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Lobulomycetales, a new order in the *Chytridiomycota*

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ABSTRACT

The *Chytridiales*, one of the four orders in the *Chytridiomycetes* (*Chytridiomycota*), is polyphyletic, but contains several well-supported clades. One of these clades is referred to as the *Chytriomyces angularis* clade, and the phylogenetic placement of this group within the *Chytridiomycetes* is uncertain. The morphology and zoospore ultrastructure of *C. angularis* have been studied using LM and were shown to differ from those of the type species of *Chytriomyces*, which is in the *Chytridiaceae* and is phylogenetically distinct from the *C. angularis* clade. In this study, chytrids with morphologies or rDNA sequences similar to *C. angularis*, including two isolates of the morphologically similar *C. poculatus*, were isolated and their phylogenetic relationships determined using molecular sequence data. Results of Bayesian and MP analyses of nuSSU and partial nuLSU rDNA sequences grouped the new isolates and the type isolate of *C. angularis* in a monophyletic clade within the *Chytridiomycota* but distinct from the *Chytridiaceae*. Zoospores of isolates examined using TEM had ultrastructural features similar to those of *C. angularis*. Genetic analyses, ultrastructural data, and morphology support the establishment of a new order *Lobulomycetales*, placement of *C. angularis* and *C. poculatus* in a new genus (*Lobulomyces*), and description of additional taxa, which we have named *Clydaea vesicula* and *Maunachytrium keaense*.

Introduction

Some species of chytrids have characteristic morphologies that make them easily recognisable; *Chytriomyces poculatus*, a chytrid reported from three continents, is an example of these. The *C. poculatus* zoosporangium is monocentric, operculate, elongate, and surrounded by a 'ghostly, loose veil' (Sparrow & Lange 1977), or cupules (Willoughby & Townley 1961). Researchers noted non-cupuled forms of *C. poculatus* (Booth 1971a, 1971b; Dogma 1969), but it was not until *C. angularis* (Longcore 1992) was brought into pure culture and determined not to form cupules that these two species were recognised as being distinct. Because of its similarity to *C. poculatus*, Longcore (1992) provisionally placed *C. angularis* in the genus *Chytriomyces*, but noted that the zoospore ultrastructure and morphology of *C. angularis* differed from *C. hyalinus*, the type of the genus (Letcher *et al.* 2005). Longcore (1992) hypothesised that *C. angularis* might represent a new genus, but waited to describe such a genus until more chytrids were isolated that had light microscopic-level and ultrastructural characters in common with the species.

Analyses of sequence data (James *et al.* 2000; Letcher *et al.* 2005) consistently place *C. angularis* by itself, within the *Chytridiomycota*, but outside of phylogenetically well-supported groups that coincide with groups based on ultrastructural data (Barr 1980, 1990). James *et al.* (2006) included one GenBank submission that grouped with *C. angularis*, *Chytridium*

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polysiphoniae. This marine chytrid isolate has since been lost (Küpper, pers. comm.) and no zoosporic ultrastructural data have been published. Küpper *et al.* (2006) sequenced *C. polysiphoniae* and included five sequences of uncultured organisms in a nuSSU rDNA phylogeny that grouped these isolates with *Chytriomyces angularis*.

Analyses of molecular and ultrastructural data have supported major taxonomic changes in the *Chytridiales*, including the description of the *Rhizophydiales* from the *Chytridiales* (Letcher *et al.* 2006), the amendment of the *Chytridiaceae* to exclude taxa not related to *C. hyalinus* (Letcher *et al.* 2005), and the recognition of additional clades within the order (James *et al.* 2006; Letcher *et al.* 2005). Further changes in the *Chytridiomycota* include the elevation of the *Blastocladiales* (James *et al.* 2006) and the *Neocallimastigales* (Hibbett *et al.* 2007) to phylum level and the *Monoblepharidales* to class level (Hibbett *et al.* 2007).

We cultured the type isolate of *C. angularis*, two *C. poculatus* isolates, and six morphologically similar chytrids. We compared their morphology by LM and, when possible, zoospore ultrastructure, and determined their phylogenetic relationships based on analyses of the rDNA operon. *C. angularis* and *C. poculatus* are monophyletic as hypothesised from their morphology, and we place them in a new genus. Our additional taxon sampling provided strong molecular support for a clade that contains these two species plus others. We describe the '*C. angularis* clade' (James *et al.* 2006) as a new order, the *Lobulomycetales*, and name one family and three genera.

Materials and methods

Chytrid isolates, medium optimisation and temperature maxima

Isolates examined in this study are listed in Table 1. Because little is known about habitat preferences of members of this group, other than that *Chytriomyces poculatus* has been reported from acidic, peaty, or bog soils, we searched for them by baiting a variety of soil samples and brought them into pure culture using chytrid-isolation techniques (Barr 1987). Stock cultures were maintained on nutrient agar slants in screw-topped culture tubes at 5 °C and transferred every three months.

We first isolated strains on PmTG (1 g l^{-1} peptonized milk, 1 g l^{-1} tryptone, 5 g l^{-1} glucose, 10 g l^{-1} agar) nutrient agar (Barr 1986). In an attempt to optimise growth of the isolates we compared growth in liquid medium without glucose (PmT) or with glucose substituted with 5 g l^{-1} cellobiose or 5 g l^{-1} keratin. The PmT medium, with no added carbohydrate beyond that present in the peptonised milk and tryptone, provided the best growth conditions for all isolates except *C. angularis*. Subsequently, we used PmT nutrient agar or broth for isolation and growth of all clade members except *C. angularis*, which did not grow on PmT. To determine maximum temperature for growth, we incubated duplicates of all isolates in 75 ml liquid medium in 125 ml flasks at 17, 20, 25, 30, and 35 °C for two weeks in the dark.

