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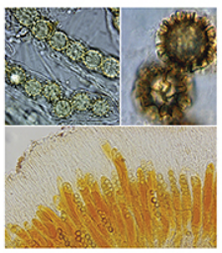

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Novel taxa in Cladochytriales (Chytridiomycota): *Karlingiella* (gen. nov.) and *Nowakowskiella crenulata* (sp. nov.)

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ABSTRACT

Six *Nowakowskiella* species from Brazil were identified and purified on corn meal agar (CMA) plus glucose and Peptonized Milk-Tryptone-Glucose (PmTG) media and placed into a phylogenetic framework for the genus. New sequence data are presented for four species: *N. elongata, N. multispora*, and *N. ramosa* and the new species *N. crenulata*. Our maximum likelihood and Bayesian analyses of combined 18S, 5.8S, and 28S subunits of nuc rDNA showed that *Nowakowskiella* is not a monophyletic clade because of the position of *Nowakowskiella elongata*, which is more related to *Cladochytrium*. We reclassify *N. elongata* as the type of a new genus, *Karlingiella*.

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Received 5 November 2018
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KEYWORDS
Chytrid; phylogeny; 3 new taxa

INTRODUCTION

Molecular phylogenetics have deeply influenced the systematics of several groups of fungi, and in the Chytridiomycota (chytrid fungi, or chytrids), DNA sequences, primarily of the nuc rDNA operon, have corroborated the relationships suggested by zoospore ultrastructure and have revealed that the classical taxonomic system, based on convergently derived thalli structures, is phylogenetically inaccurate (Letcher et al. 2006; Letcher and Powell 2014). Thus, numerous nomenclatural changes have been proposed, including the description of new species, genera, families, and orders (e.g., Letcher and Powell 2005; James et al. 2006; Letcher et al. 2006; Mozley-Standridge et al. 2009; Simmons et al. 2009; Longcore and Simmons 2012; Vélez et al. 2013; Karpov et al. 2014), and there have been major revisions and reclassifications of polyphyletic groups (e.g., Vélez et al. 2011; Letcher and Powell 2014).

The description of the Cladochytriales (Mozley-Standridge et al. 2009) segregated some genera classically included in Chytridiales based on molecular phylogenies of the partial 18S and 28S subunits of nuc rDNA. Additionally, of those species examined by transmission electron microscopy, all produce zoospores with a similar constellation of ultrastructural characters (Lucarotti 1981). Representatives of the order have different types of thallus development, reproductive structures, and ecological preferences. The polycentric genera *Nowakowskiella* J. Schröt. and *Cladochytrium* Nowak. are the largest in the order, but only a few species have been isolated, cultured, and included in molecular phylogenies, keeping their interspecific relationships, and indeed the monophyly of the generic concept, uncertain.

Nowakowskiella was proposed by J. Schröter to include the type species *N. elegans*, previously named *Cladochytrium elegans* Nowak. (Schröter 1893). Several species have been described, and currently the genus contains 18 described species (www.mycobank.org), which are morphologically recognized by the production of polycentric thalli with operculate zoosporangia, interconnected by gradually attenuated hyphal branches called rhizomycelia that contain occasional, nonseptate swellings (Sparrow 1960; Karling 1977). Originally included in the Nowakowskiellaceae by Sparrow (1942), this genus was transferred shortly thereafter to Megachytriaceae (Sparrow 1943). Neither family was validly published (International Code of Nomenclature for algae, fungi, and plant [ICN], Art. 39.1; Turland et al. 2018), and Mozley-Standridge et al. (2009) later validated and reinstated the original Nowakowskiellaceae. Although families of the Cladochytriales have been reshuffled by Mozley-Standridge et al. (2009) based on molecular phylogenetics, the most recently described species, *N. keratinophila* Hassan and Batko, was published over 30 years ago (Hassan and Batko 1988), indicative of the lack of attention that this order has received.

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During our work in Brazil, we found and cultured several species of *Nowakowskiella*, allowing a phylogenetic and morphological analysis to be performed. Considering this, we (i) describe the new species *N. crenulata* based on the distinctive morphology of the zoosporangia and resting spores, along with its phylogenetic placement; (ii) include *N. crenulata, N. elongata* Karling, *N. multispora* Karling, and *N. ramosa* Butler in phylogenetic analyses; (iii) transfer *N. elongata* to the new genus *Karlingiella*, based on its disparate phylogenetic position as sister to the Cladochytriaceae; and (iv) enhance knowledge about phylogenetic relationships within *Nowakowskiella* and the Cladochytriales.

MATERIALS AND METHODS

Isolation and culture.—We collected sediment surface, soil, and water samples from lentic and lotic freshwater habitats in several locations in the Brazilian Atlantic rainforest (TABLE 1) and baited samples with cellulosic substrates (onion skin, corn leaves, and cellophane) sterilized in ultraviolet light (2 h). Infected substrates were aseptically removed and placed on agar medium, on which chytrids were further isolated into pure culture by the collection of zoospores and/or rhizomycelium onto fresh media, including Peptonized Milk-Tryptone-Glucose (PmTG) with penicillin (Barr 1986; 1 g peptonized milk, 1 g tryptone, 5 g dextrose, 12 g agar, 0.1 g streptomycin sulfate, 0.1 g penicillin G, 1 L deionized water), corn meal agar (CMA) plus glucose (16 g BD [Franklin Lakes, New Jersey] BLL corn meal agar, 2.5 g glucose, 1 L deionized water), or yeast phosphate soluble starch (YPSS)/8 (Emerson 1958; 0.125 g yeast extract, 2.5 g soluble starch, 0.125 g K_2HPO_4, 0.062 g $MgSO_4 \cdot 7\ H_2O$, 12 g agar, 1 L deionized water). Pure cultures were deposited in the Coleção de Cultura de Algas, Fungos e Cianobácterias do Instituto de Botânica culture collection (CCIBt). Additionally, the strains were cryopreserved following the protocol of Boyle et al. (2003) and kept in −80 C conditions in the CCIBt culture collection. The voucher numbers, localities, geographic coordinates, kinds of samples, and habitats collected are shown in TABLE 1.

