lobulomycetales": "Lobulomycetaceae"	EF443134	EF443139
"*Lobulomyces poculatus*"	JEL374	"Lobulomycetales": "Lobulomycetaceae"	EF443135	EF443140
"*Lobulomyces* sp."	JEL178	"Lobulomycetales": "Lobulomycetaceae"	EF443136	EF443141
"*Clydaea vesicula*"	JEL369	"Lobulomycetales": "Lobulomycetaceae"	EF443137	EF443142
"*Clydaea vesicula*"	PL70	"Lobulomycetales": "Lobulomycetaceae"	EF443138	EF443143

TABLE 2. Continued.

Species	Isolate ID	Order: Family/Clade	GenBank Accession No.	
			nucSSU	nucLSU
"*Maunachytrium keaensis*"	AF021	"Lobulomycetales": "Lobulomycetaceae"	EF432822	EF432822
Unknown chytrid	AF007	"Lobulomycetales": "Lobulomycetaceae"	EF432820	EF432820
Unknown chytrid	AF011	"Lobulomycetales": "Lobulomycetaceae"	EF432819	EF432819
Unknown chytrid	AF017	"Lobulomycetales": "Lobulomycetaceae"	EF432821	EF432821
Cyllamyces aberensis	EO14	Neocallimastigales: Neocallimastigaceae	DQ536481	DQ273829
Neocallimastix sp.	GE13	Neocallimastigales: Neocallimastigaceae	DQ322625	DQ273822
Hyaloraphidium curvatum	SAG235-1	Monoblepharidales: *incertae cedis*	Y17504	DQ273771
Monoblepharella mexicanum	UCB78-1	Monoblepharidales: Monoblepharidaceae	AF164337	DQ273777
Monoblepharella sp.	M15	Monoblepharidales: Monoblepharidaceae	AY546682	AY546687
Oedogoniomyces sp.	CR84	Monoblepharidales: Oedogoniomycetaceae	AY635839	DQ273804
Batrachochytrium dendrobatidis	JEL197	Rhizophydiales: *incertae cedis*	AH009052	AY546693
Entophlyctis helioformis	JEL326	Rhizophydiales: *incertae cedis*	AY635826	DQ273784
Endochytrium sp.	JEL174	Rhizophydiales: *incertae cedis*	AY635824	DQ273782
Rhizophlyctis harderi	JEL171	Rhizophydiales: *incertae cedis*	AF164272	DQ273775
Rhizophydium macrosporum	PLAUS21	Rhizophydiales: Terramycetaceae	DQ322622	DQ273823
Rhizophydium brooksianum	JEL136	Rhizophydiales: Rhizophydiaceae	AY601710	DQ273770
Rhizophydium sp.	JEL151	Rhizophydiales: *incertae cedis*	AF164270	DQ273774
Rhizophydium sp.	JEL317	Rhizophydiales: *incertae cedis*	AY635821	DQ273779
Rhizophydium sphaerotheca	JEL299	Rhizophydiales: *incertae cedis*	AY635823	DQ273781
Catenomyces sp.	JEL342	Spizellomycetales: *Rhizophlyctis* clade	AY635830	DQ273789
Rhizophlyctis rosea	JEL318	Spizellomycetales: *Rhizophlyctis* clade	AY635829	DQ273787
Gaertneriomyces semiglobiferus	UCB91-10	Spizellomycetales: *Spizellomyces* clade	AF164247	DQ273778
Powellomyces sp.	JEL95	Spizellomycetales: *Spizellomyces* clade	AF164245	DQ273776
Spizellomyces punctatus	ATCC48900	Spizellomycetales: *Spizellomyces* clade	AY546684	AY546692
Spizellomycetales chytrid	JEL355	Spizellomycetales: *Spizellomyces* clade	DQ536477	DQ273821
Triparticalar arcticum	BR59	Spizellomycetales: *Spizellomyces* clade	DQ536480	DQ273826
Rozella allomycis	AFTOL297	*incertae cedis*: *Rozella* clade	AY635838	DQ273803
Rozella sp.	JEL347	*incertae cedis*: *Rozella* clade	AY601707	DQ273766

generations. Trees were sampled every 1000 generations, with 10,001 trees sampled overall. A consensus of 7,501 trees left after a burnin of 2,500 trees was used to compute the posterior probability values on a 50 % majority rule tree. The consensus tree was optimized under the likelihood criterion to compute a phylogram with the most likely branch lengths.

Results

Morphology and Growth

Isolates JEL178, JEL343, JEL369, JEL374, PL70, and AF021 were grown on PmT agar and JEL45 was grown on PmTG agar to document morphology. All isolates exhibited endogenous development with thin, isodiametric rhizoids. Zoospores were not enclosed in a vesicle after release from the zoosporangium. Isolates JEL343 and JEL374 were identified as isolates of *C. poculatus*, and isolates JEL369 and PL70 had the same features and were determined to be duplicate isolates of one fungus. Isolates JEL178 (FIG. 3), AF021 (FIG. 4), and JEL369 (FIG. 6) had light-level morphologies that differed from *C. angularis* and *C. poculatus* (TABLE 3). Temperature maxima and light-level morphological features of the isolates varied among the isolates examined. Isolates AF007, AF011, and AF017 were not examined in detail for light-level morphology and development.

TABLE 3. Temperature maxima and morphological and ultrastructural features of species within the "Lobulomycetales."

	Species				
	"*Lobulomyces angularis*"	"*Lobulomyces poculatus*"	"*Lobulomyces* sp."	"*Maunachytrium keaensis*"	"*Clydaea vesicula*"
Isolates	JEL45	JEL343 JEL374	JEL178	AF021	JEL369 PL70
Temperature maximum	20 C	25 C	30 C	25 C	30 C
Thallus morphology					
Zoosporangium shape	Longer than wide	Longer than wide	Longer than wide and bifurcating	Spherical	Spherical
Operculation	Operculate	Operculate	Operculate	Inoperculate	Inoperculate
Avg. rhizoidal base width at maturity	1 µm	1 µm	1 µm	5 µm	1 µm
Zoospore					
Diameter	4 5 µm	-[a]	6 µm	4 µm	5 µm
Avg. flagellum length	30 µm	-	22 µm	26 µm	20 µm
No. of lipid globules	1	-	1	2	1
Zoospore ultrastructure					
Fibrillar bridge	Dense	-	-	Simple	Simple
Flagellar plug extensions	Anterior and posterior	-	-	Anterior and posterior	Anterior
Vesicles near kinetid	No	-	-	No	Yes

[a] indicates no data.

