FUNGAL BIOLOGY 115 (2011) 381–392

British Mycological Society promoting fungal science

journal homepage: www.elsevier.com/locate/funbio

Molecular phylogeny of the *Blastocladiomycota* (Fungi) based on nuclear ribosomal DNA

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ARTICLE INFO

Article history:
Received 22 November 2010
Received in revised form
3 February 2011
Accepted 4 February 2011
Available online 13 February 2011
Corresponding Editor:
Joseph W. Spatafora

Keywords:
Blastocladiales
Blastocladiomycota
Molecular phylogenetic systematics
rDNA

ABSTRACT

The *Blastocladiomycota* is a recently described phylum of ecologically diverse zoosporic fungi whose species have not been thoroughly sampled and placed within a molecular phylogeny. In this study, we investigated the phylogeny of the *Blastocladiomycota* based on ribosomal DNA sequences from strains identified by traditional morphological and ultrastructural characters. Our results support the monophyly of the *Coelomomycetaceae* and *Physodermataceae* but the *Blastocladiaceae* and *Catenariaceae* are paraphyletic or polyphyletic. The data support two clades within *Allomyces* with strains identified as *Allomyces arbusculus* in both clades, suggesting that species concepts in *Allomyces* are in need of revision. A clade of *Catenaria* species isolated from midge larvae group separately from other *Catenaria* species, suggesting that this genus may need revision. In the *Physodermataceae*, *Urophlyctis* species cluster with a clade of *Physoderma* species. The algal parasite *Paraphysoderma sedebokerensis* nom. prov. clusters sister to other taxa in the *Physodermataceae*. *Catenomyces persicinus*, which has been classified in the *Catenariaceae*, groups with the *Chytridiomycota* rather than *Blastocladiomycota*. The rDNA operon seems to be suitable for classification within the *Blastocladiomycota* and distinguishes among genera; however, this region alone is not suitable to determine the position of the *Blastocladiomycota* among other basal fungal phyla with statistical support. A focused effort to find and isolate, or directly amplify DNA from additional taxa will be necessary to evaluate diversity in this phylum. We provide this rDNA phylogeny as a preliminary framework to guide further taxon and gene sampling and to facilitate future ecological, morphological, and systematic studies.

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1878-6146/$ – see front matter
doi:10.1016/j.funbio.2011.02.004

Introduction

The *Blastocladiomycota* contains only the *Blastocladiales* (Petersen 1909; Petersen 1910), an order of zoospore-producing true fungi that contains both saprobes, several of which were once model research species (e.g., *Allomyces*, *Blastocladiella*), and obligate parasites of plants and animals. Although some members of the order do not seem to reproduce sexually, others are noted for having isomorphic or heteromorphic alternation of generations. In these species meiosis takes place during germination of resistant sporangia, leading to zoospores that develop into haploid thalli that produce gametes. Currently included within the order are five families (Barr 2001): (1) *Blastocladiaceae* Petersen (1909), which contains only saprobic species; (2) *Catenariaceae* Couch (1945), which contains both saprobes and pathogens; (3) *Coelomomycetaceae* Couch ex Couch (1962), which contains pathogens of invertebrates; (4) *Physodermataceae* Sparrow (1952), which contains obligate parasites of plants; and (5) *Sorochytriaceae* Dewel *et al*. (1985), which contains a pathogen of tardigrades; *Polycaryum laeve* Stempell (1903), a pathogen of *Daphnia*, has not been placed in a family.

Experienced viewers can often distinguish members of the *Blastocladiales* from other zoospore-producing fungi by observing their zoospores by light microscopy. Typically, blastocladian zoospores have a distinctive ribosomal nuclear cap and, in some species, a large side body containing lipid globules. With the rise of electron microscopy, classification shifted to emphasize zoospore ultrastructure (Fuller 1977; Barr 1980). The ultrastructure of zoospores, which is conserved and informative for defining the *Blastocladiales* and orders in the *Chytridiomycetes* (Fuller 1977; Barr 1978, 1980, 1981; Powell 1978; Lange & Olson 1979; James *et al*. 2000; Letcher *et al*. 2006; Letcher *et al*. 2008; Mozley-Standridge *et al*. 2009; Simmons *et al*. 2009; Vélez *et al*. in press) led to the transfer of the *Physodermataceae* from the *Chytridiales* to the *Blastocladiales* (Lange & Olson 1980b). Ultrastructural zoospore features also led to the classification of *Sorochytriaceae* within the *Blastocladiales* (Dewel *et al*. 1985) and, along with molecular evidence led to placing *Polycaryum laeve* in the *Blastocladiales* (Johnson *et al*. 2006).

Although earlier molecular analyses yielded uncertain results about the relationship of the *Blastocladiales* with other zoosporic fungi (James *et al*. 2000), the *Blastocladiales* recently was reclassified from the *Chytridiomycota* to a new phylum, the *Blastocladiomycota*. The new phylum is based on a molecular rDNA phylogeny and ultrastructural characters (James *et al*. 2006). Taxon sampling in earlier work that included the *Blastocladiales* was small because the studies were designed to determine the phylogenetic placement of the order within the greater fungal phylogeny. Here, our objective was to produce a molecular phylogeny with a widespread sampling from the major families and genera within the phylum. For the first time, we analyzed 18S-5.8S-28S rDNA sequences from 11 genera in four families and assessed the correspondence of current classification with our rDNA phylogeny. This new phylogeny indicates that a few families and genera are in need of revision and provides a framework that can inform taxon and gene sampling in future systematic work.

Materials and methods

Culture collection and isolates

Methods for collecting zoosporic parasites from aquatic insects have been described (Martin 1987). Larvae parasitized by *Coelomomyces* and *Coelomycidium* isolates were initially fixed and stored in 80–95 % ethanol or 2× CTAB buffer. *Blastocladia* species were isolated from blueberry baits according to the methods of Whisler (1987), cultured in Petri dishes in GY5 broth (Emerson 1958) or yeast protein soluble starch growth medium (YpSs) broth (Emerson 1941), and maintained under anaerobic conditions in a BBL GasPak anaerobic system. *Catenaria*, *Catenophlyctis*, *Allomyces*, *Microallomyces*, and *Blastocladiella* isolates were maintained on YpSs agar. *Physoderma* and *Urophlyctis* samples were obtained from dried plant material or herbarium collections at the University of Michigan Herbarium.

