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Revisiting the aquatic phycomycete biota of the Douglas Lake region since the time of Dogma and Sparrow

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Abstract

Fredrick K. Sparrow and his students, including Irineo J. Dogma, conducted many surveys of the aquatic phycomycete biota of the Douglas Lake region in Michigan, United States of America. Following the tradition of Sparrow and Dogma, we undertook an inventory of the aquatic phycomycete biota of the Douglas Lake region with an emphasis on Chytridiomycota. Cognizant of the difficulties of relying solely on light microscopy, we used a combination of light microscopy, culturing, and single cell techniques. We observed a total of 42 taxa. We successfully cultured Terramycetaceae sp., *Physocladia obscura*, and *Rhizoclosmatium globosum*. Using single cell techniques, we obtained molecular sequence data for *Rhopalophlyctis sarcoptoides*, *Rhizophydium echinocystoides* and an unidentified parasite of *Desmidium*. Our inferred maximum likelihood phylogeny placed *Rhopalophlyctis sarcoptoides* in the Chytridales as sister to *Chytriomyces hyalinus*. *Rhizophydium echinocystoides* was placed in the Rhizophydiales but did not group with the type of the genus. The unidentified parasite of *Desmidium* surprisingly grouped with members of *Synchytrium*. Our results provide a pilot study for demonstrating how light microscopy, culturing, and single cell techniques to obtain molecular sequences of chytrid taxa could be used to create a local aquatic fungal inventory based on molecular techniques, discover novel taxa, and potentially revise current taxonomy.

Keywords: Chytridiomycota, single cell, Smith's Fen, Bryant's Bog

Introduction

The Douglas Lake region in northern Michigan, United States of America (USA) is one of the most well studied areas in terms of aquatic phycomycete diversity. Surveys in this area began in 1952 with F. K. Sparrow and continued into the 1970s with his students, including Irineo J. Dogma. Historically, aquatic phycomycetes included lineages no longer recognized as Fungi, such as oomycetes. Today, the fungal aquatic phycomycetes are spread across 5 phyla: Blastocladiomycota, Chytridiomycota, Cryptomycota/Rozellomycota, Neocallimastigomycota, and Zoopagomycota. Counting only Fungi, 130 taxa have been recorded from the Douglas Lake region (Table S1) with quite a few undescribed species awaiting rediscovery (e.g., the *Physoderma* spp. in Sparrow 1961). Irineo J. Dogma, himself, observed 13 taxa from the region (Table S1) and first described *Irineochytrium annulatum* (Dogma) Letcher, Longcore, & M. J. Powell as *Chytriomyces annulatus* (Dogma 1969) and *Arkaya serpentina* (Dogma) Longcore & D. R. Simmons as *Rhizophlyctis serpentina* (Dogma 1973).

During their time, Sparrow and his students relied on light microscopy and baiting and focused mainly on chytrid fungi. However, most chytrid species lack sufficient morphological distinction for accurate identification due to both convergent and plastic morphology (Miller 1968; Longcore 2005; Powell and Koch 1977). More recent taxonomic and ecological studies of chytrids have used culture-based methods (e.g., Booth 1971; Barr 1986, 1989; Letcher et al. 2006, 2008; Longcore 2004, 2005; Jerônimo et al. 2015). Using zoospore ultrastructural characters and rDNA sequences from cultured strains, researchers found cryptic phylogenetic diversity within common morphological species, such as *Rhizophlyctis rosea* (Barr and Hartmann 1977; Barr and Désaulniers 1986; Letcher et al. 2008a), the *Entophlyctis variabilis* species complex (Booth 1971; Powell and Koch 1977; Longcore et al. 1995; Simmons 2011), and *Chytriomyces hyalinus* (Davis et al. 2013). However, culture-based methods are limited to chytrids capable of growing under laboratory conditions (Lozupone and Klein 2002; Davis et al. 2016). Culture-free methods, such as

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environmental cloning and next generation sequencing, can detect more chytrid taxa than culture-based methods (e.g., Lèfevre et al. 2012; Monchy et al. 2011). However, since molecular databases, such as GenBank, are largely populated with sequences derived from culturable chytrids, culture-free methods are limited in their ability to confidently identify lineages that are unculturable or difficult to culture (Davis et al. 2018). A promising approach is the use of single cell genomic techniques to obtain whole genome data for taxa that are not easily cultured.

For example, Ishida et al. (2015) isolated colonies of chytrid infected diatoms through a series of micro-washes before using the HotSHOT method for DNA extraction and PCR. The results were the discovery of novel phylotypes in currently described orders and three completely novel clades that potentially represent undescribed orders (Ishida et al. 2015). However, they did not analyze morphology; so, it cannot be determined if these are novel taxa or described taxa for which we do not have sequence data. Additionally, the level of phylogenetic information of these uncultured lineages needs to be increased beyond single gene sequences in order to produce robust phylogenies, and single cell genomic methods would provide an opportunity to do so (Ahrendt et al. 2018). Ahrendt et al. (2018) successfully lysed cells of *Blyttiomyces helices* and *Caulochytrium protosteliodes*, chytrids that currently cannot be axenically cultured, and amplified and sequenced the genomic DNA for use in whole genome phylogenetic analysis. Davis et al. (2019a) lysed cells of currently unculturable taxa in Zoopagales; they used multiple displacement amplification (MDA, Gawad et al. 2016) to amplify the whole genome before sequencing the nuc 18S rDNA (18S) for phylogenetic analysis. The benefit to a single cell approach is that taxa that are identified by 18S sequences as phylogenetically interesting can be subjected to whole genome sequencing (Davis et al. 2019b).

We inventoried the aquatic phycomycete biota of the Douglas Lake region in 2016 and 2017 with a focus on Chytridiomycota. We combined light microscopy, culturing, and single cell genomic techniques with the goal of obtaining sequences of known but hitherto unculturable taxa. We observed 42 taxa, cultured 3 taxa, and successfully obtained sequence data for 3 previously uncultured taxa using single cell techniques.

Materials and Methods

Collection of samples

In 2016, we sampled water and decaying vegetable matter from Bryant's Bog (45.566851 -84.711864), Smith's Fen (45.553126 -84.648285), and Carp Lake (45.682832, -84.733608) of the Douglas Lake region of Michigan (Cheboygan County). Samples were transported on ice to the University of Michigan (Ann Arbor, Michigan, USA) and monitored for 1 month. In 2017, we collected samples of water, vegetable matter, and insect exuviae from Bryant's Bog, Smith's Fen, Carp Lake, Mud Lake bog (45.606983, -84.595539), Hebron Swale, Lille Pond, and Daly pond (45.625, -84.871). Samples were stored on ice and transported to the University of Michigan Biological Station (Pellston, Michigan, USA) for processing. Phytoplankton, vegetable matter, and insect exuviae were examined using compound and stereomicroscopes. Observed fungi were photographed using a DinoCapture camera. Fungi were then processed either for single cell genomic techniques or culturing. Additional samples were transported to the University of Michigan and monitored for 1 month; observed fungi were photographed using a Zeiss AxioCam MRc camera. Images were edited and assembled into plates in GIMP 2.10 and Adobe Photoshop. Taxa were grouped into plates according to whether we attempted to obtain sequences using single cell techniques (FIG. 1), brought strains into culture (FIG. 2), or only observed them (FIGS. 3–4).