Table 1 – Culture designations, identities, origins, habitats, and rDNA accession numbers of *Lobulomycetales* isolates

Culture no.	New taxon Identification	Origin	Habitat	GenBank accession no.		
				nuSSU	nuLSU	ITS1–5.8S–ITS2
AF011	Undescribed	Peru	Alpine barren soil	EF432819	EF432819	EF432819
AF017	Undescribed	Peru	Alpine barren soil	EF432821	EF432821	EF432821
AF021[a,b]	*Maunachytrium keaense*	Hawaii, USA	Alpine barren soil	EF432822	EF432822	EF432822
JEL45	*Lobulomyces angularis*	Maine, USA	*Sphagnum* from acidic lake	AF164253	DQ273815	AY997036
JEL178[a]	Undescribed	Maine, USA	Spring under mixed hard woods	EF443136	EF443141	EU352772
JEL343	*Lobulomyces poculatus*	Maine, USA	Soil under pine trees	EF443134	EF443139	EU352770
JEL369[a,b]	*Clydaea vesicula*	California, USA	Soil under *Eucalyptus* trees	EF443137	EF443142	EU352773
JEL374	*Lobulomyces poculatus*	New Zealand	Tree canopy detritus	EF443135	EF443140	EU352771
PL70	*Clydaea vesicula*	Utah, USA	Creek bank	EF443138	EF443143	EU352774
CCW64[c]	Environmental PCR	Massachusetts, USA	Salt marsh	AY180029		
Chy Pyl IR 14[d]	*Chytridium polysiphoniae*	Shetland, UK	Marine	AY032608		
RSC-CHU-18[e]	Environmental PCR	North Rhine-Westphalia, Germany	Rhizosphere of *Zea mays*	AJ506000		
RSC-CHU-20[e]	Environmental PCR	North Rhine-Westphalia, Germany	Rhizosphere of *Zea mays*	AJ506002		
RSC-CHU-23[e]	Environmental PCR	North Rhine-Westphalia, Germany	Rhizosphere of *Zea mays*	AJ506003		
RSC-CHU-69[e]	Environmental PCR	North Rhine-Westphalia, Germany	Rhizosphere of *Zea mays*	AJ506037		

GenBank accession numbers for species in other orders are published in James *et al.* (2006).
a Taxa examined using LM.
b Taxa examined using TEM.
c Stoeck & Epstein 2003.
d Küpper *et al.* 2006.
e C. Hussels unpubl.

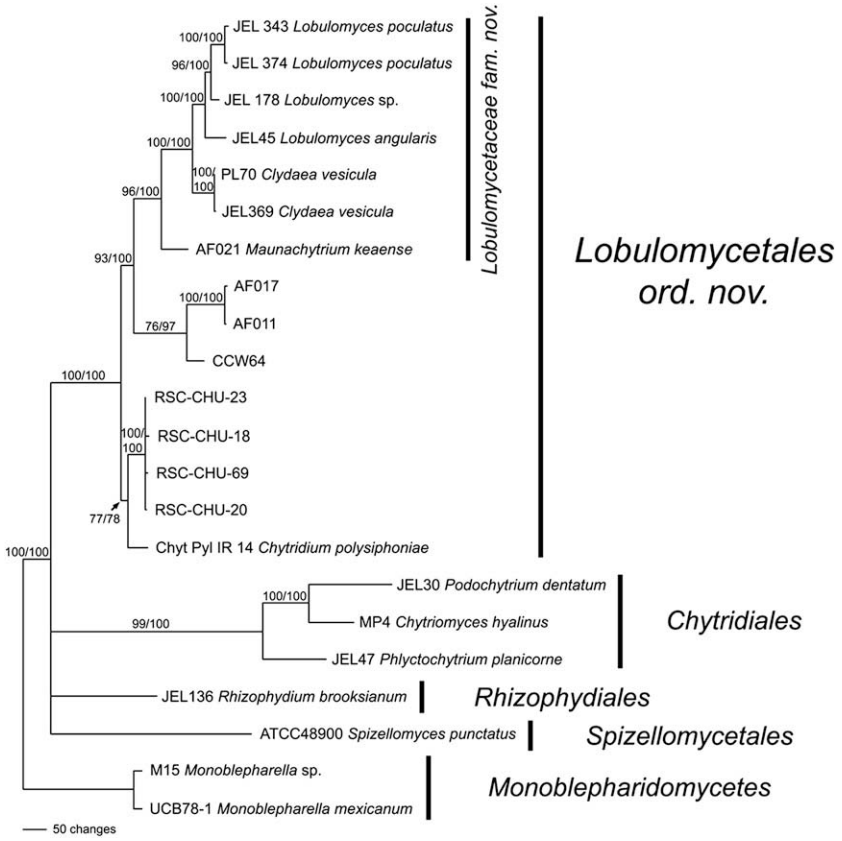

Fig 1 – Single most parsimonious tree obtained from combined nuSSU and partial nuLSU rDNA sequences. Numbers to the left of the slash indicate support above 70 % in 10K replicates with parsimony analysis. Numbers to the right of the slash are Bayesian inference estimates of PPs. MP phylogeny tree length = 2877 steps, CI = 0.7188, RI = 0.6902. Species of the *Monoblepharidomycetes* were used as outgroup taxa.

Morphology and zoospore ultrastructure

We grew isolates on agar medium incubated at 17 °C in the dark to determine morphological stages using LM. During development, thin slices of agar with living thalli were removed aseptically, examined, and photographed with a Nikon E400 microscope (Nikon Instruments, Melville, NY) equipped with phase optics and a Spot RT digital camera (Diagnostic Instruments, Sterling Heights, MI). We grew and prepared isolates JEL369 and AF021 for TEM following the methods of Longcore 1992.

DNA extraction, amplification, and sequencing

We extracted DNA from cultures (James *et al.* 2000) and followed the PCR protocol as described by Vilgalys & Hester (1990). We used primers SR1R and SR6.1 for amplification of the nuSSU rDNA region (James *et al.* 2000; Vilgalys & Hester 1990) and additionally used SR1.5 (James *et al.* 2000) and NS6 (White *et al.* 1990) for sequencing. Primers LR0R and LR5 (Vilgalys & Hester 1990) were used for amplification and sequencing of the nuLSU rDNA region, and primers BMB-CR (Lane *et al.* 1985) and ITS4 (White *et al.* 1990) were used for the ITS1–5.8S–ITS2 rDNA region. For AF011, AF017, and AF021, the rDNA operon was amplified using primers NS1 and LR5 and sequenced using NS1, NS2, NS4, NS6, NS7, NS8, ITS4, LR0R, and LR5 (Vilgalys & Hester 1990; White *et al.* 1990).

