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Morphological, molecular, and ultrastructural characterization of *Rozella rhizoclosmatii*, a new species in Cryptomycota

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ABSTRACT

Rozella is a genus of unwalled endoparasites of a variety of hosts including Oomycota (Stramenopiles), Blastocladiomycota and Chytridiomycota (Fungi), and one green alga (*Coleochaete*, Chlorophyceae). It currently includes more than 20 formally described species, and no new species of *Rozella* have been described since 1987. We discovered a new *Rozella* species parasitizing *Rhizoclosmatium globosum* (Chytridiales, Chytridiomycota) and investigated its morphology, ultrastructure, and phylogenetic position. Herein named as *Rozella rhizoclosmatii* sp. nov., the organism induces hypertrophy of the host. Its zoospore is ultrastructurally similar to that of *Rozella allomycis*, although it has a unique zoospore ultrastructural feature, a lattice of perpendicular rods about the nucleus. The 18S rDNA molecular sequence of *R. rhizoclosmatii* is similar to that of the previously sequenced '*Rozella ex Rhizoclosmatium*'. This is the first study to inclusively characterize a new species of *Rozella* with morphological, ultrastructural and molecular data. As this is only the second *Rozella* species to be examined ultrastructurally, and because it is parasitic on a member of Chytridiomycota and not Blastocladiomycota, this research supports the conservative nature of zoospore ultrastructure to help define the genus.

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Introduction

Cornu (1872) described the genus *Rozella* and included four species: *Rozella monoblepharidis polymorphae* Cornu, *Rozella rhipidii spinosi* Cornu, *Rozella apodyae brachynematis* Cornu, and *Rozella septigena* Cornu. Clements & Shear (1931) designated *R. septigena* as the lectotype. The genus is characterized by an endobiotic, holocarpic, unwalled, inoperculate thallus with one or more discharge papillae; zoospores (often elongate) with a single posterior flagellum; and thick-walled, smooth or spiny resting spores (Sparrow 1960). At present, more than 20 species have been described, but descriptions

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are incomplete for several species. To date, only one species of *Rozella*, (*Rozella allomycis* Foust) has been characterized with morphological (Foust 1937), ultrastructural (Held 1975) and molecular data (James *et al.* 2006). Two strains of *Rozella* (JEL 347 *Rozella* ex *Rhizoclosmatium* and *Rozella* ex *Pythium*, from a gross enrichment culture) have been characterized with molecular data only (James *et al.* 2006; Lazarus & James 2015).

Rozella is considered a member of the recently erected and circumscribed phylum 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, 2011b; Karpov *et al.* 2014), = Rozellomycota Doweld (2013). Cryptomycota encompasses numerous environmentally derived phylotypes as well as species of *Rozella* (Lazarus & James 2015) and the amoebae parasites *Paramicrosporidium* (Corsaro *et al.* 2014a) and *Nucleophaga* (Corsaro *et al.* 2014b, 2016). Recent research (Lazarus & James 2015; Corsaro *et al.* 2016) has shown that *Rozella* strains, the amoebae parasites, and related environmental DNA sequences occur as a monophyletic lineage whose constituents derive primarily from soil and freshwater habitats but also from some marine habitats. Karpov *et al.* (2014) defined boundaries among lineages of the clade which they considered sister to the true fungi, erecting the superphylum Opisthosporidia, which comprises the phyla Cryptomycota, Microsporidia, and Aphelidea.

Expanding our knowledge of the diversity of *Rozella*, herein we report a strain of *Rozella* isolated from a fen water sample, characterize its thallus morphology and zoospore ultrastructure, place it in a molecular phylogenetic context, and designate it as a new species, *Rozella rhizoclosmatii*. This is the first new species of *Rozella* described since 1987 (Karling 1987) and the first new species characterized on the basis of combined morphological, ultrastructural and molecular data. As this is only the second *Rozella* species to be examined ultrastructurally, and because it is parasitic on a member of Chytridiomycota and not Blastocladiomycota, this research supports the conservative nature of zoospore ultrastructure to help define the genus.

Materials and methods

Isolation and light microscopy

In the summer of 2015 we collected *Sphagnum* and organic debris monthly from the fen edge of Perch Pond (Penobscot County, Maine, USA), baited collections with spruce pollen and chitin, and surveyed for *Rozella* spp. *Rhizoclosmatium globosum*, which had previously been found to be a host of *Rozella* (James *et al.* 2006), was consistently present on baits but we found it infected with *Rozella* only from our Aug. 10 collection. We brought the host and parasite into dual culture (designated as JEL 863) on mPmTG (0.4 g L^{-1} peptonized milk, 0.4 g L^{-1} tryptone, 2 g L^{-1} glucose, 10 g L^{-1} agar with 200 mg L^{-1} Penicillin G and 200−500 mg L^{-1} Streptomycin added after autoclaving). To facilitate infection of the host in numbers great enough to harvest for transmission electron microscopy and molecular analysis we examined infected material with a stereo microscope (40×) and, with a sterile needle, picked up and moved mature infected thalli to areas on nutrient agar plates where we had added discharging host material. This assured that zoospores of host and parasite were simultaneously present. We observed and recorded stages of development with a Nikon Eclipse E400 compound microscope and photographed developmental stages with a Spot RT3 digital camera. Attempts to cryopreserve (Boyle *et al.* 2003) the dual culture failed with only the host reviving after thawing.

DNA extraction, purification, and amplification

Using a dissecting pin, approximately 100 thalli of *Rhizoclosmatium globosum* infected with the *Rozella* parasite were individually moved to a centrifuge tube. DNA Extraction was performed using the MO BIO PowerLyzer PowerSoil DNA Isolation Kit (MO BIO Laboratories) following the manufacturer's instructions. Amplification of the 18S region of nuclear rDNA (18S) was obtained using two sets of primers; the *Rozella* preferential, Rozella 1F and Rozella 1R (Lazarus & James 2015), and the eukaryotic specific, SSUI and SSU II (Corsaro *et al.* 2014b). PCR was conducted with the *Rozella* primers in 25 μL reactions with the following recipe: 0.2 μL ExTaq DNA polymerase (Takara), 10.2 μL H$_2$O, 3.2 μL Bovine Serum Albumin, 3.2 μL ExTaq Buffer, 2.6 μL ExTaq 2 mM MgCl$_2$, 2.6 μL ExTaq 10 mM dNTPs, 1 μL each primer, and 1 μL DNA. The thermocycler protocol for this amplification 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. The iProof high fidelity DNA polymerase (Bio-Rad Laboratories) and modified protocol were used for the SSU I/II primers with the following recipe for a 12.5 μL reaction: 0.16 μL iProof polymerase, 6.84 μL H$_2$O, 3 μL iProof Buffer, 0.7 μL MgCl$_2$, 0.3 μL 2.5 mM dNTPs (Qiagen), 0.25 μL each primer, and 1 μL DNA. The PCR conditions were as follows: 98 °C for 3:00 min; 35 cycles at 98 °C for 30 s, 55 °C for 30 s, 72 °C for 1:00; 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 both sets of primers was then generated and used in downstream phylogenetic analyses.