Single cell amplification techniques

We used multiple displacement amplification (MDA) to generate molecular data from single to few cells (Gawald et al. 2016). We placed fungal cells into 2 µL of UV-sterilized PBS buffer in PCR reaction tubes using either an insect needle or a dental file (Henry Schein Inc., Melville, New York, USA). PCR tubes were transported to the University of Michigan on dry ice and stored at −20 C until processed. We used half reactions of the Qiagen REPLI-g Single Cell kit (Qiagen, Maryland, USA) to extract and amplify genomic DNA (Davis et al. 2019b). MDA products were diluted 1:100. The nuc 18S rDNA (18S) was amplified using the SR1R/NS4 primer pair (Vilgalys and Hester 1990; White et al. 1990), and the nuc 28S rDNA (28S) was amplified using the LROR/LR5 primer pair (Vilgalys and Hester 1990; Rehner and Samuels 1994). Amplicons were sequenced at the University of Michigan Sequencing Core (https://seqcore.brcf.med.umich.edu/) and assembled into a contiguous sequence in SEQUENCHER 5.3. Sequences were deposited in GenBank (MH933965–MH933970).

Baiting and culturing from samples

A subset of the samples was placed in sterile glass jars, and we added sterile pollen, cellulose (onion epidermal cells), keratin (snake skin and human hair), chitin (shrimp exoskeleton) and sesame seeds. We examined baits periodically as wet mounts using a Zeiss Imager.A2 compound microscope, and

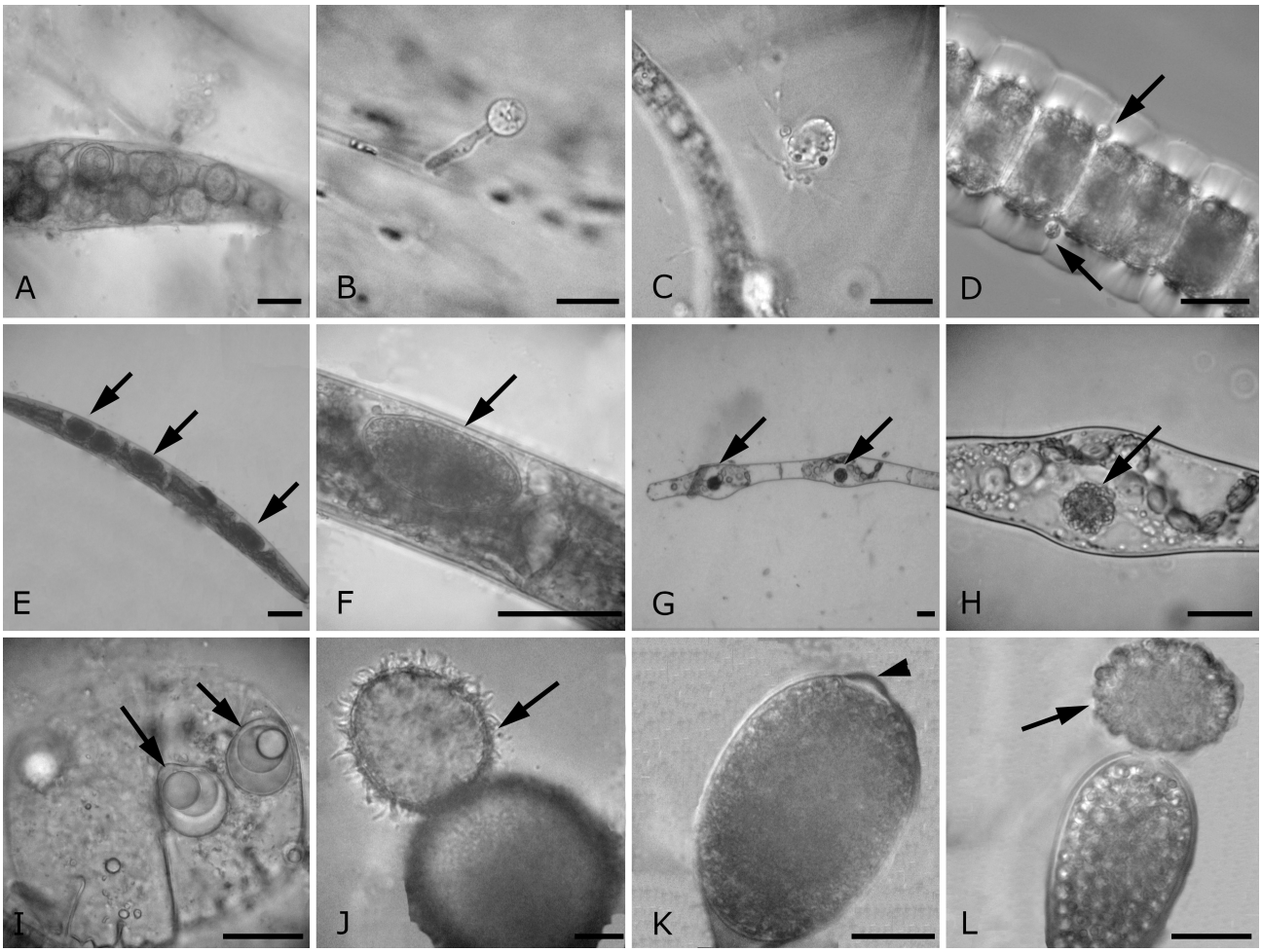

Figure 1. Taxa targeted for sequencing via single cell techniques. Images were edited in Gimp. Unless otherwise indicated, digital editing included converting to grayscale and adjusting brightness and contrast. Scale bars = 20 µm. A. *Ancylistes closterii* resting spores in *Closterium*. B. Germinating zoospore of *Coenomyces* sp. in the mucus surrounding a colony of *Gleotrichia*. C. Maturing zoosporangium of *Coenomyces* sp. embedded in the mucus surrounding a colony of *Gleotrichia*. D. Maturing chytrid zoosporangia (arrows) intercalary on a colony of *Desmidium*. E. Resting spores (arrows) of *Micromyces mirabilis* inside *Closterium*. F. Close-up of a resting spore (arrow) of *M. mirabilis*. G. Resting spores (arrows) of *Micromyces zygonii* inside a filament of *Mougeotia*. H. Close-up of a resting spore (arrow) of *M. zygonii*. I. *Olpidium* sp. resting spores (arrows) within *Micrasterias*. J. Zoosporangium of *Rhizophydium echinocystoides* (arrow) on pollen. K. Mature zoosporangium of *Rhopalophlyctis sarcoptoides* with a visible operculum (arrow). L. *Rhopalophlyctis sarcoptoides* releasing zoospores into a vesicle (arrow).

fungi were photographed using a Zeiss AxioCam MRc. Infected substrates were aseptically removed and placed on PmTG (Barr 1986: 1 g peptonized milk, 1 g tryptone, 5 g glucose, and 8 g agar per liter of water) or corn meal agar amended with glucose (16 g corn meal agar and 2.5 g glucose per liter of water). Strains were brought into pure culture through the aseptic transfer of zoospores or rhizomycelium onto fresh nutrient agar.