Sample harvest and DNA extraction

Larvae infected with *Coelomomyces* or *Coelomycidium* were stored in 2× CTAB until extraction. Individual larvae were dissected under a stereomicroscope with 'Minuten' insect pins in holders. Resting sporangia (RS) and hyphae were placed into 500 μl 2× CTAB extraction buffer [2× CTAB: 2% cetyltrimethylammonium bromide, 1.4 M NaCl, 100 mM Tris, 20 mM Na-EDTA pH 8]. Samples in extraction buffer were ground for 2–3 min with a plastic pestle (Kontes) in a 1.5 ml microcentrifuge tube. A small amount of sterile sand and 1 mm silica beads were added and the sample was vortexed for 6–9 min. Microscopic examination revealed ~90 % of the outer pigmented wall of RS had been broken with the inner wall still intact on many. Samples were subsequently incubated at 65 °C for 60 min in a water bath to further extract and hydrate the nucleic acids. Mini DNA preparations of approximately 500 μl were extracted 2–3 times with an equal volume of 24:1 chloroform:isoamyl alcohol. The final aqueous phase was precipitated with 0.6 V cold isopropanol and pelleted. Following a 1 min wash with cold 80 % EtOH the samples were dried in a speed vacuum concentrator (Savant) and resuspended in 25–50 μl of distilled water.

Specimens from pure cultures on agar were flooded with 2–3 ml water and left to sit for 15–30 min. The resulting zoospore-hyphal suspension was transferred to a 1.7 ml microcentrifuge tube, centrifuged to pellet, the liquid decanted, then the pellet resuspended in 500 μl 2× CTAB. Alternatively, broth cultures were harvested by vacuum-filtration using a Buchner funnel and flask onto Whatman No.1 paper. The sample was added to a 1.7 ml microcentrifuge tube to fill approximately half the conical portion and mixed with 500 μl 2× CTAB. Samples were ground with a disposable pestle with a small amount of sand and extracted as described above.

PCR, cloning, and sequencing

The cocktail consisted of 0.1–10 ng DNA, 1× PCR buffer (no $MgCl_2$), 250 μg bovine serum albumin, 1–3.75 mM $MgCl_2$, 10–12.5 μM each of forward and reverse primers, 5 mM dNTPs,

1 unit of Taq polymerase and water to 25 μl. In some cases, culture PCR was performed by using a sterile pipette tip to transfer a small portion of a pure culture directly into the PCR cocktail instead of adding 1 μl DNA. To amplify the SSU rDNA region from *Coelomomyces*, *Coelomycidium*, and *Blastocladia*, we used the PCR primers SR1R (Vilgalys & Hester 1990) or a newly designed 18S-Cs-1F [5′-GAGGCCTACCRTGGTGAT-3′] with NS4 or NS6 (White *et al.* 1990). To amplify the SSU rDNA region in *Catenaria*, *Catenophlyctis*, *Blastocladiella*, and *Allomyces* species, we used the primers SR1R (Vilgalys & Hester 1990) and SR6 (Vilgalys lab, Duke University, unpubl. http://www.botany.duke.edu/fungi/mycolab). To amplify the SSU rDNA region in *Physoderma* we used SR1R and NS4, and for *Urophlyctis* we used SR1R and SR6.1 (Parrent & Vilgalys 2009). We used the following thermal cycling program: 95 °C for 2 min (or 10 min for direct PCR), followed by 35–39 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 3.5 min, and a final 72 °C for 10–12 min. To amplify the ITS and 5′-LSU regions, we used the PCR primers ITS1F (Gardes & Bruns 1993) with ITS4 (White *et al.* 1990), and LR0R (Rehner & Samuels 1994) with LR5 (Vilgalys & Hester 1990). Thermal cycling conditions were as described above, but the extension time was shortened to 72 °C for 1 min. Amplicons were purified using the Qiaquick PCR purification kit (Qiagen) or treatment with five units of exonuclease I and one unit of antarctic phosphatase with 1× of each enzyme buffer and 5 μl of PCR product at 37 °C for 30 min and 80 °C for 20 min.

Samples that originated from insect larvae were cloned using a TOPO (vector 2.1) PCR subcloning kit with TOP10 chemically competent cells (Invitrogen, CA) according to the manufacturer's directions. White transformed colonies were picked and amplified by culture PCR and purified as described above. BigDye Terminator v3.1 (Applied Biosystems, CA, USA) chemistry was used and samples were submitted for sequencing using the same primers as those used for PCR, with the addition of two internal primers for 18S sequences from *Catenaria* and *Allomyces*: NS4 (White *et al.* 1990) and BMB-BR (Lane *et al.* 1985). Sequences were assembled using Sequencher version 4.8 (GeneCodes).