Phylogenetic analysis

A sequence of 18S of JEL 863 was added to a database including 18S from isolates and sequences included in Fig 2, clade XII in Lazarus & James (2015) plus the *Rozella* ex *Rhizoclosmatium* clade and additional related isolates. Combined sequences were aligned with Clustal X (Thompson *et al.* 1997) and manually edited in BioEdit (Hall 1999). The combined data had 1267 characters with 219 parsimony informative sites after uninformative characters were excluded in PAUP* (Swofford 2002). Maximum parsimony (MP) phylogenetic trees were constructed with PAUPRat (Sikes & Lewis 2001) and support values were generated as heuristic searches with 500 replicates, each with 10 random-addition replicates. Maximum likelihood (ML) trees were constructed as described in Vélez *et al.* (2011). MrModeltest 2.3 (http://mrmodeltest.joydownload.com/) was used to determine the best fit model of base substitution, and GARLI 0.951 (Zwikl 2006) was used to identify the ML tree. ML branch support was assessed with 500 bootstrapping replicates. Sequence similarity between isolates of

interest was assessed *via* pairwise alignment in BioEdit. Inferred trees were rooted with Namako-37 from a clade near to clade XII (Lazarus & James 2015).

Transmission electron microscopy

Individually infected thalli were harvested 2−5 d following inoculation of host with parasite zoospores. Thalli were processed for transmission electron microscopy following the protocol in Letcher *et al.* (2016). Additionally, embedded material was collected on 300 mesh nickel grids for random sections, and 30 nm coated slot grids (Luxfilm R TEM Specimen Support Ni-2.0-30-L-25, Luxel Corp., Friday Harbor, WA, USA) for serial sections.

Results

Phylogenetic analysis

The alignment was deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S19404). All 1005 trees derived from PAUPRat were equally parsimonious (length [L] = 733 steps, CI = 0.693, RI = 0.666) and were used to construct a majority rule consensus tree (>50 % branch support). MrModeltest indicated the most appropriate model of DNA substitution was the Transversional Substitution Model (TVM). MP and ML (−lnL = 2367.3) phylogenies were identical with similar or equal support values. The MP phylogeny (Fig 1) is presented with bootstrap values (ML/MP) indicated above branches. Isolate JEL 863 grouped with isolate JEL 347 with ≥96 % support, in a lineage containing four environmental sequences, with 100 % support. Sister to that lineage was a lineage containing two isolates of *Rozella allomycis* (97 % support), an isolate of *Rozella* ex *Pythium*, and four environmental sequences; that lineage was only marginally supported (50 %).

Morphology *via* light microscopy

Infection of *Rhizoclosmatium globosum* by the *Rozella* parasite begins with the simultaneous presence of the larger (2.5 μm × 3.5 μm) host zoospores (Fig 2A) and the much smaller (~1.4 μm × ~1.8 μm) parasite zoospores (Fig 2B). In wet mounts, *Rozella* zoospores cluster around host zoospores (Fig 2C) and adhere to swimming host zoospores (Supplemental Fig 1). Empty parasite zoospore cysts were seen on host thalli at early stages of development (Fig 2D). Infected thalli grow and develop for 2−3 d (Fig 2E) before releasing zoospores (Fig 2F) whereas adjacent, uninfected *R. globosum* thalli mature and shed zoospores within 24 h (Fig 2G, H arrows). Before, and during, discharge, the tiny *Rozella* zoospores rapidly roil (Supplemental Figs 2 and 3) within the host sporangium, a phenomenon that is not observed during discharge of host sporangia.
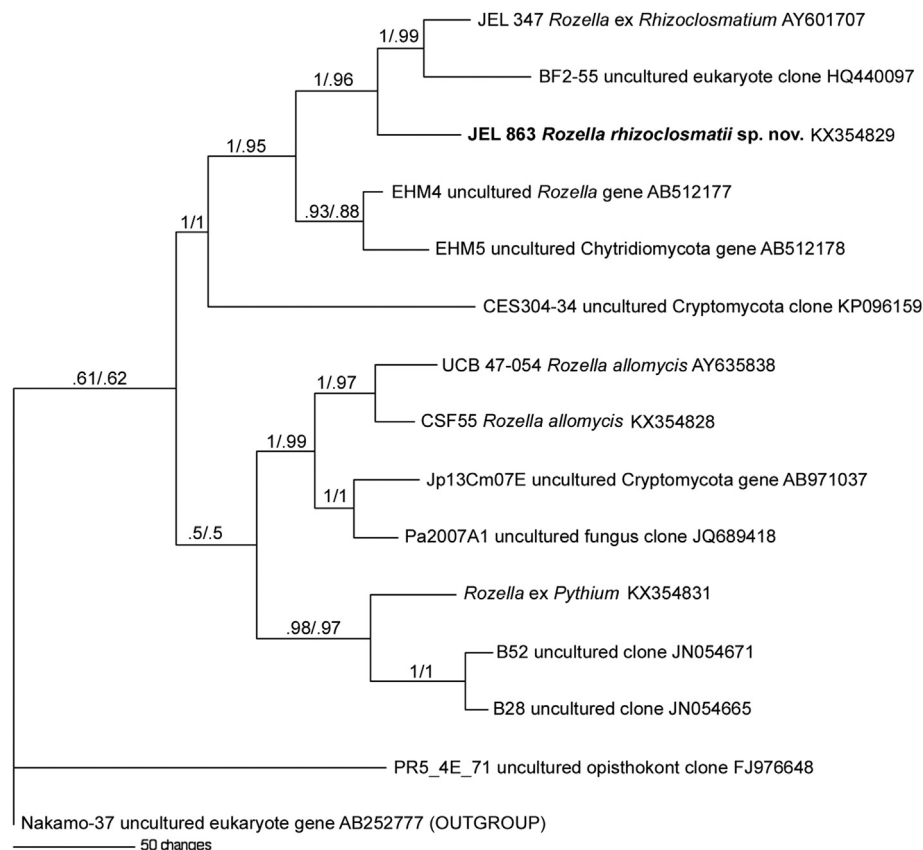