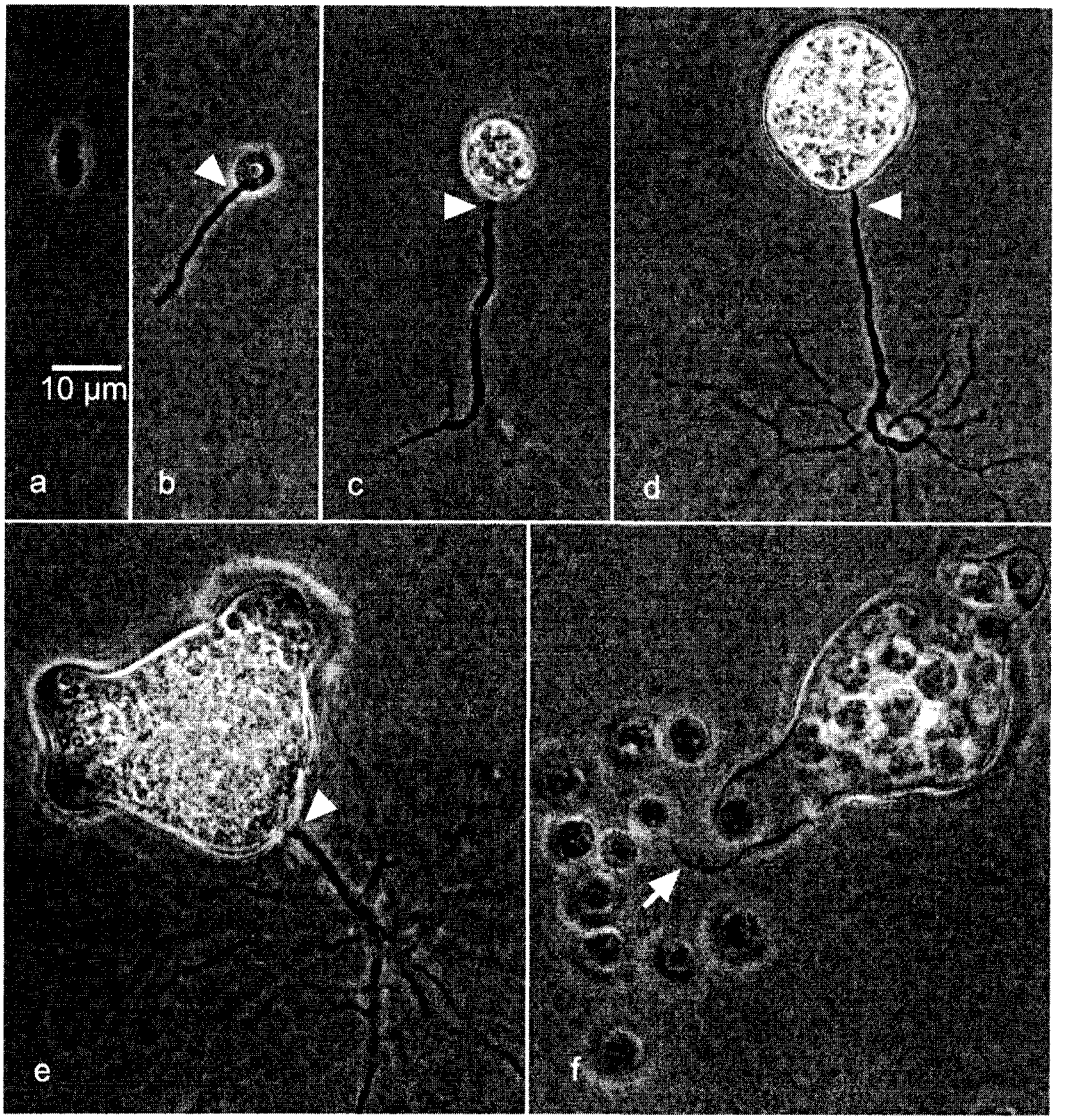

FIGURE 3. Morphology of JEL178 ("*Lobulomyces* sp.") on PmT agar at 17 C. Note lack of subsporangial swelling (arrowhead). a. Zoospore at rest. b. Germling (2 h) with single rhizoidal axis. c. Young thallus (23 h) with 90 degree branching of primary rhizoid. d. Developing thallus (47 h) with semi-spherical zoosporangium. e. Mature thallus (55 h) with bifurcating zoosporangium. f. Zoospore discharge from one discharge papilla with operculum (arrow).

