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ORIGINAL ARTICLE

Phytochytrium and *Sparrowiella*, two new polycentric genera in *Cladochytriales*

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Abstract

Cladochytriales comprise multiple monocentric and polycentric lineages of chytrids, which are commonly found as saprobes in algae and decaying plant material in aquatic and terrestrial ecosystems. Although monocentric species are more common among the *Chytridiomycota*, additional polycentric chytrids have been described in recent years. During our ongoing effort to add to the knowledge of the diversity of chytrids, we found two polycentric chytrids on onion skin bait that produce rhizomycelia like none of described cladochytrialean genera or like those of genera in other orders. The rhizomycelia of these two newly found isolates consist of isodiametric filaments that lack swellings such as those found in either *Cladochytrium* or *Nowakowskiella*. Both zoosporangia and resting spores develop on short, right-angled offshoots from these filaments. Our analyses of the small and large subunits of rDNA indicate that these two isolates belong to separate branches of a predominantly monocentric clade of the *Cladochytriales*. *Phytochytrium stagnum* gen. et sp. nov. groups with two monocentric strains and *Sparrowiella insolita* gen. et sp. nov. are in an independent lineage distantly related to a monocentric species. We have expanded the concept of *Septochytriaceae* to include this clade. The ancestral state reconstruction allowed us to hypothesize for the first time a possible growth pattern of the common ancestral that gave origin to the order, besides to corroborate the unreliable nature of thalli's development as a taxonomic character.

Keywords Chytrid · Four new taxa · Morphology · Phylogeny

Introduction

Cladochytriales was established by Mozley-Standridge et al. (2009) to accommodate a monophyletic lineage that includes both monocentric and polycentric genera whose members are commonly found as saprobes in algae and decaying plant material in aquatic and terrestrial ecosystems (Mozley-Standridge et al. 2009; Powell and Letcher 2014; Jerônimo et al. 2019). Monocentricity, in which each individual zoospore produces a single zoosporangium, is more common among the *Chytridiomycota* (chytrid fungi, or chytrids); however, additional polycentric genera have been described in recent years, including *Karlingiella* (Jerônimo et al. 2019) in the *Cladochytriales* and *Polyrhizophydium* (Simmons et al. 2021) in the *Rhizophydiales*.

Polycentric chytrids consist of a rhizomycelium from which multiple zoosporangia develop. The term rhizomycelium has not been specifically defined in textbooks and includes a variety of morphologies. Dee et al. (2015) defined a rhizomycelium as the extensive rhizoidal system of polycentric species that links multiple sporangia. Although rhizoids are typically described as being anucleate, a rhizomycelium, perforce, contains nuclei that give rise to zoosporangia. Polycentricity was once a paramount character in chytrid taxonomy (e.g., *Cladochytriaceae sensu* Karling 1977); however, we now realize that this growth form occurs in at least 5 orders of the *Chytridiomycota* (Table 1).

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Table 1 Polycentric genera confirmed to be members of the Chytridiomycota (Nowakowski 1877; Schroeter 1893; Sparrow 1932; Berdan 1939; Ajello 1942; Hanson 1944; Longcore 1993; Mozley-Standridge et al. 2009; Powell et al. 2018; Jerônimo et al. 2019; Simmons et al. 2021)

Order	Genera	Substrates
Chytridiales	*Physocladia*	Saprobic in plant debris and staminate cones of *Pinus*
	Zopfochytrium	Saprobic in plant debris
Rhizophlyctidales	*Catenomyces*	Saprobic in plant debris
Rhizophydiales	*Polyrhizophydium*	Saprobic in plant debris (*Eriocaulum aquaticum*)
Polychytriales	*Polychytrium*	Chitinophylic
	Lacustromyces	Chitinophylic
Cladochytriales	*Cladochytrium*	Saprobic in plant debris and algae
	Karlingiella	Saprobic in plant debris
	Nowakowskiella	Saprobic in plant debris and algae
	Septochytrium	Saprobic in plant debris
	***Phytochytrium* (JEL1002)**	Saprobic in plant debris
	***Sparrowiella* (JEL1004)**	Saprobic in plant debris

The most well-known and species-rich polycentric genera in the order *Cladochytriales* are *Cladochytrium* Nowak. (*sensu* Sparrow 1943) and *Nowakowskiella* J. Schröt. Although historically differentiated based on whether their zoospores discharged through operculate (*Nowakowskiella*) or inoperculate (*Cladochytrium*) openings (Sparrow 1960; Karling 1977), an additional difference is the morphology of their rhizomycelia (Mozley-Standridge et al. 2009). *Cladochytrium* rhizomycelium consists of narrow tubes with distinct intercalary swellings that are usually septate. *Karlingiella*, which is sister to *Cladochytrium*, has distinct swellings that are not septate (Jerônimo et al. 2019). The rhizomycelium of *Nowakowskiella* varies in diameter and swellings are seldom, if ever, septate. *Septochytrium*, an additional polycentric genus in the *Cladochytriales*, was described as having septae in the rhizomycelium (Berdan 1939); however, this character, as well as the polycentricity is variable and authors have observed species without septae and producing monocentric thalli (Karling 1942).