Fig 1 − Phylogenetic analysis. Maximum parsimony phylogeny derived from SSU rDNA sequences of *Rozella* strains and related environmental sequences in Cryptomycota (ingroup) and Nakamo-37 (outgroup). ML/MP bootstrap values indicated at nodes. L = 733 steps, CI = 0.693, RI = 0.666, −lnL = 2367.3.
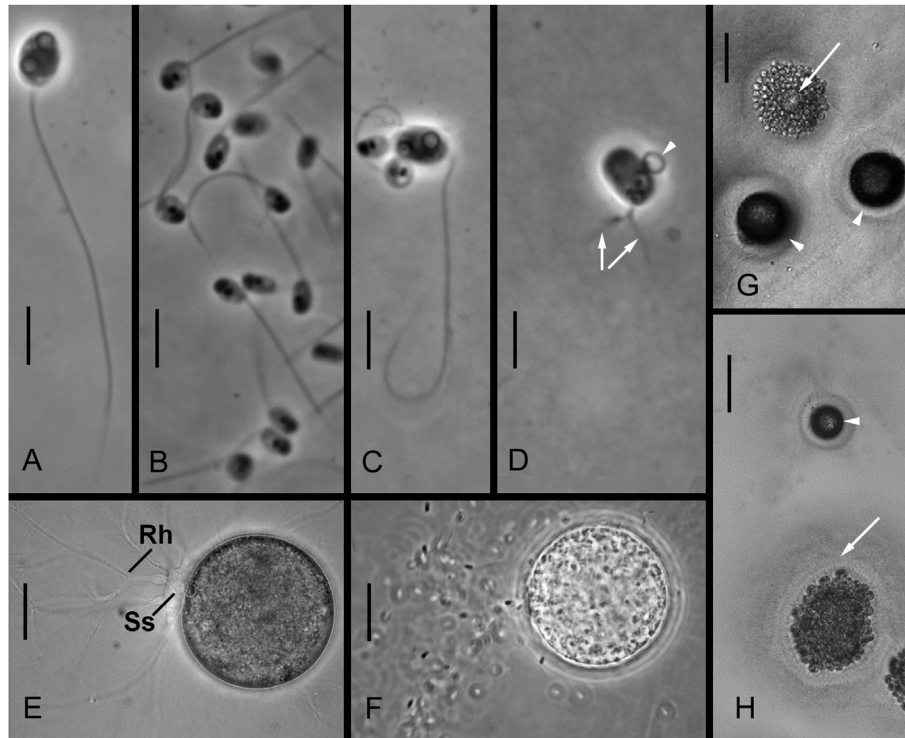
Fig 2 − **Light microscopic development of host and parasite. (A). Zoospore of host *Rhizoclosmatium globosum*. (B). Zoospores of *Rozella rhizoclosmatii*. (C). Two *Rozella* zoospores adhering to a *Rh. globosum* zoospore. (Background structures digitally removed for clarity.) (D). Empty cyst (arrowhead) of *Rozella* attached to a developing thallus of host. Arrows indicate host rhizoids. (E). Two-day old parasitized thallus. (F). Release of *Rozella* zoospores through an area that would have served for release of host zoospores. (G, H). Low magnification images illustrate relative size and rate of development of parasitized and non-parasitized host. (G). At day one *Rh. globosum* has already released zoospores (arrow); parasitized host (arrowheads) is still growing. (H). At day two *Rh. globosum* has formed a colony (arrow) and the parasitized host (arrowhead) has not yet released zoospores. Scale bars indicate 5 μm for (A−D); 20 μm for (E, F) and 100 μ for (G, H). Rh, host rhizoid, Ss, host subsporangial swelling.**

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.funbio.2016.08.008.

On agar, uninfected *R. globosum* thalli were 13−16.5 μm diameter, while infected thalli often were up to 4× the diameter (37−56 μm) of uninfected thalli (Fig 2G, H), indicating uniform hypertrophy due to infection by the parasite. At maturity the parasite occupied the entire hypertrophied host.

Ultrastructure *via* transmission electron microscopy

Uninfected *Rhizoclosmatium globosum* sporangia were spherical to oval, 13−16.5 μm diameter (Fig 3A), and the cytoplasm contained nuclei ∼3.5 μm diameter (Fig 3A, B, D), lipid globules and vacuoles (Fig 3A), and well-developed mitochondria (Fig 3E). Infected host sporangia were spherical, 37−56 μm diameter (Figs 3B and 5A). During development of the parasite, the infected host sporangium contained remnants of host cytoplasm, as well as the parasite plasmodium (Fig 3B) with multiple nuclei ∼2 μm diameter (Fig 3B, D) and mitochondria with poorly developed cristae (Fig 3F). The host−parasite interface between the unwalled thallus of *Rozella* and the host *Rhizoclosmatium* consisted of three membranes, the inner of which is the parasite's plasma membrane (Fig 3C). We have not

determined developmentally the origin of the outer two membranes of the host−parasite interface, but it is possibly host cisterna. Lobes of parasite plasmodium were evident in the host cytoplasm (Fig 3D), and serial section reconstruction confirmed the lobes to be continuous with the main plasmodium body (Fig 4).