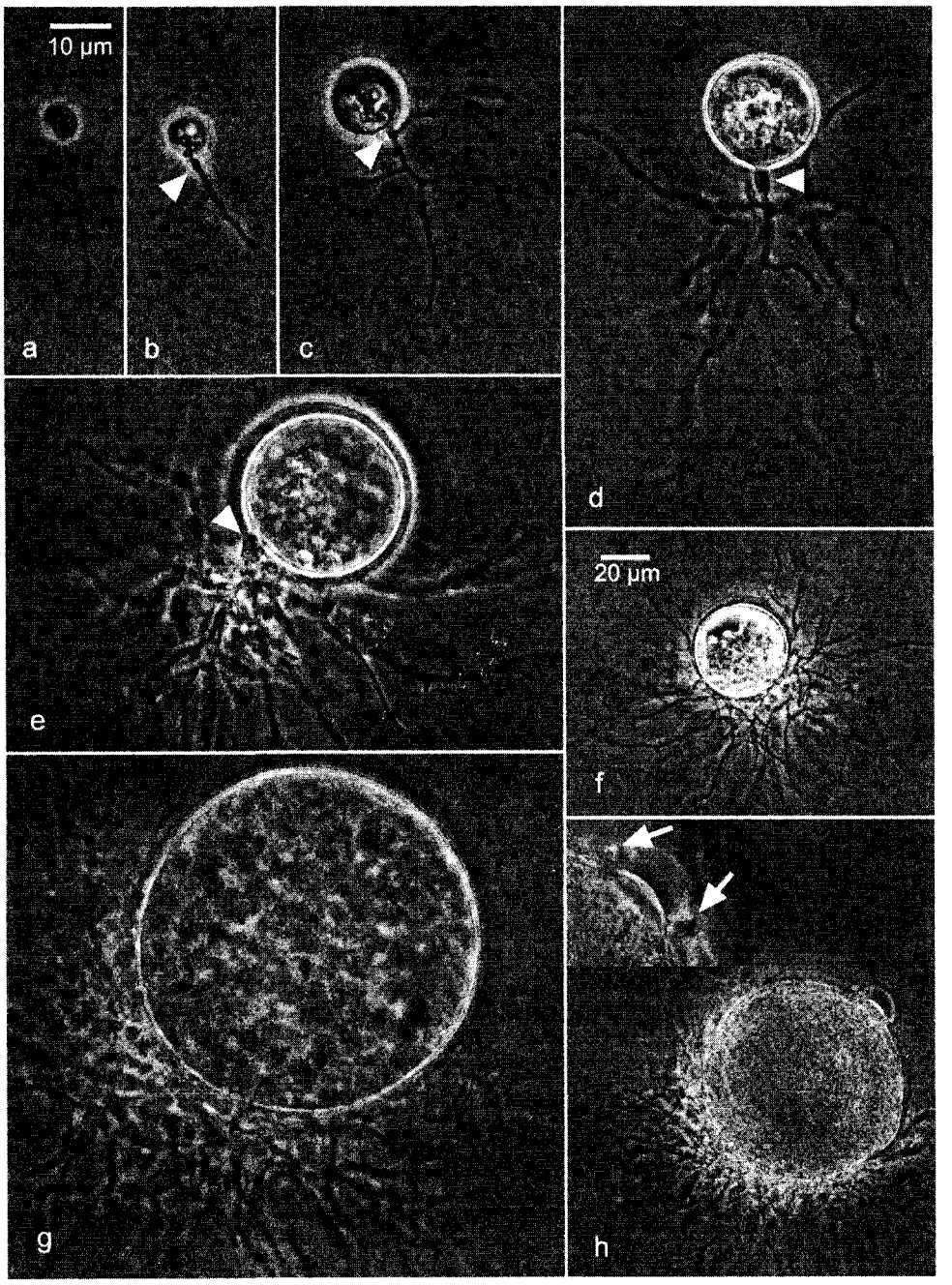

FIGURE 4. Morphology of "*Maunachytrium keaensis*" (AF021) on PmT agar at 17 C. Note lack of subsporangial swelling in young thalli with partial swelling during development (arrowhead). Scale for a–e, g, h inset on a, scale for f & h are same. a. Zoospore with two lipid globules. b. Germling (6 h) with single rhizoidal axis. c. Young thallus (28 h) with multiple branches of thin primary rhizoid at various intervals. d & e. Developing thalli (48 & 75 h) with enlarging subsporangial swelling. f. Growing thallus (75 h) with thin, profusely branching rhizoids. g. Thallus (98 h) with large, spherical zoosporangium and thin, isodiametric rhizoids. Initial rhizoid obscured. h. Mature thallus (145 h) with membrane protruding. Cell wall material indicated by arrows in inset.

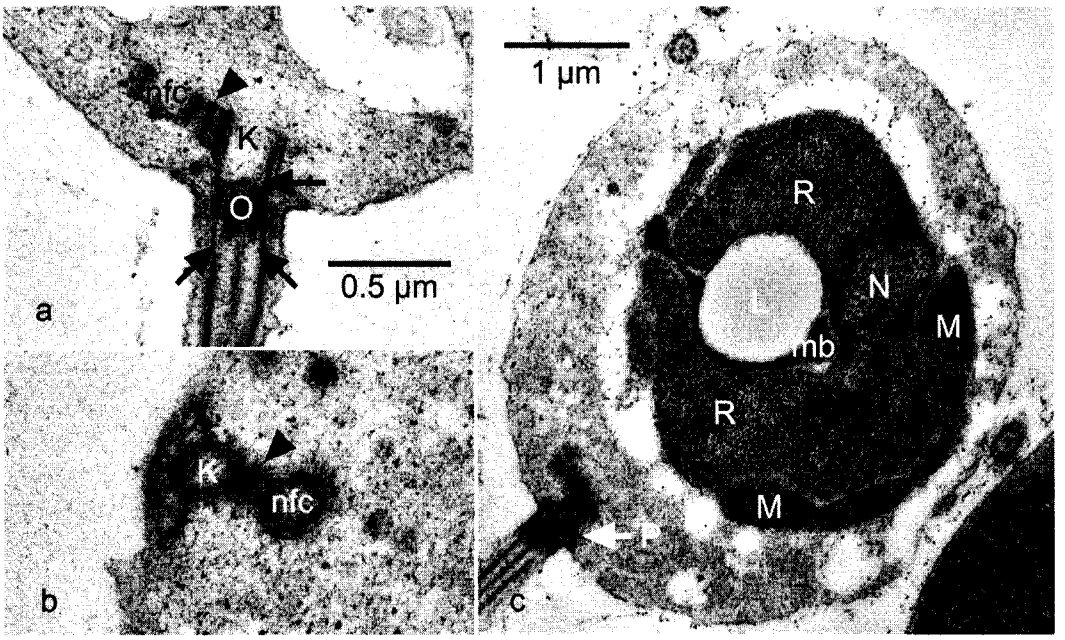

FIGURE 5. Ultrastructure of "*Maunachytrium keaensis*" (AF021) zoospore. Scale for a & b are same. a. Longitudinal section through kinetosome, nonflagellated centriole, and flagellar plug with anterior and posterior extensions (black arrows). b. Cross section of the fibrillar bridge (black arrowhead) between the kinetosome and the nonflagellated centriole. c. Longitudinal section of zoospore. KEY to FIG. 5 on FIG. 1.