During our ongoing effort to add to the knowledge of the diversity of chytrids, we found two polycentric chytrids from onion skin bait that produced rhizomycelia unlike any cladochytrialean genera or genera of other chytrid orders. Instead of intermittent swellings, the newly found chytrids have a rhizomycelium that does not have distinct swellings. Our analyses of the small and large subunits of rDNA indicated that these two isolates belong to separate branches of a predominantly monocentric clade of the *Cladochytriales*, and their morphology seems to be undescribed in the classical literature (e.g., Zopf 1884; Sparrow 1960; Karling 1977). We performed an ancestral state reconstruction of the growth pattern by Bayesian Binary MCMC and hypothesized for the first time a possible growth pattern of the common ancestral that gave origin to the order. Herein, we also described these two novel rhizomycelial *Cladochytriales* genera and species by morphological and molecular characterizations, the latter of which provided with the intent that similar organisms may be identified from molecular markers detected during environmental studies.

Materials and methods

Isolation

We collected a water sample with plant debris from a pond located in the Littlefield Ornamental Garden on the University of Maine campus on September 26, 2020, added small bits of boiled, white onionskin to the samples (1 cm × 0.5 cm), and incubated the baited samples at room temperature (~23 °C). After 1 week, we found chytrids by microscopic observation and isolated them into pure culture on mPmTG agar (peptonized milk, 0.4 g/L; tryptone, 0.4 g/L; glucose, 2.0 g/L; agar, 10 g/L) plus antibiotics (200 mg/L of penicillin and 200–500 mg/L of streptomycin sulfate). JEL1002 was isolated from zoosporangia that we found on the onionskin, but JEL1004 grew as a contaminant on an isolation plate when we attempted to isolate a monocentric chytrid (JEL1003). Cultures are archived and available from CZEUM (https://czeum.herb.lsa.umich.edu/), the Collection of Zoosporic Eufungi at the University of Michigan.

Morphology

For morphological studies, we incubated isolates on mPmTG agar plates at room temperature (~23 °C) and photographed growth stages with a Spot RT3 camera (Diagnostic Instruments, Inc., Sterling Heights, MI). We also photographed early stages of JEL1002 growing in weak TCL agar (tryptone 0.2 g/L; cellobiose 0.5 g/L; agar 10g/L; lake water 50%, distilled water 50%) in depression slides. Depression slides were placed in damp chambers and

autoclaved before drops of weak TCL were added to the depressions. After the agar was firm, we added small bits of culture to one side of the agar, and then added water to the damp chamber as needed to maintain the humidity of the small amount of agar on the open slide. After growth was evident, we added coverslips to the agar-filled wells and photographed the growing fungi.

DNA collection, sequencing, and phylogenetic analyses

We transferred small pieces of agar from JEL1002 and JEL1004 to Falcon tubes (Corning, New York, NY) containing 30 mL of sterile mPmTG liquid medium and incubated the cultures at room temperature for 4–6 weeks. We then pipetted the tissue from each culture from the liquid medium and placed the biomass into its own microcentrifuge tube and centrifuged at 13000 RPM for 5 min, after which we removed the supernatant. We followed a 2× CTAB extraction protocol (James et al. 2008) to extract genomic DNA and a GoTaq Green master mix protocol (Promega, Madison, Winsconsin) to amplify the small and large subunits of ribosomal DNA using the primer pairs LR0R/LR5 (Vilgalys and Hester 1990; Rehner and Samuels 1994) for partial 28S nc rDNA and NS1.5/NS6 (White et al. 1990) for 18S nc rDNA. Thermocycling steps and amplicon purification using ExoSAP-IT (Thermo Fisher Scientific) followed methods described in James et al. (2006). Sequencing was performed at Genewiz, NJ, USA.

