MYCOLOGIA

Mycologia

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/umyc20

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To cite this article: D. Rabern Simmons, Joyce E. Longcore & Timothy Y. James (2021) *Polyrhizophydium stewartii*, the first known rhizomycelial genus and species in the Rhizophydiales, is closely related to *Batrachochytrium*, Mycologia, 113:3, 684-690, DOI: 10.1080/00275514.2021.1885206

To link to this article: https://doi.org/10.1080/00275514.2021.1885206

Published online: 13 Apr 2021.

MYCOLOGIA
2021, VOL. 113, NO. 3, 684–690
https://doi.org/10.1080/00275514.2021.1885206

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Polyrhizophydium stewartii, the first known rhizomycelial genus and species in the Rhizophydiales, is closely related to *Batrachochytrium*

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ABSTRACT

Molecular and ultrastructural investigations of the Chytridiomycota during the last 20 years have led to the separation of new orders, including the Rhizophydiales. Most species in this order are morphologically similar, possessing monocentric, eucarpic, spherical thalli. Here, based on analysis of nuc 18S and 28S rDNA, we add the new genus and species *Polyrhizophydium stewartii* to the order. This saprobe of moribund aquatic plant leaves is the first known rhizomycelial species in the order. In our molecular phylogeny, *P. stewartii* groups with the amphibian pathogens *Batrachochytriuim dendrobatidis* and *B. salamandrivorans*, making it of particular interest to investigators studying evolutionary pathways associated with host-switching and morphological adaptation.

ARTICLE HISTORY
Received 28 July 2020
Accepted 1 February 2021

KEYWORDS
Chytrid; Chytridiomycota; polycentric; *Rhizophydium*: saprobe; 2 new taxa

INTRODUCTION

The Rhizophydiales (Chytridiomycota, Chytridiomycetes) contains many species of simple eucarpic, endogenously developing chytrid fungi, several of which were once classified in the genus *Rhizophydium* Schenk. Letcher et al. (2006) segregated the order from the Chytridiales based on ultrastructural characters and nuc 28S rDNA (28S) and nuc 5.8S rDNA (5.8S) sequences, and it now contains 28 molecularly verified species in 26 genera and 18 families (Letcher et al. 2006; Longcore et al. 2011; Seto and Degawa 2018). Taxonomic placement of an unidentified Rhizophydiales fungus often requires molecular characterization because of the morphological similarities within the order.

To date, no rhizophydialean species exhibits rhizomycelial polycentricity, i.e., forming multiple sporangia connected by mycelial-like connective tissue. Indeterminate growth, because it is one of the few obvious morphological characters detectable at the light microscopic level, at one time was deemed of utmost importance, second in Sparrow's (1960) classification only to the presence or absence of an operculum at the zoospore release site. For indeterminate growth to be efficient, the substrate, e.g., moribund leaves of an aquatic plant, needs to be large enough to make it worthwhile for resources to go into rhizomycelium construction instead of zoospore production. As part of an annual sampling of *Eriocaulon aquaticum* plant tissue from Williams Pond in Hancock County, Maine, we isolated a chytrid in 2016 (JEL0888) and 2018 (JEL0932) with polycentric growth that was unlike the growth of *Cladochytrium* and *Nowakowskiella* species, the most commonly encountered polycentric chytrids that grow on cellulosic substrates.

Molecular sequence information for isolates JEL0888 and JEL0932 was produced during a project to barcode the Collection of Zoosporic Eufungi at the University of Michigan (CZEUM; Simmons et al. 2020). This project made available long-read molecular sequences of the nuc rDNA operon for over 400 cultures, and analysis of these sequences placed the two isolates of this novel polycentric chytrid fungus in the Rhizophydiales, making this the first discovered rhizomycelial lineage in the order. Here, we describe this organism as a new genus and species and illustrate its morphology and phylogenetic position within the Rhizophydiales.