Nowakowskiella species produce thick-walled resting spores that do not germinate on standard agar media, and these structures can be diagnostic for certain species. To stimulate resting spore production, we cultivated our *Nowakowskiella* strains on YPSS/8 medium prepared with soil water as described by Lucarotti (1981). We added 7 g of garden soil and 0.05 g calcium carbonate to 250 mL of distilled water (dH_2O), steamed this mixture for 2 h on two consecutive days, and then filtered the solution twice with a Buchner funnel and Whatman No. 1 filter paper to remove all particulate material. After that, the solution was diluted in the ratio of two parts of dH_2O to 1 part of filtered solution, autoclaved, and used to prepare the YPSS/8 culture medium (Emerson 1958).

Table 1. Species, collection culture or voucher numbers, geographic coordinates, and types of samples and habitats collected for the 14 Cladochytriales strains isolated during this study.

Species	Collection culture or voucher number	Municipality in Sao Paulo State/Brazil	Geographic coordinates	Sample (habitat)
Cladochytrium replicatum	CCIBt 4014	Cananéia	25°03′05″/25°18′18″S, 47°53′48″/48°05′42″W	Water (stream)
Cladochytrium replicatum	CCIBt 3845	São Paulo	23°38′20.6″S, 46°37′34.3″W	Water (stream)
Cladochytrium replicatum	CCIBt 4263	Votorantim	23°25′33.72′′S, 47°35′42.66′′W	Water column (reservoir)
Cladochytrium replicatum	CCIBt 4390	Peruíbe	24°30′25.09″S, 47°15′42.08″W	Waterfall (stream)
Nowakowskiella crenulata	CCIBt 4258	Votorantim	23°34′49.92′′S, 47°25′32.64′′W	Sediment surface (reservoir)
Nowakowskiella crenulata	CCIBt 4259	Ibiúna	23°34′54.30′′S, 47°26′12.12′′W	Sediment surface (reservoir)
Nowakowskiella elongata (= *Karlingiella elongata*)	CCIBt 4016	Cananéia	25°03′05″/25°18′18″S, 47°53′48″/48°05′42″W	Water (stream)
Nowakowskiella elongata (= *Karlingiella elongata*)	ALJ09	Peruíbe	24°30′04.07″S, 47°15′58.0″W	Submerged leaves (river)
Nowakowskiella hemisphaerospora	GHJ13	Cananéia	23°25′40.86′′S, 47°35′31.14′′W	Sediment surface (reservoir)
Nowakowskiella multispora	CCIBt 4015	Cananéia	25°03′05″/25°18′18″S, 47°53′48″/48°05′42″W	Water (stream)
Nowakowskiella multispora	CCIBt 3864	São Paulo	23°38′18.8″S, 46°37′31.7″W	Water (stream)
Nowakowskiella ramosa	CCIBt 4294	Votorantim	23°34′49.92′′S, 47°25′32.64′′W	Water column (reservoir)
***Nowakowskiella* sp. 1**	CCIBt 4260	Votorantim	23°34′58.56′′S, 47°25′50.52′′W	Sediment surface (reservoir)
***Nowakowskiella* sp.**	ALJ23	Pedro de Toledo	24°22′51.04″S, 47°20′39.0″W	Soil
Polychytrium aggregatum	CCIBt 4017	Cananéia	25°03′05″/25°18′18″S, 47°53′48″/48°05′42″W	Soil
Polychytrium aggregatum	ALJ30	Iguape	24°32′28.1″S, 47°12′38.08″W	Water (river)

Note. The isolates highlighted in bold have never been cultured, sequenced, or included in phylogenetic analyses.

Morphology.—We observed the development of the strains on agar media (PmTG, CMA plus glucose, and/or YPSS/8) and colonized cellulosic substrates (onion skin, cellophane, and corn leaves). The type of development and the shapes and sizes of zoosporangia, resting spores, rhizomycelial swellings, and zoospores were examined using a Leica DMLB2 compound microscope and photographed with a Leica MC170 HD camera using Leica Qwin 3.1 software (Hessen, Wetzlar, Germany). We used the Sparrow (1960) and Karling (1977) monographs along with the original descriptions of the species (Butler 1907; Shanor 1942; Karling 1944, 1963) to identify our isolates.

DNA extraction, amplification, and sequencing.—For DNA extraction, a small piece of agar with zoosporangia or rhizomycelium was transferred to Erlenmeyer flasks containing 50 mL of PmTG liquid medium prepared with autoclaved reverse-osmosis water. After incubation for 10–20 d at 21 C, the entire biomass was aspirated with a pipette and then transferred to 2 mL microfuge tubes. These tubes were centrifuged for 15 min at 13 000 rpm to remove excess culture medium, and the resulting pellet was used for DNA extraction. The extraction of genomic DNA followed the protocol described in the PureLink Genomic DNA kit (Invitrogen, Carlsbad, California). Genomic DNA was visualized by electrophoresis of the extracts on an 0.8% agarose gel. Ribosomal subunits were amplified with the polymerase chain reaction (PCR) SuperMix kit (Invitrogen) at a final volume of 25 µL using the primer pairs (i) LR0R/LR5 (Vilgalys and Hester 1990) for partial 28S nuc rDNA; (ii) SR1R/NS4 (Vilgalys and Hester 1990) for 18S nuc rDNA; and (iii) ITS4/ITS5 (White et al. 1990) for complete internal transcribed spacer region (ITS1-5.8S-ITS2 = ITS) nuc rDNA. Thermocycling profiles followed cycles described by Marano et al. (2014). Amplicons were purified with the AxyPrep PCR Clean-up kit (Axygen, Corning, New York) or according to the protocol described by Schmitz and Riesner (2006). Sequencing was performed in an ABI 3730 DNA Analyzer (Life Technologies, Carlsbad, California) at the Centro de Pesquisa sobre o Genoma Humano e Células Tronco, Universidade de São Paulo, Brazil.