Our PCR products were purified with exonuclease I and shrimp alkaline phosphatase using the SBE Clean-Up Reagent protocol (USB Corporation, Cleveland, OH), QIAquick PCR Purification Kits, or QIAquick Gel Extraction Kits (Qiagen, Valencia, CA). The PCR products of the ITS1–5.8S–ITS2 rDNA amplifications were cloned following the TOPO TA Kit protocol (Invitrogen Corporation, Carlsbad, CA). The purified PCR products were sequenced by the University of Maine Sequencing Lab (Orono, ME), Functional Biosciences (Madison, WI), the Department of Molecular, Cellular, and Developmental Biology at Colorado University (Boulder, CO), or the Biology Department at Duke University (Durham, NC).

Phylogenetic analysis

We analysed chromatograms of our nuSSU and nuLSU rDNA sequences using ChromasPro 1.32 (Technelysium Pty). Using ClustalX 1.8 (Thompson *et al.* 1997), we aligned our sequences to selected taxa of the *Chytridiomycota* from the Assembling the Fungal Tree of Life (AFTOL) project (http://aftol.org) and nuSSU rDNA sequences of uncultured organisms grouping with *Chytriomyces angularis* (Küpper *et al.* 2006). Sequences of

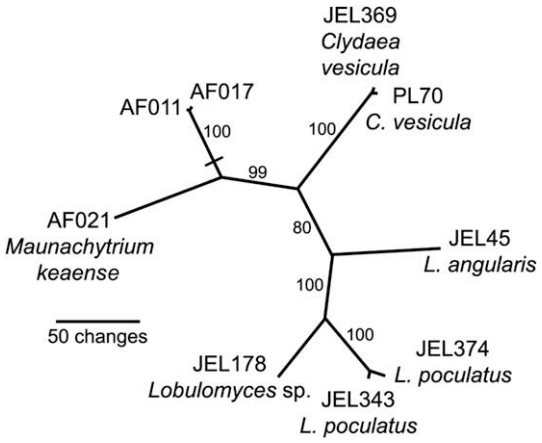

Fig 2 – Single most parsimonious tree obtained from ITS1–5.8S–ITS2 rDNA sequences. Numbers on branches indicate support above 50 % in 1K replicates with parsimony analyses. MP phylogeny tree length = 746, CI = 0.8954, RI = 0.8641. A hash mark indicates branch length has been shortened by 250 changes to aid viewing.

the ITS1–5.8S–ITS2 rDNA region did not align sufficiently between the *C. angularis* clade and other taxa to be used in phylogenetic analyses across the phylum, but they were used to produce an unrooted bootstrapped phylogram in PAUP 4.0b10 (Swofford 2003) by MP analysis. Support values for MP branches were estimated with 1K BS replicates to form a 50 % majority rule consensus tree. MP analyses and the computation of Bayesian PPs of nuSSU and nuLSU rDNA sequences were also conducted in PAUP 4.0b10. Support for MP branches was estimated with 10K BS replicates to form a 70 % majority rule consensus tree. We used Modeltest 3.4 (Posada & Crandall 1998) to calculate the best-fit model of DNA substitution (TrN+I+G) and these parameters were used in a Bayesian phylogenetic analysis with MrBayes v3.1.2. (Ronquist & Huelsenbeck 2003). Bayesian tree inference with MCMC sampling used two simultaneous Markov chains running 10M generations. Trees were sampled every 1K generations, with 10 001 trees sampled overall. A consensus of 7501 trees remaining after a burnin of 2.5K trees was used in PAUP 4.0b10 to compute the PP values on a 50 % majority rule tree.

Results

Phylogenetic analysis

We first compared the nuSSU and nuLSU sequences of taxa from Table 1 (*Lobulomycetales*) to representatives of currently described clades and orders (James *et al.* 2006; Letcher *et al.* 2005, 2006). Based on the monophyly of our group in these comparisons (data not shown), we chose taxa from the *Chytridiaceae* and *Phlyctochytrium* clade (Letcher *et al.* 2005), *Rhizophydiales* (Letcher *et al.* 2006), and *Spizellomycetales* to illustrate the distinction of this new order (Fig 1). The data matrix had 726 parsimony-informative characters from 4764 total characters. All isolates from Table 1 formed a separate clade, and *Chytriomyces hyalinus*, the type species of *Chytriomyces*, grouped within the *Chytridiaceae* with *Podochytrium dentatum*, both of which were outside of the *C. angularis* clade. An ITS1–5.8S–ITS2 rDNA phylogram of cultured isolates (Fig 2) produced branching patterns identical to those of the *Lobulomycetales* clade in Fig 1. The ITS1–5.8S–ITS2 data matrix had 361 parsimony-informative characters from 1181 total characters.

LM and TEM morphology

Zoosporangia of all isolates developed from the expansion of the encysted zoospore (endogenous development) and had isodiametric rhizoids, rarely greater than 1.5 μm diam, that branch from a single rhizoidal axis. Some isolates discharged zoospores after dehiscence of an operculum and others were inoperculate. In none of the isolates were zoospores enclosed in a vesicle during release from the zoosporangium. The presence of cupules on the zoosporangium identified isolates JEL343 and JEL374 as *Chytriomyces poculatus*. In pure culture, JEL369 (Fig 3) and AF021 (Fig 5) had thallus morphologies that differed from *C. angularis* and *C. poculatus* (Table 2). Temperature maxima were between 20 (JEL45) and 30 °C (JEL178, JEL369, PL70).

The zoospore ultrastructural features of isolates JEL369 (Fig 4) and AF021 (Fig 6) were compared with those of *C. angularis* (Fig 7). No isolate that we examined using TEM possessed a rumposome (fenestrated cisterna) or a microtubule root leading from the kinetosome to the lipid globule; however, all of the studied isolates had tightly aggregated ribosomes, a non-flagellated centriole parallel and attached to the kinetosome, and a flagellar plug. These three features are also found in the *Chytridiaceae sensu* Letcher *et al.* (2005) and *Monoblepharidales*.