Because *Batrachochytrium dendrobatidis* Longcore, Pessier & Nichols (*Bd*; Longcore et al. 1999) and *B. salamandrivorans* Martel et al. (*Bsal*; Martel et al. 2013), two panzootic amphibian pathogens, are of global importance, their closest phylogenetic relatives are of interest. One of these related species is *Homolaphlyctis polyrhiza* Longcore, Letcher & T.Y. James (Longcore et al. 2011), which, as the closest known relative of *B. dendrobatidis*, was studied by Joneson et al. (2011) to compare enzymatic capabilities between the saprobe and the amphibian pathogen. The new genus and species, described here as *Polyrhizophydium stewartii*, are of particular interest because this new taxon is more closely related to *Batrachochytrium* species than is *H. polyrhiza*.

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Published online 13 Apr 2021

The availability of cultures of this new species presents an opportunity to expand and verify the conclusions of Joneson et al. (2011) concerning the changes in enzymatic capabilities during evolution from a plant saprobe to an animal parasite.

MATERIALS AND METHODS

Isolation and culture.—In Sep 2016 and Jul 2018, J.E. L. collected *Eriocaulon aquaticum*, a common submersed plant in Eriocaulaceae, from the shoreline of Williams Pond, a 32-hectare lake near Bucksport, in Hancock County, Maine. In the laboratory, several plants were maintained in a fingerbowl with lake water at room temperature. We examined moribund leaves under a light microscope and placed infected areas on mPmTG nutrient agar (peptonized milk, 0.4 g/L; tryptone, 0.4 g/L; glucose, 2.0 g/L; agar, 10 g/L) plates containing antibiotics (penicillin, 200 mg/L; streptomycin sulfate, 200–500 mg/L). Several species of chytrids grew from the pieces of plant tissue, and one species, represented by isolate JEL0888 in 2016 and isolate JEL0932 in 2018, had indeterminate growth. We maintained isolates on mPmTG agar plates at room temperature (they did not survive refrigeration) and cryopreserved samples (Boyle et al. 2003). To document thallus morphology, we cultivated JEL0888 on mPmTG agar, in PmTG nutrient broth (Barr 1986), and on briefly boiled wheat coleoptiles. Growth stages were photographed with a Spot RT3 camera (Diagnostic Instruments, Sterling Heights, Michigan).

Temperature trials.—To determine growth limitations, we incubated JEL0888 in three 125-mL screw-topped flasks containing 75 mL of PmTG broth at both 32 and 35 C and visually compared growth with flasks of JEL0888 prepared at the same time and incubated at room temperature.

DNA collection, sequencing, and phylogenetic analysis.—For JEL0888 and JEL0932, we placed agar plugs from 1-wk-old plates of each culture into ~20 mL of liquid mPmTG medium in 50-mL centrifuge tubes and incubated the cultures at room temperature for 2 wk. We then removed tissue from the liquid medium with a sterilized 26-gauge resistance heating wire probe and placed it into a 1.5-mL microcentrifuge tube. We centrifuged the microcentrifuge tube at 13 000 RPM for 5 min, removed the majority of the liquid medium, and then followed the 2× cetyltrimethylammonium bromide (CTAB) extraction protocol described by James et al. (2008) using chloroform:isoamyl alcohol (24:1).

We quantified DNA extracts with a Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts) and diluted working stocks for polymerase chain reaction (PCR) amplification to 0.25–0.75 ng/μL. The majority of the nuc 18S-ITS1-5.8S-ITS2-28S rDNA operon was amplified in 12.5 μL volumes composed of (i) 2.875 μL ultraviolet (UV)-radiated PCR water, (ii) 1.25 μL 10× LA Taq buffer (Takara Bio USA, Inc., Mountain View, California), (iii) 1.25 μL 25 mM $MgCl_2$, (iv) 2 μL 10 mM dNTPs, (v) 0.125 μL LA Taq polymerase, (vi) 1.25 μL each barcoded primer NS1short/RCA95m, and (vii) 2.5 μL working stock DNA template. PCR conditions included an initial denaturing at 95 C for 5 min, followed by 35 cycles of denaturing at 95 C for 30 s, annealing at 55 C for 30 s, and extension at 68 C for 4 min, and a final hold at 4 C (Wurzbacher et al. 2019). We confirmed amplification of our 4.5–6-kb products by gel electrophoresis on a 1× TAE (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA disodium salt dihydrate) gel run at 80 V for 45 min.