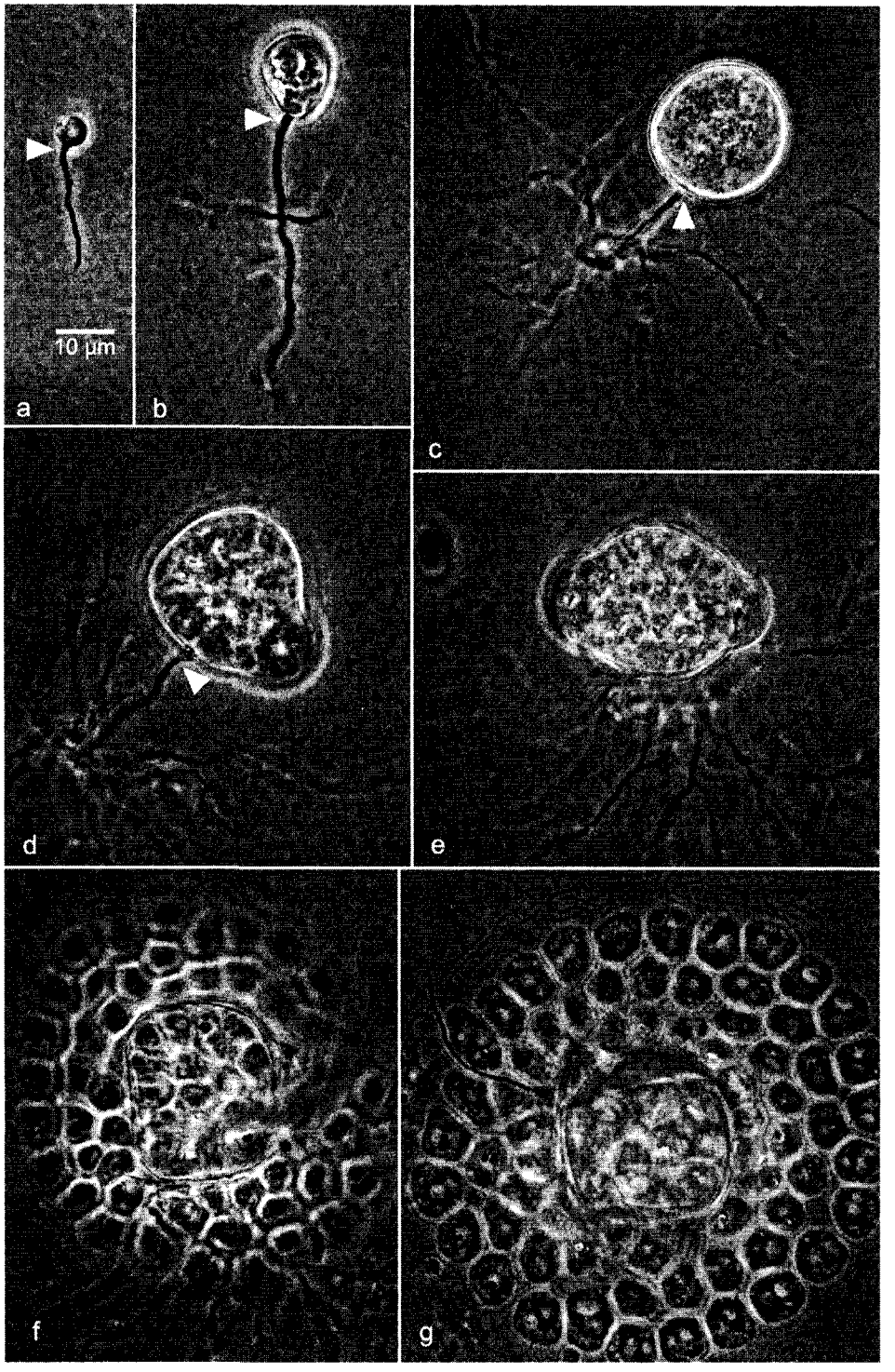

FIGURE 6. Morphology of "*Clydaea vesicula*" (JEL369) on PmT agar at 17 C. Note lack of subsporangial swelling (arrowhead). a. Germling (2 h) with single rhizoidal axis. b. Young thallus (25 h) with 90 degree branching of rhizoidal axis. c. Developing thallus (68 h) with rounded zoosporangium. d. Nearly mature thallus (74 h) with single protruding papilla. e–g. Mature thallus discharging zoospores (74 h), showing collapse of zoosporangium and lack of operculum.

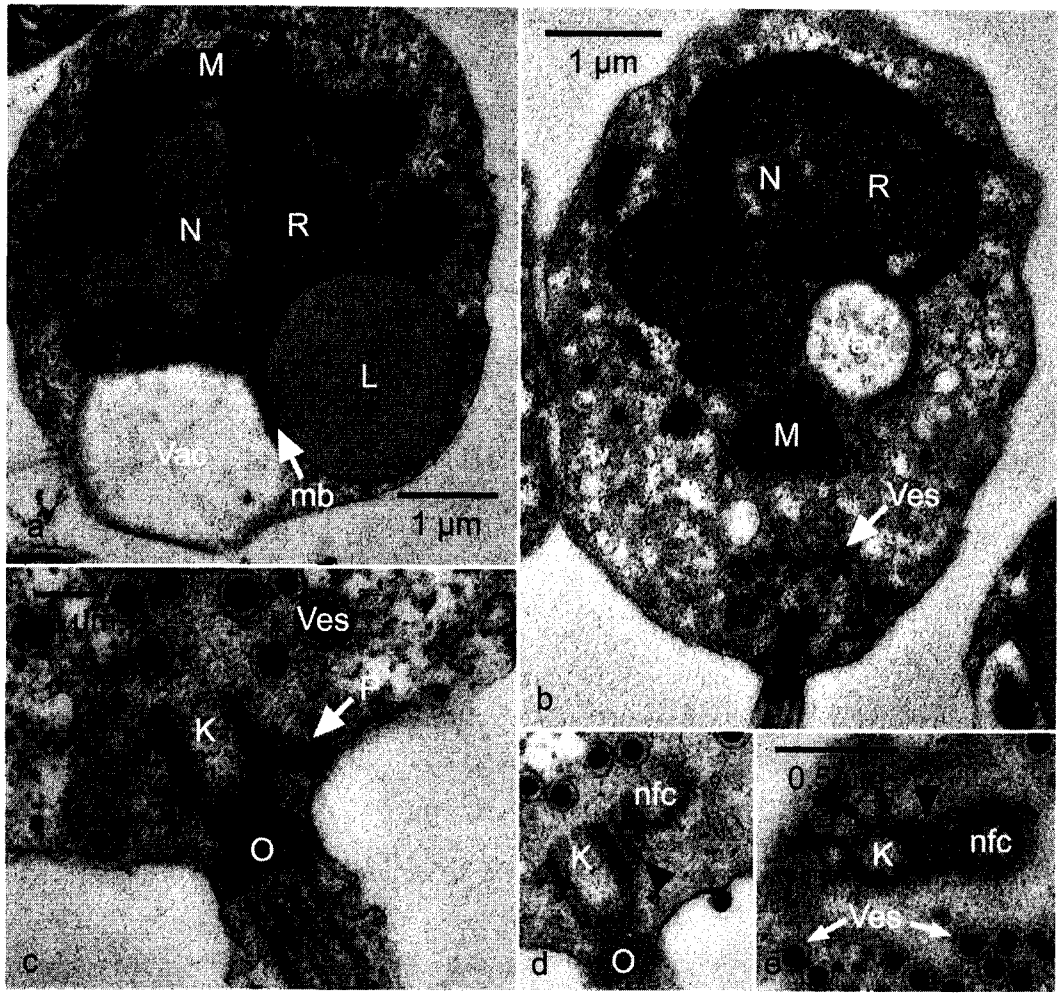

FIGURE 7. Ultrastructure of "*Clydaea vesicula*" (JEL369) zoospore. Scale for d & e are same. a. Cross section through zoospore. b. Longitudinal section through zoospore with aggregation of vesicles near the kinetid. c. Longitudinal section through kinetosome and flagellar plug with anterior extensions (black arrow). d & e. Longitudinal and cross section of zoospore showing the fibrillar bridge (black arrowhead) between the kinetosome and the nonflagellated centriole. KEY to FIG. 7 on FIG. 1.

TEM Analysis

The zoospore ultrastructural properties of isolates AF021 (FIG. 5) and JEL369 (FIG. 7) were compared to those of *C. angularis* (JEL45; Longcore 1992). Zoospore characteristics of *C. poculatus* were not available because zoospores of JEL343 and JEL374 encysted within the zoosporangium and were rarely seen to be motile, and isolate JEL178 did not produce zoospores in sufficient numbers for examination. Isolates AF007, AF011, and AF017 were not examined.

Isolate AF021 possesses extensions of the flagellar plug proximal and distal to the zoospore (FIGS. 5a & 5c), as in *Chytriomyces angularis*. A fibrillar bridge is present between the entire adjacent edges of the kinetosome and nonflagellated centriole, but it does not stain as densely as that of *C. angularis* (FIG. 1b, Longcore 1992: FIGS. 19–23). The simple bridge has a zipper-like appearance where the fibers meet in its middle (FIGS. 5a & 5b). Isolate AF021 lacks organelles and ultrastructural components that are found in true *Chytriomyces* species, as does *C. angularis*.

Isolate JEL369 also lacks structures specific to *Chytriomyces* species and *C. angularis*. Isolate JEL369 has a flagellar plug with only anterior extensions (FIGS. 7b–7d) and a fibrillar bridge with fibers meeting between the kinetosome and nonflagellated centriole to form a zipper-like connection (FIGS. 7d & 7e), as seen in isolate AF021. An aggregation of vesicles containing electron-opaque material, which is not apparent in *C. angularis* or isolate AF021, is near the kinetid (FIGS. 7b–7e). These characters of JEL369 are consistent in the genetically- and morphologically-similar isolate PL70 (Letcher unpublished data).