Phylogenetic analyses

Blastocladiomycota sequences for each rDNA region were automatically aligned using Muscle (Edgar 2004) then were manually adjusted and concatenated using Mesquite version 2.72 (Maddison & Maddison 2009). Ambiguously aligned regions were excluded from the concatenated rDNA alignment. jModelTest and MrModelTest were used to select the best model of sequence evolution (Guindon & Gascuel 2003; Nylander 2004; Posada 2008). Bayesian analyses were conducted using MrBayes v.3.1.1 with the following settings: lset nst = 6 rates = invgamma, prset statefreqpr = Dirichlet(1,1,1,1), sampling 1 tree every 100 generations (Ronquist & Huelsenbeck 2003). Analyses with four chains were allowed to continue until the burnin period represented less than 20 % of the run and the topology of two parallel runs had converged using the program Are We There Yet? (AWTY) (Nylander *et al.* 2008). The full dataset included 71 taxa, 3542 included nucleotide characters, and was allowed to run for 4 376 900 generations. The *Allomyces* dataset comprised 16 ingroup taxa, 3203 included nucleotide characters from the SSU + 5.8S + LSU rDNA region, and was allowed to run for 10 million generations. The *Physoderma* dataset comprised eight taxa, 1609 included nucleotide characters from the SSU rDNA region, and was allowed to run for 10 million generations. Maximum likelihood analyses were conducted with RAxML version 7.0.4 to obtain the best ML tree as well as to determine bootstrap support using a GTR + G + I model, with 1000 bootstrap replicates (Stamatakis 2006; Stamatakis *et al.* 2008). Trees were visualized with PAUP 4.0b10 (Swofford 2003) and edited using CanvasX version 10.5.8 (ACD Systems, Inc.). Sequences generated for this study have been deposited in GenBank with accession numbers HQ888683–HQ888760 (Table 1). Alignments are available from TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S11216).

Results

There were no strongly supported conflicts, maximum likelihood bootstrap proportion (MLBP) greater than 70 % or Bayesian posterior probability (BPP) greater than 90 %, among our best maximum likelihood and Bayesian trees. In our Bayesian rDNA (SSU + 5.8S + LSU) phylogeny for the *Blastocladiomycota* (Fig 1) 13 *Allomyces* isolates form a monophyletic group with 98 % MLBP and 1.0 BPP (Fig 1). In a separate analysis limited to the *Allomyces* group (Fig 2), *Allomyces* taxa do not group according to current subgenera, which are defined by life cycle characteristics. Two major *Allomyces* clades are recovered; two isolates of the type species *Allomyces arbusculus* (subgenus *Euallomyces*) are in one clade and four isolates identified as the same species are in the other. Two strains of *Allomyces anomalus* (subgenus *Brachyallomyces*) also do not cluster together. Two stains of *Allomyces moniliformis* and one strain of *Allomyces neomoniliformis* (subgenus *Cystogenes*) form a monophyletic group nested among other *Allomyces* isolates.

The two *Blastocladia* isolates form a monophyletic group sister to the *Allomyces* clade with 92 % MLBP and 1.0 BPP (Fig 1). *Microallomyces* is a monotypic genus and *Microallomyces dendroideus* CR74, the type-isolate from Costa Rica (Emerson & Robertson 1974), clusters basal to *Allomyces* and *Blastocladia* with 82 % MLBP and 1.0 BPP. *Catenaria uncinata* and *Catenaria spinosa* form a monophyletic group with 99 % MLBP and 1.0 BPP; this clade is separated from the *Catenaria* + *Catenophlyctis* clade. Seven isolates of *Catenaria* spp. and *Catenophlyctis* are strongly supported as a monophyletic group but *Catenomyces* did not cluster within the phylum. Three *Blastocladiella* isolates cluster with 82 % MLBP and 1.0 BPP; *Blastocladiella emersonii* clusters separately from the other *Blastocladiella* isolates with 86 % MLBP and 1.0 BPP.

The *Coelomomycetaceae* is recovered sister to the *Catenariaceae* and *Blastocladiaceae* with 1.0 BPP; however, there is no maximum likelihood bootstrap support for this relationship. This family, represented by four strains of *Coelomomyces* and two *Coelomycidium* isolates, are on long branches that form a clade with 0.99 BPP; however, there is also no maximum likelihood bootstrap support for this relationship. *Coelomomyces* species have about six large insertions varying in size from about 50–275 bp in their 18S rDNA sequences. This is particularly noticeable in the 18S

Table 1 – List of strains used in this study, the source of sequences used or a description of where isolates were collected in this study, and GenBank accession numbers. Vouchers and cultures are available directly from the culture collections, herbaria, or authors listed below

Taxonomic group	Sample	Source/availability	GenBank accession		
			18S	5.8S	25S
Blastocladiaceae	*Allomyces javanicus* IndiaB2	From collections of R. Emerson and L.W. Olson. Available from Fungal Genetics Stock Centre, MO, USA.	–	–	HQ888738
	Allomyces javanicus Cuba S7	(As above)	–	HQ888720	HQ888739
	Allomyces anomalus ATCC 10982	(As above)	–	HQ888721	HQ888740
	Allomyces arbusculus His-B 35C Type species	(As above)	HQ888683	–	HQ888741
	Allomyces anomalus British East Africa 2	(As above)	HQ888684	–	HQ888742
	Allomyces anomalus California 6	(As above)	HQ888685	HQ888722	–
	Allomyces macrogynus Australia 3	(As above)	HQ888686	HQ888723	–
	Allomyces arbusculus Texas 1 Type species	(As above)	HQ888687	HQ888724	HQ888743
	Allomyces macrogynus WT Burma 3-35	(As above)	EF014364	–	–
	Allomyces moniliformis India 3	(As above)	HQ888688	HQ888725	HQ888744
	Allomyces neomoniliformis CBS 105.52	(As above)	HQ888689	–	HQ888745
	Allomyces arbusculus Belgian Congo 1 Type species	(As above)	HQ888690	HQ888726	HQ888746
	Allomyces arbusculus Denmark 1 Type species	(As above)	HQ888691	HQ888727	HQ888747
	Allomyces moniliformis WM105	Isolated from hemp seed bait in straw infusion, commercial straw imported from Mexico, Sep. 18, 2007 by W. Martin.	HQ888693	HQ888728	HQ888748
	Allomyces arbusculus MAR CW16	Isolated from summer cropping soil, Narrabri, NSW, Australia, 2007 by F. Gleason.	HQ888694	HQ888729	HQ888749
	Allomyces arbusculus Brazil 2 Type species	(Genbank)	AY552524	AY997028	AY552525
	Blastocladia ramosa WM101	Isolated from blueberry bait in water, stream crossing Monte Vista Rd. at junction with Asbury Rd., Candler, NC, USA, Nov. 17, 2005 by W. Martin.	HQ888695	–	–
	Blastocladia pringsheimii WM102	(As above)	HQ888696	–	–
	Microallomyces dendroideus CR74 Type isolate	(Genbank)	AY635840	AY997059	DQ273805
	Blastocladiella sp. JEL440	Isolated from tallus from Niwot Ridge, CO, USA by J. Longcore.	HQ888697	–	–
	Blastocladiella sp. JEL363	Isolated from soil form Orono, ME, USA by J. Longcore.	HQ888698	–	–
	Blastocladiella britannica Barr 214	Originally isolated by G. Willoughby from Esthwaite Water, Lake District, UK. Available from Canadian Collection of Fungal Cultures, Ottawa, Canada.	HQ888699	HQ888730	HQ888750
	Blastocladiella emersonii BK49-1	(Genbank)	AY635842	AY997032	DQ273808
Catenariaceae	*Catenaria uncinata* WM103B	Isolated from *Glyptotendipes lobiferus* egg masses, Hanover School for Boys Lake, Hanover County, VA, USA, Oct. 5, 2008 by W. Martin.	HQ888700	–	HQ888751
	Catenaria uncinata WM103	Isolated from *Glyptotendipes lobiferus* egg masses, Hanover School for Boys Lake, Hanover County, VA, USA, Oct. 10, 2005 by W. Martin.	HQ888701	–	HQ888752
	Catenaria spinosa WM100	Isolated form *Chironomus decorus* egg masses, temporary pond, Asheville, NC, USA, Aug. 18, 2005 by W. Martin.	HQ888702	–	HQ888753
	Catenaria sp. JEL339	Isolated from soil from Athens, GA, USA by J. Longcore.	HQ888703	HQ888731	HQ888754

