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Letcher et al.---A New *Rozella* From *Pythium*

Morphology, Ultrastructure, and Molecular Phylogeny of *Rozella multimorpha*, a New Species in Cryptomycota

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ABSTRACT

Increasing numbers of sequences of basal fungi from environmental DNA studies are being deposited in public databases. Many of these sequences remain unclassified below the phylum level because sequence information from identified species is sparse. Lack of basic biological knowledge due to a dearth of identified species is extreme in Cryptomycota, a new phylum widespread in the environment and phylogenetically basal within the fungal lineage. Consequently, we are attempting to fill gaps in the knowledge of *Rozella*, the best-known genus in this lineage. *Rozella* is a genus of unwalled, holocarpic, endobiotic parasites of hosts including Chytridiomycota, Blastocladiomycota, Oomycota, Basidiomycota, and a green alga, with most species descriptions based on morphology and host specificity. We found a *Rozella* parasitizing a *Pythium* host that was a saprobe on spruce pollen bait placed with an aquatic sample. We characterized the parasite with light microscopy, TEM of its zoospores and sporangia, and its 18S/28S rDNA. Comparison with other *Rozella* species indicates that the new isolate differs morphologically, ultrastructurally, and genetically from *Rozella* species for which we have data. Features of the zoospore also differ from those of previously studied species. Herein we describe the *Rozella* as a new species, *R. multimorpha*.

Keywords

Monosporangiate; parasite; polymorphic; polysporangiate; *Pythium*; zoospore.

CRYPTOMYCOTA (M. D. M. Jones & T. A. Richards) emend Karpov & Aleoshin in the superphylum Opisthosporidia Karpov, Aleoshin & Mikhailov (Lara et al. 2010; Jones et al. 2011a, b; Karpov et al. 2014) is a phylum phylogenetically basal within the fungal lineage, and is composed of numerous environmentally derived phylotypes and relatively few described species. The lineage contains *Rozella* and the microsporidian-like parasites *Paramicrosporidium saccamoebae*, *P. vannellae* (Corsaro et al. 2014a), *Nucleophaga amoebae*, *N. terricolae* (Corsaro et al. 2014b, 2016), *Mitosporidium daphniae* (Grossart et al. 2016), and *Antonospora locustae* (Tedersoo et al. 2017). Most members of this lineage derive from soil and freshwater habitats, but also from some marine habitats. Because of its phylogenetic position, genetic affiliations, mode or modes of nutrition, and cell wall composition, an ongoing debate places Cryptomycota

(=Rozellomycota) either with the Fungi (e.g., James & Berbee 2012; Corsaro et al. 2014; Tedersoo et al. 2017) or with non-fungal organisms in Holomycota (e.g., Karpov et al. 2014, 2017 as Rozellosporidia). With the exception of *Rozella*, we know little about the species that comprise this lineage.

The genus *Rozella* Cornu was described almost 150 years ago (Cornu 1872) to include four species of endobiotic, holocarpic parasites of Oomycota and Chytridiomycota. Today the genus encompasses 25 described species of which the majority have a thallus that produces a single sporangium (monosporangiate), whereas three species are composed of multiple sporangia separated from each other by cross walls (polysporangiate). Characteristically, species of *Rozella* produce posteriorly uniflagellate zoospores and thick-walled, spherical, spiny (occasionally smooth) resting spores (Sparrow 1960). *Rozella* was long considered a member of Chytridiales (Sparrow 1960), but a multigene phylogeny placed it as the earliest diverging lineage in the fungi (James et al. 2006).

Our research here expands our knowledge of Cryptomycota generally, through the addition of unidentified environmental sequences to the lineage, and *Rozella* more specifically, with our discovery of a new *Rozella*, strain JEL 883 described here as *R. multimorpha* sp. nov. *Rozella multimorpha* is primarily monosporangiate, but occasionally produces a pair of sporangia separated by a cross wall that might be construed as "occasionally polysporangiate". In our inferred molecular phylogeny strain JEL 883 is in a lineage separate from lineages that appear to be either singularly monosporangiate or polysporangiate. Herein we examine the morphology, ultrastructure, and molecular phylogenetic placement of our new species of *Rozella* and compare its ultrastructure with that of other examined *Rozella* species.

MATERIALS AND METHODS

Isolation

In the summer of 2016 we collected a water sample from a pond located on the campus of the University of Maine, and baited it with spruce pollen to detect zoosporic fungi. An oomycete, later verified as *Pythium*, grew on the spruce pollen; we observed unusual (for oomycetes) zoosporangia and later discharge of minute, posteriorly uniflagellate, *Rozella* zoospores from swollen, polymorphic structures with long discharge tubes. We maintained the *Rozella*, designated strain JEL 883, in gross culture by weekly to biweekly adding a subsample of

microscopically verified, actively infected host on pollen to a small dish of distilled water with freshly added, spruce pollen grains. We did not attain a pure culture.

Light microscopy

For light microscopy, crude cultures of strain JEL 883 infecting *Pythium* sp. on spruce pollen were examined for morphology and development using either a Nikon Eclipse E400 compound microscope with a Spot RT3 digital camera or a Zeiss Axioskop equipped with a Zeiss Axiocam MRc camera.

Transmission electron microscopy

For transmission electron microscopy (TEM) of thallus features, individual sporangia at various stages of development were prepared following the protocol in Letcher et al. (2016). For TEM analysis of the zoospore, a small mass of pollen grains colonized with the *Pythium* host that was infected with strain JEL 883 was placed under a coverslip. The mass was periodically hydrated with deionized water. When one or more sporangia were observed discharging zoospores, the droplet of water under the coverslip was pipetted off and placed in a solution of 2.5% glutaraldehyde in 0.1 M sym-collidine buffer. This procedure was repeated approximately 100 times over a period of six days to accumulate an adequate sample of zoospores for TEM examination. The glutaraldehyde/buffer/zoospore suspension was processed as described in Letcher et al. (2016).

DNA extraction, purification, and amplification

For strain JEL 883, approximately 0.1 g of infected *Pythium* on pollen was removed from gross culture and aseptically examined on a depression slide in DNAse-free water. After 30 min, posteriorly-uniflagellate zoospore activity was observed in the water. The zoospore solution was aspirated from the slide, and DNA was extracted with CTAB buffer (James et al. 2008). For strains JEL 880 and JEL 882, DNA was extracted with the E.Z.N.A.®Fungal DNA Mini kit (Omega Bio-tek, Norcross, GA USA) following the manufacturer's instructions. For all three strains, amplification of the 18S region of nuclear rDNA (18S) was obtained with primers Rozella 1F and Rozella 1R (Lazarus & James 2015) or SR1R and NS4 (Vilgalys & Hester 1990; White et al. 1990), and amplification of the 28S region of nuclear rDNA (28S) was obtained with

primers LR0R/LR5 (Rehner & Samuels 1994, Vilgalys & Hester 1990). PCR was conducted with the Rozella 1F and Rozella 1R primers in 25 µL reactions with the following recipe: 0.2 µL ExTaq DNA polymerase (Takara Bio, Inc., Shiga, Japan), 10.2 µL H2O, 3.2 µL Bovine Serum Albumin, 3.2 µL ExTaq Buffer, 2.6 µL ExTaq 2 mM MgCl2, 2.6 µL ExTaq 10 mM dNTPs, 1 µL each primer, and 1 µL DNA. PCR was conducted with all other primers in 12.5 µL reactions with the following recipe: 6.25 µL GoTaq Green Master Mix (Promega, Madison, WI USA), 0.625 µL each primer (10µM), and 5 µL DNA. The thermocycler protocol for all amplifications was 94 °C for 3 min; 35 cycles at 94 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min; 72 °C for 7 min. PCR products were purified using ExoSAP (Promega) and sequenced at the University of Michigan DNA Sequencing Core. A consensus sequence of the products from primers was then generated in Geneious 9.1.7 (Biomatters Ltd., Auckland, NZ) and used in downstream phylogenetic analyses.