Extraction and sequencing of cultures

Strains were grown on cellulose overlaid on nutrient agar, and DNA was extracted using a modified CTAB extraction (James et al. 2008). Briefly, fungal cells were ground with sand in 2x CTAB. Two extractions of chloroform removed cellular debris. DNA was precipitated with isopropanol, cleaned with 70% ethanol, and resuspended in TE buffer. DNA was diluted 1:25 for PCR. The 28S was amplified using the LROR/LR5 primer pair (Vilgalys and Hester 1990; Rehner and Samuels 1994). Amplicons were sequenced at the University of Michigan Sequencing Core (https://seqcore.brcf.med.umich.edu/) and assembled into a contiguous sequence in SEQUENCHER 5.3. Sequences were deposited in GenBank (MH919298-MH919300).

Phylogenetic analyses

We downloaded reference 18S and 28S sequences of Chytridiomycota from GenBank with a focus on strains and taxa

observed and/or isolated from the region. We also included previously unpublished sequences representing strains Joyce E. Longcore isolated from the region (GenBank: MK228863-MK228866). The 28S and 18S loci were aligned separately using the automatic function in MAFFT 7 (Katoh et al. 2002; Katoh et al. 2017) and filtered using GBLOCKS 0.91b (Castresana 2000). For the 18S alignment, the minimum number of sequences and the number of flanking positions was set to 28, the maximum number of contiguous non-conserved positions was set to 8, the minimum block length was set to 5, and gaps were allowed. For the 28S alignment, the minimum number of sequences and the number of flanking positions was set to 28, the maximum number of contiguous non-conserved positions was set to 8, the minimum block length was set to 5, and gaps were allowed. The alignments were concatenated in SEAVIEW 4.7 (Gouy et al. 2010). A maximum likelihood tree was inferred with RAXML 8.2.8 (Stamatakis 2006) under the GTR + gamma model of sequence evolution with rapid bootstrapping (Stamatakis et al. 2008) and rooted with representatives of Monoblepharidomycetes.

Results and Discussion

Diversity observed

We observed a total of 42 taxa (FIGS. 1–4). Thirty-six taxa belonged to Chytridiomycota with both classes—Chytridiomycetes and Monoblepharidomycetes—represented. Two taxa belonged to Blastocladiomycota, and four taxa belonged to Zoopagomycota. Counting only taxa we identified to the species level, we observed 23 of the taxa previously recorded from the area. Sixteen taxa were observed from Bryant's Bog; 11 taxa were observed from Smith's Fen; six taxa were observed from Carp Lake; four taxa were observed from Daly Pond; three taxa were observed from Mud Lake Bog; and three taxa were observed from other localities (Table 1). Twenty-six of the taxa we observed were saprobic; 10 were parasites; one was a predator (*Zoophagus insidians*); and five could not clearly be labelled a saprobe or a parasite. We targeted eight taxa for sequencing via single cell techniques (FIG. 1) but successfully obtained sequence data for only three of those taxa. We were successful in obtaining sequences from a single zoosporangium of *Rhizophydium echinocystoides* (isolate B8), two zoosporangia of *Rhopalophylctis sarcoptoides* (isolates B9 and B10), and multiple zoosporangia infecting a colony of *Desmidium* (isolate B25). We successfully cultured three taxa (FIG. 2): Terramycetaceae sp. GHJ027, *Physocladia obscura* GHJ029, and *Rhizoclosmatium globosum* GHJ028. We observed an additional 31 taxa (FIGS. 3–4).

Taxa targeted for single cell techniques

Ancylistes closterii, Zoopagomycota FIG. 1A
Resting spores observed in *Closterium* sp. from Mud Lake Bog June 2017 (https://www.inaturalist.org/observations/6747943). Sparrow often observed this species at Bryant's Bog (Sparrow 1952; Sparrow and Koch 1959). We attempted to obtain sequence data for this species using single cell techniques but were unsuccessful.

Cyanobacteria parasite, Chytridiomycota FIG. 1B–C
Growing in the gelatinous sheath of a planktonic cyanobacteria tentatively identified as *Gloeotrichia* sp. collected from Daly Pond (https://www.inaturalist.org/observations/6757883) and Carp Lake (https://www.inaturalist.org/observations/6747944) June 2017. We attempted to obtain sequence data for this species from Daly Pond using single cell techniques but were unsuccessful.

The zoosporangia we observed were ellipsoid, measuring approximately 10 µm by 20 µm, with a single rhizoidal axis. The zoosporangia were embedded in the gelatinous sheath around a cyanobacteria colony without penetrating the host cells. This partially matches the description of *Coenomyces*, a monotypic genus erected for *C. consuens*, a chytrid with ellipsoidal zoosporangia that grew in the gelatinous sheaths of marine *Calothrix* spp. and *Rivularia* spp. without penetrating the host cells (Sparrow 1960). However, *C. consuens* zoospores produce two oppositely directed germ tubes that give rise to an irregularly septated rhizomycelium with orange zoosporangia (Sparrow 1960). The chytrid observed in this study produced a single germ tube (FIG. 1B) and was monocentric; therefore, it is unlikely the observed chytrid is a member of *Coenomyces*. Additionally, the morphology of the observed chytrid differs significantly from other chytrid taxa that parasitize cyanobacteria. For example, *Rhizosiphon* produces two thick rhizoids that penetrate multiple cells of the host; *Rhizophlyctis mastigotrichis* has larger (40 µm diameter) zoosporangia with ornamentation around the apex; *Rhizophlyctis tolypotrichis* is papillate; *Chytridium cornutum* has ornamentation on the zoosporangia; *Rhizophydium microsporum* has larger (30–50 µm diameter) sporangia (Sparrow 1960). Thus, we likely have observed a novel taxon and further data will be needed to describe it.

Desmidium parasite, Chytridiomycota FIG. 1D
Growing intercalary on filaments of *Desmidium* in water collected from Smith's Fen June 2017 (https://www.inaturalist.org/observations/6958469). We were successful at obtaining sequences for this chytrid using single cell techniques

Figure 2. Taxa for which strains were brought into pure culture. Images were edited in Gimp. Unless otherwise indicated, digital editing included converting to grayscale and adjusting brightness and contrast. Scale bars = 20 µm. A. Terramycetaceae sp. GHJ027 zoosporangia (arrows) on pine pollen. B. *Physocladia obscura* GHJ029 growing on onion skin bait. C. Zoosporangium (arrow) of *Rhizoclosmatium globosum* GHJ028 on chitin bait.