	Catenaria anguillulae BR105 Type species	Originally isolated by D.J.S. Barr. Obtained from the Canadian Collection of Fungal Cultures by W. Martin.	HQ888704	HQ888732	HQ888755
	Catenaria sp. WM099	Isolated from dead ostracod, Hanover School for Boys Lake, Hanover County, VA, USA. Aug. 10, 2007 by W. Martin.	HQ888705	HQ888733	HQ888756
	Catenaria sp. JEL589	Isolated from Unity Pond, ME, USA by J. Longcore.	HQ888706	HQ888734	HQ888757
	Catenophlyctis variabilis JEL298 Type species	Isolated from soil from Tempe, AZ, USA by J. Longcore.	HQ888707	AY997034	DQ273789
	Catenaria sp. Poly Ad 2-0	Isolated from cropping soils, Narrabri, NSW, Australia, 2007, by F. Gleason.	HQ888708	HQ888735	HQ888758
	Catenaria anguillulae JEL194 Type species	Originally isolated by R. Emerson from infected nematodes provided by R. Mankau, Citrus Experiment Station, University of California, Riverside, CA. Available from J. Longcore.	HQ888709	HQ888736	HQ888759
	Catenaria sp. APO4	Isolated from soil baited with cat hair. Soil from donkey/goat pen at the North Carolina Museum of Life Science, Nov. 11, 2005 by T. James.	HQ888710	HQ888737	HQ888760
Coelomomycetaceae	*Coelomomyces stegomyiae* DUH0008925	(Genbank)	AF322406	AY997038	DQ273767
	Coelomomyces quadrangulatus	Collected from *Anopheles quadrimaculatus* larvae, Fluvanna Ruritan Lake, Fluvanna County, VA, USA, Jul. 21, 2005 by W. Martin.	HQ888711	–	–
	Coelomomyces punctatus	Collected from *Anopheles quadrimaculatus* larvae, Hanover School for Boys Lake, Hanover County, VA, USA, Jul. 17, 2005 by W. Martin.	HQ888712	–	–
	Coelomomyces lativittatus	Collected from *Anopheles spp*. larvae, Cemetery Pond, Milford, PA, USA, Aug. 9, 2005 by W. Martin.	HQ888713	–	–
	Coelomycidium sp.1	Collected from *Simulium angustipes* larvae, Sweden, May 5, 2006 by P.H. Adler.	HQ888714	–	–
	Coelomycidium sp.2	Collected from *Simulium latipes* larvae, Sweden, May 5, 2006 by P.H. Adler.	HQ888715	–	–
Physodermataceae	*Paraphysoderma sedebokerensis* nom. prov.	(Genbank)	EF565163	–	–
	Physoderma dulichii BL060	(Genbank)	DQ536472	–	–
	Physoderma maydis DUH0007932	(Genbank)	AY601708	AY997072	DQ273768
	Physoderma maculare BL135 Type species	(Genbank)	DQ536489	–	–
	Physoderma lycopi	Collected from *Lycopus americanus*, Cheboygan County, MI, USA, Jul. 1, 1957 by R.M. Johns. Available from the University of Michigan Herbarium.	HQ888716		
	Urophlyctis trifolii	Collected from *Trifolium repens*, Camden, SC, USA, Apr. 10, 1944 by R.E. Atkinson. Available from the University of Michigan Herbarium.	HQ888717		
	Urophlyctis pulposa ex. *Atriplex* Type species	Collected from *Atriplex patula*, Ann Arbor, MI, USA by Y. Lingappa, 1958. Available from the University of Michigan Herbarium.	HQ888718		
	Urophlyctis pulposa ex. *Chenopodium* Type species	Experimentally inoculated onto *Chenopodium album*, R.M. Johns, 1958. Available from the University of Michigan Herbarium.	HQ888719		

(*continued on next page*)

Table 1– (*continued*)