Phylogenetic analyses.—For phylogenetic reconstruction, we selected 24 isolates of Cladochytriales (TABLE 2), which represent the major genera with sequences in GenBank; three *Polychytrium aggregatum* strains (CCIBt 4017, ALJ30, JEL109) were used as one outgroup. We included sequences derived from our 14 new Cladochytriales strains isolated from different habitats at the Brazilian Atlantic rainforest (TABLES 1 and 2). The contiguous sequences were assembled using Sequencher 4.1.4 (Gene Codes, Ann Arbor, Michigan), and alignment was performed online using MAFFT 7.058 (Katoh et al. 2017). The 18S, 5.8S, and 28S nuc rDNA sequences were concatenated through SequenceMatrix 1.8 (Vaidya et al. 2010), resulting in a final length of 2383 base pairs. The maximum likelihood (ML) analyses was conducted in GARLI 2.01 (Bazinet and Cummings 2008) and Bayesian inference (BI) in MrBayes 3.2.2 (Ronquist et al. 2012) on the CIPRES Science Gateway platform (https://phylo.org), adding the partition models generated by jModelTest 0.1.1 (Posada 2008). We performed the ML analysis with 1000 bootstrap replicates and the BI using the Markov chain Monte Carlo (MCMC) methodology to calculate posterior probabilities (PPs). The parameters for BI were 5 million generations, with the first 10% of iterations discarded as burn-in, then sampled every 1000th iterations from the remainder. Values <50% (ML) or <0.50 (BI) are omitted from the final tree. The character matrix (concatenated alignment), ML tree, and BI tree are deposited in TreeBASE (study TB2:S23854).

RESULTS

We isolated 10 *Nowakowskiella* strains (TABLES 1 and 2), 6 of which were identified as described species and 2 as unknown species. The YPSS/8 soil medium was effective at stimulating resting spore production of *N. crenulata*, but the same was not observed with two unidentified strains, *Nowakowskiella* sp. 1 CCIBt 4260 and *Nowakowskiella* sp. ALJ23, which only produced zoosporangia. Because of this, we decided to restrict our identification of these two strains to the generic rank for now. Brief morphological comments, in addition to some pictures, are included below in consideration of the sparse information available for these taxa.

Morphology.—*Nowakowskiella ramosa* produces spheri cal to pyriform zoosporangia and resistant structures with a parenchymal region (Pr), which supports a single or as many as three resting spores with small incrustations in the wall (FIG. 1A–C). *Nowakowskiella hemisphaerospora* Shanor produces ovoid to pyriform zoosporangia (FIG. 1D) and septate resting spores, which form as two hemispheres, in which all contents migrate to one side, leaving the other side empty (FIG. 1E). *Nowakowskiella multispora* produces subspherical to pyriform zoosporangia (FIG. 1F) and intercalary resistant structures, formed by consecutive and linked resting spores with a homogenous content (FIG. 1G). *Nowakowskiella* sp. ALJ23 (FIG. 1H–J) has spherical and piriform

Table 2. Species, strains/vouchers, and GenBank accession numbers.

Taxon	Strain/voucher	GenBank accession number		
		SSU	ITS	LSU
Allochytridium luteum	ATCC 60989	JN940948	NA	AY439066
Catenochytridium sp.	JEL 145	EU828475	NA	EU828503
Catenochytridium sp.	JEL 044	EU828478	NA	EU828506
Cladochytrium replicatum	**CCIBt 4263**	**MH590084**	**MH590046**	**MH590071**
Cladochytrium replicatum	JEL 303	EU828461	NA	EU828488
Cladochytrium replicatum	**CCIBt 3845**	**MH590085**	**MH590047**	**MH590072**
Cladochytrium replicatum	**CCIBt 4390**	**MH590086**	**MH590048**	**MH590073**
Cladochytrium replicatum	JEL 180	NG017169	NA	NG027614
Cladochytrium replicatum	WJD 123	NA	NA	KC691378
Cladochytrium replicatum	**CCIBt 4014**	**KJ464414**	**NA**	**KJ464415**
Cladochytrium sp.	SMS 013	EU828459	NA	EU828486
Cladochytrium sp.	JEL 153	EU828458	NA	EU828485
Cladochytrium sp.	BR 696	JN940946	JN943816	JN941001
Cylindrochytridium johnstonii	JEL 596	JF796051	NA	JF796052
Endochytriaceae	JEL 072	EU828470	NA	EU828497
Endochytrium ramosum	JEL 402	EU828484	NA	EU828513
Endochytrium sp.	JEL 324	AY635844	AY997044	DQ273816
Nephrochytrium sp.	JEL 125	AH009049	NA	EU828511
Nowakowskiella elegans	M 29	NA	AY353257	AY349080
Nowakowskiella elegans	JEL 046	EU828463	NA	EU828490
Nowakowskiella elegans	UCB 50 1	EU828464	NA	EU828491
Nowakowskiella elegans	JEL 157	EU828465	NA	EU828492
Nowakowskiella elongata (= *Karlingiella elongata*)	**CCIBt 4016**	**MH590087**	**MH590049**	**MH590074**
Nowakowskiella elongata (= *Karlingiella elongata*)	**ALJ 09**	**NA**	**MH590050**	**MH590075**
Nowakowskiella hemisphaerospora	**GHJ 13**	**MH590088**	**MH590051**	**MH590076**
Nowakowskiella multispora	**CCIBt 3864**	**MH590089**	**MH590052**	**MH590077**
Nowakowskiella multispora	**CCIBt 4015**	**KJ539147**	**KJ539148**	**KJ539149**
Nowakowskiella ramosa	**CCIBt 4294**	**MH590090**	**NA**	**MH590078**
***Nowakowskiella* sp.**	**ALJ 23**	**MH590091**	**MH590053**	**MH590079**
Nowakowskiella elegans	JEL 127	EU828466	NA	DQ273798
Nowakowskiella sp.	JH HBR	EU828469	NA	EU828496
Nowakowskiella sp.	JH CC2	EU828481	NA	EU828509
Nowakowskiella sp.	JH SA	EU828482	NA	EU828510
Nowakowskiella crenulata	**CCIBt 4258**	**MH590092**	**MH590054**	**MH590080**
Nowakowskiella crenulata	**CCIBt 4259**	**MH590093**	**MH590055**	**MH590081**
***Nowakowskiella* sp. 1**	**CCIBt 4260**	**MH590094**	**MH590056**	**MH590082**
Polychytrium aggregatum	JEL 109	NG017168	AY997074	AY349084
Polychytrium aggregatum	ALJ 30	MH590095	MH590057	MH590083
Polychytrium aggregatum	CCIBt 4017	KJ464408	KJ464409	KJ464410
Septochytrium sp.	JEL 177	EU828474	NA	EU828502
Septochytrium variabile	JEL 191	EU828483	NA	EU828512