We selected 68 isolates of *Cladochytriales* for phylogenetic analyses (Fig. 1), most of which had sequences available in a public database (e.g., GenBank). Three *Polychytrium aggregatum* strains (CCIBt4017, ALJ30, JEL109) served as an outgroup. We assembled the contiguous sequences derived from JEL1002 and JEL1004 with Sequencher 4.1.4 (Gene Codes, Ann Arbor, MI), and aligned them with the other sequences in MAFFT version 7 web tool (http://mafft.cbrc.jp/alignment/server/) (Katoh et al. 2017). The 18S and 28S nc rDNA sequences were concatenated through SequenceMatrix 1.8 (Vaidya et al. 2010) and the ambiguously aligned regions were removed manually on BioEdit (Hall 1999), resulting in a final length of 4689 base pairs. As the partitioned and unpartitioned analyses resulted in identical topologies, we proceeded with the analysis using the unpartitioned data. Maximum likelihood (ML) analyses were conducted in RaxML-HPC v.8 on XSEDE (Stamatakis 2014) and Bayesian inference (BI) in MrBayes 3.2.6 (Ronquist et al. 2012) on the CIPRES Science Gateway platform (https://phylo.org), with default settings and partition models generated by jModelTest 0.1.1 (Posada 2008). A Bayesian MCMC ancestral state reconstruction of growth pattern (i.e., monocentric or polycentric) was conducted across the best ML tree with the *make.simmap* functionality from *phytools* (Revell 2012) in R. Node probabilities were computed based on 1,000 simulations where all rates were allowed to be different (i.e., under an ARD model). Node probabilities were mapped onto the best ML tree with *ggtree* (Yu et al. 2017). The character matrix (concatenated alignment), ML tree, and BI tree are deposited in TreeBASE (study TB2:S29000).

Results

Phylogenetic analyses and ancestral state reconstruction

The combined sequence data (18S and 28S of the nc rDNA) contained 4689 characters. jModelTest indicated that the most appropriate model of DNA substitution was TIM2+I+G, according to Akaike information criterion (AIC). In our phylogenetic analyses, JEL1002 and JEL1004 form two distinct branches in the monocentric-polycentric clade of the *Cladochytriales* that we have labeled *Septochytriaceae* (Fig. 1). JEL1002 is in a group containing JEL050 and JEL931, two monocentric strains with long, isodiametric rhizoids and operculate zoosporangia. JEL1004 formed an independent lineage that, in our analyses, is sister to a group that contains a clade whose members produce carotenoid pigments (Fig. 1) plus the clade that contains *Septochytrium* and JEL1002.

The ancestral state reconstruction of growth pattern (monocentric or polycentric) (Fig. 1) indicated that the common ancestral of *Cladochytriales* was probably a monocentric chytrid. In addition, *Endochytriaceae*, *Nowakowskiellaceae*, and *Septochytriaceae* probably evolved from monocentric ancestors, while *Cladochytriaceae* from an ancestral with a polycentric thallus. Besides that, the two new polycentric genera proposed here evolved independently from distinctive monocentric ancestors.

Morphology

On mPmTG agar, JEL1002 germinates from a zoospore with a lipid droplet (Fig. 2A). The rhizomycelium is made up of filaments that are up to ~2-μm diam. (Fig. 2B–D). Although these filaments may occasionally be bead-like (Fig. 2C–D), they do not possess expanded areas like those of *Nowakowskiella* or intermittent septate swellings as does *Cladochytrium*. Zoosporangia formed from a pear-shaped extension from the rhizomycelium (Fig. 2C–D). As the zoosporangium expands, it becomes spherical and rests directly on the rhizomycelium (Fig. 2E–F). Mature zoosporangia can be more than 60-μm diam. and produce broad, operculate discharge tubes (Fig. 2G–H) that can extend to multiple times the diameter of the zoosporangium. Resting spores (Fig. 2I) formed like the zoosporangia and are thick-walled with a large lipid globule. The morphology of JEL1004 (Fig. 3A–J) is