Taxonomy

Lobulomycetales D. R. Simmons, **ord. nov.**
MycoBank no.: MB511804
Etym.: *lobulus* (Lat.): referring to the lobed zoosporangia of *Lobulomyces* species.

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

Zoospore with opaque flagellar plug, anterior or posterior plug extensions; one or two lipid globules; lacking microtubule root, Golgi apparatus, striated inclusion, electron-opaque bodies near kinetosome, and rumposome associated with lipid globule (Fig 7). Monophyletic clade within the *Chytridiomycota*.

Lobulomycetaceae D. R. Simmons, **fam. nov.**
MycoBank no.: MB511805
Supra; monocentricus thallus, eucarpicus, cum incremento endogeno. Rhizoidia isodiametrica, 0.5–1.5 *μm* in amplitudine.

Typus: *Lobulomyces* D. R. Simmons

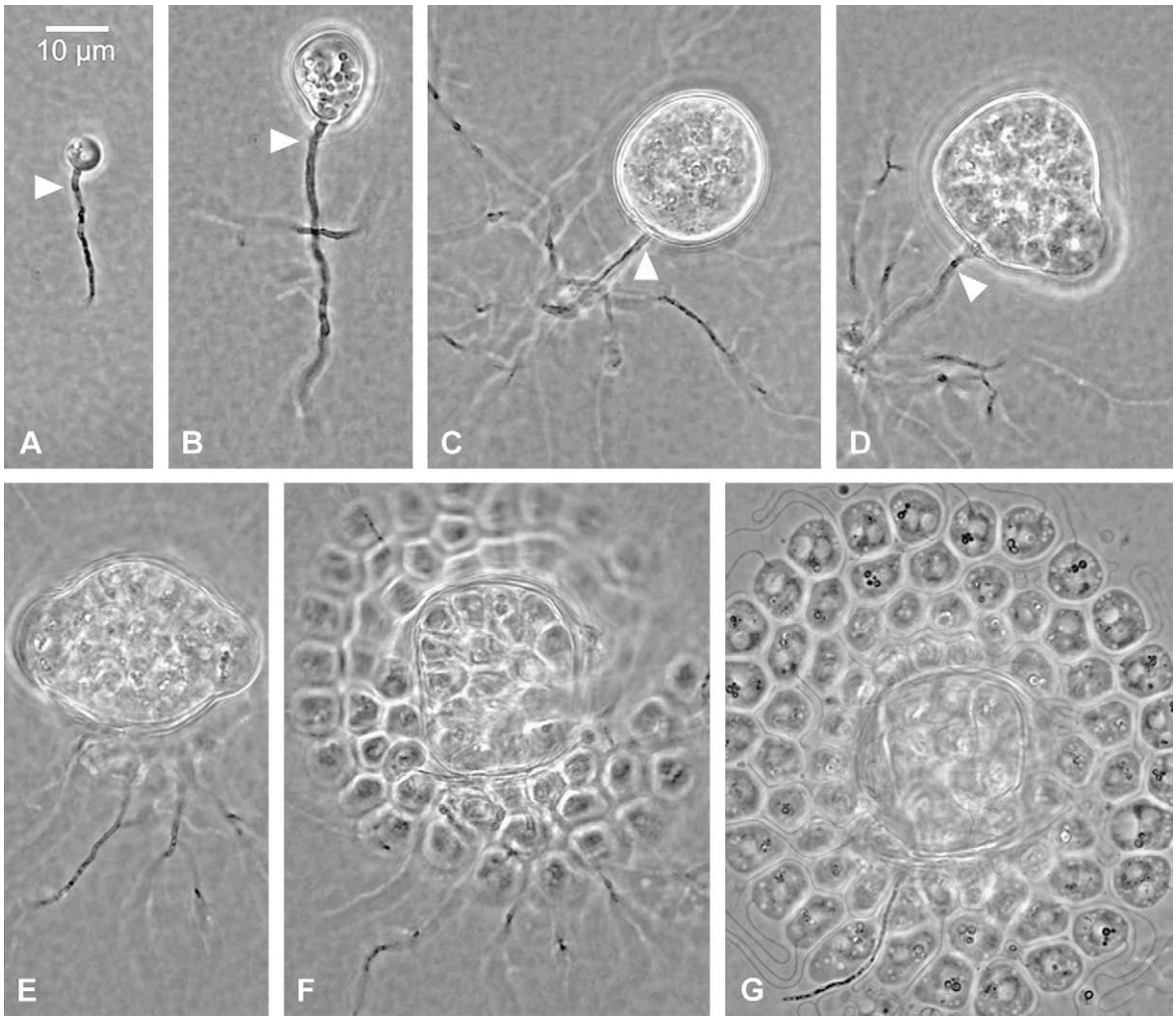

Fig 3 – Morphology of *Clydaea vesicula* (JEL369) on PmT agar at 17 °C. Note lack of subsporangial swelling (arrowhead). (A) Germling (2 h) with single rhizoidal axis. (B) Young thallus (25 h) with 90° branching of rhizoidal axis. (C) Developing thallus (68 h) with rounded zoosporangium. (D) Nearly mature thallus (74 h) with single protruding papilla. (E–G) Mature thallus discharging zoospores (74 h), showing collapse of zoosporangium and lack of operculum.

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

Three new genera, *Lobulomyces*, *Clydaea*, and *Maunachytrium* are described. They are distinguished by ultrastructural characters and rDNA sequences. At this time, we do not recognise the uncultured isolates, AF011, AF017, and *Chytridium polysiphoniae* as members of this family.

Lobulomyces D. R. Simmons, **gen. nov**.
MycoBank no.: MB511806

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

Typus: Lobulomyces angularis (Longcore) D. R. Simmons, **comb. nov.**

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

Lobulomyces angularis (Longcore) D. R. Simmons, **comb. nov**.
MycoBank no.: MB512107
Basionym: *Chytriomyces angularis* Longcore, *Mycologia* **84**: 443 (1992).
Type: Longcore, *Mycologia* **84**: 443, figs 1–28 (1992). We emend this species as follows: nuSSU rDNA, GenBank accession no. AF164253; ITS1–5.8S–ITS2 rDNA, GenBank accession no. AY997036; nuLSU rDNA, GenBank accession no. DQ273815.