Taxonomic group	Sample	Source/availability	GenBank accession		
			18S	5.8S	25S
Chytridiomycota	*Catenomyces persicinus* JEL342	(Genbank)	AY635830	AY997033	DQ273789
	Chytriomyces hyalinus	(Genbank)	DQ536487	DQ536499	DQ273836
	Endochytrium spp. JEL324	(Genbank)	AY635844	AY997044	DQ273816
	Rhizophydium brooksianum	(Genbank)	AY601710	AY997079	DQ273770
	Spizellomyces punctatus	(Genbank)	AY546684	AY997092	AY546692
	Cladochytrium replicatum	(Genbank)	AY546683	AY997037	AY546688
	Gonapodya spp. JEL183	(Genbank)	AH009066	AY349112	AY349059
	Monoblepharella mexicana	(Genbank)	AF164337	AY997061	DQ273777
Ascomycota	*Emericella nidulans*	(Genbank)	U77377	AY373888	AF454167
	Saccharomyces cerevisiae	(Genbank)	GQ458028	GQ458028	GQ458028
	Schizosaccharomyces pombe	(Genbank)	Z19578	Z19578	Z19136
Basidiomycota	*Coprinopsis cinerea*	(Genbank)	M92991	AF345819	AF041494
	Puccinia graminis	(Genbank)	AY125409	AF468044	AF522177
	Ustilago maydis	(Genbank)	X62396	AY854090	AF453938
Glomeromycota	*Scutellospora heterogama*	(Genbank)	AY635832	AY997088	DQ273792
	Glomus intraradices	(Genbank)	DQ322630	AY997054	DQ273828
	Paraglomus occultum	(Genbank)	DQ322629	AY997069	DQ273827
Mucoromycotina	*Mortierella verticillata*	(Genbank)	AF157145	AY997063	DQ273794
	Endogone pisiformis	(Genbank)	DQ322628	AY997046	DQ273811
	Rhizopus oryzae	(Genbank)	AF113440		AF113481
Zygomycota	*Coemansia reversa*	(Genbank)	AF007533	AY997039	AY546689
	Rhopalomyces elegans	(Genbank)	AY635834	–	DQ273795
	Dimargaris bacillispora	(Genbank)	AB016020	AY997043	DQ273791
	Smittium culisetae	(Genbank)	AF007540	AY997089	DQ273773
	Basidiobolus ranarum	(Genbank)	AY635841	AY997030	DQ273807
Entomophthorales	*Conidiobolus coronatus*	(Genbank)	AF113418	AY997041	AY546691
Outgroup	*Nuclearia simplex*	(Genbank)	AF484687	AF484687	AY148095
	Monosiga brevicollis	(Genbank)	AF100940	–	AY026374

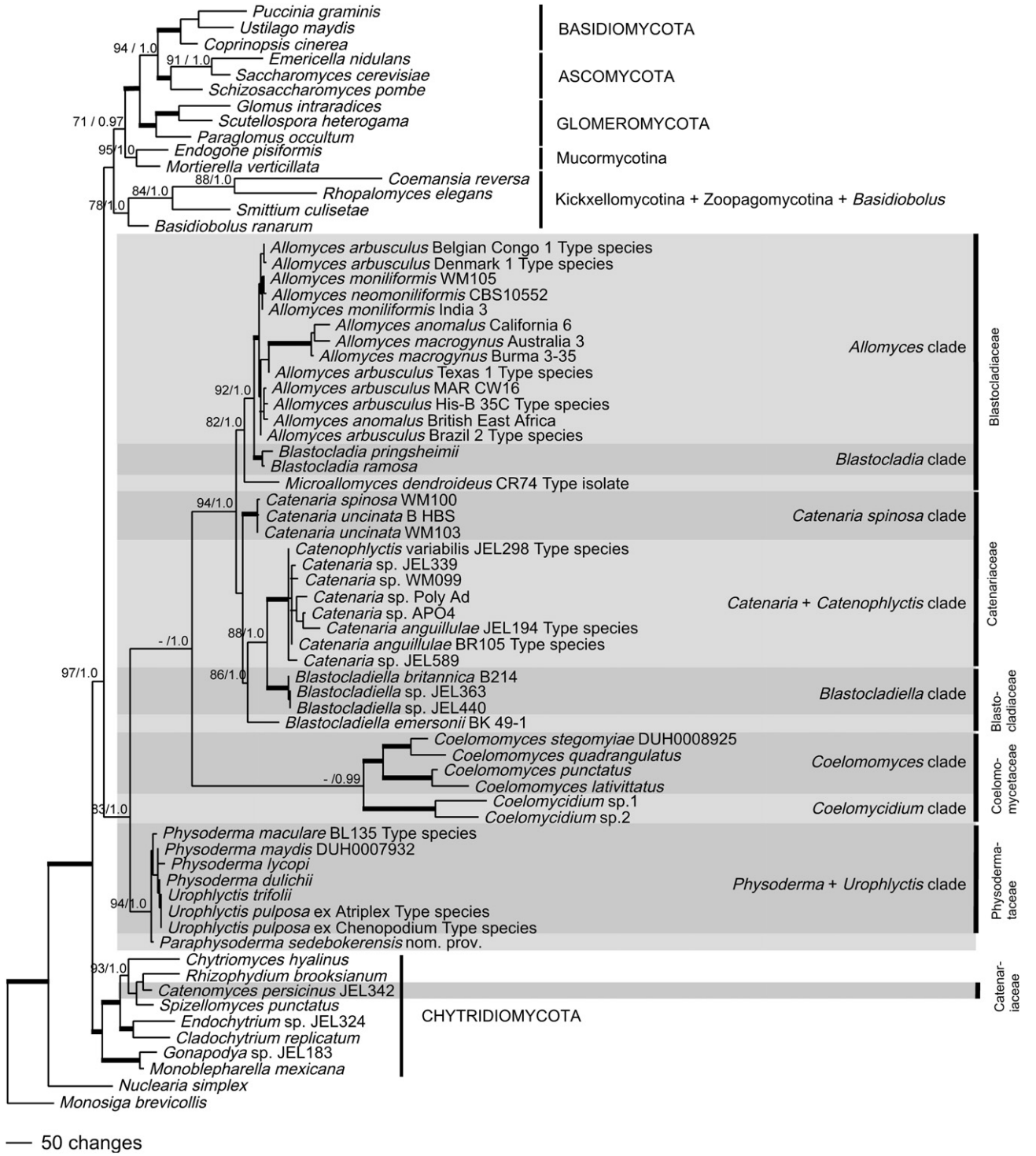

Fig 1 – Bayesian rDNA phylogeny for the *Blastocladiomycota*. The analysis included 71 taxa and 3542 included nucleotide characters from SSU + 5.8S + LSU rDNA. A MLBP equal to or greater than 98 % and BPP equal to or greater than 0.98 are shown as thickened branches. If the MLBP is equal to or greater than 70 % and BPP is equal to or greater than 0.90, the values are shown at the nodes (MLBP/BPP). A dashed line '–' indicates that this branch was not statistically supported in maximum likelihood analyses. Statistical support on short branches is omitted for clarity.