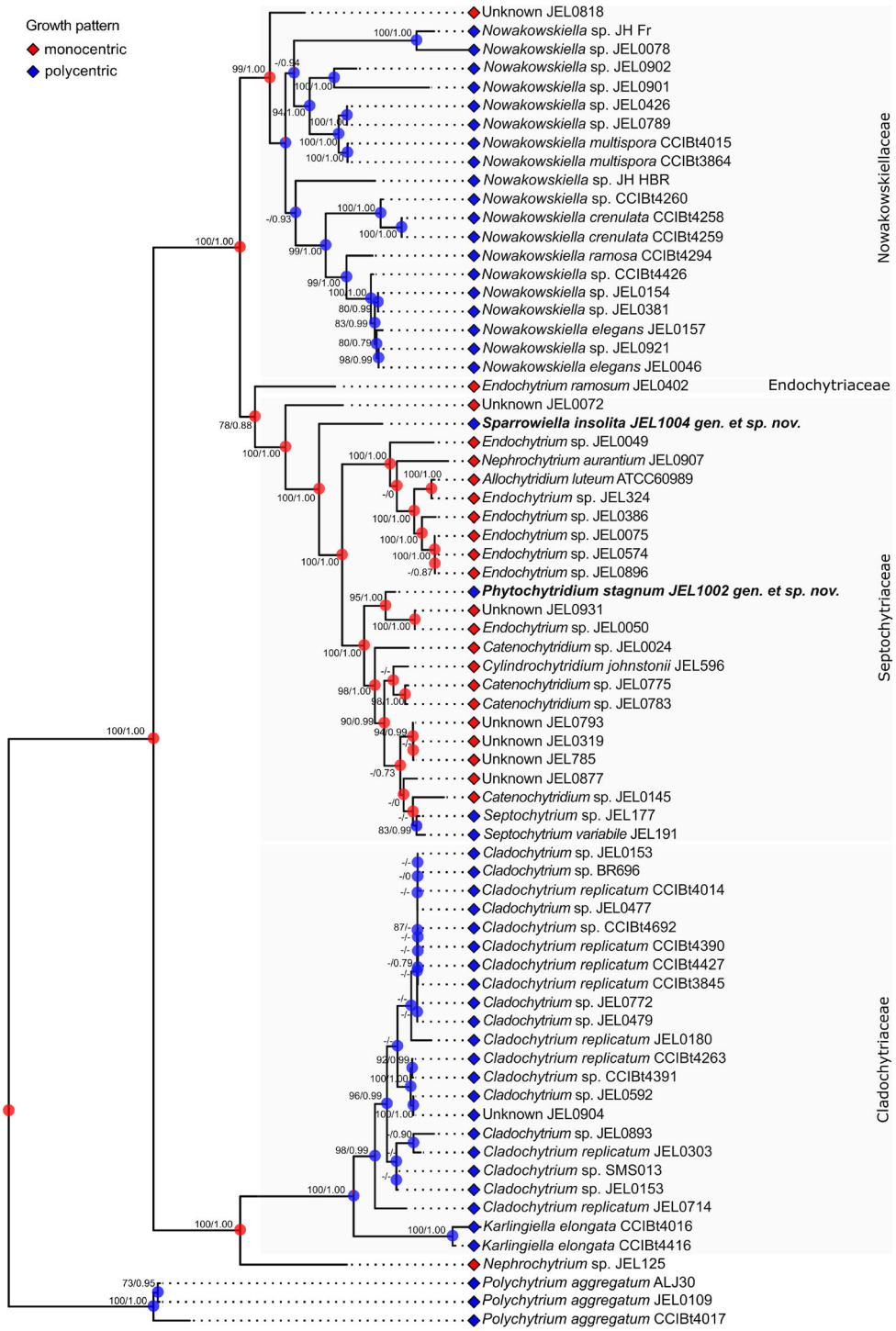

Fig. 1 Ancestral states reconstructed by Bayesian Binary MCMC plotted in the best maximum likelihood tree generated by 68 ingroup isolates of *Cladochytriales* through combined (SSU and LSU) sequences data. Maximum likelihood bootstrap support values <70% are indicated with (-). Bayesian posterior probability values >0.50 are labeled numerically. The clades that were not recovered in the Bayesian tree are indicated with (0)

much like that of JEL1002, except on a smaller scale. The rhizomycelium filaments are ~1-µm diam. and taper but do not have broad swellings or intermittent, septate swellings (Fig. 3A–C). Zoosporangia arise from stalked, ovate swellings. As the zoosporangium develops, the stalk swells and becomes a sub-sporangial swelling for the mature zoosporangium. This causes the zoosporangium to resemble those of apophysate, monocentric chytrids (Fig. 3C). Mature zoosporangia around 20-µm diam. and narrow discharge tube length extend greater than the diameter of the zoosporangia (Fig. 3D–H), with operculum to discharge of the zoospores (Fig. 3H). The ca 20-µm diam. resting spores are formed like the zoosporangia, are thick-walled, and possess a central lipid globule (Fig. 3I–J).