Mature zoospores were dispersed in the sporangium (Fig 5A). Fixed zoospores were 1.3−1.4 μm wide and 1.8−2.0 μm long (Fig 5B). The anterior end of the zoospore contained a helmet-shaped nucleus (Held 1975) ∼0.75 μm wide × 0.6 μm high, anteriorly convex and posteriorly concave (Fig 5B−E). Appressed to the surface of the nucleus was a lattice composed of perpendicular rods, each rod ∼13 nm diameter (Fig 5C−E). We interpret this as a lattice, based on tangential sections at a surface of the nucleus (Fig 5E). Serial sections indicate these are overlapping rods that envelop the nucleus and not a fenestrated cisterna that is found among some Chytridiomycota (Letcher & Powell 2014). Posterior to the nucleus was a single well-developed mitochondrion (0.54−0.60 μm diameter) nestled into the concave surface of the nucleus (Fig 5B, C, E). Posterior to the mitochondrion, a striated rhizoplast capped the kinetosome at the anterior end of the flagellum (Figs 5B and 6E). The flagellum extended from the kinetosome to the posterior end of the zoospore (Figs 5B
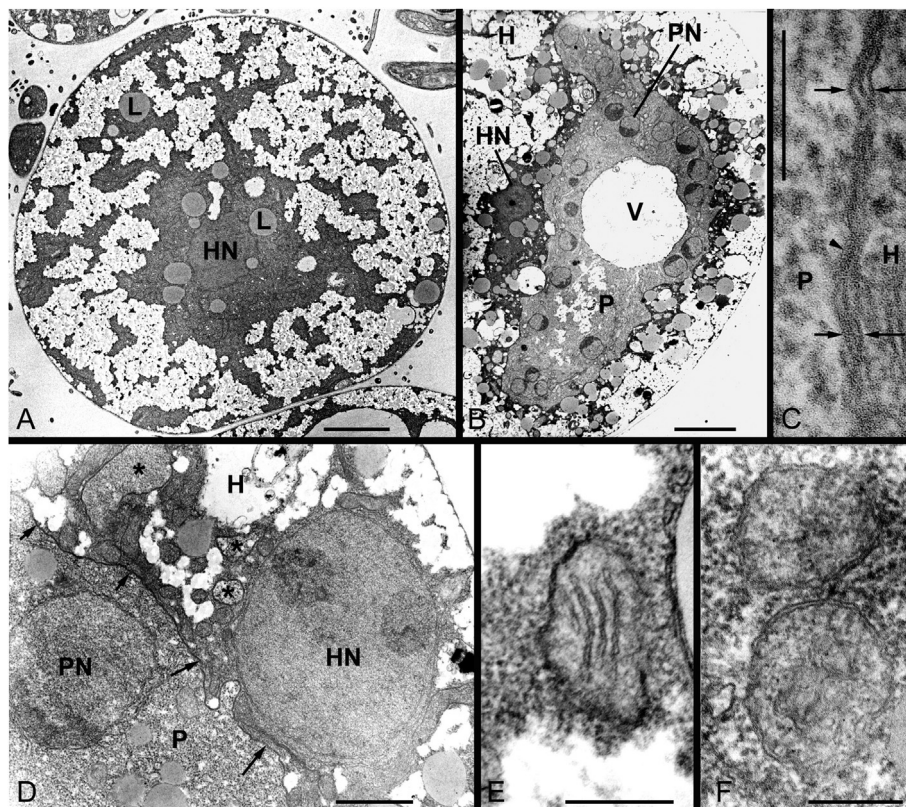
Fig 3 − Ultrastructure of *Rozella rhizoclosmatii* thallus. (A). Uninfected sporangium of host *Rhizoclosmatium globosum*, with a central nucleus and scattered lipid globules. (B). Portion of host sporangium infected with *Rozella rhizoclosmatii*. A host nucleus and multiple parasite nuclei are visible; parasite has a central vacuole. (C). Membrane interface between host and parasite (between arrows); inner membrane (arrowhead) is parasite's plasma membrane. (D). Host nucleus in residual host cytoplasm, and parasite nucleus in parasite cytoplasm; asterisks indicate lobes of parasite plasmodium; host and parasite delineated by membrane interface (arrows). (E). Mitochondrion of host. (F). Mitochondria of parasite. Scale bars in (A) = 25.0 μm; in (B) = 5.0 μm; in (C) = 0.12 μm; in (D) = 1.0 μm; in (E), (F) = 0.25 μm. L, lipid globule; H, host; HN, host nucleus; P, parasite; PN, parasite nucleus; V, vacuole.

and 6E). A complex of 4−5 perikinetosomal microtubules radiated anteriorly from one side of the kinetosome, around the mitochondrion (Fig 6A, B). A non-flagellated centriole was adjacent and at a slight angle to the kinetosome (Fig 6H, I). The flagellum was attached to the plasma membrane *via* flagellar props (Fig 6C, D). In the central region of the zoospore was a microbody-lipid globule complex (MLC) composed of two or more lipid globules, a granular microbody, and a backing membrane composed of a flat stack of membranes (Fig 6E, F). Ribosomes were dispersed in the cytoplasm. Vesicles (0.18−0.2 μm diameter) with or without cores, and vesicles with electron-opaque contents (0.17−0.2 μm diameter) occurred in the cytoplasm (Fig 6G). A schematic of the zoospore illustrates its features (Fig 7).

Taxonomy

Rozella rhizoclosmatii Letcher and Longcore, sp. nov.
MycoBank MB817338.
Etymology: The epithet reflects the genus name of the host of this fungus, *Rhizoclosmatium globosum*.