Phylogenetic analysis

Concatenated sequences of partial 18S/partial 28S rDNA of strains JEL 882 and JEL 883, and a partial 18S sequence of strain JEL 880 were incorporated in a database of strains of *Rozella*, *Mitospordium daphnia*, Aphelida, and related environmental sequences, 23 of which were comprised of only 18S rDNA sequence data. The separate loci alignments were produced in Geneious, and the Akaike information criterion in jModeltest 0.1.1 (Guindon & Gascuel 2003, Posada 2008) was used to select the nucleotide substitution model for the two loci. Maximum likelihood (ML) phylogenetic analyses were conducted in GARLI 2.01 (Zwickl 2006) with these model parameters to find the best tree topology and bootstrap support values from 500 replicates, summarized in SumTrees (Sukumaran & Holder 2010). Bayesian posterior probabilities (BPP) were determined with the same model parameters with MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) in two runs of four chains, each executing 5,000,000 generations with sampling every 500 generations for 10,001 trees, with BPP consensus values calculated after a burn-in of the first 2,500 trees.

RESULTS

Thallus morphology

Uninfected host hyphae were ~ 2.2 µm diam. and isodiametric (Fig. 1A). Infection of the host by zoospores of strain JEL 883 produced terminal and intercalary swellings (Fig. 1B) as sporangial initials. Some infected host hyphae became extensively hypertrophied (Fig. 1C). Developing parasite sporangia were initially elongate (Fig. 1D) and at maturity were primarily spherical, ~ 15–25 µm diam., usually with one (occasionally two or three) discharge tubes (Fig. 1E–I). The discharge tube terminated with a slight constriction and bulb (Fig. 1G, I, J).

Although the majority of sporangia were spherical, polymorphic variation was common (Fig. 2). Some sporangia were irregularly shaped, occasionally with multiple discharge tubes (Fig. 2A). Some sporangia consisted of two (occasionally three) swellings, with one or more discharge tubes (Fig. 2B–D, F). Rarely, adjacent sporangia separated by a cross wall were observed (Fig. 2E). Numerous sporangia of all morphological variations contained a spherical vacuole (Fig. 2F).

The degree of development of the sporangium could be determined by the appearance of the sporangial contents (Fig. 3A–C). Prior to cleavage the contents appeared coarsely granular (Fig. 3A); as the cytoplasm continued to mature it became finely granular (Fig. 3B), and upon zoospore cleavage and just prior to discharge, individual lipid globules, each associated with an individual zoospore, were visible (Fig. 3C). Zoospore discharge followed cleavage within minutes (Fig. 3D). Empty sporangia did not collapse, and the bulb at the tip of the discharge tube before discharge was absent (Fig. 3E). Zoospores did not swarm in the sporangium before discharge but upon discharge, the majority of zoospores rapidly emerged as a mass and immediately dispersed; zoospores remaining in the sporangium emerged individually moments after the mass was released (Fig. S1). Pockets of flagella were observed among mature zoospores at discharge (Fig. 3F).

Ultrastructure

The zoospore of strain JEL 883 (Fig. 4) is 1–1.2 µm diam. and 1.6–1.8 µm long (Fig. 4A). A helmet-shaped nucleus (Held 1975) occupies the anterior end of the zoospore (Fig. 4A, B); posterior to the nucleus is a spherical mitochondrion ~ 0.5 µm diam. (Fig. 4A–D). A striated rhizoplast connects the mitochondrion and kinetosome (Fig. 4A). The flagellum exits the body of the zoospore through an elongate cavity (Fig. 4D). Lateral to the mitochondrion is a microbody (Fig. 4C, D) appressed to two lipid globules (Fig. 4A, C, D), and appressed to the lipid globules

on the side facing the plasma membrane is a backing membrane (Fig. 4C, D). In the cytoplasm, and adjacent to the mitochondrion, is a vacuole (Fig. 4B, C) and a multi-vesicular body (Fig. 4B).

Phylogenetic analysis

The best ML tree from GARLI analysis (Fig. 5) revealed four lineages containing *Rozella* strains. Lineage 1 (the "*Rhizoclosmatium*" lineage) contained strains JEL 863 *R. rhizoclosmatii*, JEL 347, and JEL 882, the three being parasites of *Rhizoclosmatium* (*Rh.*) *globosum*, and strain JEL 880, a parasite of *Rh. aurantiacum*. Lineage 2 (the "*Pythium* 1" lineage) contained a *Rozella* from *Pythium* from a gross enrichment culture. Lineage 3 (the "*Allomyces*" lineage) contained two strains (UCB 47-054 and CSF55) of *Rozella allomycis*, parasites of *Allomyces*. Lineage 4 (the "*Pythium* 2" lineage) contained strain JEL 883 as sister to a clade of three uncultured environmental sequences, but with no ML bootstrap or BPP support for this branch/lineage. Alternatively, the Bayesian consensus tree (Fig. S2) placed strain JEL 883 sister to the "*Allomyces*" lineage with 100% BPP. Regardless, both analyses indicated JEL 883 represented a new taxon. Analysis of the 10 strains represented by concatenated 18S/28S rDNA placed strain JEL 883 in a lineage with *R. allomycis* (Fig. S3).

TAXONOMY

Rozella multimorpha Letcher and Longcore, sp. nov.

Diagnosis/description. Sporangia are monosporangiate, spherical, typically 15–20 µm diam., rarely to 50 µm diam., pyriform, or irregularly-shaped, with (typically) one to three discharge tubes ~ 2 µm diam. x 5–40 µm long, the discharge tube terminating with a constriction and bulb-shaped structure. Zoospores are elongate, 1–1.2 µm diam. x 1.6–1.8 µm long, containing a helmet-shaped nucleus, a microbody-lipid globule complex composed of a single mitochondrion, a pair of lipid globules, a microbody, and a backing membrane. Zoospores are immotile in the sporangium prior to release, and are released as a mass of motile spores that rapidly disperse. Resting spores not observed.

Type species. *Rozella multimorpha* sp. nov. Letcher and Longcore

Type material. ICBN: Fig. 1 this publication (HOLOTYPE).

Type host. *Pythium* host has a 99.8% rDNA ITS1-5.8S-ITS2 sequence similarity to *P. catenulatum* (GenBank LC150553).

Type habitat. Pond associated with The Lyle E. Littlefield Ornamentals Trial Garden, located on the University of Maine campus, Orono, Maine USA.

Type locality. UNITED STATES, MAINE: Orono, 44° 53’ 50.85”N, 68° 40’ 07.64”W, ~40 m., collected June 2016.

Etymology. The epithet *multimorpha* refers to the multiplicity of sporangium forms. This epithet should not be confused with the similarly-named species *R. monoblepharidis-polymorphae* Cornu or *R. pseudomorpha* (Scherffel) Sparrow (current name *Olpidium pseudomorphum* Scherffel [Scherffel 1926]).

Gene sequence. GenBank accession MF196185 (18S), MF196182 (28S).

