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To cite this article: Joyce E. Longcore , Shan Qin , D. Rabern Simmons & Timothy Y. James (2020) *Quaeritorhiza haematococci* is a new species of parasitic chytrid of the commercially grown alga, *Haematococcus pluvialis* , *Mycologia*, 112:3, 606-615, DOI: [10.1080/00275514.2020.1730136](https://doi.org/10.1080/00275514.2020.1730136)

To link to this article: <https://doi.org/10.1080/00275514.2020.1730136>

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Quaeritorhiza haematococci is a new species of parasitic chytrid of the commercially grown alga, *Haematococcus pluvialis*

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ABSTRACT

Aquaculture companies grow the green alga *Haematococcus pluvialis* (Chlorophyta) to extract the carotenoid astaxanthin to sell, which is used as human and animal dietary supplements. We were requested to identify an unknown pathogen of *H. pluvialis* from an alga growing facility in the southwestern United States. To identify this zoospore fungus and determine its phylogenetic placement among other chytrids, we isolated it into pure culture, photographed its morphology and zoospore ultrastructure, and sequenced and analyzed portions of nuc rDNA 18S and 28S genes. The organism belongs in the Chytridiomycota, but a comparison of rDNA with available representatives of the phylum did not convincingly place it in any described order. The unique zoospore ultrastructure supports its indeterminate ordinal position, and the morphology, as determined by light microscopy, did not match any described species. Consequently, we have placed this chytrid in the new genus, *Quaeritorhiza*, and described it as the new species *Q. haematococci* in the family Quaeritorhizaceae but otherwise incertae sedis in the Chytridiomycetes. This new taxon is important because it increases the known diversity of Chytridiomycota and the organism has the ability to disrupt agricultural production of an algal monoculture.

ARTICLE HISTORY

Received 28 October 2019
Accepted 12 February 2020

KEYWORDS

Alga parasite;
Chytridiomycota;
commercial algae
production;
Quaeritorhizaceae; TEM; 3
new taxa

INTRODUCTION

The green alga *Haematococcus pluvialis* (Chlorophyta; Volvocales) is grown commercially for harvesting the carotenoid astaxanthin, which is used as a dietary supplement for humans and other animals and as a color enhancer for fish such as farm-raised salmon (Guerin et al. 2003; Shah et al. 2016). As with other crops grown in monoculture, *H. pluvialis* attracts fungal pathogens. Both the widespread *Aquamyces chlorogonii* (Rhizophydiales; Carney et al. 2016) and *Paraphysoderma sedebokerensis* (Blastocladales; James et al. 2011) interfere with mass production of *Haematococcus* (Carney and Lane 2014). Crop losses have led to research to detect and control fungal pathogens in aquatic agriculture (e.g., Carney et al. 2016). Recently, we found another chytrid (Chytridiomycota) pathogen of *H. pluvialis* from an aquaculture facility in the southwestern United States and isolated it into pure culture. We could not find any description of this chytrid in the literature, as growing either on *Haematococcus* or on other algae. Phylogenetic analysis of portions of nuc rDNA 18S and 28S gene regions placed it within the Chytridiomycetes but with uncertain affinity to described chytridiomycete orders or

families. Because genetic analysis of its position placed it outside of known taxonomic orders, we also studied the ultrastructure of its zoospores. Herein, we describe isolate JEL0916 as *Quaeritorhiza haematococci*, sp. nov., in the new genus *Quaeritorhiza* and place it in the new family Quaeritorhizaceae.

MATERIALS AND METHODS

Isolation and culture.—We collected infected *Haematococcus* during a population crash at an alga growing facility in the southwestern United States and plated the infected algal cells onto various media commonly used to grow members of Chytridiomycota (all containing penicillin G at 200 mg/L and streptomycin at 200–500 mg/L). Media included PmTG (Barr 1986), modified PmTG (mPmTG; Longcore 2004), Cd (Longcore and Simmons 2012), and ¼ strength Emerson's YpSs medium (Fuller and Jaworski 1987). We incubated isolation attempts at room temperature or at 30 C. After the fungus did not grow on these media, we tried PmTG without glucose (PmT; peptonized milk, 1 g; tryptone, 1 g; agar 10 g; distilled water, 1000 mL), and

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a monocentric chytrid with large zoosporangia grew from clumps of infected *Haematococcus*. This chytrid discharged zoospores on isolation plates, and we gradually separated clumps of thalli from planctomycete bacteria and host cells. We maintained the cultured fungus, designated JEL0916, at 30 C in PmT liquid or agar medium and cryopreserved samples according to Boyle et al. (2003). Frozen samples are archived in CZEUM, which is part of the University of Michigan Herbarium (MICH; Thiers [continuously updated]).