Description: Sporangium monocentric, holocarpic, filling host cell and causing uniform hypertrophy of host as indicated by larger size of infected host sporangium (45 μm av. diameter) relative to that of uninfected host sporangium (15 μm av. diameter). Zoospores motile in the sporangium before they release through a single exit pore. Zoospores oblong or oval, 1−1.3 μm wide × 1.8−2 μm long, with a single posterior flagellum 10−12 μm long. Zoospores have a *Rozella*-type ultrastructure (Held 1975), plus a lattice of perpendicular rods surrounding the nucleus. Resting spores not observed.
Specimen examined: USA, Maine, Penobscot County, Orono, Perch Pond, 44.2952, −68.7779. Fungus parasitic within the sporangium of *Rhizoclosmatium globosum* (Chytridiales) saprophytic on pollen and isolated from a fen water sample containing sphagnum and organic debris, Aug. 2015, J.E. Longcore 863 (**holotype** Fig 2E, this paper). Ex-type strain JEL 863 preserved in a metabolically inactive state at −80 °C or lower in cryoprotectant at UACCC (University of Alabama Chytrid Culture Collection); GenBank KX354829 (18S), KX354831 (ITS).
Note: 'Rozella ex *Rhizoclosmatium*' JEL 347 has been included previously in molecular phylogenies (e.g. James *et al.* 2006),
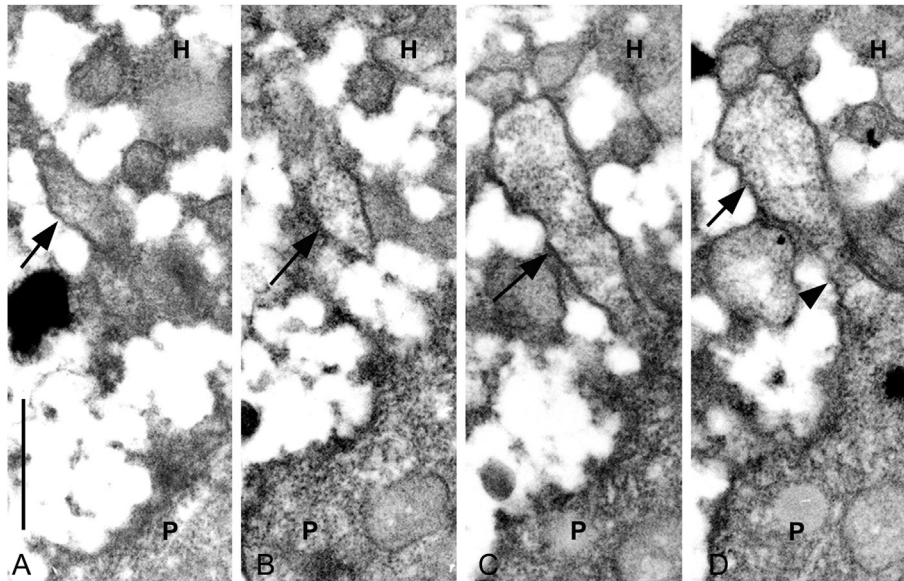
Fig 4 – Continuity of parasite plasmodium. (A–D). Serial sections through parasite plasmodium. Arrows (A–D) indicate a lobe of the plasmodium in host cytoplasm, and arrowhead (D) indicates continuity of lobe of plasmodium to main body of plasmodium. Scale bars in (A–D) = 0.5 μm. H, host; P, parasite.

although its ultrastructure was not investigated. The 18S rDNA sequence for our new species JEL 863 *R. rhizoclosmatii* is 97.4 % similar to that of JEL 347 'Rozella ex *Rhizoclosmatium*'. This indicates the two strains are closely related but may not be the same species.

Discussion

Phylogenetic analysis and ecological considerations

We included in our phylogenetic analysis all sequenced *Rozella* strains as well as related environmental sequences. Because we isolated strain JEL 863 *Rozella rhizoclosmatii* from the same fen as strain JEL 347 *Rozella* ex *Rhizoclosmatium*, we expected the two strains to be genetically identical. Both isolates came from the same habitat, a fen around a small, rural lake, and ∼20 m apart. Notably, their ITS1-5.8S-ITS2 rDNA (ITS) sequences were only 68 % similar, indicating that they are likely not conspecific, and this agrees with the 2.6 % 18S divergence, where 1 % divergence is roughly equivalent to 250 million years (Berbee & Taylor 1993). Surprisingly, partial 28S rDNA sequences of the hosts of JEL 863 *R. rhizoclosmatii* and JEL 347 *Rozella* ex *Rhizoclosmatium* (designated as JEL 863h [KX354826] and JEL 347h [DQ273769] respectively) were identical. This suggests that there are at least two very similar *Rozella* parasites of the same host, *Rhizoclosmatium globosum*. *Rhizoclosmatium globosum* is one of the most common chytrids, and is considered 'the most ubiquitous of the exuviae-inhabiting chytrids' (Sparrow 1960). Unfortunately, the strain JEL 347 is no longer available for study.

Included in the *R. rhizoclosmatii* clade was an environmental sequence (clone BF2-55) recovered from a pharmaceutical effluent and municipal wastewater treatment plant in China (Deng *et al.* 2012), and 18S sequence similarity with *R.*

rhizoclosmatii was >99 %. Thus, our new *Rozella* taxon seems to have distant and global distribution and to occur in highly diverse habitats. Two additional environmental sequences (EHM4, EMH5) in the clade were derived from a natural microbial biofilm community that developed in a bioreactor (Nishio *et al.* 2010), while a fourth environmental sequence (clone CES304-34) was recovered from a freshwater sediment sample from Florida (Lazarus & James 2015). As more environmental sequences are recorded we will be able to see how widespread this organism (and its close relatives) is. Our working hypothesis is that these environmental sequences are also derived from parasites of *Rhizoclosmatium* or related monocentric chytrids that are likely to occur in such habitats, which are almost exclusively a broad array of aquatic environments.