Phylogenetic Analysis

I constructed a molecular phylogeny (FIG. 8) using sequences derived from the SSU and partial LSU rDNA of the ten isolates of chytrids in this study (TABLE 1), which were compared to sequences provided by the Assembling the Fungal Tree of Life (AFTOL) project (http://aftol.org/) (TABLE 2). The data matrix had 851 parsimony-informative characters of a total of 2532 characters. My analysis supported all previously described clades within the core chytrids (James et al 2006b), indicating that the Chytridiales is a polyphyletic order. These clades had posterior probabilities of 100 % and bootstrap values of ≥71 % in my analysis. The *C. angularis* clade (= "Lobulomycetales") grouped exterior to other clades and orders with a posterior probability and a bootstrap value of 100 %. Each node of the *C. angularis* clade had a posterior probability of 100 % except the grouping of AF007 and AF011, which had 64 % support. Bootstrap values were ≥88 % with the exceptions of the monophyly of JEL45, JEL178, JEL343 and JEL374 and the grouping of AF007 and AF011.

Under the MP and Bayesian hypotheses, the *Chytriomyces angularis* clade represents a monophyletic clade within the Chytridiomycota. *Chytriomyces hyalinus*, the type species of *Chytriomyces*, lies within the Chytridiaceae, which is well outside of the *C. angularis* clade. This phylogeny (FIG. 8) defines monophyletic groupings within the Chytridiomycota and is not intended to clarify deep phylogenetic relationships.

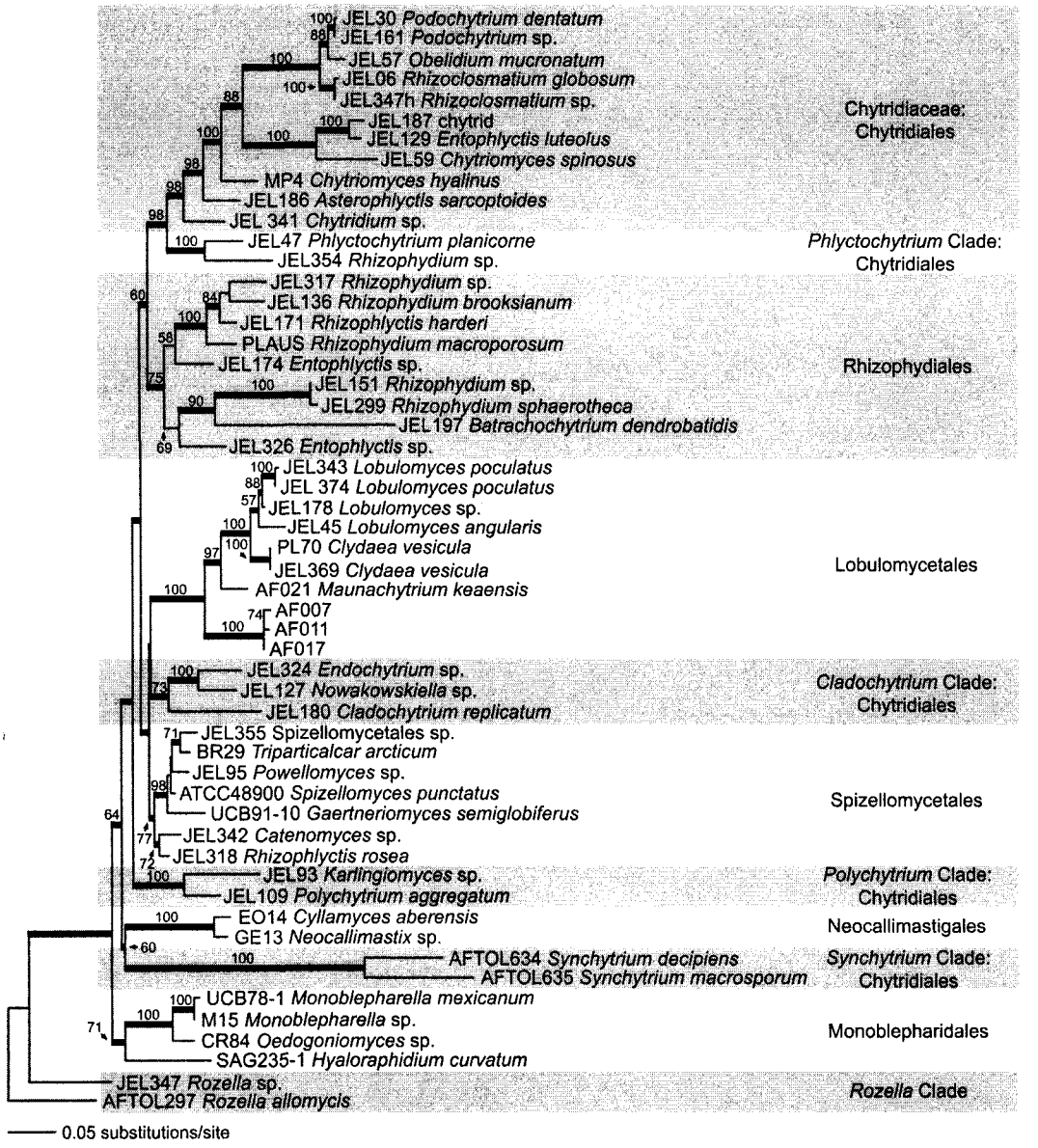

FIGURE 8. 50 % Majority rule consensus tree of 7501 trees used to compute Bayesian phylogeny from nucSSU and partial nucLSU rDNA sequences of 54 taxa in the Chytridiomycota and Neocallimastigomycota. Values are bootstrap values (1000 replicates) and bold lines indicate ≥98 % Bayesian inference estimates of posterior probabilities. Maximum parsimony phylogeny tree length = 4788 steps, consistency index = 0.379, retention index = 0.610. Species of *Rozella* were used as the outgroup because they were basal and outside of the Chytridiomycota in the phylogeny produced by James et al 2006b.

Taxonomy

Publication of Taxonomy

The following description of new taxa within the Chytridiomycota is not intended to be valid. Under the restrictions of Articles 29–31 of the International Code of Botanical Nomenclature, names are valid only after publication in a freely accessible manuscript, such as a peer-reviewed journal. Therefore, I have placed the new names of taxa in quotation marks; genera and species have been left italized for emphasis of Latin words.

"Lobulomycetales" D. R. Simmons, ord. nov.

Zoospora cum caeco flagelliano obturamento, anterioribus atque posterioribus obturamenti extensionibus; unus aut duo globi lipoidei; sine microtubulo, Golgi apparatus, striatum clausum, electron-caeca somata iuxta kinetosomam, atque cisterna usa globo lipoideo. Monophyleticus klados intra Chytridiomycotam.

Zoospore with opaque flagellar plug, anterior and posterior plug extensions; one or two lipid globules. Lacking microtubule root, Golgi apparatus, striated inclusion, electron-opaque bodies near kinetosome, and cisterna associated with lipid globule. Monophyletic clade within the Chytridiomycota.