We produced fast5 nanopore sequencing reads from PCR products with an Oxford Nanopore Technologies (ONT) MinION device and MinKNOW software (Oxford Nanopore Technologies, Oxford, United Kingdom) after preparation of a multiplexed sample, of up to 96 pooled barcoded amplicons, with the ONT Ligation Sequencing Kit (LSK-109), following the manufacturer's protocol. The fast5 reads were basecalled to fastq reads following the LSK-109 parameters in Guppy 3.2.4 (ONT), and fastq reads were quality filtered with NanoFilt (De Coster et al. 2018) to include fragments 4–7 kb in length with quality scores of ≥10. The resulting fastq files were converted to fasta files for downstream processing with Seqtk (https://github.com/lh3/seqtk), and fasta files were demultiplexed by barcode combination recognition with Minibar (Krehenwinkel et al. 2019). We assembled sequences in Canu 1.9 (Koren et al. 2017) following the search criteria settings described in Simmons et al. (2020) that first yielded an assembly composed of ≥20 reads. Assemblies were polished in Medaka (https://github.com/nanoporetech/medaka), and barcodes were removed in Geneious 9.1.7 (Biomatters, Auckland, New Zealand) to obtain final rDNA operon sequences.

We constructed a phylogeny by gathering 18S and 28S molecular data from GenBank for 37 Rhizophydiales taxa, representing 15 families of the order, plus one taxon each of Spizellomycetales and Rhizophlyctidales as outgroup taxa (Simmons et al. 2020), and JEL0888 and JEL0932, for a total of 41 operational taxonomic units (OTUs) (FIG. 1). GenBank accession numbers are indicated on FIG. 1. The data matrix of these 41 OTUs was aligned in MAFFT 7.310, and ambiguously aligned regions were removed in TrimAl 1.2 under the

"automated1" setting. The Akaike information criterion in jModeltest 0.1.1 (Guindon and Gascuel 2003; Posada 2008) selected the GTR+I+G nucleotide substitution model for the entirety of the alignment. The parameters of the model were used in PhyML 3.0 (Guindon et al. 2010) to produce the best maximum likelihood (ML) tree and to calculate bootstrap percentages from 500 replicates. We also used these parameters to calculate Bayesian inference posterior probabilities from a consensus of 66 002 trees generated from two runs of 5 million generations, sampling every 100 generations and dismissing the first 17 000 trees in MrBayes 3.2.6 (Ronquist et al. 2012) until the average standard deviation of split frequencies was below 0.01. We submitted the TrimAl alignment and best ML tree with bootstrap support to TreeBASE as submission S27036.

RESULTS

Phylogeny.—The alignment of sequences from 39 Rhizophydiales cultures, including samples from 15 families and three lineages of uncertain placement, and two outgroup taxa from the Spizellomycetales and Rhizophlyctidales resulted in a data matrix of 41 OTUs and 6683 characters. A TrimAl analysis of ambiguously aligned sites reduced the alignment to include 3220 characters, which were used for further analyses. JEL0888 and JEL0932 long-read rDNA sequences were 99.96% identical (4672 and 4674 bp, respectively, before deletion of ambiguous alignments), and they grouped sister to *Batrachochytrium* with 86% ML support and 0.82 Bayesian inference posterior probability in our ML phylogeny (FIG. 1). The Rhizophydiales clade that contains *Batrachochytrium*, JEL0888 and JEL0932, plus *Homolaphlyctis polyrhiza* and *Entophlyctis helioformis* was supported by 0.97 Bayesian inference posterior probability but only 51% ML bootstrap support. Polyphyly of the Alphamycetaceae and Halomycetaceae (Simmons et al. 2020) and their poor support values (Letcher et al. 2015) are consistent with other published results.