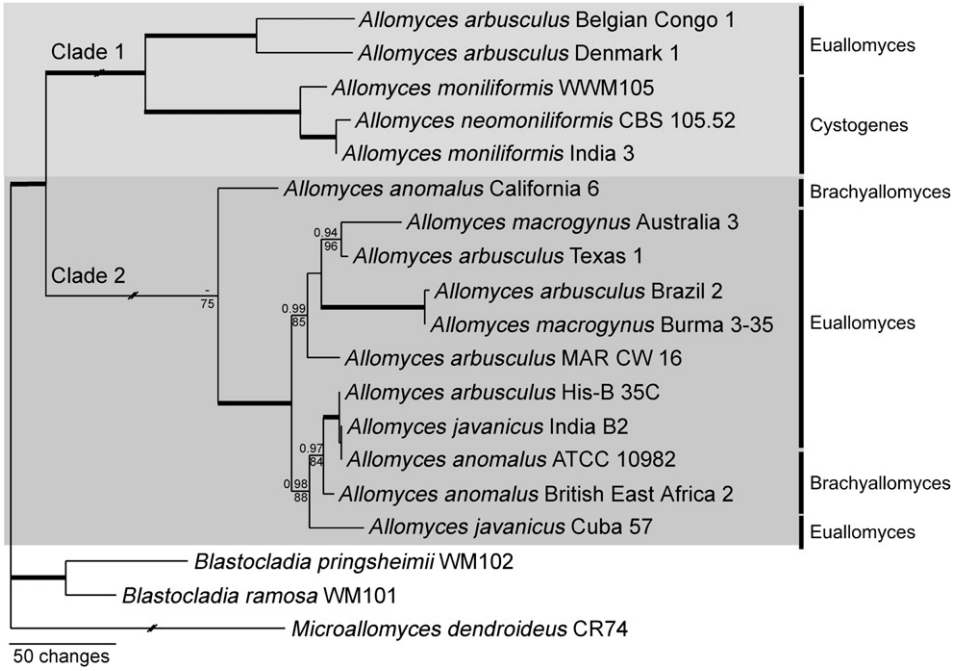

Fig 2 – Bayesian rDNA phylogeny for *Allomyces*. The analysis included 19 taxa and 3451 included nucleotide characters from SSU + 5.8S + LSU rDNA. A BPP equal to or greater than 0.98 and MLBP equal to or greater than 98 % are shown as thickened branches. If the BPP is equal to or greater than 90 % and MLBP is equal to or greater than 70 % the values are shown at the nodes. Statistical support on short branches is omitted for clarity. Long branches shortened by the length equivalent to 100 changes are indicated by two parallel lines (//).

rDNA sequence for *C. punctatus* that contains an additional 14 insertions that vary in size from about 50–2000 bp. Despite the presence of numerous introns, conserved domains could still be identified and aligned with other *Blastocladiomycota* taxa.

Specimens of the plant parasites *Physoderma* and *Urophlyctis* group together with the algal parasite *Paraphysoderma* nom. prov. with 94 % MLBP and 1.0 BPP (Fig 1). In separate analyses focused on these isolates, *Physoderma* and *Urophlyctis* appear reciprocally monophyletic with 99 % MLBP and 1.0 BPP for the *Physoderma* clade and 97 % MLBP and 0.99 BPP for the *Urophlyctis* clade (Fig 3).

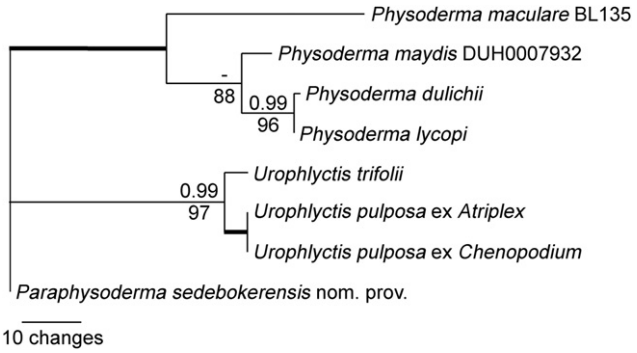

Fig 3 – Bayesian SSU rDNA phylogeny for the *Physodermataceae*. The analysis included eight taxa and 1609 included nucleotide characters from SSU rDNA. A BPP equal to or greater than 0.98 and MLBP equal to or greater than 98 % are shown as thickened branches. If the BPP is equal to or greater than 90 % and MLBP is equal to or greater than 70 % the values are shown at the nodes.

Discussion

Comparison of rDNA phylogeny with traditional taxonomy of the Blastocladiales

The *Blastocladiaceae* currently includes the genera *Allomyces*, *Microallomyces*, *Blastocladia*, *Blastocladiella*, and *Blastocladiopsis* (Karling 1977). The genera *Allomyces*, *Microallomyces*, and *Blastocladia* form a statistically well-supported clade in this family, but the *Blastocladiella* isolates grouped with the *Catenariaceae*. The type species of *Blastocladiella*, *Blastocladiella simplex* V.D. Matthews 1937, was not available for this study. Figures of *B. simplex* resemble unbranched *Allomyces* or *Blastocladia* species; however because we were not able to include *B. simplex* in our phylogeny we cannot say whether the type species is actually closely related to *Allomyces* and *Blastocladia*. Because *Blastocladiella* has been the only genus comprised of taxa with monocentric thalli in the *Blastocladiales*, some later described species with monocentric thalli may have been put in the genus in spite of lacking the strong stalked nature and *Allomyces*-like morphology of the zoosporangium of *B. simplex*. It is possible that the *Blastocladiella* spp. that grouped with the *Catenariaceae* in our study constitutes a second monocentric lineage within the phylum and may

represent a new genus. Isolates of *B. simplex* are needed to resolve this taxonomic problem. *Blastocladiopsis* is a small genus in the *Blastocladiaceae* containing *Blastocladiopsis elegans* and *Blastocladiopsis parva* that we were unable to sample in this study; they also need to be found to determine the placement of this genus.