"Lobulomycetaceae" D. R. Simmons fam. nov.

Supra; monocentricus thallus, eucarpicus, cum incremento endogeno. Rhizoidia isodiametrica, 0.5-1.5 μm *in amplitudine.*

Description as for "Lobulomycetales"; thallus monocentric, eucarpic, with endogenous development. Rhizoids isodiametric, 0.5–1.5 μm wide.

Type. "**Lobulomyces"** D. R. Simmons gen. nov.

Commentary. Three new genera, "*Lobulomyces*," "*Maunachytrium*," and "*Clydaea*" are described. They are distinguished by ultrastructural and rDNA (nucSSU + partial nucLSU) characters.

"Lobulomyces" D. R. Simmons gen. nov.

Zoospora cum densa fibrata colligatione inter kinetosoma et nonflagellatum centriolum; flagelliani obturamenti extensiones anteriorae atque posteriorae; unus globus lipoidus. Monophyleticus klados intra "Lobulomycetales."

Zoospore with dense fibrillar connection between kinetosome and nonflagellated centriole; flagellar plug extensions anterior and posterior; one lipid globule. Monophyletic clade within the "Lobulomycetales."

Type. "**Lobulomyces angularis"** (Longcore) D. R. Simmons comb. nov.

≡ *Chytriomyces angularis* Longcore, *Mycologia* 84:443; FIGS. 1-29. 1992.

I emend this species as follows: nucSSU rDNA GenBank # AF164253, nucLSU rDNA GenBank # DQ273815.

Commentary. The characteristics of the zoospore include the extensions of the flagellar plug and the dense fibrillar bridge between four triplets of the kinetosome and three triplets of the nonflagellated centriole. These apomorphic features differ from character states seen in *Chytriomyces hyalinus*, the type species of

Chytriomyces. Ultrastructural characters and my molecular phylogeny mandate that this species be removed from *Chytriomyces*.

"Lobulomyces poculatus" (Willoughby & Townley) D. R. Simmons comb. nov.

≡ *Chytriomyces poculatus* Willoughby & Townley, *Trans. Brit. Mycol. Soc.* 44:183; FIG. 3, pl. 14. 1961. Emended Letcher & Powell 2002.

I emend this species as follows: nucSSU rDNA GenBank # EF443135, nucLSU rDNA partial GenBank # EF443140.

Epitype. NEW ZEALAND. SOUTH WESTLAND, SOUTH ISLAND: Haast Ecological District near mouth of Cole Creek, 45°43′S, 169°14′E. Tree canopy detritus, 22 May 2003, JEL374.

Additional material examined. USA. MAINE: Old Town, Mud Pond. Soil from spruce and fir forest, JEL343; nucSSU rDNA GenBank # EF443134, nucLSU rDNA partial GenBank # EF443139.

Commentary. I am declaring isolate JEL374 from New Zealand soil the epitype culture of "*Lobulomyces poculatus*" based on the occurrence of *Chytriomyces poculatus* in Australian soils as noted by the author of the species (Willoughby 1965). Zoosporic ultrastructural comparisons have not yet been made of isolate JEL343 or JEL374. Motile zoospores have been unavailable for fixation attempts, because, in pure culture, zoospores encyst within the zoosporangium. Analyses of genetic characters, however, dictate that the species be removed from the polyphyletic genus *Chytriomyces*. Although the two isolates of "*L. poculatus*" were from different continents, the nucSSU and partial nucLSU sequences of the two isolates used for

phylogenetic analysis had a 98 % similarity. Because of its genetic and morphological similarity to "*Lobulomyces angularis*," I am placing this species in the genus "*Lobulomyces*."

"*Lobulomyces*" species have zoosporangia that are taller than wide, averaging 30 μm X 20 μm on PmT agar medium. All species have isodiametric rhizoids not exceeding 1.5 μm in width, and the first branching of the initial rhizoid in this genus occurs at an approximately 90 degree angle at some distance from the zoosporangium (FIGS. 2 & 3). Molecular analysis (FIG. 8) indicates that isolate JEL178 (FIG. 3) may represent a third species in the genus. This isolate is operculate (FIG. 3f), which is consistent with the operculation of "*Lobulomyces angularis*" (Longcore 1992) and "*L. poculatus*" (Willoughby & Townley 1961); however JEL178 develops a bifurcating zoosporangium (FIGS. 3e & 3f), which is ~45 μm in diameter at its widest point. Discharge of zoospores did not occur from both papillae, however, and further study is needed to describe a species from this isolate. Zoospores were not released in sufficient numbers for TEM examination. Zoospores of JEL178 are spherical and ~6 μm in diameter when in motion; flagella are 20–23 μm long.

"Maunachytrium" D. R. Simmons gen. nov.

Zoospora cum duabus globis lipoidis. simplex fibrata colligato inter kinetosoma et nonflagellatum centriolum. Clarum genus intra "Lobulomycetales."

Zoospore with two lipid globules. Simple fibrillar connection between kinetosome and nonflagellated centriole. Distinct lineage within the "Lobulomycetales."

Type. "**Maunachytrium keaensis"** D. R. Simmons sp. nov.

Commentary. This genus is distinguished from "*Lobulomyces*" by having two lipid globules in the zoospore, the absence of the dense fibrillar connection extending from four triplets of the kinetosome to the nonflagellated centriole, and differences in nucSSU and partial nucLSU rDNA sequences.

Etymology. Name from *mauna*, Hawaiian for mountain.

"Maunachytrium keaensis" D. R. Simmons sp. nov. FIGS. 4 & 5

Zoosporangium globulum cum multis, inoperculatis poris evacuationibus atque tumor ad fundamentum axis rhizoidiae non magis quam 5 um. Sporangia requiescenta non apparenta.

Zoosporangium spherical with multiple, inoperculate discharge papillae and swelling at base of rhizoidal axis not exceeding 5 μm. Resting sporangia not seen. nucSSU-ITS1-5.8S-ITS2 rDNA complete and nucLSU rDNA partial GenBank # EF432822.

Type. FIGS. 4 & 5 from isolate AF021. USA. HAWAII: Mauna Kea, permafrost-fed lake, 19°48′39″N and 155°28′40″W, 3978m. Soil, 13 Sep 2005.

Etymology. Name from Mauna Kea, Hawaii, site of soil collection.

Commentary. If judged only from morphological characters of the zoosporangium this chytrid could be placed in the genus *Rhizophydium sensu* Sparrow (1960); however, ultrastructural and molecular analyses support its placement within the "Lobulomycetaceae." Zoosporangia of "*Maunachytrium keaensis*" (FIG. 4) are round and 60-85 μm in diameter on PmT agar medium. At

maturity, one or more pores develop in the cell wall of the zoosporangium (FIG. 4h), and the membrane-bound cytoplasm protrudes. Zoospores exit through one or more of these sites. Zoospores are spherical when in motion, ~4 μm in diameter and with a flagellum ~26 μm long. The isodiametric rhizoids of "*M. keaensis*" branch profusely (FIGS. 4b–4h), from the slightly swollen (~5 μm) base of the rhizoidal axis (FIGS. 4d & 4e). In pure culture at 17 C, "*M. keaensis*" may take 7 days to reach maturity. Resting spores were not seen.