(GenBank: 18S—MH933965).

Our maximum likelihood phylogeny places this chytrid in a clade with the genus *Synchytrium* with 100% bootstrap support (FIG. 5). The phylogeny suggests our parasite on *Desmidium* is sister to *Synchytrium*, but that branch is poorly supported (37% bootstrap support). Until recently, *Synchytrium* comprised endobiotic, holocarpic species largely parasitic on higher plants, though some authors (e.g. Karling 1953) included *Micromyces*, an endobiotic, holocarpic genus parasitic on green algae, in *Synchytrium*. Longcore et al. (2016) recently described *Synchytrium microbalum*, a species that is apparently epibiotic, eucarpic, and saprobic. It will be interesting to see where *Micromyces* places relative to our *Desmidium* parasite and *Synchytrium*.

Micromyces mirabilis, Chytridiomycota FIG. 1E–F
Growing in *Closterium* cells collected from Smith's Fen June 2017. Sparrow and Koch (1959) and Sparrow et al. (1965) reported this species from Bryant's Bog. We attempted to obtain sequence data for this species using single cell techniques but were unsuccessful.

Micromyces zygononii, Chytridiomycota FIG. 1G–H
Growing in *Mougeotia* filaments collected from Daly's Pond June 2017 (https://www.inaturalist.org/observations/6757884). Sparrow (1952) reported this species in *Mougeotia* from Smith's Fen. We attempted to obtain sequence data for this species using single cell techniques but were unsuccessful.

Olpidium sp., incertae sedis FIG. 1I
Growing within *Micrasterias* and *Cosmarium* collected from Bryant's Bog (https://www.inaturalist.org/observations/6724290) and *Euastrum* collected from Smith's Fen June 2017. *Olpidium endogenum*, the type of the genus, and *O. utriculiforme* both were found in the region and parasitize similar hosts (Sparrow and Barr 1955; Sparrow and Koch 1959), but since we only observed resting spores, we were not able to determine which species we observed. We attempted to obtain sequence data for this species using single cell techniques but were unsuccessful.

Rhizophydium echinocystoides, Chytridiomycota FIG. 1J
Growing on pollen in Bryant's Bog June 2017 (https://www.inaturalist.org/observations/6724372). Dogma (1969) observed this species in soil collected from the region. We were successful at obtaining sequences for this chytrid using single cell techniques (GenBank: 18S—MH9333966; 28S—MH933969). In our maximum likelihood phylogeny, *R. echinocystoides* is sister to Terramycetaceae with 100% bootstrap support (FIG. 5). Adjusting for differences in length, our sequence of *R. echinocystoides* is 91% similar to the type strain of *Boothiomyces macrosporosum* PLAUS021.

Rhopalophlyctis sarcoptoides, Chytridiomycota FIG. 1K–L
Growing on mayfly exuviae collected from Carp Lake June 2017. We were successful at obtaining sequences for this chytrid using single cell techniques (GenBank: isolate B9, 18S—MH933968; isolate B10, 18S—MH933967; isolate B10, 28S—MH933970). First report of this chytrid from the region. In our maximum likelihood phylogeny, *Rhopalophlyctis sarcoptoides* is sister to *Chytriomyces hyalinus* with 100% bootstrap support (FIG. 5).

Taxa cultured

Terramycetaceae sp, Chytridiomycota FIG. 2A
Observed on snake skin bait placed in water collected from Bryant's Bog, June 2017. Brought into pure culture as strain GHJ027 (GenBank: 28S—MH919300). Strain GHJ027 was tentatively identified as *Boothiomyces macroporosum* based on

morphology. However, members of Rhizophydiales in general and the Terramycetaceae clade often overlap morphologically and require zoospore ultrastructure and molecular sequences to accurately identify (Letcher et al. 2006, 2008a, 2008b). In our phylogenetic tree, GHJ027 is sister to a clade containing the types of *Boothiomyces macroporosum* (PLAUS021) and *Terramyces subangulosum* (PL003) with 72% bootstrap support (FIG. 5). Adjusting for differences in length, strain GHJ027 is 97.7% similar to the type of *Boothiomyces macroporosum* (PLAUS021), 98.6% similar to the types of *Terramyces subangulosum* (PL003), and 99.2% similar to strain ARG040, which is listed as *Boothiomyces* sp. in Letcher et al. (2008a).

Physocladia obscura, Chytridiomycota FIG. 2B
Growing on onion skin bait placed in water from Bryant's Bog June 2017. Brought into pure culture as strain GHJ029 (GenBank: 28S—MH919298). Strain GHJ029 was placed in a well-supported clade (100% bootstrap support) with strains JEL137 and JEL139 (FIG. 5). Strain JEL137 is listed as *Physocladia obscura* in previous molecular phylogenies (e.g., James et al. 2006; Vélez et al. 2011) and strain JEL139 is listed as *Physocladia obscura* in GenBank. Adjusting for differences in sequence length, GHJ029 is 100% similar to JEL137 and 98% similar to JEL139.

Rhizoclosmatium globosum, Chytridiomycota FIG. 2C
Growing on onion skin bait placed in water from Lille Pond June 2017. Brought into pure culture as strain GHJ028 (GenBank: 28S—MH919299), which forms a well-supported (100% bootstrap support) clade with *Rhizoclosmatium globosum* strain JEL347h (FIG. 5).

Other taxa observed

Allomyces sp., Blastocladiomycota FIG. 3A
Growing on a sesame seed baited in soil collected from a temporary pond August 2016 located near the southeast coast of Carp Lake, Michigan, USA (https://www.inaturalist.org/observations/4928937).

Asterophlyctis sp., Chytridiomycota FIG. 3B
Observed growing on a chitin bait placed in water collected from Smith's Fen July 2016 (https://www.inaturalist.org/observations/3900584). On two separate occasions, Sparrow and his students reported *Asterophlyctis sarcoptoides* from the region (Sparrow 1952; Sparrow et al. 1965; Dogma 1969). However, using strain JEL186 isolated from Douglas Lake, Letcher et al. (2018) described *Asterophlyctis michiganensis* and separated it from *A. sarcoptoides* based on differences in development, morphology, and molecular sequence divergence. Without a pure culture to track development and obtain sequence data, we are not able to determine to which species our observation belongs.

Blyttiomyces helicus, Chytridiomycota FIG. 3C
Observed on pine pollen at Bryant's Bog (https://www.inaturalist.org/observations/6720565) and Mud Lake Bog June 2017. It is commonly observed in the region (Sparrow and Barr 1955; Sparrow and Koch 1959; Sparrow and Lange 1977).

Chytriomyces appendiculatus, Chytridiomycota FIG. 3D
Growing on chitin bait placed in water collected from Smith's Fen June 2017. This species has been previously observed growing on chitin baits from this locality (Sparrow and Koch 1959; Dogma 1969).