Morphology.—Zoospores (FIG. 2A, B) were ~4–5 µm diam and contained one or sometimes two lipid globules. Flagella were ~30 µm long. On nutrient agar (FIG. 2C–F), the polycentric thallus consisted of regular to irregularly swollen tubular hyphae, from which branching rhizoids extended (FIG. 2C, D). Within the hyphae were swellings, some of which became unevenly enlarged (FIG. 2D, E) and developed into sporangia with one (FIG. 2F) or more long (often 50 µm or more) discharge papillae. On wheat coleoptiles, thalli grew within plant cells (FIG. 2G), and zoosporangia produced multiple, inoperculate discharge tubes (FIG. 2H) that emerged beyond the surface of host cells (FIG. 2I). After deliquesence of the apex of the discharge tubes, zoospores were free to exit the zoosporagium. Thick-walled resting spores formed on wheat coleoptiles (FIG. 2J). JEL0888 grew well as rhizomycelia in PmTG broth at 32 C but did not grow at 35 C. Refrigerated cultures died, but cultures survived cryopreservation; cultures on nutrient agar in Petri dishes sealed with Parafilm and kept at room temperature remained viable for several months.

TAXONOMY

Polyrhizophydium Longcore & D.R. Simmons, gen. nov. MycoBank MB837540

Type: Polyrhizophydium stewartii Longcore & Simmons, sp. nov. (described below).

Description: Thallus polycentric with interspersed, often irregular, nonseptate swellings. Some swellings unevenly enlarged and developing into sporangia. Zoosporangia produce multiple discharge tubes several times longer than the diameter of the zoosporangium. Zoospore discharge inoperculate.

Etymology: polýs (Greek) = multiple; referring to polycentric growth of the thallus and *rhizophydium* referring to placement within the Rhizophydiales.

Comments: Based on distinct differences in thallus morphology and rDNA phylogenetic analyses, we conclude that the Batrachochytriaceae (Doweld 2013) with monocentric or colonial thalli is not appropriate for *Polyrhizophydium*. Rather than emend the Batrachochytriaceae or describe new families for each of the genera related to *Batrachochytrium*, we suggest that assignment to a taxonomic family should await discovery and analysis of additional members of the clade.

The diagnostic characteristic of *Polyrhizophydium* is the production of consistently long, inoperculate discharge tubes emanating from zoosporangia, which develop directly from intercalary uneven swellings. The species resembles *Zopfochytrium polystomum* (Zopf) M. J. Powell, Longcore & Letcher (Chytridiales) in having long discharge tubes, but in pure culture on agar medium, *Z. polystomum* produces the majority of its profuse rhizomycelium from enlarged, spherical, intercalary zoosporangia that release zoospores en masse (fig. 3G in Powell et al. 2018). By contrast, the rhizomycelium of *Polyrhizophydium* is produced by indeterminate polycentric growth with no central zoosporangium; we did not observe zoospores produced in pure culture on agar.