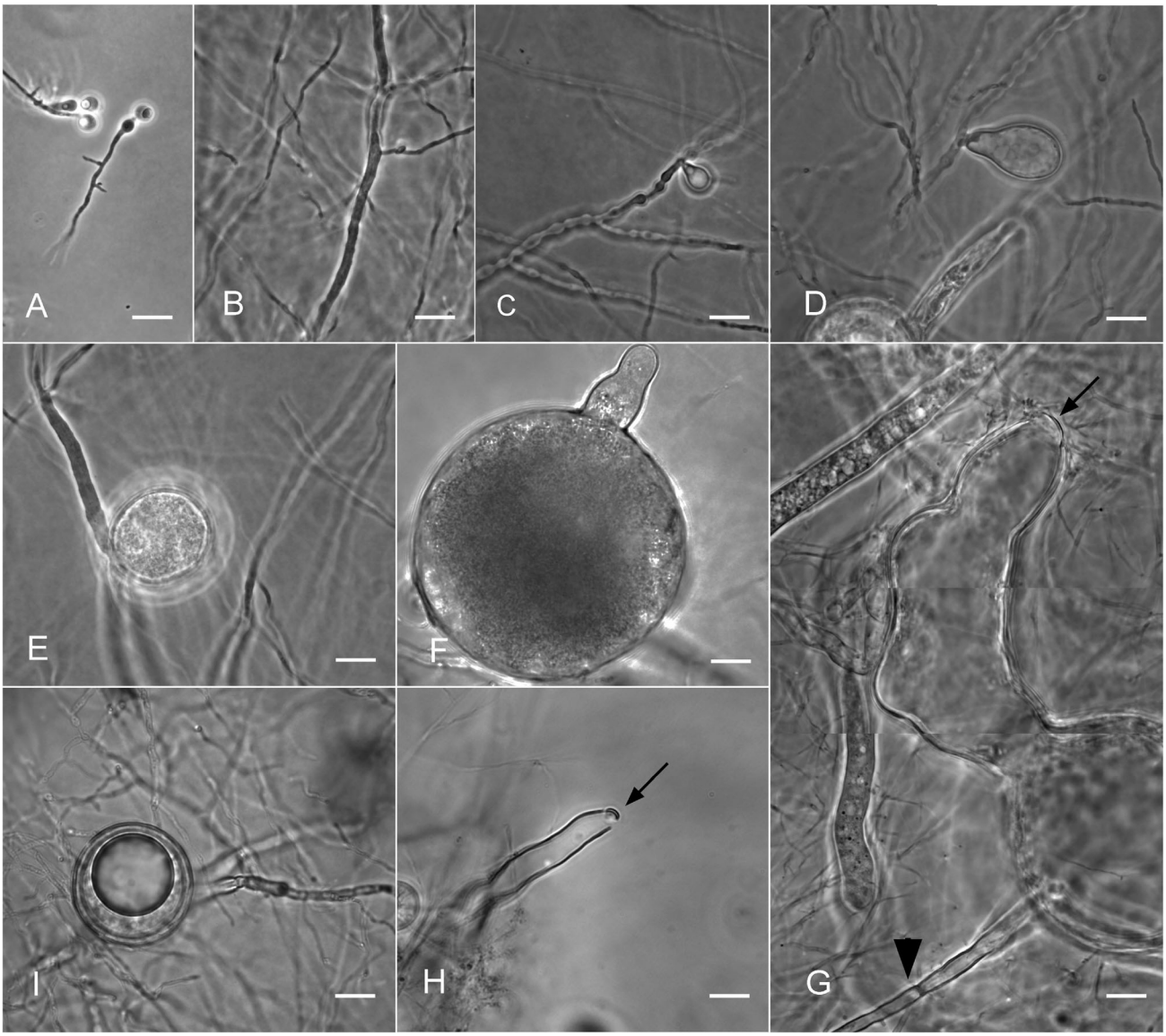

Fig. 2 *Phytochytrium stagnum* JEL1002 (holotype). **A** Germlings. **B** Rhizomycelium. **C**, **D** Rhizomycelium is sometimes bead-like (arrow). Zoosporangium begins as a pear-shaped swelling perpendicular to the rhizomycelium. **E** As zoosporangium becomes larger, it becomes spherical and directly attached to the rhizomycelium. **F** Zoosporangia usually spherical, varying in size up to 70 μm or more in diameter. **G**, **H** Discharge papillae often longer than the diameter of the sporangium; operculate; varying in diameter. **I** Resting spore formed from rhizomycelium as the zoosporangia. Bars = 10 μm

Taxonomy

Septochytriaceae S.E. Mozley-Standridge. MycoBank no.: MB512008

The *Septochytriaceae* description states that the operculate thalli may be monocentric or polycentric. The morphologies of the species in the clade delimited in our analysis of the small and large subunits of ribosomal DNA (Fig. 1) can be included in this description, although the operculum of isolate JEL1004 is small and seldom found (Fig. 3H). The family description, however, also states that the rhizomycelium is septate, which we do not find in the new genera. We base our concept of the *Septochytriaceae* on the well-supported grouping of monocentric and polycentric chytrids in our molecular analysis (Fig. 1), which is supported by the analysis of CZEUM members (Simmons et al. 2020). The family now contains *Septochytrium*, *Allochytridium* (Mozley-Standridge et al. 2009), *Cylindrochytridium* (Steiger et al. 2011), and isolates provisionally in *Endochytrium*, *Catenochytridium*, and *Nephrochytrium* that may eventually receive alternative genus placement.