ZooBank urn:lsid:zoobank.org:act:0216CB3E-BD95-4BA7-B806-A193D3E9DF8C

Mycobank MB 821536

Species similarity

Many *Rozella* species are considered host specific (Held 1981, Karling 1965), but Johnson (1955) found isolates that spanned genera. The thallus of strain JEL 883 *R. multimorpha* does not resemble the thallus of any of the 17 described species of *Rozella* that parasitize Chytridiomycota (Chytridiomycetes and Monoblepharidomycetes), Blastocladiomycota, Basidiomycota, or the green alga *Coleochaete*. Of the eight species of *Rozella* that have been reported as parasites of *Pythium* spp., *R. longicollis* Karling (1965) somewhat resembles *R. multimorpha*. Sporangia of *R. longicollis* are described as spherical, 20–60 µm diam., pyriform, or ovoid, with no mention or illustration of the sporangial polymorphism that is present with *R. multimorpha*. Both species have long discharge tubes, with *R. longicollis* having 1–5, whereas *R. multimorpha* usually has a single, occasionally two, and rarely three. As an additional difference, discharge tubes of *R. multimorpha* sporangia, before discharge, have a characteristic bulb-like constriction at the terminal end of the discharge tube. This is a consistent and easily observed feature that would have been illustrated by Karling if it were present in *R. longicollis*.

The remaining *Rozella* species that infect various species of *Pythium* have either a discharge pore or a short discharge papilla, with perhaps the exception of *R. irregularis* (E. J. Butler) Sparrow. Butler (1907) described this species as having both terminal and intercalary

sporangia of variable size and shape, each with a single, short, exit papilla from which minute zoospores exited after swarming in the sporangium for a short time before discharge; he also illustrated, but did not describe, sporangia with multiple discharge tubes. Zoospores of *R. multimorpha* do not swarm in the sporangium before discharge. Shen and Siang (1948) described a putative strain of *R. irregularis* having sporangia with one to several exit papillae, but reported the zoospores as 7–9 µm diam. (If their report of zoospore size is accurate, they were possibly studying a different species). Subsequently, Karling (1981) tentatively identified *R. irregularis* on *P. debaryanum* from a soil sample from China. Karling's strain had sporangia that "…vary so markedly that it is almost impossible to ascribe a definitive shape and size for them" (Karling 1981). Sporangia of his isolate developed 1–8 exit papillae (but not exit tubes), and minute zoospores 1.5–2.5–3 µm swarmed in the sporangium before emerging. We conclude that our strain JEL 883 differs from *R. irregularis* and other described *Rozella* species on *Pythium* in the features of sporangial morphology, discharge apparatus, and zoospore behavior at discharge; and thus, should be considered a new species.

DISCUSSION

The genus *Rozella* Cornu was described almost 150 years ago (Cornu 1872) to include four species of parasites of Oomycota and Chytridiomycota: *R. septigena* Cornu, *R. apodachlyae* Cornu (as "*R. Apodyae brachynematis*"), *R. rhipidii* Cornu (as "*R. Rhipidii spinosi*"), and *R. monoblepharidis* Cornu (as "*R. Monoblepharidis polymorphae*"). (Note: "Cornu [1872]…used the long and short versions of the specific epithets *monoblepharidis* and *monoblepharidis-polymorphae* and *rhipidii* and *rhipidii-spinosi* without preference. The selected epithet is therefore determined by 'accepted usage'…Sparrow [1938] and Karling [1942b] used the shorter versions, and their choice…is followed here" [Dick 2001]). No type species was designated. The four species had in common certain morphological features, including: (1) a plasmodial, holocarpic, endobiotic thallus; (2) posteriorly uniflagellate zoospores that escape through a pore or papilla; (3) a spherical, spiny resting spore with no companion cell. However, not all of these features were observed for all four species; for example, zoospores were not observed with *R. monoblepharidis*. Three of the species (*R.apodachlyae, R. rhipidii*, and *R. monoblepharidis*) caused host hypertrophy at the point of infection, and each thallus formed a single sporangium

("monosporangiate"; = "Sporangiumgruppe" [Fischer 1882]). The fourth species, *R. septigena*, was distinguishable from the others by the absence of hypertrophy and by the formation of a linear series of sporangia separated by cross walls ("polysporangiate"; = "Septigenagruppe" [Fischer 1882]). This developmental distinction was seemingly important, such that Fischer (1892) erected the genus *Pleolpidium* for the monosporangiate members, with *P. monoblepharidis* as the type species, and retained *Rozella* for polysporangiate forms, with *R. septigena* as the type species. Clements and Shear (1931) supported Fischer's designations in their account of all published fungal genera and their types. Subsequently, with the discovery of the polysporangiate *R. allomycis* Foust (1937) and *R. achlyae* Shanor (1942), the two foremost experts on Chytridiomycota at the time retained *Rozella* in the original sense of Cornu (Sparrow 1938, Karling 1942b), suppressing *Pleolpidium* as a synonym of *Rozella*. In the ensuing decades numerous additional *Rozella* species were described. In more recent taxonomic revisions, Batko (1977) and Doweld (2014) each resurrected the generic distinction based on monosporangiate versus polysporangiate morphology. With the addition of *R. multimorpha*, of the 26 currently described species, 23 are considered monosporangiate and three are considered polysporangiate.

Until relatively recently, our knowledge of *Rozella* has been limited to light microscopic observations (Butler 1907; Canter 1950, 1969; Cornu 1872; Foust 1937; Johnson 1955; Karling 1942a, b, 1944, 1946, 1965, 1981, 1987; Minden 1916; Petersen 1910; Shanor 1942; Sörgel 1952; Sparrow 1936; Willoughby and Rigg 1983). Held (1975) produced the first electron microscopic study of a *Rozella* zoospore, and James et al. (2006) first placed *Rozella* in a molecular phylogeny, indicating it as the earliest diverging lineage in the fungi. A number of *Rozella* strains have been recently found, and with studies of these strains, we can begin to develop hypotheses about morphology, zoospore and thallus ultrastructure (Powell et al. 2017), and molecular affiliations within the lineage.

Morphology

Regardless of its multiple sporangial morphologies, we consider *R. multimorpha* to be monosporangiate. Our evidence for the monosporangiate morphology is derived from TEM observations, in which serial sections demonstrate that a single parasite plasmodium is continuous in adjacent sporangial swellings. However, we occasionally observed thalli in which

two adjacent sporangial swellings were separated by a cross wall. We hypothesize that this phenomenon may result from two zoospores entering the host and forming adjacent sporangia.

Resting spores are a feature of many species of *Rozella*, and they may be characterized as spherical to slightly flattened (e.g., *R. canterae* Sparrow), minutely spiny to smooth (*R. parva* Canter, *R. cuculus* (Butler) Sparrow, *R. laevis* Karling), and in some cases functioning as a prosporangium. Although observation of a resting spore within a host is often beneficial to taxon identification, we have not observed a resting spore for *R. multimorpha*, even in gross cultures that are many months old. Notably, however, the parasite readily revives from older gross cultures upon the addition of fresh spruce pollen grains. Thus, there may be a survival mechanism other than resting spores for this strain of *Rozella*.