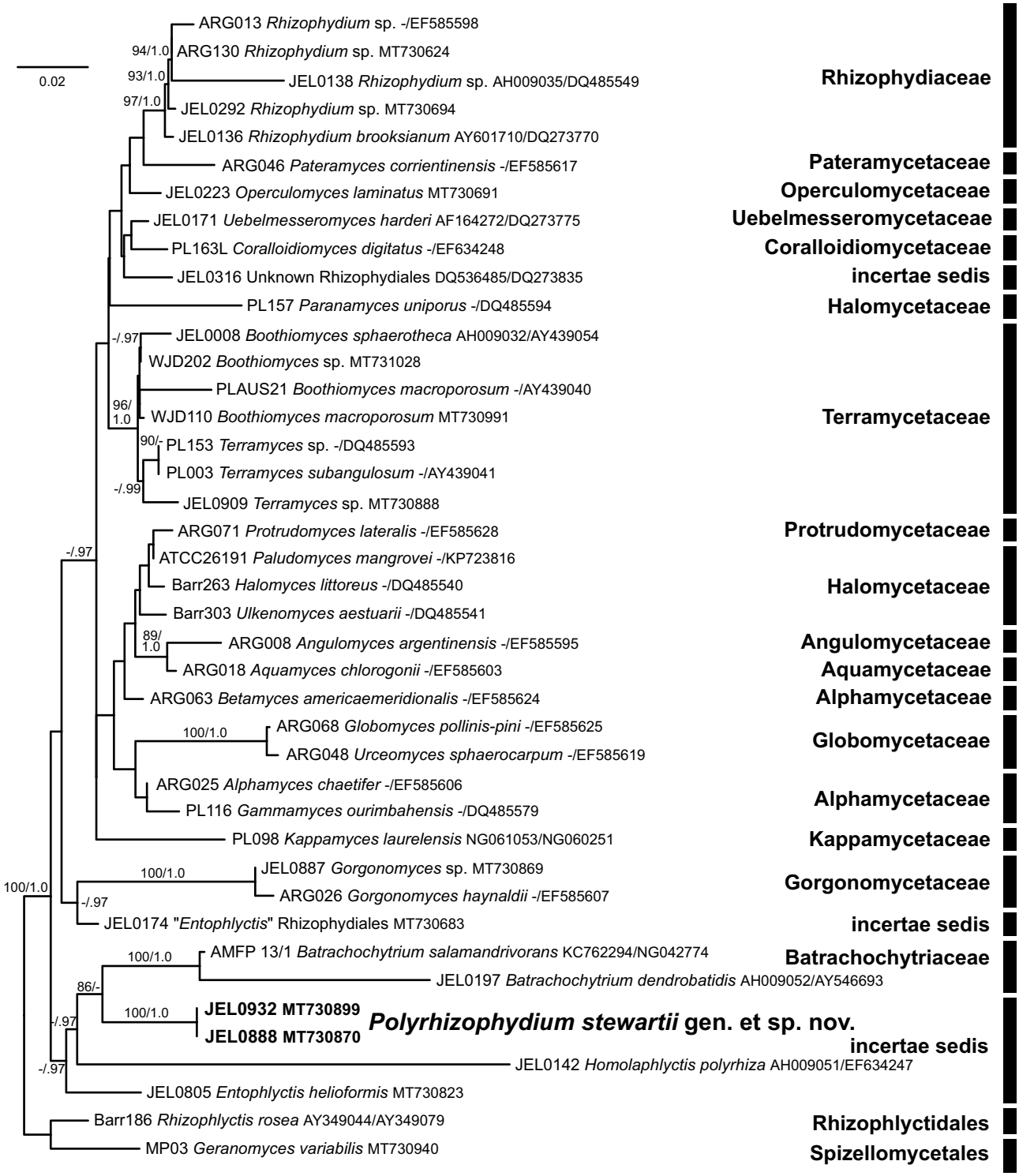

Figure 1. Best PhyML phylogeny of the Rhizophydiales produced from concatenated 18S and 28S rDNA sequences or long-read rDNA operon sequences of 39 cultures representing 15 families, plus one taxon each of Rhizophlyctidales and Spizellomycetales as outgroups. Values at nodes represent ≥70% ML bootstrap support/≥0.95 Bayesian inference posterior probability percentages. When divided by "/", GenBank accession numbers represent 18S/28S rDNA sequences; when only a single GenBank accession is listed, the long-read rDNA sequence contains both regions.

Polyrhizophydium stewartii Longcore & D.R. Simmons, sp. nov. FIG. 2A–J
MycoBank MB837544

Typification: USA. MAINE: Hancock County, Stewart camp on Williams Pond, isolated from moribund *Eriocaulon aquaticum* leaf, 17 Sep 2016, *J.E.*