Phytochytrium Longcore & G.H. Jerônimo gen. nov. MycoBank: MB 841930

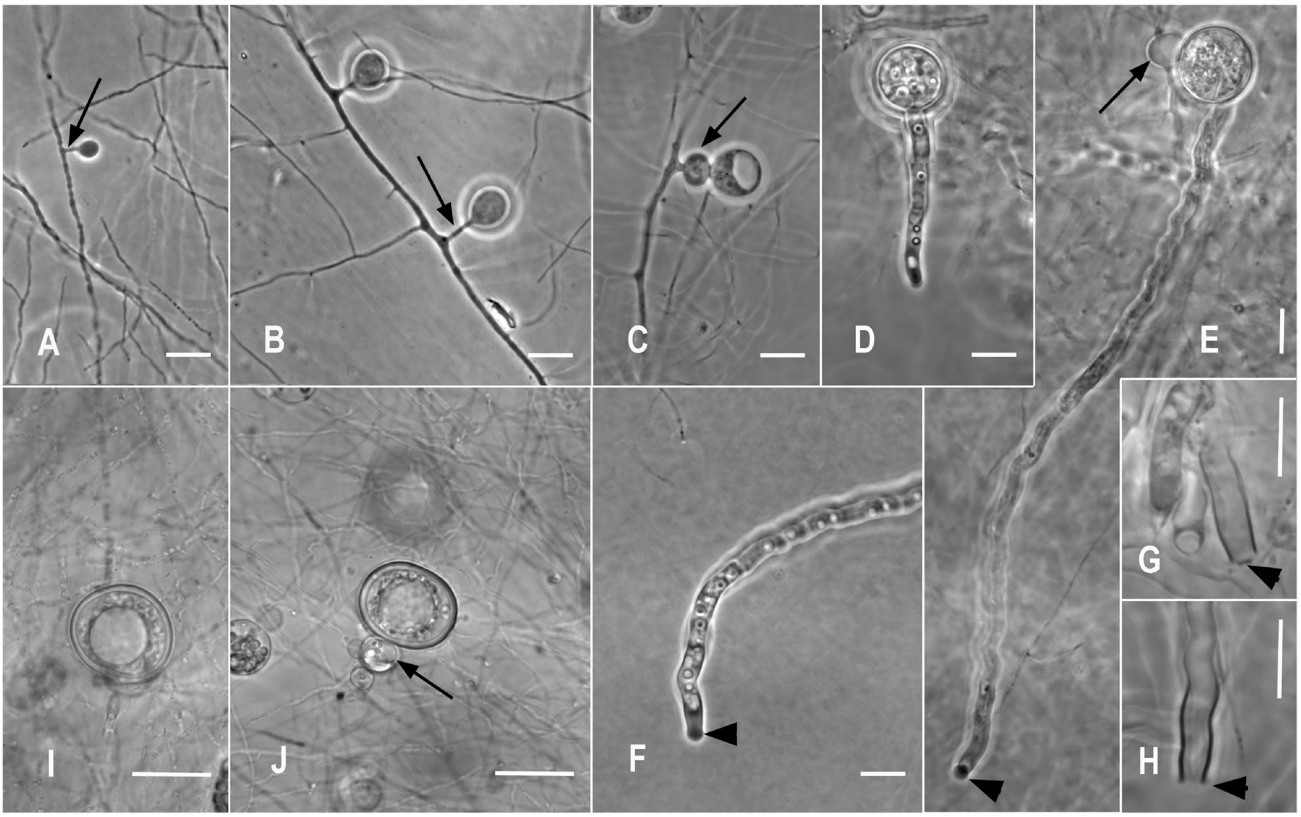

Fig. 3 *Sparrowiella insolita* JEL 1004 (holotype). **A** Rhizomycelium with incipient, stalked (arrow) zoosporangium. **B** Expanding zoosporangia connected by stalk (arrow) to the rhizomycelium. **C** Developing stalk becoming swollen (arrow). **D** Discharge papilla extends to greater than the diameter of the zoosporangium. **E** Arrow indicates subsporangial swelling and arrowhead indicates the tip of a long, nearly mature discharge papilla. **F** Discharge papilla of mature papilla. **G** Arrowhead indicates tip of discharged papillae with no obvious operculum. **H** Arrowhead indicates possible small operculum. **I**, **J** Resting spores. Arrow indicates subsporangial swelling. Bars = 10 μm

Type: *Phytochytrium stagnum* Longcore & G.H. Jerônimo sp. nov. (described below).