Note. The taxa highlighted in bold represent the strains isolated during this study.

zoosporangia, which are supported by terminal or intercalary spherical apophysis (FIG. 1H–I). The zoosporangia produce a long discharge tube (FIG. 1J), and zoospores contain a single, hyaline lipid globule. Resting spores were not observed. *Nowakowskiella* sp. 1 CCIBt 4260 (FIG. 1K–N) forms intercalary or terminal zoosporangia, which are commonly spherical, subspherical, or pyriform (FIG. 1K–N), with a small discharge tube (FIG. 1N). Additionally, zoosporangia are subtended by prominent apophyses, which are produced on both sides of zoosporangia when development is intercalary (FIG. 1M). The zoospores have a single, hyaline lipid globule and are released in a membrane-bound mass (FIG. 1K), in which the zoospores remain immobile for a short period before becoming mobile and rupturing the binding membrane. The new species *N. crenulata* (FIG. 3) produces spherical and subspherical zoosporangia (FIG. 3C–E) with an operculum at the apex (FIG. 3E). The apophyses are prominent and clavate (FIG. 3C, D), and the resting spore produces singular crenulated ornamentations in the wall (FIG. 3J). Additionally, the branch that supports the resting spore is septate (FIG. 3H). The zoospores produce a single and hyaline lipid globule (FIG. 3B).

Phylogenetic analyses.—The combined sequence data (18S, 5.8S, and 28S of the nuc rDNA) had 2383 characters, with 1343 parsimony-informative sites. jModelTest indicated the most appropriate models of DNA substitution were TrNef+I (18S), TPM1uf+G (5.8S), and TIM3ef+I+G (28S), according to Akaike information criterion (AIC). The phylogenetic reconstruction contains two strongly supported clades that represent the Nowakowskiellaceae and Cladochytriaceae families proposed by Mozley-Standridge et al. (2009), although internal configurations and support values for these families were altered (FIG. 2). In the