Molecular segregation

Multiple lineages are apparent in our inferred phylogeny. The "*Rhizoclosmatium*" lineage contains four strains of *Rozella* (JEL 347 *Rozella* ex *Rhizoclosmatium* (*Rh*.) *globosum*, JEL 863 *Rozella rhizoclosmatii* ex *Rh. globosum*, JEL 882 *Rozella* ex *Rh. globosum*, and JEL 880 *Rozella* ex *Rh. aurantiacum*); all of these strains are monosporangiate and parasitic on members of Chytriomycetaceae, and originated from Maine, USA. Sister to the "*Rhizoclosmatium*" lineage is a second lineage (the "*Pythium* 1" lineage) that contains *Rozella* ex *Pythium* KX354831 from a crude culture from soil in Maine. Observations of the morphology of *Rozella* ex *Pythium* KX354831 indicate it to be monosporangiate (unpub. data). A third lineage (the "*Allomyces*" lineage) contains two strains of *Rozella allomycis*, and both of these strains are polysporangiate, UCB 47-054 from the Philippine Islands and CSF55 from a roadside ditch, Hattiesburg, MS, USA. Strain JEL 883 *R. multimorpha* is in a fourth lineage (the "*Pythium* 2" lineage) that is sister to the other three lineages and native to Maine in northeastern North America. It is interesting that *Rozella* strains that parasitize *Pythium* occur in two separate lineages and that three lineages of *Rozella* are present in Maine. Finding three lineages of *Rozella* from our limited sampling area indicates that we are just beginning to realize the diversity among these parasites. The placement of *R. multimorpha* in the "*Pythium* 2" lineage with environmental sequences from lake water in France (ESS270706.089), soil from China (GL9833-096-S189), and a peat bog in Switzerland (PRS_4E_71) extends our knowledge about the biology of *Rozella* to a broader region of the tree. In doing so, it helps clarify the morphology and biology of the parasite and

identifies at least one type of host. Given the host fidelity of clades in our 18S/28S phylogeny of *Rozella*, we predict that the "*Pythium* 2" lineage would largely infect *Pythium* and perhaps other Oomycota.

TEM: zoospore ultrastructure

Our observations of the zoospore of *R. multimorpha* permit comparison with zoospore ultrastructure of other examined species of *Rozella* (Table 1).

The zoospores of strains in three lineages ("*Rhizoclosmatium*", "*Allomyces*", and "*Pythium* 2") have similar morphologies that we collectively refer to as the "*Rozella* zoospore", which has its basis in the morphology of the zoospore of *R. allomycis* as elucidated by Held (1975). The zoospore of *R. allomycis*, ~ 2 × 3.5 µm, has unique ultrastructural variation on the basic pattern of posteriorly uniflagellate zoospores that includes an anterior helmet-shaped nucleus subtended by a robust spherical mitochondrion, which is anterior to a fibrillar rhizoplast connected to the kinetosome, a flagellar cavity that surrounds the base of the flagellum, and a lateral microbody/lipid globule/backing membrane complex. Thus, in the "*Allomyces*" lineage we have examined Held's photographs of *R. allomycis* strain UCB 47-054 (Held 1972; 1975); our strain CSF55 *R. allomycis* has the same ultrastructural configuration. In the "*Rhizoclosmatium*" lineage, the zoospore of JEL 863 *Rozella rhizoclosmatii* (Letcher et al. 2017) and JEL 880 *Rozella* ex *Rh. aurantiacum* (unpub. data) has the same basic configuration and arrangement of organelles as that of *R. allomycis*, although it is smaller, being ~ 1.2 × 1.6 µm, and has a lattice of perpendicular rods about the nucleus, which is a distinct ultrastructural feature that has not been observed in any other zoosporic fungus. As to the zoospore of strain JEL 883 *Rozella multimorpha* in the "*Pythium* 2" lineage, it is similar in size and morphology to that of the "*Rhizoclosmatium*" lineage, but the lattice of rods about the nucleus is absent. Thus, each molecular lineage has a distinct zoospore morphology, although the zoospores of all three lineages are characteristic of *Rozella*.

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LITERATURE CITED

Batko, A. 1977. Further observations on *Nellymyces megaceros* and its parasite- *Rozellopsis uliginosa* sp. nov. *Acta Mycol.*, 13:313-324.

Butler, E. J. 1907. An account of the genus *Pythium* and some Chytridiaceae. *Mem. Dept. Agr. India, Bot. Ser*., 1:1-160.

Canter, H. M. 1950. Studies on British chytrids. XI. *Chytridium oedogonii* Couch. *Trans. Brit. Mycol. Soc*., 33:354-358.

Canter, H. M. 1969. Studies on British chytrids. XXIX. A taxonomic revision of certain fungi on the diatom *Asterionella*. *Bot. J. Linn. Soc*., 62:267-278.

Clements, F. E. & Shear, C. L. 1931. The Genera of Fungi. H. W. Wilson, New York.

Cornu, M. 1872. Monographie des Saprolégniées: étude physiologie et systématique. *Ann. Sci. Nat. Bot*. V, 15:1-198.

Corsaro, D., Walochnik, J., Venditti, D., Steinmann, J., Müller, K-D. & Michel, R. 2014a. Microsporidia-like parasites of amoebae belong to the early fungal lineage of Rozellomycota. *J. Parasitol. Res.*, 113:1909-1918.

Corsaro, D., Walochnik, J., Venditti, D., Müller, K-D., Hauröder, B. & Michel, R. 2014b. Rediscovery of *Nucleophaga amoebae*, a novel member of the Rozellomycota. *J. Parasitol. Res.*, 113:4491-4498.

Corsaro, D., Michel, R., Walochnik, J., Venditti, D., Müller, K-D, Hauröder, B. & Wylezich, C. 2016. Molecular identification of *Nucleophaga terricolae* sp. nov. (Rozellomycota), and new insights on the origin of the Microsporidia. *J. Parasitol. Res.*, 115:3003-3011.

Dick, M. W. 2001. Systematics of the Peronosporomycetes including accounts of the marine staminipilous protists, the plasmodiophorids and similar organisms. Kluwer Academic Publishers, Dordrecht/Boston/London.

Doweld, A. B. 2014. Nomenclatural novelties. Skirgielliaceae. *Index Fungorum*, 129:1.

Fischer, A. 1882. Untersuchungen über die Parasitender Saprolegnieen. *Jahrb. Wiss. Bot*., 13:286-371.

Fischer, A. 1892. Phycomycetes. Die Pilze Deutschlands, Oesterreichs und der Schweiz. *Rabenhorst. Kryptogamen-Fl.*, 1:1-490. Leipzig.

Foust, F. K. 1937. A new species of *Rozella* parasitic on *Allomyces*. *J. Elisha Mitchell Sci. Soc*., 53:197-204.

Grossart, H-P., Wurzbacher, C., James, T.Y. & Kagami, M. 2016. Discovery of dark matter fungi in aquatic ecosystems demand a reappraisal of the phylogeny and ecology of zoosporic fungi. *Fungal Ecol.*, 19:28-38.

Guindon, S. & Gascuel, O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.*, 52:696-704.

Held, A. A. 1972. Improved culture methods for *Rozella* and for *Olpidiopsis*. *Mycologia*, 64:871-886.

Held, A. A. 1975. The zoospore of *Rozella allomycis*: ultrastructure. *Canad. J. Bot*., 53:2212-2232.

Held, A. A. 1981. *Rozella* and *Rozellopsis*: naked endoparasitic fungi which dress-up as their hosts. *Bot. Rev.*, 47:451-515.

James, T. Y., Letcher, P. M., Longcore, J. E., Mozley-Standridge, S. E., Porter, D., Powell, M. J., Griffith, G. W. & Vilgalys, R. 2006. A molecular phylogeny of the flagellated fungi (Chytridiomycota) and description of a new phylum (Blastocladiomycota). *Mycologia*, 98:860-871.