Description: Rhizomycelium branched, profuse, without septate or nonseptate swellings, saprobic on plant material. Zoosporangia initially pear-shaped, non-stalked, directly attached to the rhizomycelium wall, producing broad, operculate discharge tubes. Resting spores thick-walled with a large lipid globule, formed like zoosporangia.

Etymology: *phyto* (Greek) = plant refers to the substrate and *chytrium* refers to chytrid.

Phytochytrium stagnum Longcore & G.H. Jerônimo sp. nov. Fig. 2A–I

MycoBank: MB 841933

Typification: USA. MAINE: Orono, pond in Littlefield Ornamental Garden, University of Maine campus, isolated from onion skin bait placed with a water collection containing plant detritus collected 26 Sep 2020, *J.E. Longcore*. (Holotype Fig. 2). The photographs were based on the strain JEL1002 deposited at CZEUM-MICH. GenBank: OL473637; OL473639.

Etymology: *stagnum* (Latin)= pond, referring to the habitat.

Description: Rhizomycelium branched, profuse, without septate or nonseptate swellings, saprobic on plant material. Zoosporangia initially pear-shaped, non-stalked, directly attached to the rhizomycelium wall, producing broad, operculate discharge tubes. Resting spores thick-walled with a large lipid globule, formed like zoosporangia. In pure culture, zoosporangia up to 70 μm or greater diam.; operculate discharge tubes may extend to greater than the diameter of the zoosporangia and be narrow or broad. Resting spores approximately 30-μm diam.

Sparrowiella Longcore & G.H. Jerônimo gen. nov.

MycoBank: MB 841931

Type: *Sparrowiella insolita* Longcore & G.H. Jerônimo sp. nov. (described below).

Description: Fungus saprophytic. Polycentric rhizoidal system, branched, profuse, without septate or nonseptate swellings. Zoosporangia lateral, originated from stalked, ovate swellings and producing an extensive discharge tube. Operculum smooth, wall thin, colorless. Zoospores spherical, with a single and hyaline lipid globule. Resting spores thick

walled with a central lipid globule, spherical or subspherical, originated from stalked, ovate swellings.

Etymology: The prefix "Sparrow" is to honor the researcher Frederick Kroeber Sparrow Jr., who dedicated his academic career to the study of zoosporic fungi.

Sparrowiella insolita Longcore & G.H. Jerônimo sp. nov. Fig. 3A–J

MycoBank: MB 841932

Typification: USA. MAINE: Orono, pond in Littlefield Ornamental Garden, University of Maine campus, isolated from onion skin bait placed with a water collection containing plant detritus collected 26 Sep 2020, *J.E. Longcore*. (Holotype Fig. 3). The photographs were based on the strain JEL1004 deposited at CZEUM-MICH. GenBank: OL473638; OL473640.

Etymology: *insolita* (Latin) = uncommon, referring to the rarity of finding this species.

Description: Fungus saprophytic. Polycentric rhizoidal system, branched, profuse, without septate or nonseptate swellings. Zoosporangia lateral, originated from stalked, ovate swellings and producing an extensive discharge tube. Operculum smooth, wall thin, colorless. Zoospores spherical, with a single and hyaline lipid globule. Resting spores thick walled with a central lipid globule, spherical or subspherical, originated from stalked, ovate swellings. In pure culture, zoosporangia about 23-μm diam.; narrow discharge tubes extend to greater than the diameter of the zoosporangia; opercula obscure. Resting spores approximately 25-μm diam. subtended by sub-sporangial swelling.

Discussion

With the addition of our two new genera, 12 polycentric genera are verified by DNA analyses as being in six orders of the *Chytridiomycota* (Table 1). Of these, *Cladochytrium*, *Nowakowskiella*, *Septochytrium*, *Karlingiella*, and now *Phytochytrium* and *Sparrowiella* are in the *Cladochytriales*. Polycentricity and operculation were the pillars of classical chytrid taxonomy, having been used by Sparrow (1960) and Karling (1977) as primary criteria to group or to separate species, genera, and families. The availability of genetic tools during the last 20 years, however, has allowed the rDNA of many taxa with polycentric development to be sequenced and analyzed, revealing that polycentric growth is not a reliable taxonomic character reflective of phylogenetic relatedness. Although polycentricity and operculation are particularly common in the *Cladochytriales*, these characters have evolved independently in other orders of the *Chytridiomycota* (James et al. 2006).