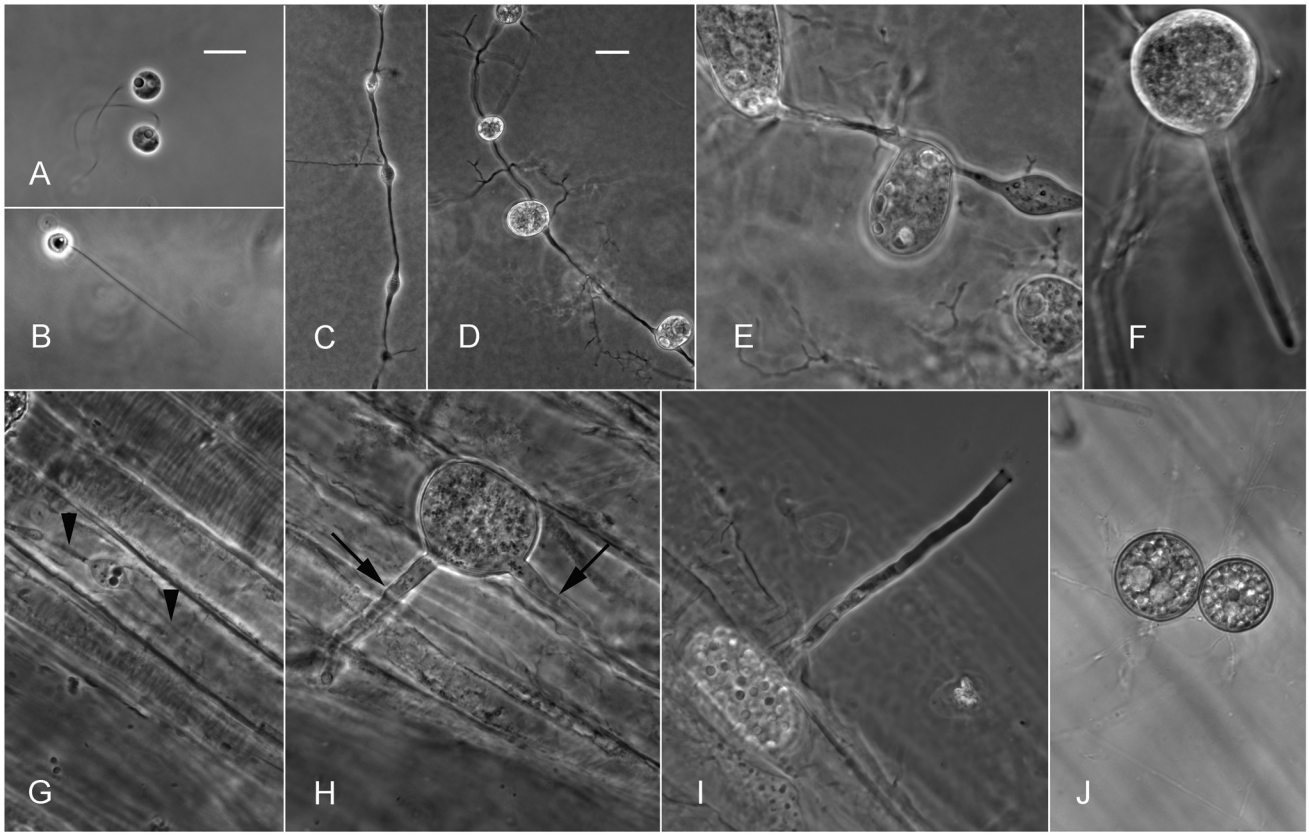

Figure 2. *Polyrhizophydium stewartii* JEL0888 (holotype). A. Zoospores showing single lipid globule and nucleus surrounded by shadowy area that is probably aggregated ribosomes. B. Zoospore with extended flagellum. C. Rhizomycelium with intercalary swellings. D. Rhizomycelium with unevenly swollen swellings. E. Expanded swellings and unexpanded swelling. F. Zoosporangium with discharge tube. G. Rhizomycelium (arrowheads) on either side of swelling within onionskin cells. H. Zoosporangium within onionskin cells producing two discharge tubes (arrows). I. Mature zoosporangium with open discharge papilla extending from onionskin substrate. J. Two resting spores formed in onionskin substrate. Bar in A = 10 µm for A, B, E–J. Bar in D = 20 µm for C, D.

Longcore (**holotype** FIG. 2A–J). Ex-type culture JEL0888 (CZEUM-MICH). GenBank: 18S-ITS1-5.8S-ITS2-28S = MT730870.

Etymology: stewartii, named for the Stewart family, from whose camp on Williams Pond, Hancock County, Maine, the substrate containing the fungus was collected.

Description: Zoospores spherical, ~4–5 µm diam, usually containing a single lipid globule; flagella ~30 µm long. Zoosporangia form from unevenly enlarged swellings in the rhizomycelium and discharge zoospores through 1–4 long (often 50 µm or more), inoperculate discharge tubes. Thick-walled resting spores develop when growth is on plant tissue.

Other specimen examined: USA. MAINE: Same locality as the type, isolated from *E. aquaticum*, 4 Jul 2018, *J. E. Longcore* (JEL0932 CZEUM-MICH). GenBank: 18S-ITS1-5.8S-ITS2-28S = MT730899.