Chytriomyces hyalinus, Chytridiomycota FIG. 3E
Growing on the exoskeleton of a microcrustacean collected from Mud Lake Bog June 2017. This species is commonly observed growing on chitin substrates in the region (Sparrow and Koch 1959; Sparrow et al. 1965; Sparrow and Lange 1977).

Dangeardia sp., Chytridiomycota FIG. 3F
Growing on an unidentified algal cell in water collected from Daly Pond June 2017 (https://www.inaturalist.org/observations/6757885).

Sparrow and Barr (1955) described *Dangeardia laevis* growing on *Gleodinium* from Smith's Fen. They distinguished it from *Dangeardia mammillata* based on larger size, the lack of ornamentation on the resting spore, lack of sexuality, and the host algae (Sparrow and Barr 1955). Since we cannot identify the algal host, we did not observe resting spores, and we could not obtain molecular sequences to compare it to those available for *D. mammillata* (Van den Wyngaert et al. 2018), we are not able to determine to which of these two species our observation corresponds.

Diplophlyctis sp., Chytridiomycota FIG. 3G–H
Orange zoosporangia growing on onion skin bait placed in water from Smith's Fen collected June 2017. Two *Diplophlyctis* species, *D. intestina* and *D. laevis*, were reported from the Douglas Lake region (Sparrow 1962), but both produce hyaline zoospores, which suggests our isolate is a different taxon. To date, there are no described species of *Diplophlyctis* that produce orange zoospores, which leads us to believe this could be a new species. However, it is necessary to obtain more details about its morphology, development and molecular placement to test this hypothesis.

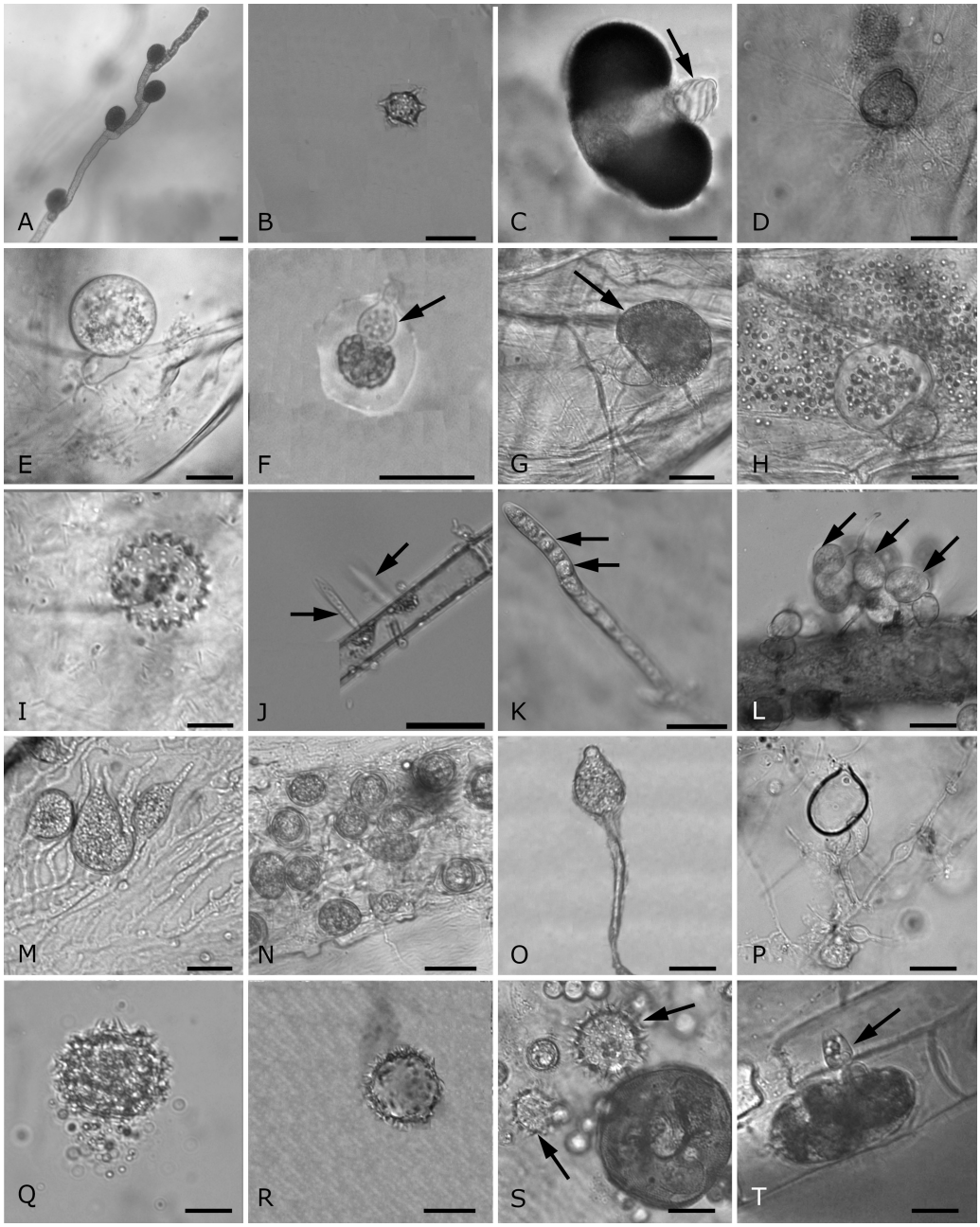

Figure 3. Other taxa observed. Images were edited in Gimp. Unless otherwise indicated, digital editing included converting to grayscale and adjusting brightness and contrast. Scale bars = 25 μm unless noted otherwise. A. *Allomyces* sp. hypha with sporangia. Scale bar = 50 μm. B. Zoosporangim of *Asterophlyctis* sp. on chitin bait. To make it easier to view the sporangium, surrounding debris was edited out of the image. C. Zoosporangium (arrow) of *Blyttiomyces helicus* on pine pollen. D. *Chytriomyces appendiculus* zoosporangium on chitin bait. E. *Chytriomyces hyalinus* zoosporangium on a microcrustacean. F. *Dangeardia* sp. zoosporangium (arrow) on an alga. To make it easier to view the sporangium, surrounding debris was edited out of the image. G. Orange zoosporangium (arrow) of *Diplophlyctis* sp. on onion skin bait. H. Zoospore release of *Diplophlyctis* sp. I. Empty zoosporangium of *Fayochytriomyces spinosus* on onion skin bait. J. Zoosporangia (arrows) of *Harpochytrium* sp. growing on moribund algal cells. To make it easier to view the sporangium, surrounding debris was edited out of the image.K. Zoosporangium of *Monoblepharis* sp. with cleaved zoospores (arrows). L. Thalli (arrows) of *Neozygites* sp. on an aphid leg. M. Zoosporangia of *Nowakowskiella multispora* on onion skin bait. N. Resting spores of *Nowakowskiella multispora* on onion skin bait. O. Maturing zoosporangia of *Nowakowskiella* sp. 1. P. Empty zoosporangium of *Nowakowskiella* sp. 2. Q. Detached zoosporangium of *Odontochytrium milleri*. R. Zoosporangium of *Phlyctochytrium aureliae* on chitin bait. S. Zoosporangia (arrows) of *Phlyctochytrium furcatum*. T. Zoosporangium (arrow) of *Phlyctochytrium planicorne*.