The current described families of the *Cladochytriales* include the *Cladochytriaceae* J. Schröt., the *Nowakowskiellaceae* Sparrow ex S.E. Mozley-Standridge 2009, the *Septochytriaceae* S.E. Mozley-Standridge, and the *Endochytriaceae* Sparrow ex D.J.S. Barr. While the *Nowakowskiellaceae* is characterized by having unevenly expanded rhizomycelia, the *Cladochytriaceae* have isodiametric, narrow rhizomycelia with interspersed swollen regions that, except in *Karlingiella* (Jerônimo et al. 2019), are usually septate. Although it leaves the *Endochytriaceae* as a single-genus family based on the type species of *Endochytrium* (Barr 1980), we chose to expand the *Septochytriaceae* to include the monocentric-polycentric clade that is at a similar level of phylogenetic relatedness, by our reconstruction methods, as the *Cladochytriaceae* and the *Nowakowskiellaceae* (Fig. 1).

Phytochytrium and *Sparrowiella* are in different subclades of the *Septochytriaceae* but both produce a morphologically similar type of polycentric thallus. *Phytochytrium stagnum* is sister to monocentric strains with endobiotic thalli and operculate zoosporangia (JEL050 and JEL931), whereas *S. insolita* forms an independent lineage within the family. Although their rhizomycelia are similar, the two genera can be distinguished microscopically, not only by the subsporangial swelling in *S. insolita*, but also by their difference in scale. *Phytochytrium stagnum* is consistently larger than *S. insolita*. We have kept the same magnification for all photos (Figs. 2C, 3B) to make evident the size differences of the two new species.

The rhizoidal systems of monocentric *Septochytriaceae* differ from those of most other orders of the *Chytridiomycota* in that they tend to be long, sometimes extending many times the diameter of the developing zoosporangium, thus resembling the extended rhizomycelium of the two new polycentric species. This may be because the species so far discovered in this group grow on extensive substrates such as decaying plant leaves, where the ability to grow indeterminately allows organisms to make use of larger substrates.

The JEL isolates in our molecular analyses labeled as *Catenochytridium*, *Endochytrium*, or "unknown" (Fig. 1) were determined by the isolator based on morphological and substrate characteristics at the time that were isolated into pure culture. Therefore, these isolates serve to illustrate that convergent morphologies have masked phylogenetic diversity within the *Cladochytriales*. As can be seen by their distribution in the *Septochytriaceae*, these monocentric chytrids need to be re-examined and compared to type characteristics of genera and species to access and further justify taxonomic revisions.

Our ancestral state reconstruction allowed us to hypothesize for the first time a possible growth pattern of the common ancestral that gave origin to the order, besides to corroborate the unreliable nature of thalli's development as a taxonomic character. Considering that, our study brings important information about the evolution of *Cladochytriales* besides to expand the diversity

knowledge of the phylum and the plasticity morphology of polycentric development.

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Author contribution All authors contributed to the study conception and design. Sample collection and strain isolation were performed by Joyce E. Longcore, while cryopreservation and inclusion in CZEUM collection culture by D. Rabern Simmons and Timothy Y. James. Morphological characterization and description of new taxa were conducted by Joyce E. Longcore, Gustavo Henrique Jerônimo, D. Rabern Simmons, and Carmen L.A. Pires Zottarrelli. The DNA extraction and sequencing were performed by Gustavo Henrique Jerônimo and phylogenetic analyses by Gustavo Henrique Jerônimo, Kevin R. Amses, Kensuke Seto, and Timothy Y. James. The first draft of the manuscript was written by Gustavo Henrique Jerônimo, Joyce E. Longcore, Carmen L.A. Pires Zottarrelli and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability DNA sequences used in the present study are available in GenBank. Alignments and topologies were deposited in TreeBase (study TB2:S29000). Fungal specimens are stored in CZEUM collection culture (as indicated in specimens examined).

Declarations

Ethics approval Not applicable.

Consent to participate and consent for publication All authors have contributed to this work. They have also read and commented on the manuscript and accepted its publication in the *Mycological Progress* journal. This manuscript represents original work that is not being considered for publication, in whole or in part, in another journal, book, conference proceedings, or government publication with a substantial circulation.

Conflict of interest The authors declare no competing interests.

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