A sister clade to the *R. rhizoclosmatii* lineage just discussed contained two lineages, with one lineage containing two strains of *Rozella allomycis*, while the other contained a strain of *Rozella* ex *Pythium*. It is interesting that the 18S sequences of the two strains of *R. allomycis* (UCB 47-054 [AY635838] and CSF55 [KX354828]) were 96 % similar, and, as with the strains parasitizing *Rhizoclosmatium*, sequences for the ITS locus for these *R. allomycis* strains (UCB 47-054 [AY997087] and CSF55 [KX354827]) were 69 % similar. These data on parasites of *Rhizoclosmatium* and *Allomyces* suggest that the phylogenetic relatedness is a strong indicator of host affinity, but that the *Rozella* species identified on the basis of host are likely to be comprised of multiple cryptic species. Finally, 18S sequence similarity among JEL 863 *R. rhizoclosmatii* (KX3548290), UCB 47-054 *R. allomycis* (AY635838), and *Rozella* ex *Pythium* (KX354831) is no greater than 88 %. This indicates significant divergence in what is considered a conservative, slow-evolving locus, among species in this genus. All of these data point to ancient, stable associations between host and parasite over millions of years.
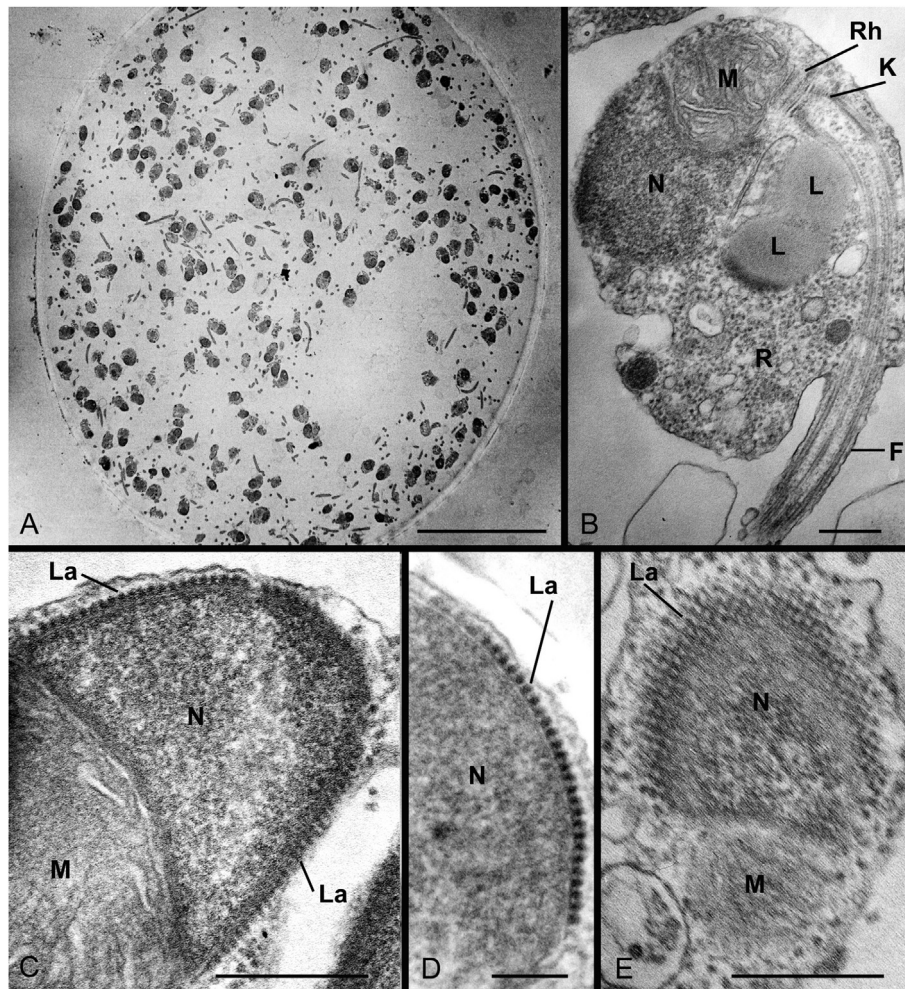
Fig 5 − Ultrastructure of *Rozella rhizoclosmatii* zoospore. (A). Cleaved zoospores in sporangium. (B). Longitudinal section (LS) of zoospore, with helmet-shaped nucleus, single mitochondrion anterior to a fibrillar rhizoplast, which is anterior to the kinetosome; two lipid globules, and dispersed ribosomes. (C). LS through nucleus and mitochondrion, illustrating a portion of the lattice appressed to the nucleus. (D). Medial transverse section through the lattice of perpendicular rods. E. Grazing section through lattice. Scale bars in (A) = 10 μm; in (B), (C), (E) = 0.25 μm; in (D) = 0.125 μm. F, flagellum; K, kinetosome; L, lipid globules; La, lattice; M, Mitochondrion; N, nucleus; R, ribosomes; Rh, rhizoplast.

Morphology

One aspect of infection by many species of *Rozella* is the induction of local hypertrophy in the host at the site of infection, or septation of the host hyphae (e.g., *Rozella septigena*, *Rozella allomycis*). It is on the basis of presence or absence of septation in the host that the genus has been divided into two groups (see Karling 1942). The 'non-septigenous group', composed of mono-sporangiate species, exhibits marked local hypertrophy of the host, and each thallus results in a single sporangium or resting spore. This is the case with our new *Rozella* species, as with all described species of *Rozella* that parasitize members of Chytridiomycota. Alternatively, in the 'septigenous group' the thallus or plasmodium putatively divide, resulting in a linear series of sporangia. In most species of this group, the infection also causes slight hypertrophy as well as the formation of crosswalls in the host thallus, which separate parasite sporangia from each other. This is the case with three *Rozella*

species: *R. septigena* (the type), *Rozella achlyae* Shanor, and *R. allomycis*. As a nomenclatural aside, Batko (1978) illegitimately (McNeill *et al.* 2012, Art. 52.1, 52.2) moved the type *R. septigena* (see Clements & Shear 1931, p. 234) as well as *R. achlyae* and *R. allomycis* to *Skirgiellia*, delineated to accommodate the septigenous species of *Rozella*. Doweld (2014) compounded the issue, erecting family Skirgielliaceae to accommodate the illegitimate *Skirgiellia*. Beyond the illegitimacy, our molecular phylogeny indicates that removal of *R. allomycis* from the genus would leave *Rozella* as polyphyletic.

A diagnostic feature of *Rozella rhizoclosmatii*-infected host thalli, besides the longer generation time, is the presence of enlarged (hypertrophied) host sporangia distributed among smaller, or already discharged, uninfected host thalli. This visual discrimination between infected and uninfected host thalli facilitates isolation of infected thalli for ultrastructural and molecular studies, by allowing the observer to select individual infected sporangia for fixation or DNA extraction. A
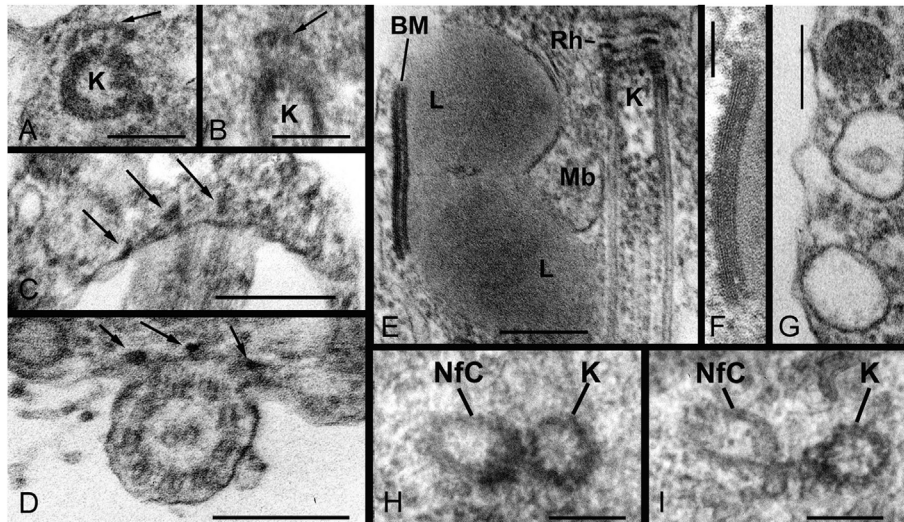
Fig 6 – Ultrastructure of *Rozella rhizoclosmatii* zoospore. (A, B). Sections through kinetosome, with adjacent microtubular root (arrows). (C). Longitudinal section through flagellar props (arrows). (D). Transverse section through flagellum and flagellar props (arrows). (E). Microbody-lipid globule complex, composed of backing membrane, lipid globules, and microbody. (F). Magnification of backing membrane (G). Electron opaque (upper), cored (center), and non-cored (lower) vesicles found in zoospore cytoplasm. (H, I). Transverse serial sections through kinetosome and non-flagellated centriole. Scale bars in (A), (B), (C), (D), (E), (G), (H), (I) = 0.25 μm; in (F) = 0.1 μm. BM, backing membrane; K, kinetosome; L, lipid globule; Mb, microbody; NfC, non-flagellated centriole; Rh, rhizoplast.