"Clydaea" D. R. Simmons gen. nov.

Simplex fibrata colligato inter kinetosoma et nonflagellatum centriolum; aggretatio electron-caecorum vesiculorum circumiens kinetidum. flagelliani obturamenti extensiones anteriorae; unus globus lipoidus. Clarum genus intra "Lobulomycetales."

Simple fibrillar connection between kinetosome and nonflagellated centriole; aggregation of electron-opaque vesicles around the kinetid; flagellar plug extensions anterior; one lipid globule. Distinct lineage within the "Lobulomycetales."

Type. **"Clydaea vesicula"** D. R. Simmons sp. nov.

Commentary. This genus is distinguished from others in the "Lobulomycetaceae" by the lack of posterior extensions of the flagellar plug and lack of a dense fibrillar bridge and possession of an accumulation of vesicles around the kinetid (FIG. 7b–7e, Letcher pers. comm.).

Etymology. Name in memory of Clyda Rae Simms Simmons, mother of D. R. Simmons.

"Clydaea vesicula" D. R. Simmons sp. nov. FIGS. 6 & 7

Zoosporangium inaequabile globosum cum 1 ad 4 poris evacuationibus eminentibus; inoperculatum. Sporangia requiescenta non apparenta.

Zoosporangium irregularly spherical with 1 to 4 protuberant discharge papillae; inoperculate. Resting zoosporangia not seen. nucSSU rDNA GenBank # EF443137, nucLSU rDNA partial GenBank # EF443142.

Type. FIGS. 6 & 7 from isolate JEL369. USA. CALIFORNIA: Goleta, *Eucalyptus* grove. Duff, Dec 2001.

Additional material examined. USA. UTAH: Big Cottonwood Creek, Wasatch County, 40°38′292″N and 111°41′896″W, 6280′. Creek bank mud, 2 Aug 2001, PL70.

Etymology. Name from accumulation of vesicles around the kinetid.

Commentary. "*Clydaea vesicula*" has a zoosporangium ~35 µm in diameter at maturity that is round with slight protrusions (FIGS. 6d & 6e) when grown on PmT agar medium. The isodiametric rhizoids of this species are nearly identical to those of "*Lobulomyces*". At maturity, one of the protrusions of the zoosporangium breaks and zoospores are violently expelled, which causes a collapse of the zoosporangium (FIGS. 6e–6g). Zoospores are spherical in motion, ~5 µm in diameter, and have flagella ~20 µm long. An operculum was not observed. Resting spores were not seen. Although the isolates of this species originated from widely separated geographic areas, the sequences used for phylogenetic analysis were 98 % similar.

Discussion

Taxonomy and phylogeny of the "Lobulomycetales"

Order "Lobulomycetales". Recent molecular phylogenies (James et al 2000, 2006b) have illustrated the genetic diversity within the Chytridiomycota. With this new information, authors (James et al 2006b, Hibbett et al in press) have limited the Chytridiomycota to the classes Monoblepharidomycetes and Chytridiomycetes, the latter of which includes the Spizellomycetales, Rhizophydiales (Letcher et al 2006), and the polyphyletic Chytridiales. The lineage represented by *Chytriomyces angularis* did not fall within any monophyletic clade in previous phylogenies. Genetic and ultrastructural evidence presented here supports the establishment of the "Lobulomycetales," a new order within the Chytridiomycota that includes the lineage represented by "*L. angularis*," "*L. poculatus*," and other genetically similar species that group with these two species. My analyses that include increased taxon sampling of this clade beyond that in recent Chytridiomycota phylogenies (James et al 2000, 2006b) clearly demonstrate that "*L. angularis*" is representative of a well-supported and separate lineage that is distant from *Chytriomyces hyalinus*.

Although the "Lobulomycetales" is well supported by the molecular phylogeny, it is difficult to assemble an ultrastructural ordinal concept for the group. Since the description of the Spizellomycetales and the amendment of the Chytridiales (Barr 1980), zoospore ultrastructure features have defined orders (Letcher et al 2006). All isolates examined by TEM in this study retain a flagellar plug and all have lost the microtubule root and rumposome that are common in most other orders of the Chytridiomycota *sensu* Powell (Hibbett et al in press). All other clades of the phylum

except the Spizellomycetales have some species that possess a rumposome and microtubule roots. These structures may eventually be found within the "Lobulomycetales" even though they are not currently in any described species in this order. The lineage represented by the current "Lobulomycetales" species has lost these plesiomorphic structures, but it is possible that undescribed species within the clade may retain them.

The negative character states of the "Lobulomycetales", namely the lack of certain ultrastructural components, also leave few characters on which to define genera. It is more probable that "*Clydaea vesicula*" has lost the posterior extensions of the flagellar plug than that "*Lobulomyces angularis*" and "*Maunachytrium keaensis*" evolved posterior extensions independently. The dense fibrillar bridge of "*L. angularis*" may be a derived character state in that species or possibly the genus, but the unique connection could just as likely have been lost in the other taxa. Study of the more ancestral lineage represented by isolates AF007, AF011, and AF017 may aid in determining the plesiomorphic states of the fibrillar bridge and flagellar plug.

Though my taxon sampling of the "Lobulomycetales" is larger than previous studies, it likely does not represent the true diversity of light-level morphology or zoospore ultrastructural features within the clade. Morphological characters in all isolates examined include the thin, isodiametric rhizoids, extensions in the flagellar plug, and the connection of the kinetosome to the nonflagellated centriole. Because they are distinct from the type of *Chytriomyces*, "*L. angularis*" and "*L. poculatus*" need a new genus. Other organisms in the clade differed from the two described

species morphologically and genetically and warrant the creation of two monospecific genera.

Genus "*Lobulomyces*". "*Lobulomyces angularis*" is distinct from *C. hyalinus* in the lack of several features (Longcore 1992, Letcher et al 2005). The zoospore of "*L. angularis*" possesses character states in the fibrillar bridge structure and flagellar plug that were novel to the Chytridiomycota at the time of the description of the species (Longcore 1992). The bridge connecting four microtubule triplets of the kinetosome to three triplets of the nonflagellated centriole is densely electron-opaque throughout the connection, and the flagellar plug possesses anterior and posterior extensions. "*Lobulomyces angularis*" lacks a microtubule root and all associated structures, including the rumposome, and also lacks a paracrystalline body in the cytoplasm of the zoospore. Longcore (1992) suggested that "*L. angularis*" might be related to the morphologically similar "*L. poculatus*" given that the latter species was often noted to lack cupules of cell wall material (Dogma 1969, Booth 1971a, 1971b) that distinguish it from "*L. angularis*." Longcore (1992) was not able to investigate this relationship because no isolates of "*L. poculatus*" existed. In contrast to *C. hyalinus*, however, both species have isodiametric rhizoids not more than 1.5 μm wide, and both release zoospores that are not enclosed in a vesicle.