James, T. Y., Stenlid, J., Olson, A. & Johannesson, H. 2008. Evolutionary significance of imbalanced nuclear ratios within heterokaryons of the basidiomycete fungus *Heterobasidion parviporum*. *Evolution*, 62:2279-2296.

James, T. Y. & Berbee, M. L. 2012. No jacket required- new fungal lineage defies dress code. *Bioessays*, 34:94-102.

Johnson, T. W. 1955. Inoculation studies with a polysporangiate *Rozella* parasitic in *Dictyuchus anomalus*. *Amer. J. Bot*., 42:119-123.

Jones, M. D. M., Forn, I., Gadelha, C., Egan, M. J., Bass, D., Massana, R. & Richards, T. A. 2011a. Discovery of novel intermediate forms redefines the fungal tree of life. *Nature*, 474:200-203.

Jones, M. D. M., Richards, T. A., Hawksworth, D. L. & Bass, D. 2011b. Validation and justification of the phylum name Cryptomycota. *IMA Fungus*, 2:173-175.

Karling, J. S. 1942a. Parasitism among chytrids. *Amer. J. Bot*., 29:24-35.

Karling, J. S. 1942b. A synopsis of *Rozella* and *Rozellopsis*. *Mycologia*, 34: 193-208.

Karling, J. S. 1944. Brazilian chytrids. IV. Species of *Rozella*. *Mycologia*, 36:638-647.

Karling, J. S. 1946. Two new chytrid parasites of *Chytriomyces*. *Mycologia*, 38:103-109.

Karling, J. S. 1965. Some zoosporic fungi of New Zealand. I. *Sydowia*, 19:213-226.

Karling, J. S. 1981. *Rozella irregularis* (?) Butler and some other zoosporic fungi from China. *Nova Hedwigia*, 34:633-644.

Karling, J. S. 1987. Ross Biological Reserve aquatic fungi III. Additional species. *Nova Hedwigia*, 45:3-4.

Karpov, S. A., Mamkaeva, M. A., Aleoshin, V. V., Nassonova, E., Lilje, O. & Gleason, F.H. 2014. Morphology, phylogeny, and ecology of the aphelids (Aphelidea, Opisthokonta) and proposal for the new superphylum Opisthosporidia. *Frontiers Microbiol.*, 5:112, https://doi.org/10.3389/fmicb.2014.00112

Karpov, S. A., Torruella, G., Moriera, D., Mamkaeva, M. A. & Lopez-Garcia, P. 2017. Molecular phylogeny of *Paraphelidium letcheri* sp. nov. (Aphelida, Opisthosporidia). *J. Eukar. Microbiol.*, 0:1-6 doi:10.1111/jeu.12389

Lara, E., Moriera, D. & Lopez-Garcia, P. 2010. The environmental clade LKM11 and *Rozella* form the deepest branching clade of fungi. *Protist*, 161:116-121.

Lazarus, K. L. & James, T. Y. 2015. Surveying the biodiversity of the Cryptomycota using a targeted PCR approach. *Fungal Ecol.*, 14:62-70.

Letcher, P. M., Lee, P. A., Lopez, S., Burnett, M., McBride, R. C. & Powell, M. J. 2016. An ultrastructural study of *Paraphysoderma sedebokerense* (Blasocladiomycota), an epibiotic parasite of microalgae. *Fungal Biol.*, 120:324-337.

Letcher, P. M., Longcore, J. E., Quandt, A. A., Leite, D. S., James, T. Y. & Powell, M. J. 2017. Morphological, molecular, and ultrastructural characterization of *Rozella rhizoclosmatii*, a new species in Cryptomycota. *Fungal Biol.*, 121:1-10.

Minden, M. von. 1916. Beitrage zur Biologie und Systematik einheimischer submerse Phycomyceten. In: Falck, R. 1916. *Mycologische Untersuchungen und Berichte*. Jena, Verlag von Gustav Fischer.

Petersen, H. E. 1910. An account of Danish freshwater phycomycetes, with biological and systematic remarks. *Ann. Mycol.*, 8:494-560.

Posada, D. 2008. jModeltest: phylogenetic model averaging. *Molec. Biol. Evol.*, 25:1253-1256.

Powell, M. J., Letcher, P. M. & James, T. J. 2017. Ultrastructural characterization of the host-parasite interface between *Allomyces anomalus* (Blastocladiomycota) and *Rozella allomycis* (Cryptomycota). *Fungal Biol.*, 121:561-572.

Rehner, S. A. & Samuels, G. J. 1994. Taxonomy and phylogeny of *Gliocladium* analysed from nuclear large subunit ribosomal DNA sequences. *Mycol. Res.*, 98:625-634.

Ronquist, F. & Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572-1574.

Scherffel, A. 1926. Beitrãge zur Kenntnis der Chytridineen, Teil III. *Arch. Protistenk.*, 54:510-528.

Shanor, L. 1942. A new *Rozella* of the polysporangiate series. *J. Elisha Mitchell Sci. Soc.*, 58:99-101.

Shen, S. C. & Siang, W.N. 1948. Studies in the aquatic phycomycetes of China. *Sci. Rept. Nat. Tsing Hua Univ. Ser. B Biol. and Psych. Sci.*, 5:179-206.

Sörgel, G. 1952. Dauerorganbildung bei *Rozella allomycis* Foust, ein Beitrag zur Kenntnis der Sexualität der niederen Phycomyceten. *Arch. Mikrobiol.*, 17:247-254.

Sparrow, F. K. 1936. A contribution to our knowledge of the aquatic phycomycetes of Great Britain. *J. Linn. Soc. London (Bot.)*, 50:417-478.

Sparrow, F. K. 1938. Remarks on the genus *Rozella*. *Mycologia*, 30:375-378.

Sparrow, F. K. 1960. Aquatic Phycomycetes, 2nd edn. University of Michigan Press, Ann Arbor.

Sukumaran, J. & Holder, M. T. 2010. DendroPy: a python library for phylogenetic computing. *Bioinformatics*, 26:1569-1571.

Tedersoo, L., Bahram, M., Puusepp, R., Nilsson, R. H. & James, T. Y. 2017. Novel soil-inhabiting clades fill gaps in the fungal tree of life. *Microbiome*, 5:42.

Vilgalys, R. & Hester, M. 1990. Rapid identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *J. Bacteriol.*, 172:4238-4246.

White, T. J., Bruns, T., Lee, S. & Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In*: Innis, M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J., eds. *PCR protocols: a guide to methods and applications*. New York: Academic Press. P. 315-322.

Willoughby, L. G. & Rigg, E. 1983. Lower aquatic fungi in the soils of Majorca. *Nova Hedwigia*, 37:369-383.

Zwikl, D. J. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under maximum likelihood criterion [doctoral dissertation]. Austin: Univ. Texas. 115 p.

FIGURE LEGENDS

Figure 1. Light microscopic morphology; most typical morphology of strain JEL 883, *Rozella multimorpha*. A. Uninfected *Pythium* hypha. B. Early infection, with terminal and intercalary sporangial initials (arrows). C. Host hyphal hypertrophy. D. Early to mid-stage parasite sporangial development. E. Spherical, terminal mature sporangium with single discharge tube. F. Spherical, intercalary mature sporangium with discharge tube G. Spherical, mature sporangium on host hypha originating from spruce pollen grain; arrow indicates characteristic bulb at tip of discharge tube. H, I. Successive images through a sporangium with two discharge tubes (arrows). J. Detail of discharge tube and sporangium. Scale bars in A–J = 10 µm. dt = discharge tube; hh = host hypha; sp = sporangium.