second diagnostic feature is the presence of smaller parasite zoospores among larger host zoospores. Indeed, in the absence of distinct or unique parasite sporangial morphology (as a result of the parasite thallus being endobiotic relative to the host), host hypertrophy and zoospore size distinction are diagnostic features for most *Rozella* species. A few species do not induce hypertrophy (e.g., *Rozella apodyae brachynematis* Cornu), and for a few species, zoospores have not been observed (e.g., *Rozella blastocladiae* (Minden) Sparrow, *Rozella monoblepharidis polymorphae*). A third diagnostic feature is the presence of a spiny (rarely but occasionally smooth) resting spore or spores in the sporangium, although resting spore formation has not been observed in our species or a few described species [*Rozella polyphagi* (Sparrow) Sparrow, *Rozella endochytrii* Karling, *Rozella rhizophydii* Karling, *Rozella longisporangia* Willoughby and Rigg].

Ultrastructure

In its ultrastructural configuration the zoospore of *Rozella rhizoclosmatii* is quite similar to that of *Rozella allomycis* (Held 1975), the only other species of *Rozella* for which the zoospore has been examined ultrastructurally. Although the zoospore of *Rozella rhizoclosmatii* is only about half the size of that of *R. allomycis*, occurrence and distribution of organelles and structures are remarkably consistent between the two. The zoospore of R. *rhizoclosmatii*, however, has a distinct ultrastructural feature not present in *R. allomycis*, or for that matter, in any other fungal zoospore. The nucleus of R. *rhizoclosmatii* is surrounded by a lattice of distinct perpendicular rods, which are readily apparent *via* TEM. We do

not consider this structure comparable to a rumposome/ fenestrated MLC cisterna (Fuller 1966; Powell 1978), as the lattice occurs with the nucleus. We have examined the ultrastructure of a strain of *R. allomycis*, CSF55, and find no evidence for or indication of the lattice structure.

Although we did not observe a dictyosome in the zoospore, we did observe a region of vesicle-like material (our Fig 6G) that may correspond with Held's (1975, Fig. 13d) interpretation of a Golgi apparatus in the zoospore of *R. allomycis*. Alternatively, the region may represent inflated cisternae with smooth surfaces, as reported for the zoospore of *Caulochytrium protostelioides* Olive (Powell 1981).

Conclusions

Rozella is an interesting organism because of its phylogenetic position, obligately parasitic and endobiotic habit, and dearth of molecular and ultrastructural knowledge of its various species. As the basis for Cryptomycota, *Rozella* is a lineage of organisms that diverged early from traditional fungi. Revealing morphological, ultrastructural, and molecular features of members of the genus is key to reconstructing early fungal evolution. Through examination of species in the two groups-septiginous and non-septiginous-we can begin to assess the monophyly of the genus and the evolution of parasitic strategies. As a larger issue, and because its members have intermediate features between true fungi and protists, is *Rozella* more fungal in nature, having a trophic phase without a chitinous cell wall but making a resting spore with a chitinous wall (James & Berbee 2012), or more protistan in nature, having possible phagotrophic nutrition
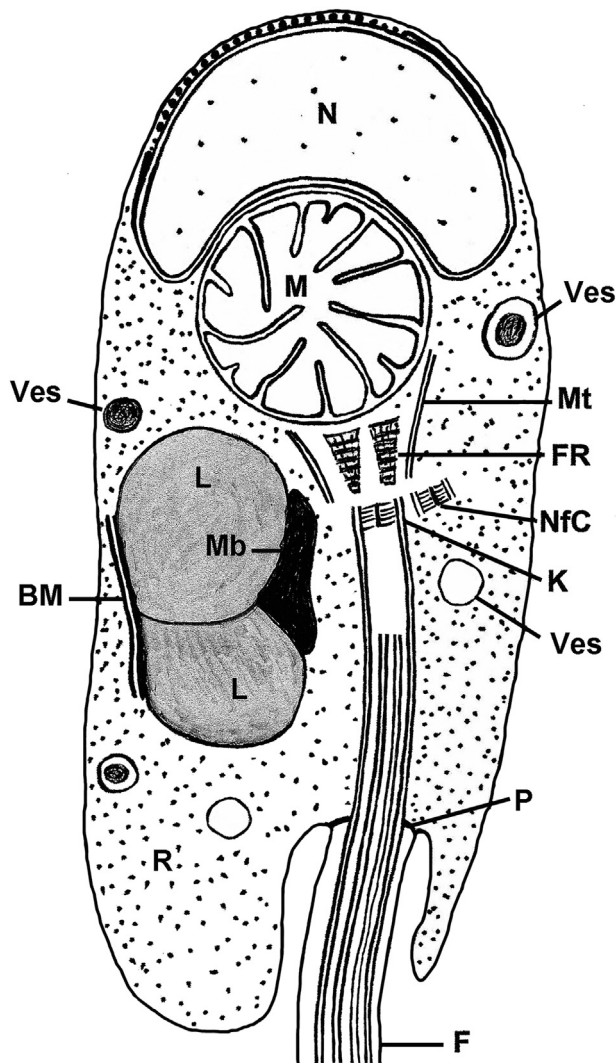
Fig 7 — Schematic of *Rozella rhizoclosmatii* zoospore. BM, backing membrane; F, flagellum; FR, fibrillar rhizoplast; K, kinetosome; L, lipid globule; M, mitochondrion; Mb, microbody; Mt, microtubule; N, nucleus; NfC, non-flagellated centriole; P, flagellar props; R, ribosomes; Ves, vesicle. Lattice surrounds nucleus.