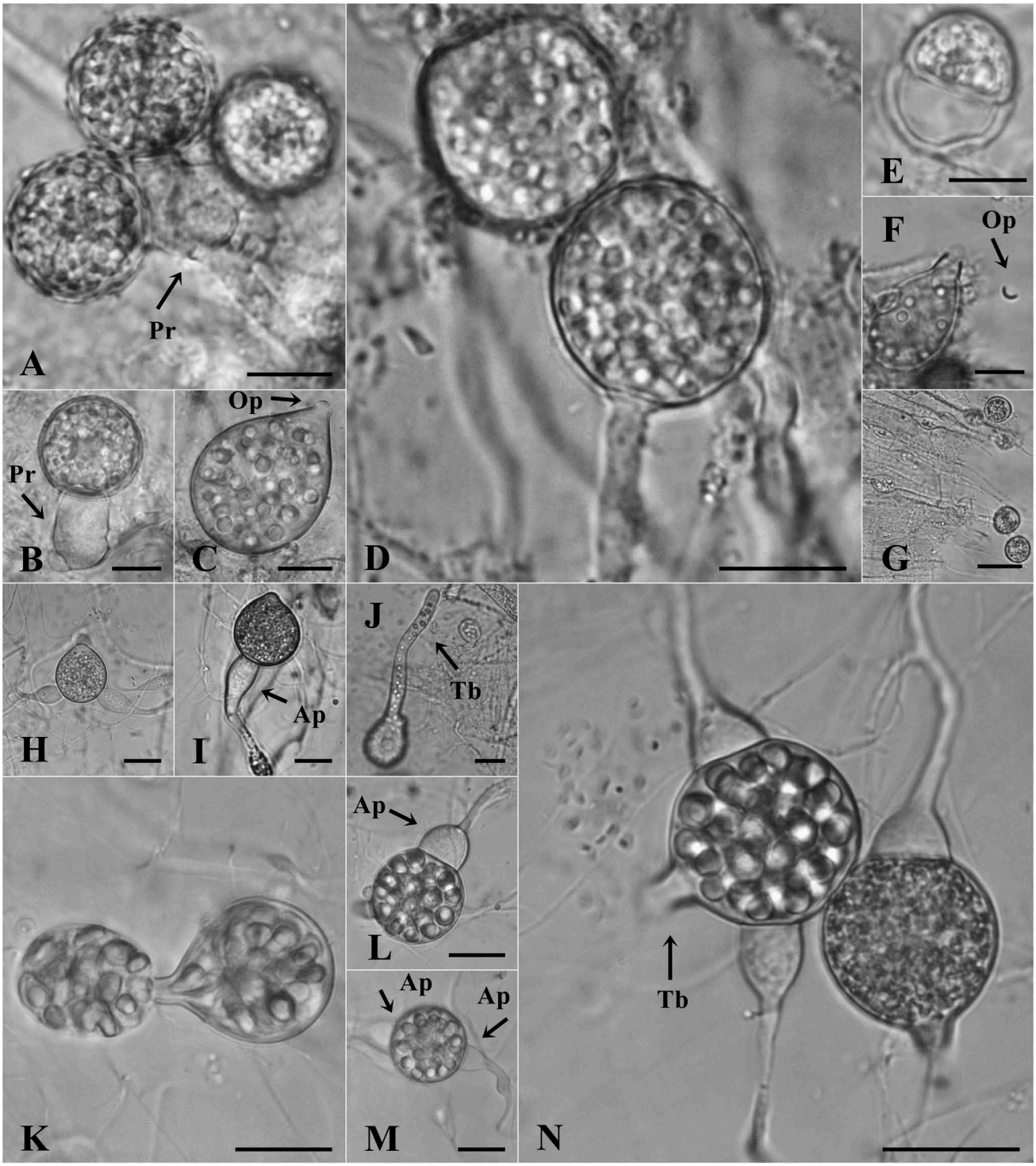

Figure 1. Zoosporangia and resting spores morphology of *Nowakowskiella ramosa, N. hemisphaerospora, N. multispora, Nowakowskiella* sp. 1 CCIBt 4260, and *Nowakowskiella* sp. ALJ23. A–C. *Nowakowskiella ramosa* CCIBt 4294. A–B. Ornamented resting spores with a parenchymal region (Pr). C. Operculate (Op) zoosporangia. D–E. *Nowakowskiella hemisphaerospora* GHJ13. D. Spherical zoosporangia. E. Septate resting spores. F–G. *Nowakowskiella multispora* CCIBt 4015. F. Operculate (Op) zoosporangia. G. Resting spores. H–J. *Nowakowskiella* sp. ALJ23. H. Intercalary apophysate zoosporangium. I. Terminal apophysate (Ap) zoosporangium. J. Zoosporangium with a long discharge tube (Tb). K–N. *Nowakowskiella* sp. 1 CCIBt 4260. K. Zoospores discharge. L. Terminal apophysate (Ap) zoosporangium. M. Intercalary apophysate (Ap) zoosporangia. N. Mature and immature intercalary zoosporangia with a discharge tube (Tb). Ap = apophysis; Op = operculum; Pr = parenchymal region; Tb = discharge tube. Bars = 10 µm.

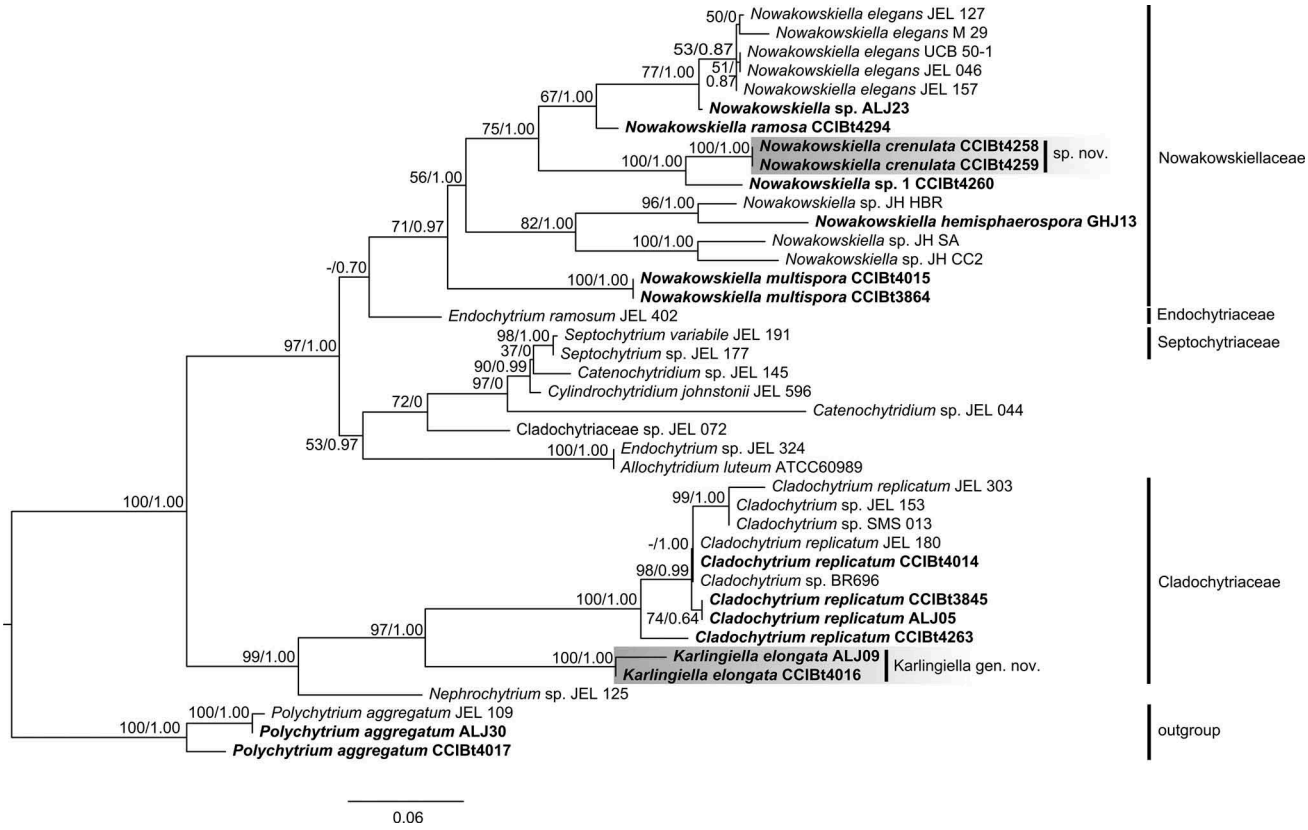

Figure 2. Phylogram inferred from maximum likelihood (ML) analysis of 37 ingroup isolates in Cladochytriales order based on combined (SSU+5.8S+LSU) sequences data. Maximum likelihood bootstrap support values <50% are indicated with (-). Bayesian posterior probability values >0.50 are labeled numerically. The clades that were not recovered in the Bayesian tree are indicated with (0). The bar indicates the number of substitutions per site. Strains highlighted in bold were isolated during this study, and those highlighted in gray represent the taxonomic novelties.

Nowakowskiellaceae, *N. ramosa* and *Nowakowskiella* sp. (ALJ23) are included in a supported clade with the type species, *N. elegans*, and the new species *N. crenulata* and *Nowakowskiella* sp. 1 (CCIBt 4260). *N. hemisphaerospora*, along with undescribed species, is sister to this clade. *Nowakowskiella multispora* is a basal lineage within the genus. Finally, the phylogenetic placement of our *N. elongata* strains indicates that this species is not related to the monophyletic genus, and considering that, we transfer the species to a new genus *Karlingiella*, within Cladochytriaceae.