The characteristics of the zoospore include the extensions of the flagellar plug and the dense fibrillar and amorphous 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 our molecular phylogeny mandate that this species be removed from *Chytriomyces*.

Table 2 – Temperature maxima, developmental morphology, and ultrastructural features of species within the *Lobulomycetales*

Species	Maximum temperature for growth (°C)	Thallus morphology			Zoospore			
		Zoosporangium shape on nutrient agar	Operculation	Diam of rhizoidal base at maturity (μm)	Zoospore diam. (μm)	Flagellum length (μm)	Flagellar plug extensions	Distinct ultrastructure
Clydaea vesicula	30	Spherical	No	1	5	20	Anterior	Vesicule cluster near kinetid
Lobulomyces angularis	20	Longer than wide, lobulate	Yes	1	4–5	30	Anterior and posterior	Dense fibrillar bridge, without vesicle cluster
Lobulomyces poculatus	25	Longer than wide, lobulate, with cupules	Yes	1	ND	ND	ND	ND
Maunachytrium keaense	25	Spherical	No	5	4	26	Anterior and posterior	Like *L. angularis* without dense fibrillar bridge

ND, no data.

The morphological similarities of representatives of *Lobulomyces* are consistent, supporting the molecular phylogeny. All isolates of the genus exhibit (1) operculation, (2) avesicular zoospore discharge, (3) angular zoosporangia longer than wide, (4) isodiametric rhizoids that branch at near-right angles from the initial rhizoid and are rarely larger than 1.5 μm, and (5) a major rhizoidal axis that does not enlarge greater than 1.5 μm (see Supplementary Material).

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

MycoBank no.: MB512108

Basionym: *Chytriomyces poculatus* Willoughby & Townley, *Trans. Brit. Mycol. Soc.* **44**: 183; fig 3, pl. 14 (1961).

Type: Herb. I.M.I. 80977, collected 11 Dec 1959. Emended Letcher & Powell 2002.

We emend this species as follows: nuSSU rDNA, GenBank accession no. EF443135; ITS1–5.8S–ITS2 rDNA, GenBank accession no. EU352771; nuLSU rDNA partial, GenBank accession no. EF443140.

Specimens examined: **New Zealand**: *South Westland*: South Island, Haast Ecological District, Cole Creek, 45°43′S, 169°414′E, tree canopy detritus, 22 May 2003, (JEL374) — **United States**: *Maine*: Old Town, 44°56′N, 68°46′W, soil from spruce and fir forest, Oct 2001, (JEL343; nuSSU rDNA, GenBank accession no. EF443134; ITS1–5.8S–ITS2 rDNA, GenBank accession no. EU352770; nuLSU rDNA partial, GenBank accession no. EF443139).

Although we have not yet examined the zoospores of *Lobulomyces poculatus*, genetic characters dictate that the species be removed from the polyphyletic genus *Chytriomyces*. The two isolates of *L. poculatus* were from different continents, and yet the nuSSU and partial nuLSU sequences of the two isolates used for phylogenetic analysis had 98 % similarity. Because of its genetic and morphological similarity to *L. angularis*, we place this species in the genus *Lobulomyces*.

Clydaea D. R. Simmons, **gen. nov.**

MycoBank no.: MB512109

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

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

Typus: *Clydaea vesicula* D. R. Simmons

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

This genus is distinguished from others in the *Lobulomycetaceae* by the lack of posterior extensions of the flagellar plug, the lack of a dense fibrillar bridge, and the possession of an accumulation of vesicles around the kinetid (Fig 4B–E) (Letcher pers. comm.). In contrast *Lobulomyces angularis* has similar vesicles scattered throughout the cytoplasm.

Clydaea vesicula D. R. Simmons, **sp. nov.** (Figs 3 and 4)

MycoBank no.: MB512110

Etym.: *vesicula* (Lat.): referring to accumulation of vesicles around the kinetid.

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

Typus: **United States**: *California*: Goleta, 34°25′N, 119°53′W, duff from *Eucalyptus* grove, Dec 2001 (Figs 3 and 4 from isolate JEL369).

Zoosporangium irregularly spherical with 1 to 4 protuberant discharge papillae; inoperculate. Resting zoosporangia not seen. nuSSU rDNA, GenBank accession no. EF443137;