DISCUSSION

As chytrid biodiversity is explored and taxonomic revisions continue, rhizomycelial fungi are becoming more widely known in the Chytridiomycetes. The Chytridiales, as understood by Sparrow (1960), was divided and expanded into 13 orders based on ultrastructural comparisons and molecular congruence. Rhizomycelial taxa now are found in five of these orders, the Rhizophydiales (this study), Chytridiales (Powell et al. 2018), Cladochytriales (Mozley-Standridge et al. 2009), Polychytriales (Longcore and Simmons 2012), and Spizellomycetales (James et al. 2006). Rhizomycelium is also a growth form shared with other phyla in the superphylum Chytridiomyceta, the Monoblepharidomycota, and the Neocallimastigomycota (Dee et al. 2015; Hanafy et al. 2018). This phylogenetic distribution suggests that the ability to produce indeterminant growth is pervasive in the Chytridiomyceta, and we

expect that additional taxonomic discovery will reveal rhizomycelial forms in other orders of Chytridiomycota.

The Rhizophydiales is a large and varied order with species native to aquatic, soil, and marine habitats. Most species have been retrieved from pollen baits, but some species grow on a variety of other substrates, including moribund plant material, snakeskin, and algae. Species related to *P. stewartii* in our phylogeny include *Homolaphlyctis polyrhiza* (Longcore et al. 2011), which was isolated on onionskin bait placed with *Eriocaulon aquaticum* in lake water, and *Entophlyctis helioformis* (Dangeard) Fischer, which was directly isolated from the green alga *Nitella*. *Batrachochytrium*, which includes the infamous pathogens that grow in amphibian skin (Longcore et al. 1999; Martel et al. 2013), is also in this otherwise plant-associated clade. Based on the substrate of *Eriocaulon aquaticum* or the morphological similarity to the chytridialean *Zopfochytrium polystomum* (Powell et al. 2018), *P. stewartii* was initially assumed to be a polycentric member of the Cladochytriales or Chytridiales. As investigations into the biodiversity of chytrid fungi continue, finding exceptions to our assumptions based on substrate and morphological similarities has become the rule rather than exception.

Comparison of *P. stewartii* to taxa in the Batrachochytriaceae or related uncertain taxa indicates that, although the other taxa are predominantly monocentric, the group shares the ability to produce elongated zoospore discharge tubes. *Polyrhizophydium stewartii* produces elongated, inoperculate discharge tubes both in pure culture and when growing within wheat coleoptiles, which allows for dispersal of zoospores beyond the substrate surface. In the exogenously developing and predominantly monocentric *Batrachochytrium* (some thalli are colonial) and *E. helioformis*, elongated discharge tubes similarly allow for the release of zoospores to the exterior of the substrate, in which the chytrid thallus matured. Elongated discharge tubes are present in the endogenously developing *H. polyrhiza*, but, in our current understanding, their production does not seem functional given that the zoosporagium develops epibiotically on agar substrates and thus elongated discharge tubes seem unnecessary. However, elongated discharge tubes are not a unique morphological feature to this clade of the Rhizophydiales. *Gorgonomyces haynaldii* (Schaarschm.) Letcher in the Gorgonomycetaceae also produces numerous elongated discharge tubes, especially when grown on PmTG agar (Letcher et al. 2008).

Investigations into the zoosporic ultrastructure of *P. stewartii* and *E. helioformis*, which have thus far been unexamined, could provide additional morphological characters to segregate or combine taxa within the Rhizophydiales. Future isolation efforts could yield molecularly verified cultures of these taxa that produce sufficient zoospores in culture to allow for ultrastructural comparisons. In addition, genome sequencing will be useful to resolve these poorly supported relationships within the Rhizophydiales and help understand the genetic and physiological changes occurring during the transition from a plant degrading saprotroph to a parasite of living amphibian skin cells.

ACKNOWLEDGMENTS

We thank Allison A. Geagan and Gregory F. Geagan for permission to sample aquatic plants from their property on Williams Pond, Hancock County, Maine. We appreciate the time and effort of the reviewers and editor, which improved this work.

FUNDING

This work was supported by the National Science Foundation through research grants DBI-1910720 and DEB-1929738.

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