Figure 2. Light microscopic morphology. Polymorphic, monosporangiate forms of strain JEL 883, *Rozella multimorpha*. A, D. Sporangium with two discharge tubes. B–D, F. Sporangium with two swellings. E. Two adjacent sporangia separated by a cross wall (arrow). F. Spherical vacuole (arrow) in sporangium. Scale bars in A–F = 10 µm. dt = discharge tube; hh = host hypha; sp = sporangium.

Figure 3. Light microscopic and TEM morphology of strain JEL 883, *Rozella multimorpha*. A–D. Sequential images of a sporangium during maturation through discharge, which occurred over 2–3 hours. Notice the coarse granular appearance of the protoplasm prior to cleavage (A), the fine granular contents during cleavage (B), the individual lipid globules of individual zoospores following cleavage (C), followed by zoospore discharge (D). E. An empty sporangium, in which continuity between the sporangial wall and discharge tube is apparent. F. Mature sporangium at discharge, with pockets of flagellar profiles and mature zoospores. Scale bars in A–F = 5 µm. dt = discharge tube; fp, flagellar pockets; hh = host hypha; sp = sporangium; z = zoospore.

Figure 4. Transmission electron microscopy morphology of strain JEL 883, *Rozella multimorpha*; ultrastructure of zoospore. A. Longitudinal section, with anterior helmet-shaped

nucleus, spherical mitochondrion, rhizoplast, kinetosome, flagellum, and lipids. B. Nucleus, mitochondrion, multivesicular body, and vacuole. C. Mitochondrion, lipid globules, microbody, backing membrane, and vacoule. D. Posterior of zoospore, and the elongate cavity through which the flagellum emerges. Scale bars in A–D = 0.25 µm. BM = backing membrane; Cav = posterior cavity; F = flagellum; K = kinetosome; L = lipid globule; M = mitochondrion; Mb = microbody; MVB = multivesicular body; N =nucleus; Vac = vacuole.

Figure 5. Phylogenetic analysis. Best ML tree from GARLI analysis, with GenBank accessions after taxon or environmental sequence. GARLI ML bootstrap support values $\geq$ 70% are indicated at nodes, and bold branches indicate MrBayes BPP $\geq$ 95%. Vertical bars numbered 1–4 at nodes indicate lineages: 1: "*Rhizoclosmatium*" lineage; 2: "*Pythium* 1" lineage; 3: "*Allomyces*" lineage; 4: "*Pythium* 2" lineage.

Table 1. Comparison of zoospore ultrastructural features for three strains of *Rozella*.

SUPPORTING INFORMATION

Figure S1. Discharge of *R. multimorpha* zoospores [109 Mb video clip].

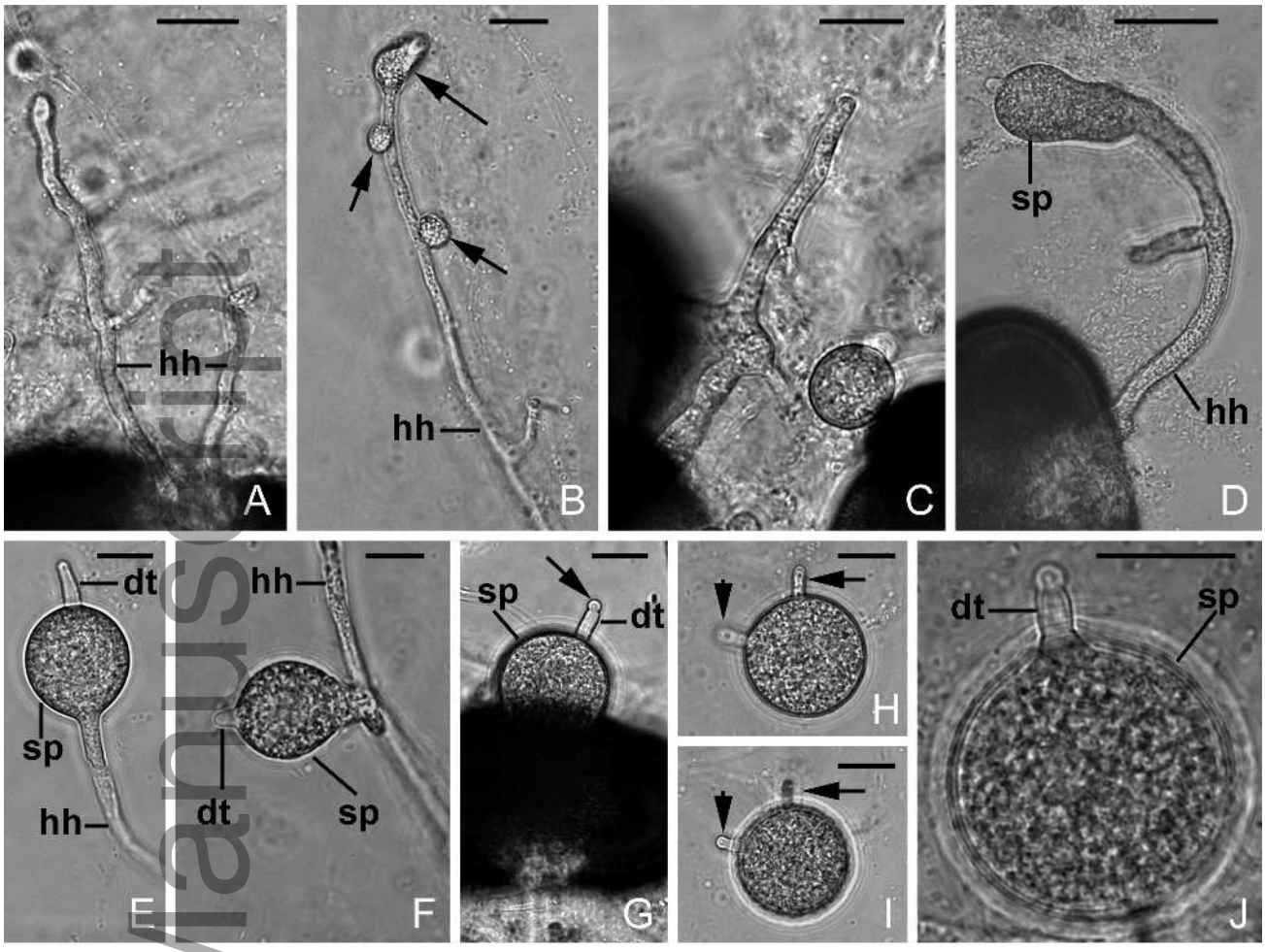
Figure S2. Bayesian consensus phylogeny; all BPP support values shown.

Figure S3. Best ML tree of 10 strains and environmental phylotypes, 18S/28S; bootstrap values > 70% indicated at nodes; bold branches $\geq$ 95% BPP.