TAXONOMY

Nowakowskiella crenulata G.H. Jerônimo & C.L.A. Pires-Zottarelli, sp. nov. FIG. 3A–J

MycoBank MB829117

Typification: BRAZIL. SÃO PAULO: Votorantim, Santa Helena reservoir, 23°34′54.30″S, 47°26′12.12″W, from sediment samples baited with onion skin and corn leaves, 20 Mar 2014. **Holotype** (FIG. 3A–J). Diagnosis based on *CCIBt 4258*. **Ex-type**, *G.H. Jerônimo CCIBt 4258*. GenBank (CCIBt 4258): 18S = MH590092; ITS = MH590054; LSU = MH590080.

Other material examined: BRAZIL. SÃO PAULO: Ibiúna, Itupararanga reservoir, 23°34′54.30″S, 47°26′12.12″W, from sediment samples baited with onion skin and corn leaves, 20 Mar 2014. *CCIBt 4259*. GenBank (CCIBt 4259): SSU = MH590093; ITS = MH590055; LSU = MH590081.

Etymology: From *crenulatus* (Latin), referring to the morphology of the resting spores, which have small crenulated invaginations in their walls.

Fungus saprophytic. Polycentric rhizoidal system, branched, profuse, with numerous nonseptate swellings, broadly fusiform, 4.5–5 × 3–4.5 µm, or spherical and 3–5 µm. Zoosporangia terminal, occasionally intercalary, hyaline, smooth, spherical, 20–23 µm, subspherical, 19–24 × 20–27 µm, or ovoid, 21–25 × 23–30 µm, usually apophysate, without a discharge tube; operculum smooth, wall thin, smooth, colorless. Apophysis clavate, 10–25 × 5–12 µm, or nearly spherical, 10–14 µm. Zoospores spherical, 2–3 µm diam, with a single, hyaline lipid globule. Resting spores spherical, 7–7.5 µm, or subspherical and 7–7.5 × 7–7.5 µm

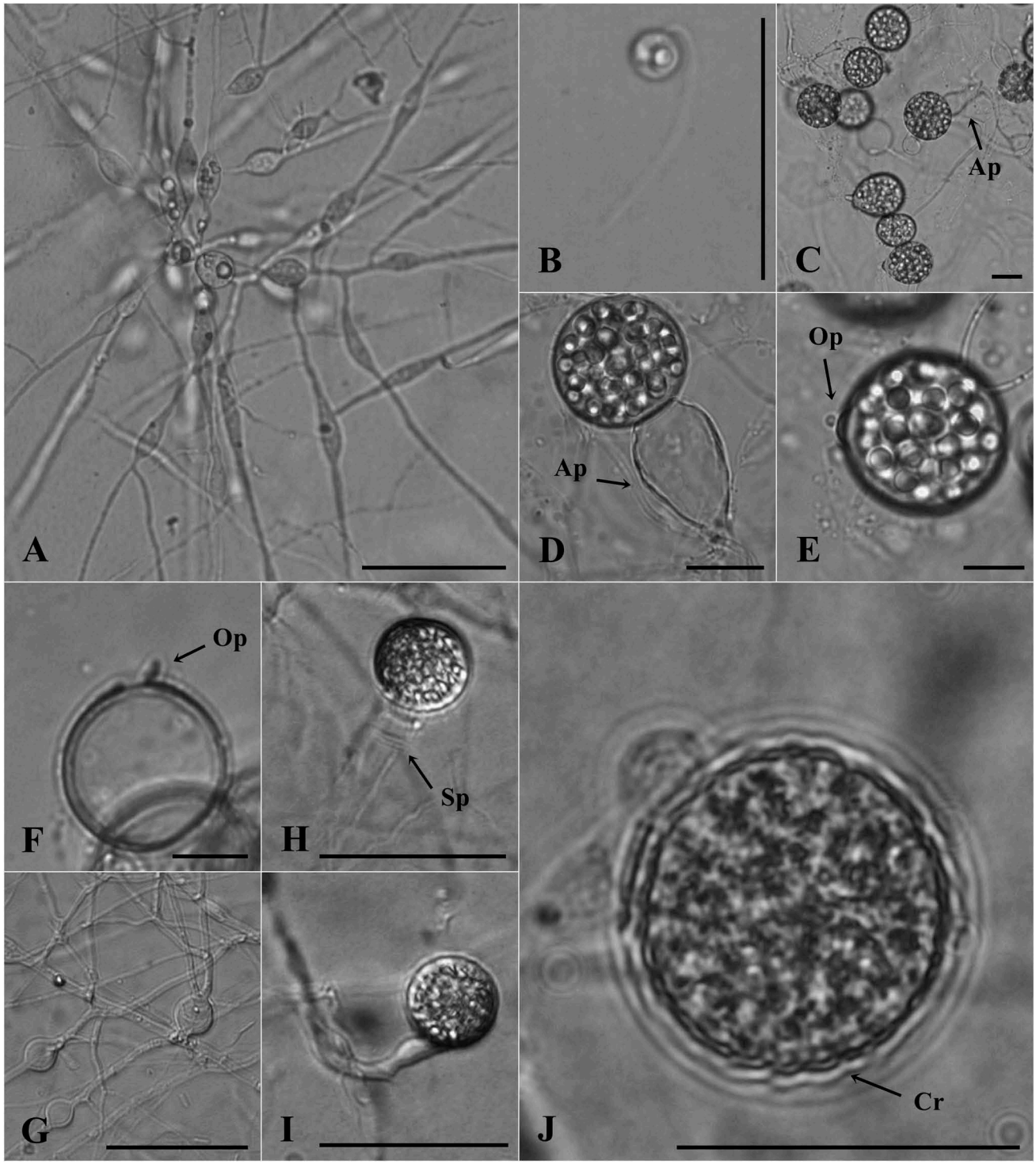

Figure 3. Morphology of *Nowakowskiella crenulata* CCIBt 4258 on onion skin and YPSS/8 broth. A. Early development stages in YPSS/8 broth. B. Encysted zoospores in YPSS/8. C–D. Zoosporangia and prominent clavate apophysis (Ap) on onion skin. E–F. Operculate zoosporangia (Op) on onion skin. G. Rhizomycelium with nonseptate swellings in YPSS/8 broth. H–J. Crenulate resting spores with a delimiting septum (Sp) in YPSS/8. Ap = apophysis; Op = operculum; Sp = septum; Cr = crenulated ornamentations. Bars = 10 µm.

diam, ornate with crenulated invaginations in the wall, supported by a septate branch.