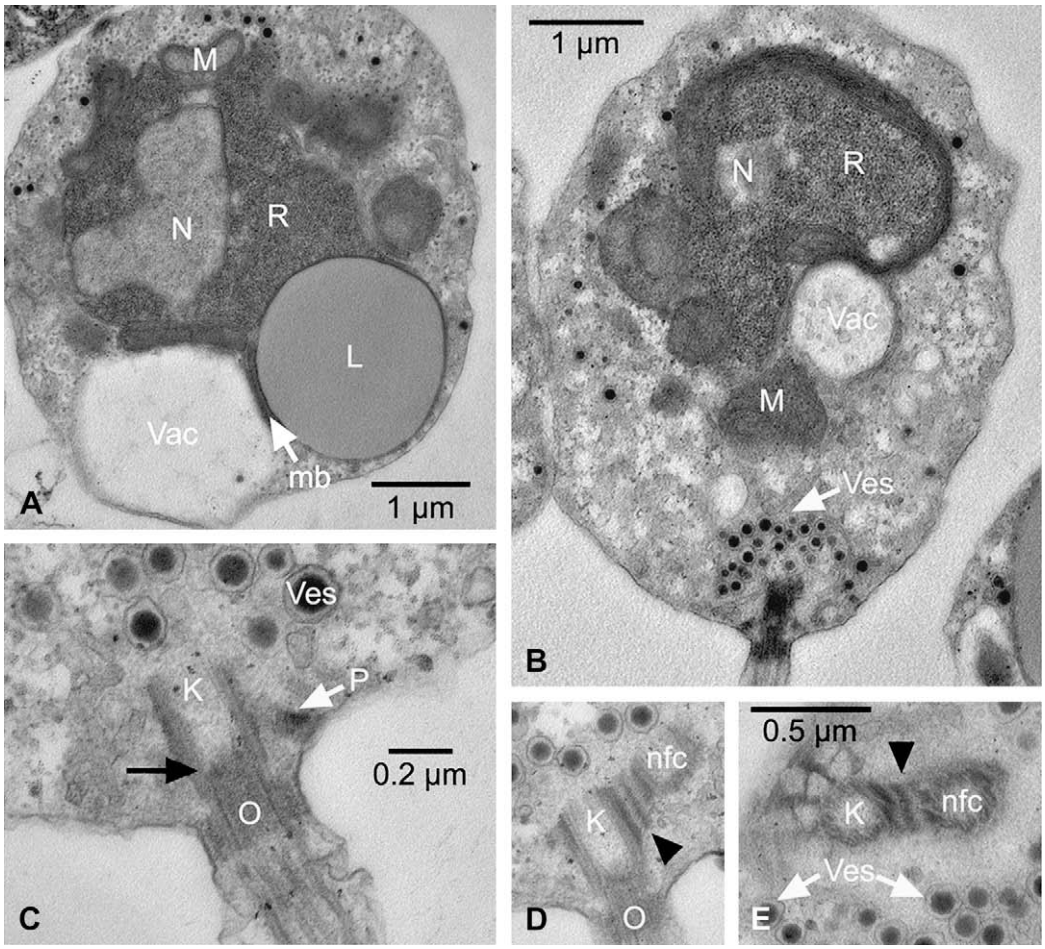

Fig 4 – Ultrastructure of *Clydaea vesicula* (JEL369) zoospore. (A) Cross-section through zoospore. (B) Longitudinal section through zoospore with aggregation of vesicles near the kinetid. (C) Longitudinal section through kinetosome and flagellar plug with anterior extensions (black arrow). (D & E) Longitudinal and cross-section of zoospore showing the fibrillar bridge (black arrowhead) between the kinetosome and the nonflagellated centriole. Scale same for (D & E). Key to Figs 4, 6 and 7: endoplasmic reticulum (ER), kinetosome (K), lipid globule (L), mitochondria (M), microbody (mb), nucleus (N), nonflagellated centriole (nfc), electron-opaque plug (O), props (P), ribosomal mass (R), vacuole (Vac), vesicles (Ves).

ITS1–5.8S–ITS2 rDNA, GenBank accession no. EU352773; nuLSU rDNA partial, GenBank accession no. EF443142.

Additional specimen examined: Specimens retained in the JEL culture collection at the University of Maine, **United States**: *Utah*: Big Cottonwood Creek, Wasatch County, 40°38′N, 111°41′W, creek bank mud, collected on pollen, 2 Aug 2001, *P. M. Letcher* (PL70: nuSSU rDNA, GenBank accession no. EF443138; ITS1–5.8S–ITS2 rDNA, GenBank accession no. EU352774; nuLSU rDNA partial, GenBank accession no. EF443143).

When grown on PmT agar medium, *Clydaea vesicula* has a zoosporangium *ca* 35 μm diam at maturity that is round with slight protrusions (Fig 3D–E). 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 (Fig 3E–G). Zoospores are spherical in motion, *ca* 5 μm diam, and have flagella *ca* 20 μm long. An operculum was not observed. Resting spores were not seen. Although the isolates of this species originated from different geographic areas and habitats, the sequences used for phylogenetic analysis were 98 % similar.

Maunachytrium D. R. Simmons, **gen. nov**.
MycoBank no.: MB512111
Etym.: *mauna*: Hawaiian for mountain.

Zoospora cum duabus globis lipoidis; simplex fibrata colligato inter kinetosoma et nonflagellatum centriolum; flagelliani obturamenti extensiones anteriorae atque posteriorae. Clarum genus intra *Lobulomycetales*.

Typus: Maunachytrium keaense D. R. Simmons

Zoospore with two lipid globules; simple fibrillar connection between kinetosome and nonflagellated centriole; flagellar plug extensions anterior and posterior. Distinct lineage within the *Lobulomycetales*.