Table 1. Comparison of zoospore ultrastructural features for three strains of *Rozella*.

species	zoospore size rounded to nearest 0.5 µm	TEM nucleus lattice	TEM perikinetosomal tubules *
R. allomycis	2 × 3.5	-	+
R. rhizoclosmatii	1 × 2	+	-
R. multimorpha	1 × 2	-	-

*Held 1975



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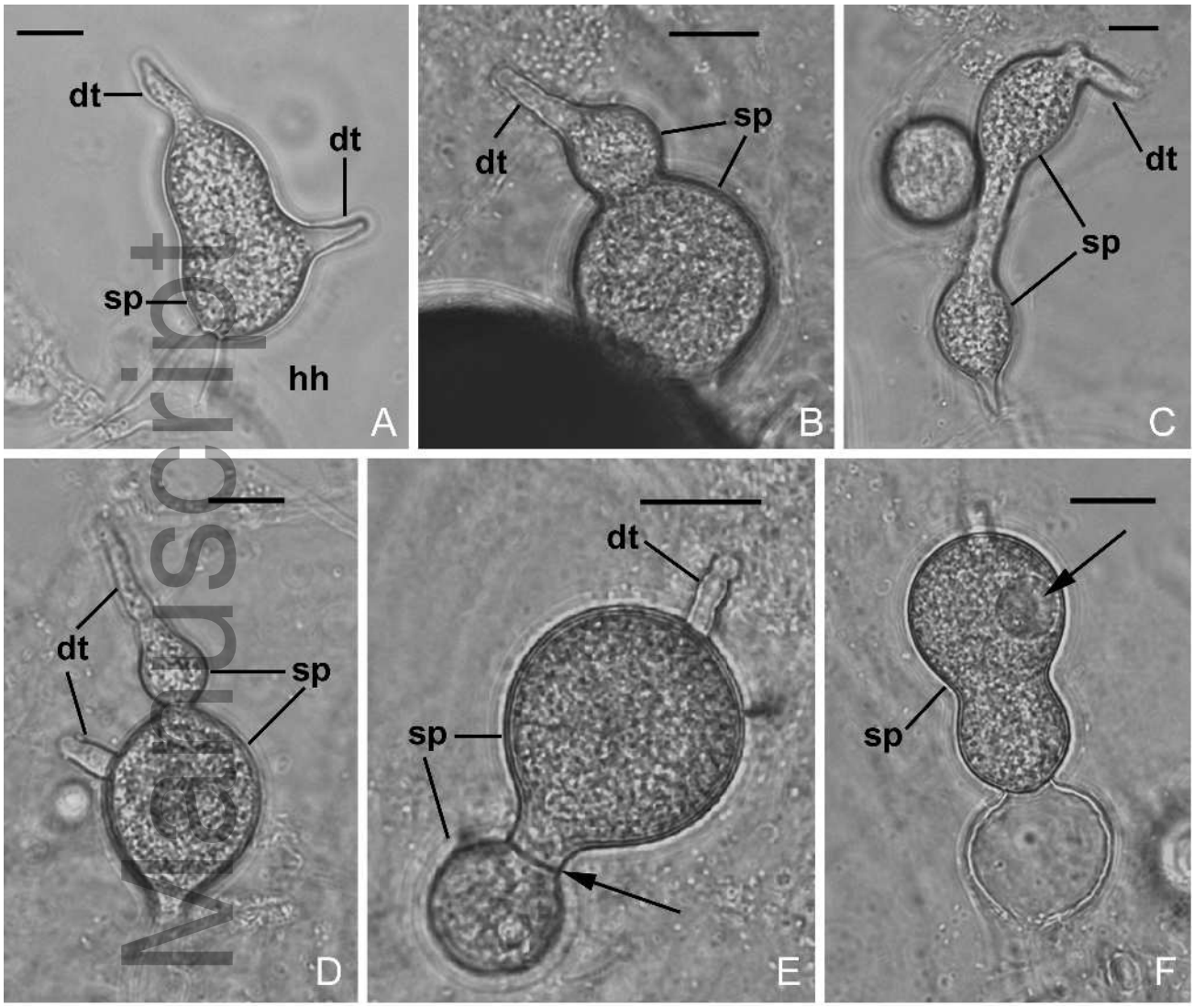

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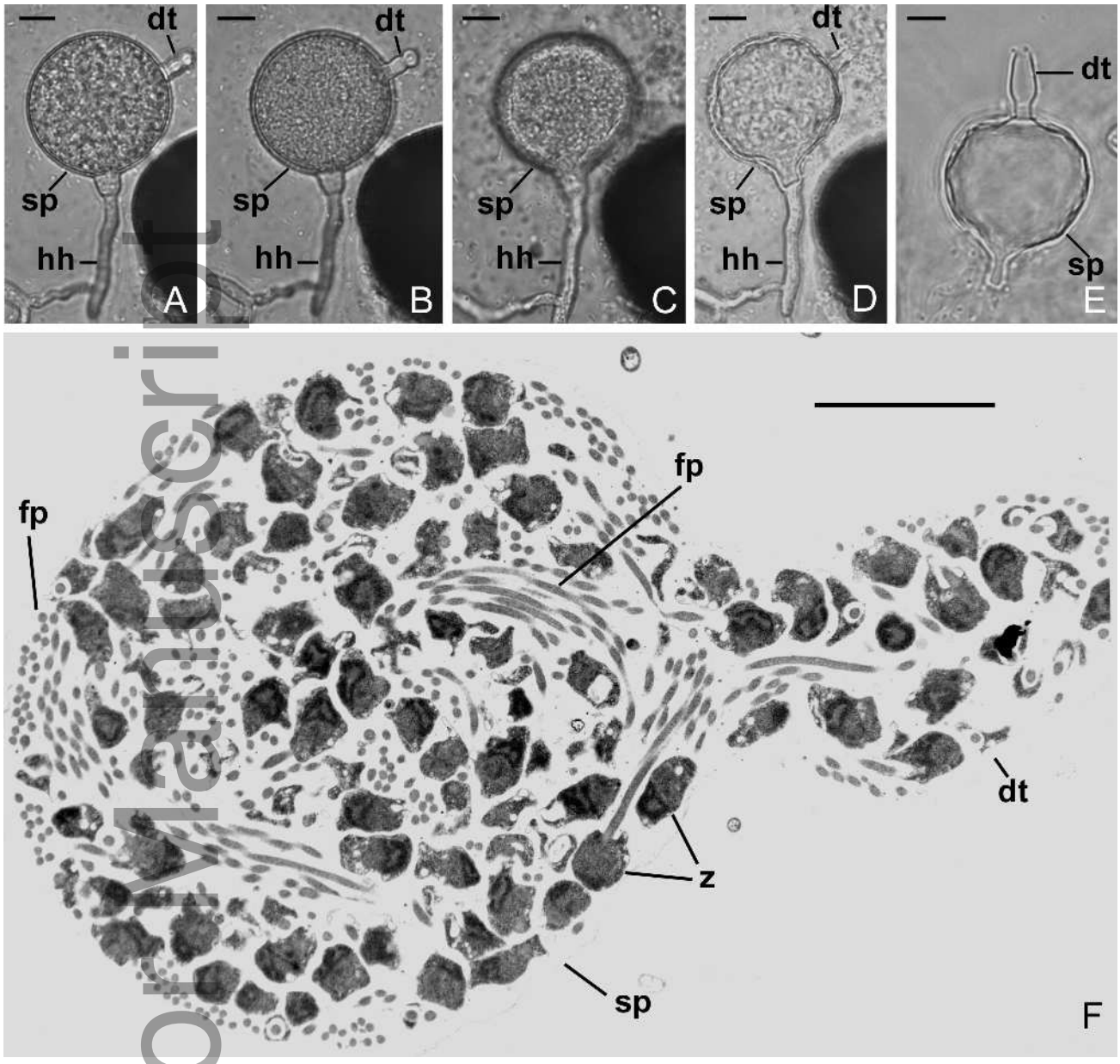