Fayochytriomyces spinosus, Chytridiomycota FIG. 3I

Growing on onion skin bait and *Closterium* in water from Smith's Fen collected June 2017.

Harpochytrium sp., Chytridiomycota FIG. 3J

Growing on empty cells of a filamentous green alga in water from Bryant's Bog collected June 2017 (https://www.inaturalist.org/observations/7296961).

Monoblepharis sp., Chytridiomycota FIG. 3K

Growing on twigs collected and incubated from Carp Lake June 2017. Sparrow (1952) reported *M. macrandra* and Sparrow and Barr (1955) reported *M. polymorpha* from Carp Lake, but without observing sexual reproduction, we were unable to determine to which species our observation belongs.

Neozygites sp., Zoopagomycota FIG. 3L

Parasitizing aphids infesting milkweed plants growing between the University of Michigan Lakeside Lab and Douglas Lake July 2016 (https://www.inaturalist.org/observations/3900408). First report of this genus from the region.

Nowakowskiella multispora, Chytridiomycota FIG. 3M–N

Growing on onion skin bait placed in water collected from Bryant's Bog June 2017. Species in *Nowakowskiella* are distinguished based on resting spore morphology as zoosporangia are remarkably similar across species. Our identification as *N. multispora* is based on the terminal zoosporangia and the production of spherical resting spores connected in series along the rhizomycelium.

Nowakowskiella sp. 1, Chytridiomycota FIG. 3O

Growing on onion skin bait placed in water collected from Lille Pond June 2017. Since resting spores were not observed, we cannot determine the species.

Nowakowskiella sp. 2, Chytridiomycota FIG. 3P

Growing on onion skin bait placed in water collected from Carp Lake June 2017. Since resting spores were not observed, we cannot determine the species.

Odontochytrium milleri, Chytridiomycota FIG. 3Q

Growing on pine pollen in Bryant's Bog June 2017.

Phlyctochytrium aureliae, Chytridiomycota FIG. 3R

Growing on chitin bait placed in water collected from Bryant's Bog June 2017. Sparrow and Barr (1955) also observed this species on chitin baits placed in Bryant's Bog, and it was frequently encountered in other bogs in the region (Sparrow and Koch 1959; Sparrow and Lange 1977).

Phlyctochytrium furcatum, Chytridiomycota FIG. 3S

Growing on the test of a testate amoeba in water collected from Bryant's Bog June 2017. Sparrow (1966) first described this species from another bog in the region.

Phlyctochytrium planicorne, Chytridiomycota FIG. 3T

Growing on a filamentous conjugating green alga collected from Bryant's Bog July 2016 (https://www.inaturalist.org/observations/3754158). Sparrow (1952) and Dogma (1969) often encountered this chytrid in the region.

Phlyctochytrium sp., Chytridiomycota FIG. 4A

Growing on moribund cell of *Closterium* in Smith's Fen June 2017 (https://www.inaturalist.org/observations/7001806).

Physoderma dulichii, Blastocladiomycota

Parasitizing the leaves of *Dulichium arundinaceum* at Smith's Fen September 2016 (https://www.inaturalist.org/observations/4143578).

Podochytrium sp., Chytridiomycota FIG. 4B

Parasitizing a diatom collected from Daly Pond June 2017 (https://www.inaturalist.org/observations/6757886).

Polycentric sp., Chytridiomycota FIG. 4C

Growing on onion skin bait placed in water collected from a stream on private property belonging to Buck Castillo June 2017.

Pseudorhizidium endosporangiatum, Chytridiomycota

Growing on pine pollen in water from Bryant's Bog June 2017 (https://www.inaturalist.org/observations/6724373).

Rhizophydium coronum, Chytridiomycota FIG. 4D

Growing on organic debris in water from Bryant's Bog collected June 2017 (https://www.inaturalist.org/observations/7296960).

Unknown chytrid species 1, Chytridiomycota FIG. 4E–F

Growing on chitin bait placed in water collected from Bryant's Bog June 2017. Based on the large zoosporangia, the width of the rhizoids, and the multiple axes of rhizoidial growth, it is tempting to place this observation in the genus *Rhizophlyctis*. However, chitinophilic members of this genus were moved into new genera in the Polychytriales (Longcore and Simmons 2012) so this is likely a member of that order.