Molecular phylogenies based on nucSSU and partial nucLSU rDNA sequences of isolates in this study resulted in a consensus tree (FIG. 8) that placed "*Lobulomyces angularis*" and "*L. poculatus*" within a monophyly in the "Lobulomycetales." "*Lobulomyces angularis*" is the only member of this genus

whose zoospore ultrastructure is currently described in the literature. Neither isolate JEL178 nor the *L. poculatus* isolates had sufficient zoospore discharge to allow TEM analyses. Further TEM work on these isolates and genetically similar isolates could test the hypothesis that the dense fibrillar bridge between the kinetosome and nonflagellated centriole is a derived character within this lineage that is not present in other genera in the "Lobulomycetales." The light-level morphological similarities of representatives of "*Lobulomyces*," however, remain consistent, supporting the molecular phylogeny. All isolates exhibit 1) operculation, 2) avesicular zoospore discharge, 3) angular zoosporangia longer than wide, 4) thin, isodiametric rhizoids that branch at near-right angles from the initial rhizoid, and 5) a major rhizoidal axis that does not enlarge greater than 1.5 μm.

Genus "*Maunachytrium*". "*Maunachytrium keaensis*" dwarfs the other species of the order with a spherical zoosporangium between 60–85 μm in diameter, which is twice as wide as that of "*Clydaea vesicula*." "*Maunachytrium keaensis*" is inoperculate, and zoospores are released through pores in the cell wall where membrane-bound cytoplasm has erupted through areas of dehisced wall. "*Maunachytrium keaensis*" possesses thin rhizoids that differ from those of other species in the "Lobulomycetales" by a slight swelling of the base of the rhizoidal axis, not exceeding 5 μm. The ultrastructural characters of "*M. keaensis*" differ from those of "*L. angularis*" only in the lack of the dense fibrillar bridge. All previous character states seen in "*L. angularis*," including the anterior and posterior extensions of the flagellar plug, exist in this species. Given the placement of "*M. keaensis*" in the

molecular phylogeny, the presence of the flagellar plug extensions might be a plesiomorphic character in the more derived lineages of "Lobulomycetales." Future analyses of isolates AF007, AF011, and AF017, which are a basal, distinct lineage within the "Lobulomycetales," may provide more information on the ultrastructural ancestral characters of the order.

"*Maunachytrium keaensis*" is placed in its own monospecific genus for several reasons. Its placement in the nucSSU and partial nucLSU rDNA phylogeny is well-supported and distinct from the other two genera. The simple zipper-like connection between the kinetosome and nonflagellated centriole and the large, inoperculate zoosporangium with a slight swelling in the rhizoidal base are also features that differentiate "*Maunachytrium*" from the other genera. With increased sampling of alpine soils, more species in this genus might be detected; however, this and other species of "*Maunachytrium*" may not be restricted to cold habitats because "*M. keaensis*" grows at temperatures up to 25 C. Major morphological characters of both *Rhizophydium* and "*Maunachytrium*" include inoperculate, multipored zoosporangia with extensively branched rhizoids. Consequently, it is also plausible that, without ultrastructural and genetic data, previously observed chytrids that possess spherical zoosporangia with multiple discharge papillae may have been erroneously identified as species of *Rhizophydium*.

Genus "*Clydaea*". "*Clydaea vesicula*," which is sister to the "*Lobulomyces*" clade, lacks an operculum and has a rounded zoosporangium with one or more discharge papillae. In ultrastructural comparisons, "*C. vesicula*" is similar to "*M. keaensis*" in

its lack of a dense fibrillar bridge between the kinetosome and nonflagellated centriole, but it differs from both "*M. keaensis*" and "*L. angularis*" by possessing an aggregation of vesicles with electron-opaque contents around the kinetid and lacking the posterior extensions of the flagellar plug. The cluster of vesicles seen in both isolates JEL369 and PL70 may be a derived character within this lineage, and this, in conjunction with the molecular phylogeny, distinguishes this genus and species.

Key to Species in the "Lobulomycetales"

Although the establishment of the new taxa of the "Lobulomycetales" relies heavily on genetic characters, the species in the order are also distinguished from each other by morphology. Below is a taxonomic key to the genera and species of "Lobulomycetales" based on light-level morphological features of first generation thalli grown on PmT agar, except "*Lobulomyces angularis*", which is grown on PmTG agar.

1. Zoosporangium longer than wide, operculate, with rhizoids 0.5 1.5 μm wide.....2

   Zoosporangium spherical, inoperculate, with rhizoidal base up to 5 μm wide.....4

2. Zoosporangium bifurcating.....“*Lobulomyces* sp.” (JEL178)

   Zoosporangium not bifurcating.....3

3. Zoosporangium with cell wall forming cupules.....“*Lobulomyces poculatus*”

   Zoosporangium without cupules.....“*Lobulomyces angularis*”

4. Enlargement of major rhizoidal axis present, zoosporangium 60 85 μm in diameter.....*"Maunachytrium keaensis"*

Enlargement of major rhizoidal axis not present, zoosporangium ~35 μm in diameter.....*"Clydaea vesicula"*

Currently, all species in the "Lobulomycetales" can be keyed by morphological features observed by light microscopy. The diagnostic flagellar plug and its extensions, the connection of the kinetosome and nonflagellated centriole, the isodiametric rhizoids produced by all isolates, and the correlation of these and other characters with genetically defined groups in the "Lobulomycetales" support molecular phylogenetic hypotheses in restructuring the Chytridiomycota while still relying on morphology for lower-level taxonomic identification. Light-level morphology can vary on different substrates, however, necessitating study in pure culture on a stated medium for proper comparison and identification. The goal of chytrid systematics is to make the complex, esoteric world of chytrid taxonomy as accessible to non-specialists as possible while representing the diversity of zoosporic fungi found with molecular tools. It is with this in mind that I describe the order "Lobulomycetales" based on light-level morphology, zoospore ultrastructure, and molecular phylogenetic evidence.

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David Rabern Simmons was born on February 16, 1982 in Big Stone Gap, Virginia. He graduated from Powell Valley High School in 2000 and earned a Bachelor of Science degree (cum laude) from the University of Virginia's College at Wise in 2004. In the summer and fall of 2004, he served as research technician for Kevin G. Jones in the Fellowship in the Natural Sciences program at the University of Virginia's College at Wise in a project that concerned host specificity of myxomyceticolous fungi. In January of 2005, he enrolled for graduate study in chytrid systematics at the University of Maine. He has served as research assistant and teaching assistant for the Department of Biological Sciences.

Mr. Simmons was elected to Pi Kappa Phi in 2003, serving as secretary from January 2003 to January 2004, and to Sigma Zeta Academic Honor Society in 2004. He is a member of the Mycological Society of America. He received the Fay Hyland-Hilborn Prize in Plant Biology from the Department of Biological Sciences at the University of Maine in 2007. He is a candidate for the Master of Science degree in Botany and Plant Pathology from the University of Maine in May, 2007.