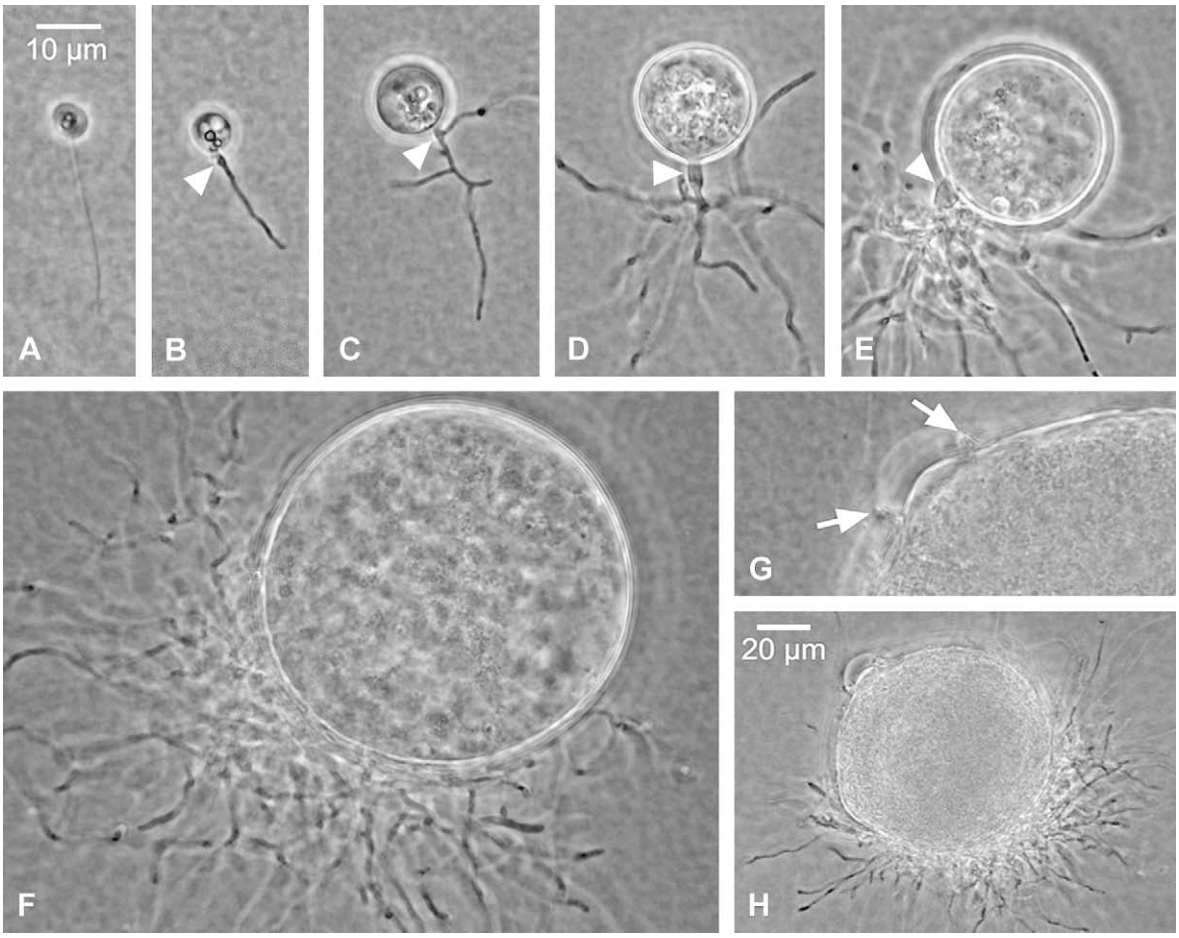

Fig 5 – Morphology of *Maunachytrium keaense* (AF021) on PmT agar at 17 °C. Note lack of subsporangial swelling in young thalli with swelling during development (arrowhead). (A) Zoospore with two lipid globules. (B) Germling (6 h) with single rhizoidal axis. (C) Young thallus (28 h) with multiple branches off primary rhizoid at various intervals. (D & E) Developing thalli (48 and 75 h) with enlarging subsporangial swelling. (F) Thallus (98 h) with large, spherical zoosporangium and isodiametric rhizoids. Base of rhizoidal axis obscured. (G) Cell wall material (arrows) deliquescence around discharge pore. (H) Mature thallus (145 h) with membrane protruding. Scale same for (A–G).

This genus is distinguished from *Lobulomyces* by the absence of the dense fibrillar and amorphous connection extending from four triplets of the kinetosome to the nonflagellated centriole and from *Clydaea* by the lack of vesicles around the kinetid (Fig 6).

Maunachytrium keaense D. R. Simmons, **sp. nov**. (Figs 5 and 6)
MycoBank no.: MB512112
Etym.: Name from Mauna Kea, Hawaii, site of soil collection.

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

Typus: **United States**: Hawaii: Mauna Kea, near permafrost-fed lake, 19°48′N, 155°28′W, soil, 13 Sept 2005, *A. Martin* (Figs 5 and 6 from isolate AF021).

Zoosporangium spherical with multiple, inoperculate discharge papillae and swelling at base of rhizoidal axis not exceeding 5 μm. Resting sporangia not seen. nuSSU–ITS1–5.8S–ITS2 rDNA complete and nuLSU rDNA partial GenBank accession no. EF432822.

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 keaense* (Fig 5) are spherical and 60–85 μm diam on PmT agar medium. At maturity, one or more pores develop in the cell wall of the zoosporangium (Fig 5G–H), and the membrane-bound cytoplasm protrudes. Zoospores exit through one or more of these sites. Zoospores are spherical when in motion, *ca* 4 μm diam, and with a flagellum *ca* 26 μm long. The isodiametric rhizoids of *M. keaense* branch profusely (Fig 5C–F, 5H), from the slightly swollen (*ca* 5 μm) base of the rhizoidal axis (Fig 5D–E). In pure culture at 17 °C, *M. keaense* may take 7 d to reach maturity. Resting spores were not seen.

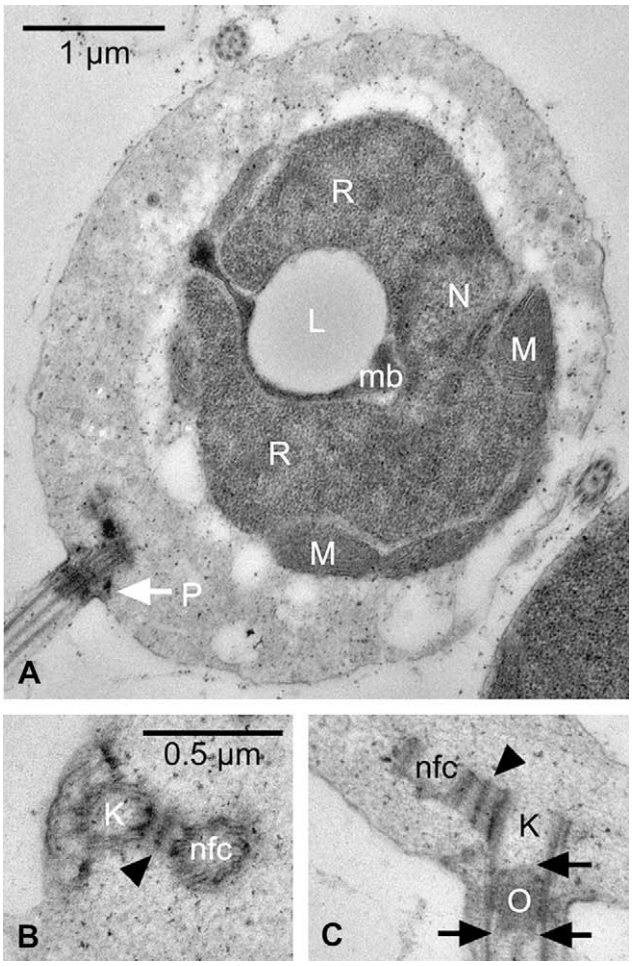

Fig 6 – Ultrastructure of *Maunachytrium keaense* (AF021) zoospore. (A) Longitudinal section of zoospore. (B) Cross-section of the fibrillar bridge (black arrowhead) between the kinetosome and the nonflagellated centriole. (C) Longitudinal section through kinetosome, nonflagellated centriole, and flagellar plug with anterior and posterior extensions (black arrows). Scale same for (B & C).