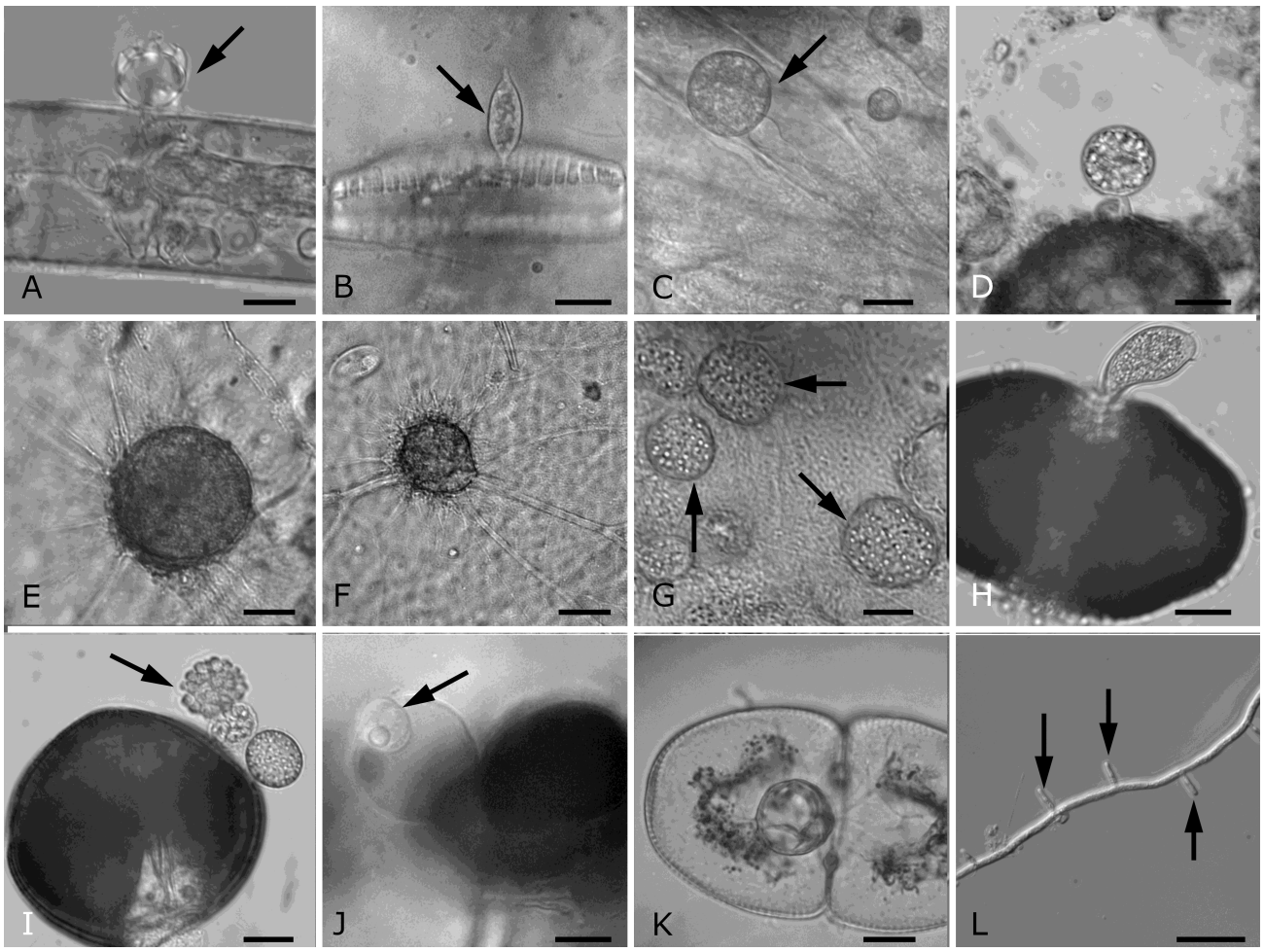

Figure 4. Other taxa observed. Images were edited in Gimp. Unless otherwise indicated, digital editing included converting to grayscale and adjusting brightness and contrast. Scale bars = 25 µm unless noted otherwise. A. Empty zoosporangium (arrow) of *Phlyctochytrium* sp. To make it easier to view the sporangium, surrounding debris was edited out of the image. B. Zoosporangium (arrow) of *Podochytrium* sp. on a diatom. C. Zoosporangium (arrow) of polycentric sp. D. Zoosporangium of *Rhizophydium cornum* on debris with characteristic clearing caused by the mucus sheath. E. Zoosporangia of unknown chytrid sp. 1. F. Zoosporangia and rhizoids of unknown chytrid sp. 1. Scale bar = 50 µm G. Mature orange zoosporangia (arrows) with cleaved zoospores of unknown chytrid sp. 2. H. Zoosporangium of unknown chytrid sp. 3. I. two zoosporangia of unknown chytrid sp. 4 with one releasing zoospores (arrow). J. Nearly empty zoosporangium of unknown chytrid sp. 5 with a large zoospore (arrow) exiting through a pore. K. Zoosporangium of unknown chytrid sp. 6. L. Hypha of *Zoophagus insidians* with characteristic peg traps (arrows). Scale bars = 20 µm.

Without observing development, zoospore discharge, and resting spores or using molecular sequences to place it in a phylogeny, we are not able to provide a confident identification for this observation.

Unknown chytrid species 2, Chytridiomycota FIG. 4G
Growing on chitin bait in water collected from Carp Lake June 2017. Given the orange coloration and substrate, it is possible that this is *Chytriomyces aureus*, *Siphonaria variabilis* or part of the *Rhizoclosmatium aurantiacum* species complex. *Chytriomyces aureus* has been reported on chitin baits from Smith's Fen (Dogma 1969), and *R. aurantiacum* species has been observed on insect exuviae across the region (Sparrow 1952; Sparrow et al. 1965). However, without observing zoospore discharge, particularly the presence/absence of an operculum, and obtaining molecular sequences, we cannot confidently assign this observation to any of the taxa.

Unknown chytrid species 3, Chytridiomycota FIG. 4H
Growing on pine pollen from Bryant's Bog collected July 2016.

Unknown chytrid species 4, Chytridiomycota FIG. 4I
Growing on organic debris collected Smith's Fen September 2016.

Unknown chytrid species 5, Chytridiomycota FIG. 4J
Growing on pollen in water collected from Bryant's Bog June 2017. Based on the large size of the zoospore, we hypothesize this is a member of Cladochytriales. Members of this order are typically found growing on cellulosic substrates (Sparrow 1960; MS et al. 2009), but species of *Cladochytrium* have been observed growing on pine pollen (e.g., https://www.inaturalist.org/observations/173209). A video of zoospore discharge was archived with figshare (https://doi.org/10.6084/m9.figshare.7963625).

Unknown chytrid species 6, Chytridiomycota FIG. 4K
Growing on moribund *Cosmarium* from Smith's Fen June 2017.

Zoophagus insidians, Zoopagomycota FIG. 4L
Observed trapping rotifers in water collected from Bryant's Bog 2016 (https://www.inaturalist.org/observations/3900383), Smith's Fen 2016 (https://www.inaturalist.org/observations/ 3960356), and Mud Lake Bog June 2017 (https://www. inaturalist.org/observations/7726474). Sparrow (1952) also observed this species in Bryant's Bog. We were successful at obtaining 18S sequences (GenBank: MG920183; Davis et al. 2019a) and a partial genome (GenBank: QZWR00000000; Davis et al. 2019b) for this fungus from the Bryant's Bog observation using single cell techniques.

Phylogenetic analysis

After Gblocks masking, the 18S alignment contained 1710 positions and the 28S alignment contained 938 positions for a total of 2648 positions with 1504 distinct alignment patterns. The inferred maximum likelihood phylogeny (−ln=30156) had well-supported relationships within orders but not among orders (FIG. 5). Chytridiales *sensu* Letcher et al. (2018) was monophyletic with 100% bootstrap support. Strain GJH028 grouped with *Rhizoclosmatium globosum* JEL347h with 100% bootstrap support. Strain GJH029 grouped with strain JEL137, which has represented *Physocladia obscura* in several molecular phylogenies (e.g., James et al. 2006; Vélez et al. 2011). Our two isolates of *Rhopalophlyctis sarcoptoides*, B9 and B10, placed sister to *Chytriomyces hyalinus* with 100% bootstrap support (FIG. 5). Synchytriales *sensu* Longcore et al (2016) was also monophyletic but with poor support (37%) unless our *Desmidium* parasite (isolate B25) is included in the order (100% bootstrap support). However, the genus *Rhizophydium* was not monophyletic as our isolate of *Rhizophydium echinocystoides* B8 was placed sister to the family Terramycetaceae with 100% bootstrap support and not with the type *Rhizophydium globosum*. *Rhizophydium* was proposed and described to accommodate inoperculate members of *Chytridium* (Schenk 1858; Rabenhorst 1868 *cf.* Letcher et al. 2006), but morphological characters overlap with other genera, and species were delineated based on substrate or host rather than morphology (Sparrow 1960; Barr 1969; Letcher et al. 2006). Using zoospore ultrastructure and molecular phylogenetics, Letcher et al. (2006, 2008a, 2008b) delineated a new order and several new genera based on former members of the genus. Our results indicate that further revision of the genus is needed and that single cell genomic techniques could aid in that revision.