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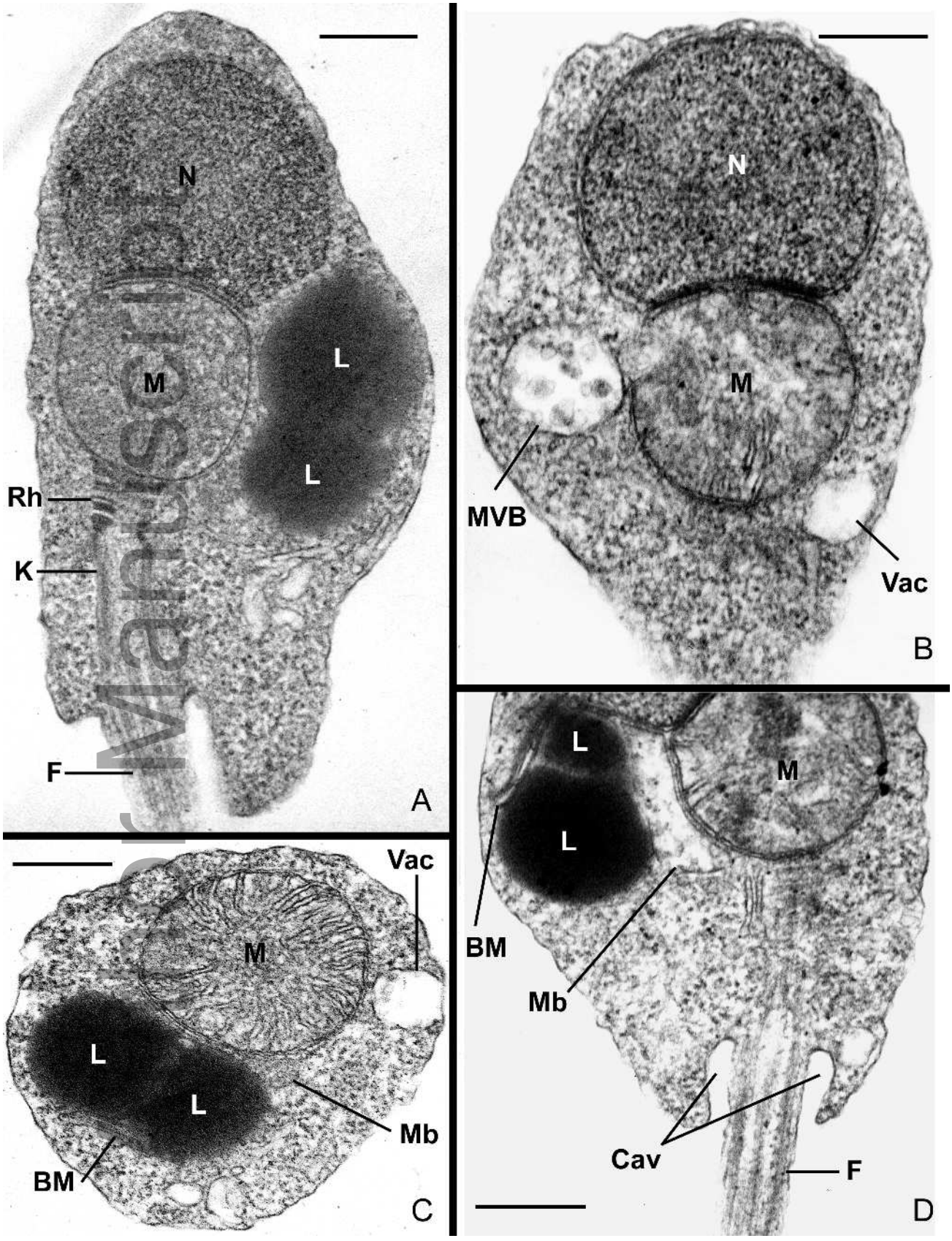

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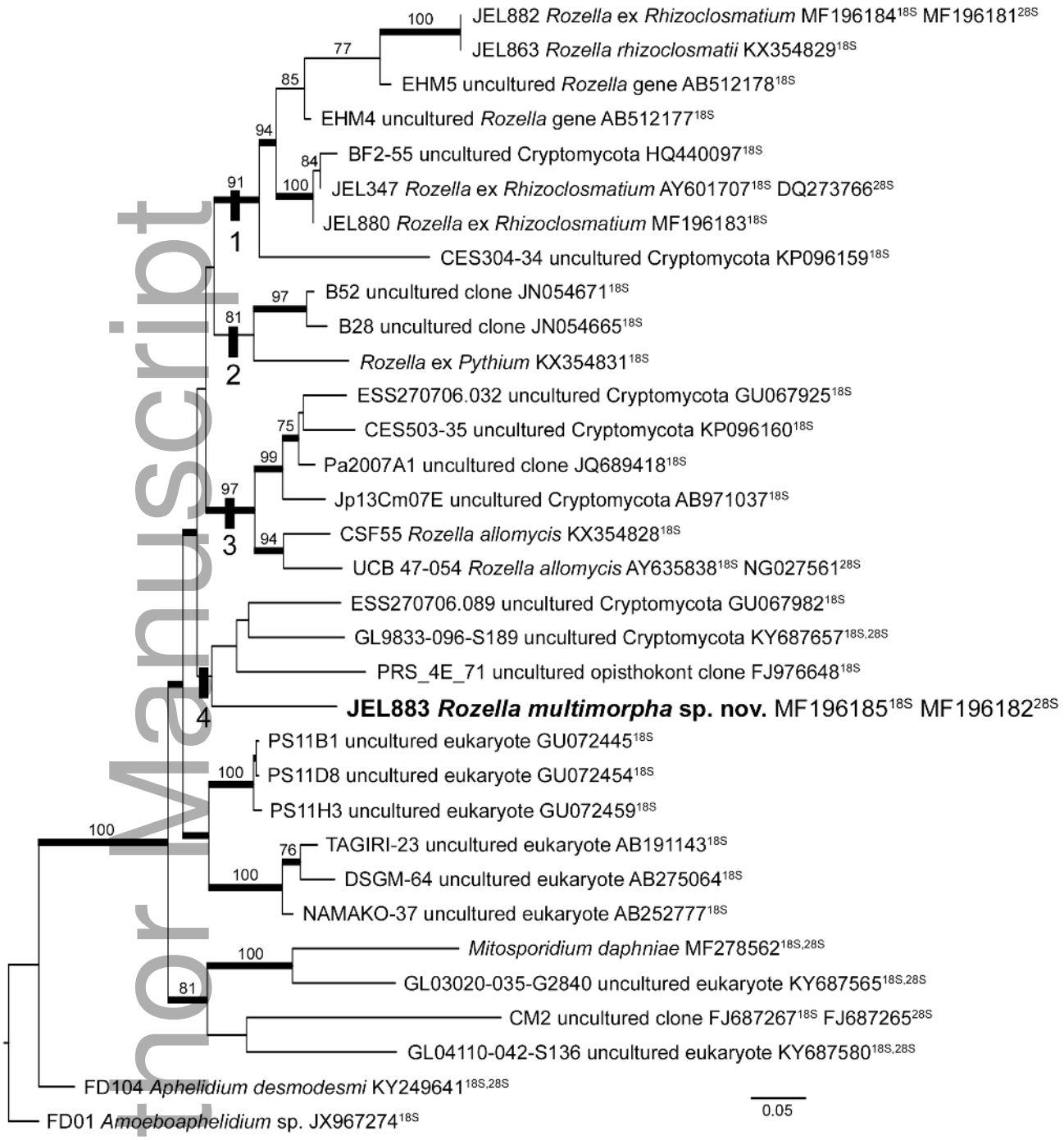

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