Notes: The strains CCIBt 4258 and CCIBt 4259 grow indeterminately on PmTG, CMA plus glucose, and even on YPSS/8, but resting spores were only observed in YPSS/8 broth. Both strains were isolated from sediment surface samples collected from an oligotrophic ("Santa Helena": 23°34′49.92″S, 47°25′32.64″W) and

a mesotrophic ("Itupararanga": 23°34′54.30″S, 47°26′12.12″W) reservoir. We were also able to bait a third strain from sediment samples of an eutrophic reservoir ("Hedberg": 23°25′40.86″S, 47°35′31.04″W) but were unable to isolate a culture from the colonized bait. The finding of *N. crenulata* from sediment samples of reservoirs with different trophic states (oligotrophic, mesotrophic, and eutrophic) indicates that this species can survive in a wide range of limnological conditions.

Karlingiella Jerônimo, Jesus & Pires-Zottarelli, gen. nov.

MycoBank MB829118

Typification: Karlingiella elongata (Karling) Jerônimo, Jesus & Pires-Zottarelli.

Etymology: The prefix of the genus name honors the researcher John S. Karling, who originally described this species, and several other *Nowakowskiella* species after a visit to the Brazilian Amazon rainforest.

Fungus saprophytic. Polycentric rhizoidal system, branched, profuse, with numerous nonseptate swellings. Operculate zoosporangia, terminal or intercalary, elongate, cylindrical or globose, frequently producing a parenchymal basal region. Zoospores spherical, with a single, hyaline lipid globule. Resting spores intercalary, spherical or oval, hyaline, containing a large refractive globule. The genus is sister to the Cladochytriaceae in molecular phylogeny of the combined nuc rDNA genes.

Type species: Karlingiella elongata (Karling) Jerônimo, Jesus & Pires-Zottarelli.

Karlingiella elongata (Karling) Jerônimo, Jesus & Pires-Zottarelli, comb. nov. FIG. 4A–I

MycoBank MB829121

Basionym: Nowakowskiella elongata Karling, Bull Torrey Bot Club 71:375. 1944.

Typification: Karling (1944) did not designate a type in the original description. Figures 30–44 from the original description (Karling 1944) are designated here as **lectotype**, MBT 386057 (ICN, Art. F.5.4).

Specimens examined: BRAZIL. SÃO PAULO: Cananéia, Ilha do Cardoso, 25°03′05″/25°18′18″S, 47°53′48″/48°05′42″W, soil sample from an Atlantic rainforest area, baited with onion skin and corn leaves, 6 Nov 2012, *G.H. Jerônimo CCIBt 4016*. GenBank: 18S = MH590087; ITS = MH590049; 28S = MH590074. Peruíbe, Mosaico de Unidades de Conservação Juréia-Itatins, 24°30′04.07″S, 47°15′58.0″W, from submerged leaves samples in an Atlantic rainforest stream, baited with onion skin and corn leaves, 24 Aug 2016, *A.L. Jesus ALJ09*. GenBank: ITS = MH590050; 28S = MH590075.

Notes: The morphologies of our isolates (CCIBt 4016, ALJ09) fit Karling's (1944) description of *N. elongata*, with no additional or contradictory characters. The polycentric thalli produce zoosporangia that are elongate, 25–85 × 10–33 µm, and operculate and frequently with a parenchymal basal region. Resting spores are hyaline and commonly spherical, 16.5–33 µm, or oval and 20–25 × 18–23 µm with a large refractive globule surrounded by several smaller droplets. These strains grow indeterminately on PmTG, CMA plus glucose, or even YPSS/8 media. On agar media, cultures are predominantly rhizomycelial, but zoosporangia and resting spores are produced occasionally. The elongated or cylindrical zoosporangia were most commonly observed on cellulosic baits (onion skin and corn leaves) rather than on culture media.

DISCUSSION

Our phylogeny represents the largest analysis of *Nowakowskiella* to date and provides a phylogenetic backbone for the interspecific relationships within *Nowakowskiella*. Branch lengths are long, and support values are low, leaving the definitive phylogeny unknown until additional cultures can be examined. However, the inclusion of *N. crenulata, N. ramosa, N. multispora*, and *N. elongata* makes an important contribution to understanding the relationships of the species of this genus (FIG. 2). By the inclusion of these species in a molecular phylogeny, we have shown that (i) *N. ramosa* is sister to the type species *N. elegans*; (ii) *N. multispora* represents the basal lineage in the genus; (iii) *N. elongata* is within the Cladochytriaceae on a supported, basal lineage, justifying its placement into the new genus *Karlingiella*; and (iv) there is genetic variation within described species of the Cladochytriales, suggesting possible cryptic species, and genetic evidence for additional undescribed species that warrant further study of thallus and zoospore morphologies.

The strains that we isolated in Brazil contribute to enhancing our understanding of some convergent characters traditionally used in the classical taxonomy of these chytrids. In particular, the phylogenetic placement of *Karlingiella* as a sister group of *Cladochytrium* and *Nephrochytrium* sp. JEL 125 indicates that morphological characters such as operculation, thallus development, and rhizomycelial swellings septation are insufficient to determine the identification of these fungi at the genus level. The phylogenetic placement of *Karlingiella* suggests that the turbinate cells, characteristic of *Cladochytrium*, could be derived from nonseptate swellings observed in *Karlingiella*. The sharing of some morphological characters previously thought to be characteristic of specific genera, such as the turbinate cells in *Cladochytrium* in the Cladochytriales and