The *Catenariaceae* is a small family with saprotrophic or facultatively parasitic members. Thallus morphology is generally polycentric, either branched or unbranched; zoosporangia and resting spores are connected by isthmuses (Karling 1977). The *Catenariaceae* traditionally includes the genera *Catenaria*, *Catenophlyctis*, and *Catenomyces* (Sparrow 1960; Karling 1965). Our rDNA phylogeny indicates that *Catenaria* is a polyphyletic genus that includes two groups: the *Catenaria* + *Catenophlyctis* clade and the *Catenaria spinosa* clade. The saprotrophic or facultative parasites in the *Catenaria* + *Catenophlyctis* clade are a well-supported group we consider as *Catenariaceae sensu stricto* because it contains the type, *Catenaria anguillulae* (Sorokin 1876). The *C. spinosa* clade clusters separately but lacks statistical support for its placement; its position within the phylogeny will require further work. *Catenaria spinosa* (Martin 1975) and *Catenaria uncinata* (Martin 1978) are united by their common ecological role as dipteran egg parasites.

The morphology of *Catenomyces persicinus* (Hanson 1945) led to confusion over its ordinal placement. Its coarse rhizomycelium and zoospores containing multiple oil globules led it to be compared with both *Nowakowskiella* and *Catenaria*. Although *C. persicinus* was originally classified in the *Chytridiales sensu* Sparrow (1943), it was reclassified in the *Catenariaceae*, *Blastocladiales* (Sparrow 1960). The genus is represented in our study by its single species. In spite of the similarity of its morphology to *Catenaria*, our analysis agrees with a previous molecular phylogeny (James *et al.* 2006) in placing this species within the *Chytridiomycota*.

The *Coelomomycetaceae* genera *Coelomomyces* and *Coelomycidium* are united by their common ecological role as obligate parasites of dipteran larvae. *Coelomomyces* spp. have an alternation of generations with the haploid stage commonly in copepods and the diploid stage typically in mosquitoes and *Coelomycidium* spp. are parasitic in black fly larvae.

We were unable to sample *Sorochytrium* in this study, and the placement of the *Sorochytriaceae* within the *Blastocladiomycota* remains uncertain. This study suggests the need for taxonomic revision of the *Catenariaceae* and *Blastocladiaceae*.

Comparison of the rDNA phylogeny and Allomyces *subgenera defined by life cycle types*

Allomyces, characterized by a branching thallus with pseudosepta (Emerson & Robertson 1974; Karling 1977), is an obligately aerobic and facultative fermenter that grows well in culture and is relatively easy to manipulate (Ingraham & Emerson 1954). Three life cycle types have been described within this genus: (1) Euallomyces (Emerson 1938); (2) Cystogenes (Emerson 1938); and, (3) Brachyallomyces (Emerson 1941). These three life cycle types are the basis for the delimitation of three subgenera with the same names (Emerson 1941). The Euallomyces life cycle has alternating haploid gametophytic and diploid sporophytic generations such as have been described for *Allomyces arbusculus*, *Allomyces macrogynus*, and *Allomyces javanicus*. These species have gametophytic and sporophytic generations that can be cultured separately and manipulated in the lab. The type species *A. arbusculus* was originally delimited from other *Euallomyces* species by having a predominance of hypogynous male gametangia (Emerson 1941). In our *Allomyces* phylogeny, *A. arbusculus* appears to be polyphletic, either because some isolates have been misidentified or alternatively, the subgenus criteria such as life cycles and morphology do not reflect rDNA phylogenetic relationships. The Cystogenes life cycle has a large, dominant, asexual sporophyte that produces thin-walled zoosporangia and resistant sporangia whereas the sexual gametophyte is a small, spherical, thin-walled cyst. Examples are *Allomyces moniliformis*, *Allomyces cystogenus*, and *Allomyces neomoniliformis* (Emerson 1938). Primary swarmers, with or without one or more flagella, are released from the resting sporangium and quickly form cysts. Most cysts produce four isogamous, uniflagellate zoospores; however, some cysts, smaller or larger than average, may produce variable numbers of isogametes in proportion with their size. These isogametes fuse in pairs forming biflagellate zoospores and the biflagellate zoospores develop into asexual thalli (McCranie 1942; Teter 1944). In our *Allomyces* phylogeny, only members of the *Cystogenes* subgenus form a monophyletic group. The Brachyallomyces life cycle, also called short-cycled, has no gametophytic or sexual thalli (e.g., *Allomyces anomalus*). Emerson (1941) assigned isolates that never produced a gametophyte stage and reproduced only asexually to subgenus *Brachyallomyces*. Emerson suggested that the subgenus may become invalid if future researchers were able to induce sexual reproduction in the isolates identified as *A. anomalus*. Later, it was demonstrated that only mitosis occurs in the resistant sporangia of *A. anomalus* and meiosis is excluded from the life cycle (Wilson 1952). Because *A. anomalus* appears to be polyphyletic in our *Allomyces* phylogeny, it may be concluded that multiple independent origins of asexual reproduction have evolved within *Allomyces*. Further work is needed to resolve the *Allomyces* molecular phylogeny and possibly revise taxonomic relationships in the genus. Since Karling (1973) later followed the subgenus approach for *Blastocladiella* species with different life histories, a detailed look at the phylogeny within *Blastocladiella* is also warranted.