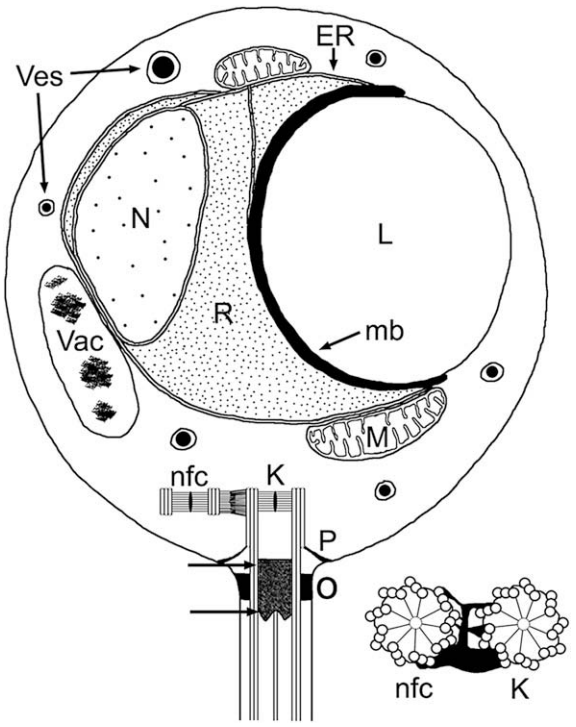

Fig 7 – Zoospore diagram of *Lobulomyces angularis*, adapted from Longcore (1992). Note arrows on anterior and posterior extensions of electron-opaque plug.

Discussion

Order Lobulomycetales

Since the description of the *Spizellomycetales* and the amendment of the *Chytridiales* (Barr 1980), zoospore ultrastructural features have defined orders (Letcher *et al.* 2006). Although the *Lobulomycetales* is well supported by our molecular phylogeny, it is difficult to assemble an ultrastructural ordinal concept for the group. All isolates of the new order examined by TEM lack the microtubule root and rumposome that are common in most other orders of the *Chytridiomycota sensu* Powell (Hibbett *et al.* 2007). All other clades of the phylum except the *Spizellomycetales* (Barr 1980) and *Rhizophlyctidales* (Letcher *et al.* 2008) have some species that possess a rumposome and microtubule roots. These plesiomorphic structures seem to have been lost in the lineage represented by the new genera, but it is possible that uninvestigated isolates in this study may retain them. Beakes *et al.* (1988) illustrated that the zoospore of the diatom parasite *Zygorhizidium affluens*, which also lacks microtubules and a rumposome, possesses a densely-stained flagellar plug. This plug seems to possess anterior projections similar to those in *Clydaea*; molecular analyses of *Z. affluens* would help determine whether this ultrastructural character is homologous. The character states of the *Lobulomycetales*, namely the lack of certain ultrastructural components, also leave few characters on which to define genera.

Genus Lobulomyces

Molecular phylogenies based on nuSSU and partial nuLSU rDNA sequences of isolates in this study resulted in a single most parsimonious tree (Fig 1) that placed *Lobulomyces angularis* and *L. poculatus* as a monophyletic clade within the *Lobulomycetales*. *L. angularis* is the only member of this genus whose zoospore ultrastructure is currently described (Longcore 1992). 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 and amorphous bridge between the kinetosome and nonflagellated centriole found in *Lobulomyces angularis* is a derived character within all species in this genus.

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. This species differs from all other examined members of the clade 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 may be a derived character within this lineage, and this, in conjunction with the molecular phylogeny, distinguishes this genus and species.

Genus Maunachytrium

Maunachytrium keaense is described in its own genus because its placement in the nuSSU and partial nuLSU rDNA phylogeny is well-supported and distinct from the other two genera. Additionally, the combination of the simple connection between the kinetosome and nonflagellated centriole and the large, inoperculate zoosporangium with a slight swelling in the rhizoidal base 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. keaense* grows at temperatures up to 25 °C. Morphological characters of both *Rhizophydium* and *Maunachytrium* include inoperculate, multipored zoosporangia with extensively branched rhizoids. Consequently, without ultrastructural and genetic data, *Lobulomycetales* isolates that possess spherical zoosporangia with multiple discharge papillae may have been, or may be, erroneously identified as species of *Rhizophydium*.

Diversity in the Lobulomycetales

Currently the *Lobulomycetales* is a small order, containing a single family with three described genera, but with further investigation of zoosporic ultrastructure additional genera may be described from our isolates. *Chytridium polysiphoniae* is in a clade separated from our isolates in culture and so cannot be placed in any of the newly described genera; we agree with Küpper *et al.* (2006) that it should be placed in a new genus when ultrastructural characters of the zoospores become available. Increased isolation attempts by chytrid specialists may also yield new species in this order. Although most of our isolates were from soil samples, *Lobulomyces angularis* was isolated from a sample taken from an acidic freshwater lake (Longcore 1992) and the *C. polysiphoniae* studied by Küpper *et al.* (2006) is a marine parasite of a brown alga. Thus members of the order may be found in aquatic, terrestrial, and marine habitats and may be saprophytes or parasites.

Species in the order can be distinguished by morphology alone; however, without isolation into pure culture and experience in viewing this group, even determining the order for some species is problematic. Because lobulomycetalean chytrids are difficult to isolate, even for chytrid specialists, we expect that members of this order will be identified primarily from molecular data. The increasing number of projects sampling DNA without isolation from the environment (e.g. Lepère *et al.* 2006; Lockhart *et al.* 2006; Lefèvre *et al.* 2007) will rely on molecular taxonomic studies, such as ours and others (James *et al.* 2006; Letcher *et al.* 2005, 2006), to identify chytrid DNA they have amplified. Without molecular data present in easily assessable databases, researchers sampling DNA from the environment may conclude that they have found novel lineages when actually sampling well-known, but unsequenced taxa (Berney *et al.* 2004). Conversely, environmental sampling will increase the known diversity within the *Chytridiomycota* and direct our attention to additional habitats to bait for chytrids.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi: 10.1016/j.mycres.2008.11.019

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