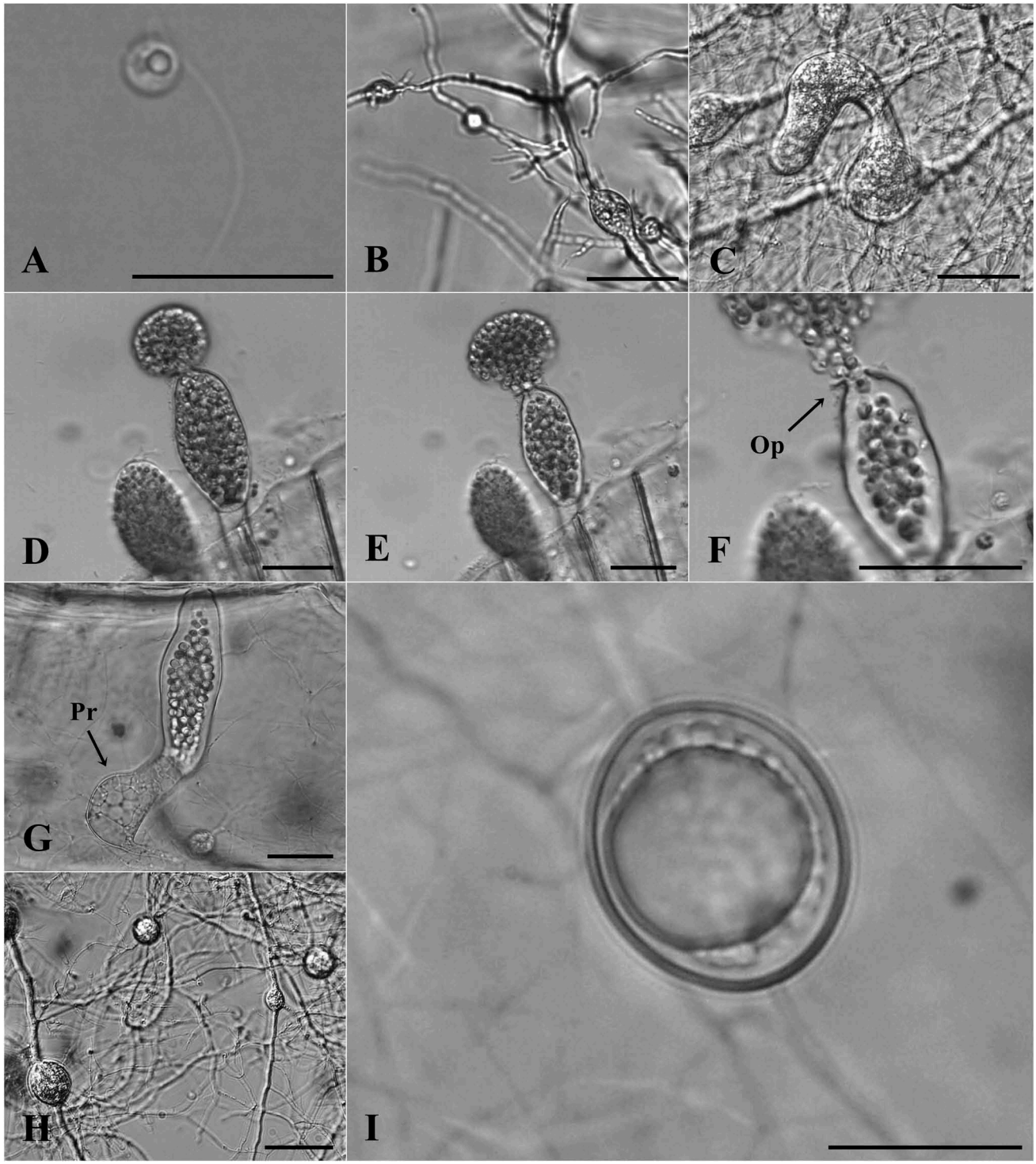

Figure 4. Morphology of *Karlingiella elongata*. A. Zoospores, each with a single hyaline lipid globule. B. Zoosporangia and rhizomycelium on PmTG medium. C. Intercalary zoosporangia with elongate tube in YPSS/8. D–F. Zoosporangium in onion skin releasing zoospores and operculum (Op). G. Zoosporangium with a parenchymal basal region (Pr) in onion skin. H. Rhizomycelium and nonseptate swellings on PmTG agar. I. Resting spores with a large lipid globule surrounded by small droplets in PmTG. Op = operculum; Pr = parenchymal region. Bars = 10µm.

the recently described *Zopfochytrium* (Powell et al. 2018) in the Chytridiales, indicates that DNA sequence data should be given priority when assigning taxa to these morphologically convergent groups.

The description of the new species *Nowakowskiella crenulata* represents the first new *Nowakowskiella* species since Hassan and Batko (1988) described *N. keratinophila* from Poland. The delimitation and classification of

N. crenulata as a new species is based on its singular morphology and its separate, supported phylogenetic lineage, whereas the new genus *Karlingiella* and new combination *Karlingiella elongata* are based on the unexpected disparate phylogenetic placement of *N. elongata* as sister group of the Cladochytriaceae. Future studies focusing on zoospore ultrastructure and DNA sequence data are necessary in order to further justify additional taxa and produce a better-resolved phylogeny of the Cladochytriales.

The genus *Nowakowskiella* is distributed worldwide (Sparrow 1960; Karling 1977; Lucarotti 1981; Hassan 1983; Hassan and Batko 1988; Czeczuga and Muszynska 1999; Marano et al. 2007, 2008; Nascimento and Pires-Zottarelli 2009; Godlewska et al. 2013; Jesus et al. 2013; Muszynska et al. 2014; Jerônimo et al. 2015). In Brazil, this genus is frequently reported from soil and water samples (Pires-Zottarelli and Gomes 2007; Jesus et al. 2013; Jerônimo et al. 2015), presenting high frequency of occurrence and abundance. *Karlingiella elongata* (as *N. elongata*) was reported from several locations in Atlantic and Amazon Brazilian rainforests (Karling 1944, 1945; Nascimento and Pires-Zottarelli 2009; Jesus et al. 2013; Jerônimo et al. 2015). It has been a common species baited from freshwater ecosystems (Nascimento and Pires-Zottarelli 2009; Jesus et al. 2013; Jerônimo et al. 2015). Furthermore, some species such as *N. granulata, N. macrospora*, and *Karlingiella elongata* were originally described from Brazil (Karling 1944, 1945), suggesting that the country could represent a rewarding region to focus future studies of the order's biodiversity and molecular systematics.

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