The Physodermataceae

The *Physodermataceae* includes two old genera, *Physoderma* Wallr. 1833 and *Urophlyctis* J. Schröt. 1886. For comparison, *Chytridium olla*, the first 'chytrid' (Braun 1851; Braun 1855), was described nearly 20 y after *Physoderma maculare*. *Physoderma* was historically confused with basidiomycete rust and smut fungi, and even with *Protomyces*, a basal ascomycete with similar host symptoms and spore colour (Karling 1950). *Physoderma* is distributed worldwide and contains parasites of a broad range of aquatic, semi-aquatic, and wetland angiosperms and ferns. Endobiotic infections produce galls, discolourations, streaks and pustules on the host (Karling 1950). The morphology of members of these two genera usually consists of an epibiotic, monocentric thallus that produces zoospores (? gametes) and an endobiotic polycentric thallus that produces thick-walled RS within the host plant. These two stages have not

unambiguously been shown to be haploid and diploid stages. The *Physoderma* and *Urophlyctis* clade shown in Fig 1 shows that the *Physodermataceae*, is a monophyletic group with a newly discovered parasite on green algae provisionally referred to as *Paraphysoderma sedebokerensis* (Hoffman *et al.* 2008; Gutman *et al.* 2009). The two species of *Urophlyctis* sampled, including the type species *U. pulposa* (Wallr.) J. Schröt, group separately from the *Physoderma* isolates, including the type, *P. maculare*, but in our focused analysis (Fig 3) the relationship between *Urophlyctis* and *Physoderma* species remains unclear based on SSU rDNA alone. The taxonomic distinction between *Urophlyctis* and *Physoderma* has been debated in the literature. Although Sparrow (1962) recognized both genera based on morphology plus host reaction, Karling (1950) monographed the genus *Physoderma*, placing *Urophlyctis* into synonymy with this genus while acknowledging the controversy in this union. The two genera are now considered synonymous (Karling 1977; Kirk *et al.* 2008). The inability to diagnose these genera is related to the difficulty in observing microscopic structures inside, and on, their obligate vascular plant hosts and the limited number of detailed observations of *Urophlyctis* spp. Additional sampling within the *Physodermataceae* will be needed to determine whether *Urophlyctis* and *Physoderma*, as previously circumscribed, are useful taxonomic distinctions.

Since the algal parasite *Paraphysoderma sedebokerensis* is sister to the remaining vascular plant parasites included in the *Physoderma* clade, it is not possible to determine whether the wall-less, flagellum-lacking propagule of *P. sedeborkerensis* or the flagellated propagules produced by *Physoderma* species are more similar to the ancestor of the *Physoderma* clade (Hoffman *et al.* 2008; Gutman *et al.* 2009). However, its ability to grow in pure culture and its position as a relative of plant parasites make it a potentially interesting organism to study genes associated with parasitism.

Major findings and directions for future work

We have provided the most extensive phylogeny of the *Blastocladiomycota* to date by sampling rDNA for 11 of the 14 genera, including multiple isolates for many. Our analyses support the monophyly of most genera and are consistent with some of the family level taxonomy. The genera *Blastocladiella* and *Catenaria* are not strictly monophyletic but together form a clade. Our analyses revealed two groups of *Catenaria* species distinguished primarily by their ecology, a large monophyletic group of saprotrophs and facultative parasites as well as a midge parasite group. Zoospore ultrastructure for taxa in the *Catenaria spinosa* clade is lacking but might help determine their taxonomic placement. Additionally, the abundance of these taxa in nature and the ability to grow these isolates in culture would seem to make this group a good model system for studying the effect of fungal parasites on insect larvae.

Coelomomyces and *Coelomycidium* taxa were on relatively long branches. Many *Coelomomyces* species have numerous large insertions in their rDNA sequences. It is possible that these unique sequences could be used to facilitate species identifications based on rDNA. Because over 65 species and 29 named varieties of *Coelomomyces* exist (Couch & Bland 1985; Kirk *et al.* 2008), it would be worthwhile to sample additional taxa to try to break up these long branches. Based on their medical and ecological importance as parasites of mosquitoes, further molecular phylogenetic work with this group is warranted and hypotheses regarding the influence of pathogens on mosquito larvae populations should be tested.

Morphological characters, and a relatively short branch length leading to the *Physodermataceae*, suggest that these extant taxa may more closely resemble the most recent common ancestor of the phylum and features in this clade may provide insight into the origins of the *Blastocladiomycota*. One obvious difference between the *Physodermataceae* and other families is the obligate parasitism of plants. All other blastocladian groups are saprotrophs or animal parasites. The presence of thallus ultrastructural characters such as the dictyosome-type of Golgi apparatus found in the *Chytridiomycota* and reported in *Physoderma maydis* (Lange & Olson 1980a), but not in other members of the *Blastocladiaceae* suggests that this trait was found in the most recent common ancestor of *Blastocladiomycota* (Lange & Olson 1980a). The relatively large size of the lipid globule in *Physoderma* is a feature that is coincident with the *Chytridiales* (*sensu* Sparrow) and might suggest that this is an ancestral character state for zoosporic fungi in general (Sparrow 1960). Though a review of zoospore ultrastructural characters goes beyond the scope of this paper, these characters seem to work well to define groups within the *Blastocladiomycota* and additional sampling of ultrastructural characters may help further define the *C. spinosa* clade.

More work is required to flesh out the phylogeny for the *Blastocladiomycota*, such as inclusion of the unsampled taxa *Blastocladiopsis*, *Sorochytrium*, and *Polycaryum*. Additionally, placement of the *Blastocladiomycota* among the other early diverging fungal lineages will require sampling of molecular data better suited to resolving these deeper nodes. Until now, no other study has included as many taxonomically diverse isolates into a single *Blastocladiomycota* phylogeny. This is the first work to combine historical isolates as well as new strains of uncultivable or difficult to cultivate isolates and should provide a solid basis for further molecular, ecological, and morphological studies.

Acknowledgements

This work was supported by the AFTOL grant NSF DEB-0732984. JEL was supported by NSF grant DEB-0529694. PML was supported by NSF grant DEB-0732599.

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