Future single-cell genomic studies

Our results and the results of other studies (e.g., Ahrendt et al. 2018) show how single cell genomic techniques can be used to further the discovery and classification of chytrid taxa. However, our results also indicate that the techniques are not without challenges. Of the eight taxa targeted, we did not obtain usable sequence data for five of them: *Ancylistes*, the cyanobacteria parasite, *Micromyces mirabilis*, *Micromyces zygonii*, and *Olpidium*. The two primary reasons for failure are likely the inability to properly lyse the cells and contamination; however, primer mismatch during the PCR step is also a possible hurdle. For *Micromyces mirabilis*, we can be certain it failed due to contamination. This sample did yield PCR amplicons, but they were to a *Malassezia* sp. common to human skin. For the cyanobacteria parasite, it is possible it failed due to a combination of co-contaminants swamping the signal and issues with the PCR primers used. PCR amplicons were also obtained for this sample, but one was to a gregarine known to the area and the other we were unable to identify phylogenetically. For the *Micromyces zygonii*, *Olpidium* sp., and *Ancylistes closterii* samples, we suspect it is an issue with cell lysis. All three of these samples were resting spores with thick walls contained within an algal cell, which could have prevented the cells from being lysed. PCR primer mismatch could also be an issue, but whole genome sequencing of the sample (e.g., on an Illumina Miseq) could be used to overcome primer and PCR biases. Future studies will need to explore whether longer lysis times or alternative lysis methods, such as freeze-thaw cycles using liquid nitrogen (Lax et al. 2018) would be more appropriate for resting spores and internal algal parasites.

Conclusions

Sparrow, Dogma, and their contemporaries used generic and species concepts based on morphology and host/substrate. We observed several of the species that Sparrow and his students did, but we also observed potentially novel species using light microscopy. Thanks to combining light microscopy with single cell techniques, we generated sequence data for one

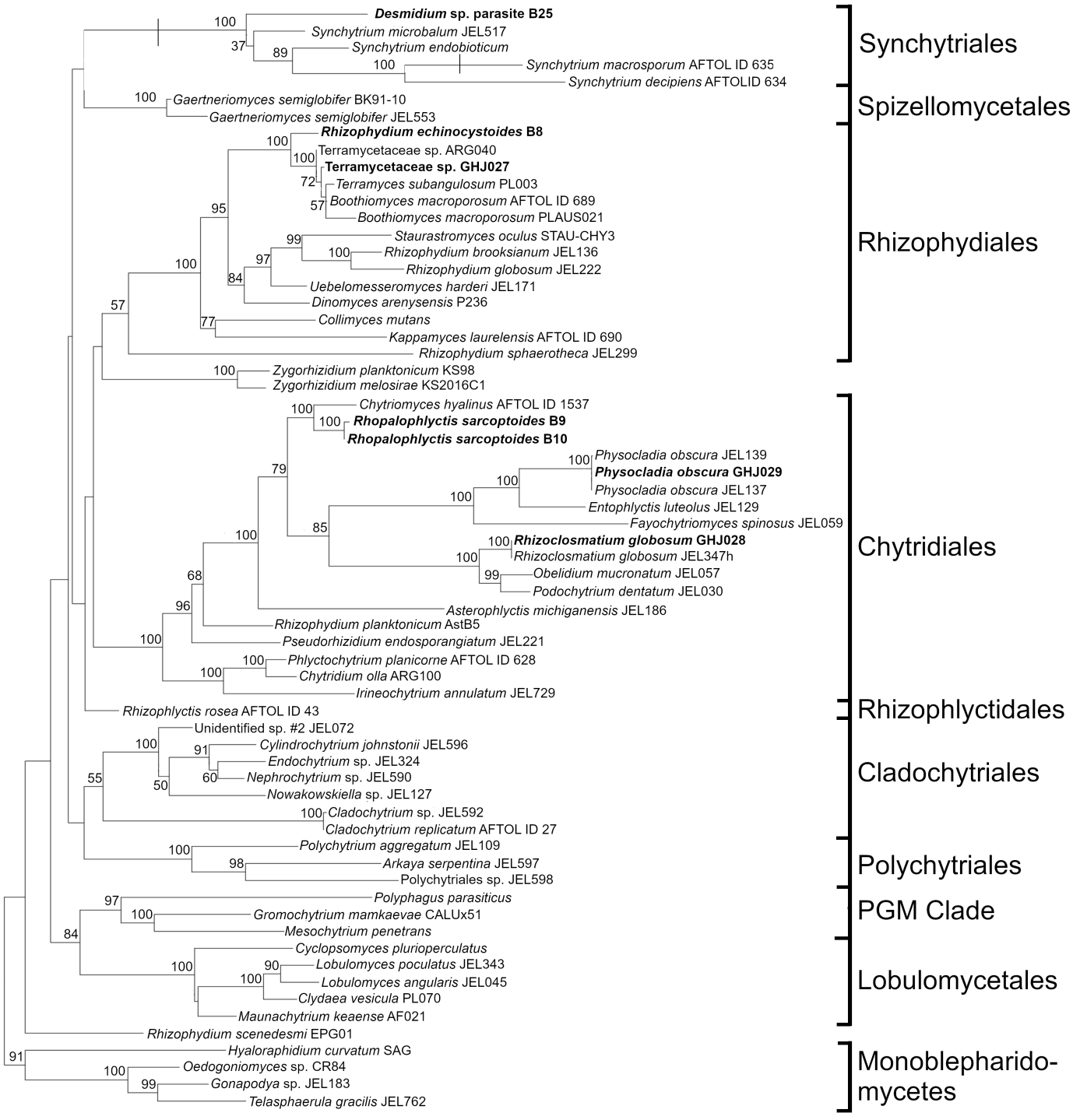

Figure 5. Maximum likelihood molecular phylogeny inferred from concatenated alignments of 18S and 28S. Taxa that we generated molecular sequences for are in **bold**. The PGM clade corresponds to Polyphagales, Gromochytriales, and Mesochytriales. For ease of viewing node labels, branch lengths were scaled by a factor of 6. Branches marked with | are displayed as half their original length. Nodes with < 50% bootstrap support are not labelled. Scale bar represents 0.03 nucleotide substitutions.

of these potentially novel taxa, the *Desmidium* parasite. As well, we generated sequence data for two described taxa that have not been cultured and therefore did have sequence data: *Rhopalophlyctis sarcoptoides* and *Rhizophydium echinocystoides*. Our results provide a pilot study for the use of single cell techniques obtain molecular sequences of chytrid taxa. Additionally, our results show that local aquatic fungal inventories can lead to the discovery of novel taxa and the potential to revise current taxonomy.

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