([Powell 1984](#))? Further investigations of *Rozella* to reveal ultrastructural and molecular features of described species and to discover new species will help put a face on Cryptomycota and further our understanding of this enigmatic group.

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REFERENCES

Batko A, 1978. Further observations on *Nellymyces megaceros* and its parasite- *Rozellopsis uliginosa* sp. nov. *Acta Mycologica Warsaw* **13**: 313–324 [1977].

Berbee ML, Taylor JW, 1993. Dating the evolutionary radiations of the true fungi. *Canadian Journal of Botany* **71**: 1114–1127.

Boyle DG, Hyatt AD, Daszak P, Berger L, Longcore JE, Porter D, Hengstberger SG, Olsen V, 2003. Cryo-archiving of *Batrachochytrium dendrobatidis* and other chytridiomycetes. *Diseases of Aquatic Organisms* **56**: 59–64.

Clements FE, Shear CL, 1931. *The Genera of Fungi*. H.W. Wilson, New York.

Cornu M, 1872. Monographie des Saprolégniées: étude physiologique et systématique. *Annales de la Société Nationale Botanique* **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 lineage Rozellomycota. *Journal of Parasitology Research* **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. *Journal of Parasitology Research* **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. *Journal of Parasitology Research*. http://dx.doi.org/10.1017/s00436-016-5055-9.

Deng Y, Zhang Y, Gao Y, Li D, Liu R, Liu M, Zhang H, Hu B, Yu T, Yang M, 2012. Microbial community compositional analysis for series reactors treating high level antibiotic wastewater. *Environmental Science and Technology* **46**: 795–801.

Doweld AB, 2013. *Nomenclatural novelties. Rozellomycota* Index Fungorum 43: 1.

Doweld AB, 2014. *Nomenclatural novelties. Skirgiellaceae* Index Fungorum 129: 1.

Foust FK, 1937. A new species of *Rozella* parasitic on *Allomyces*. *Journal of the Elisha Mitchell Scientific Society* **53**: 197–204.

Fuller MS, 1966. Structure of the uniflagellate zoospores of aquatic phycomycetes. In: Madelin MF (ed.), *The Fungus Spore*. Butterworth's, London, pp. 67–84.

Hall TA, 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Serial* (Oxford) **41**: 95–98.

Held AA, 1975. The zoospore of *Rozella allomycis*: ultrastructure. *Canadian Journal of Botany* **53**: 2212–2232.

James TY, Letcher PM, Longcore JE, Mozley-Standridge SE, Porter D, Powell MJ, Griffith GW, Vilgalys R, 2006. A molecular phylogeny of the flagellated fungi (Chytridiomycota) and description of a new phylum (Blastocladiomycota). *Mycologia* **98**: 860–871.

James TY, Berbee ML, 2012. No jacket required- new fungal lineage defies dress code. *Bioessays* **34**: 94–102.

Jones MDM, Forn I, Gadelha C, Egan MJ, Bass D, Massana R, Richards TA, 2011a. Discovery of novel intermediate forms redefines the fungal tree of life. *Nature* **474**: 200–203.

Jones MDM, Richards TA, Hawksworth DL, Bass D, 2011b. Validation and justification of the phylum name Cryptomycota phyla. nov. *IMA Fungus* **2**: 173–175.

Karling JS, 1942. Parasitism among the chytrids. *American Journal of Botany* **29**: 24–35.

Karling JS, 1987. Ross biological reserve aquatic fungi III. Additional species. *Nova Hedwigia* **45**: 529–535.

Karpov SA, Mamkaeva MA, Aleoshin VV, Nassonova E, Lilje O, Gleason FH, 2014. Morphology, phylogeny, and ecology of the aphelids (Aphelidea, Opisthokonta) and proposal for the new

superphylum Opisthosporidia. *Frontiers in Microbiology* **5** (Article 112): 1—11.

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 KL, James TY, 2015. Surveying the biodiversity of the *Cryptomycota* using a targeted PCR approach. *Fungal Ecology* **14**: 62—70.

Letcher PM, Lee PA, Lopez S, Burnett M, McBride RC, Powell MJ, 2016. An ultrastructural study of *Paraphysoderma sedebokerense* (Blastocladiomycota), an epibiotic parasite of microalgae. *Fungal Biology* **120**: 324—337.

Letcher PM, Powell MJ, 2014. Hypothesized evolutionary trends in zoospore ultrastructural characters in Chytridiales (Chytridiomycota). *Mycologia* **106**: 379—396.

McNeill J, Barrie FR, Buck WR, Demoulin V, Greuter W, Hawksworth DL, *et al.*, 2012. *International Code of Nomenclature for Algae, Fungi, and Plants (Melbourne Code)*. . In: *Regnum Vegetabile*, vol. 154. Koeltz Scientific Books.

Nishio K, Hashimoto K, Watanabe K, 2010. Light/electricity conversion by a self-organized photosynthetic biofilm in a single-chamber reactor. *Applied Microbiology and Biotechnology* **86**: 957—964.

Powell MJ, 1978. Phylogenetic implications of the microbody-lipid globule complex in zoosporic fungi. *Biosystems* **10**: 167—180.

Powell MJ, 1981. Zoospore structure of the mycoparasitic chytrid *Caulochytrium protostelioides* Olive. *American Journal of Botany* **68**: 1074—1089.

Powell MJ, 1984. Fine structure of the unwalled thallus of *Rozella polyphagi* in its host *Polyphagus euglenae*. *Mycologia* **76**: 1039—1048.

Sikes DS, Lewis PO, 2001. *Beta Software. Version 1. PAUPRat: PAUP* implementation of the parsimony ratchet*. Department Ecology Evolutionary Biology, Univ. Connecticut, Storrs, Distributed by the authors.

Sparrow FK, 1960. *Aquatic Phycomycetes*, 2nd edn. University of Michigan Press, Ann Arbor.

Swofford DL, 2002. *PAUP* 4.0b4a: phylogenetic analysis using parsimony (*and other methods)*. Sinauer Associates, Sunderland, MA.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG, 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876—4882.

Vélez CG, Letcher PM, Schultz S, Powell MJ, Churchill PF, 2011. Molecular phylogenetic and zoospore ultrastructural analyses of *Chytridium olla* establish the limits of a monophyletic Chytridiales. *Mycologia* **103**: 118—130.

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