Light and transmission electron microscopy morphology.—We photographed and measured development of the fungus with a Spot RT3 camera (Sterling Heights, Michigan) on a Nikon E400 microscope (Melville, New York) with phase-contrast and bright-field optics. We collected zoospores for fixation by inoculating plates of PmT with an actively growing culture in broth, allowing the plates to dry, incubating them at 30 C for 4 d, and initiating zoospore release by adding 3 mL of sterile distilled water to each plate. After 30 min, we collected liquid containing discharged zoospores from each plate and added an equal volume of glutaraldehyde in *s*-collidine buffer. Fixation and staining were according to Barr (1981). Briefly, we postfixed zoospores in osmium tetroxide, en bloc stained zoospores with uranyl acetate, embedded them in Epon-Araldite, stained sections with lead citrate, and examined them on a Philips CM10 transmission electron microscope (Eindhoven, The Netherlands) at 100 kV.

Inoculation of *Haematococcus*.—We tested the pathogenicity of JEL0916 by inoculating three dishes of the unialgal green stage samples of *H. pluvialis* and one sample of *H. pluvialis* red cysts with a liquid culture of the fungus that we centrifuged to remove nutrient medium and resuspended in sterile lake water. Each test consisted of 6 mL of algal culture plus 1 mL of the resuspended chytrid in a 5.5-cm Petri dish. Each inoculated dish was paired with a duplicate algal sample to which we added lake water without the chytrid. We incubated all samples at room temperature (23–25 C) in ambient light and observed them for 12 d.

DNA isolation, sequencing, and phylogenetic analyses.—We grew JEL0916 in ~30 mL of PmT liquid medium. After sufficient growth had occurred, we centrifuged the culture at 4000 rpm for 20 min, poured off the liquid until the pelleted thalli remained in ~1 mL of medium, and transferred pellet and liquid to a 1.5-mL Eppendorf microcentrifuge tube. We centrifuged the tube

at 13 000 rpm for 5 min, after which we removed the remaining medium, leaving only the pellet of thalli. We extracted DNA using cetyltrimethylammonium bromide (CTAB) buffer (James et al. 2008) and amplified the 5' ends of the 18S and the 28S rDNA regions with primers SR1R/NS4 and LR0R/LR5 (Vilgalys and Hester 1990) with GoTaq Green Master Mix (Promega, Madison, Wisconsin) as in Letcher et al. (2018). We cleaned products with ExoSAP (Promega), sequenced amplicons at the University of Michigan DNA Sequencing Core, and generated consensus sequences in Geneious 9.1.7 (Biomatters, Auckland, New Zealand). For comparison with major lineages within the Chytridiomycota, we selected taxa from Seto et al. (2017, 2020) and Karpov et al. (2016) and rooted trees with the basal Cryptomycota taxon *Rozella* sp., JEL0347. We aligned each rDNA locus in Geneious, performed initial phylogenetic maximum likelihood (ML) analyses in RAxML 8.2.8 to determine the best tree, and determined bootstrap support values from 500 replicates. We continued analyses of the concatenated data matrix in RAxML, as above, and determined Bayesian posterior probabilities of the combined alignment in MrBayes 3.2.6 from two runs of one million generations sampling every 1000 generations. We calculated posterior probabilities after a burn-in of 2500 trees in SumTrees (Sukumaran and Holder 2010). GenBank accession numbers for 18S and 28S rDNA sequences used in the analyses are indicated in the figure for the phylogenetic tree. The alignment for the combined 18S and the 28S rDNA analysis was deposited in TreeBASE (submission 25230).

RESULTS

Morphology and culture conditions.—JEL0916 grew on PmT agar when in clumps. Scattered individual zoosporangia did not develop well unless near growing clumps. In PmT liquid medium, the culture grew at room temperature (23–25 C), 30 C, and 35 C and grew somewhat, but not well, at 40 C. On nutrient agar, time to development varied from about 3 d to more than 1 wk, depending on how crowded individuals were and how large they became. Development on PmT agar is illustrated in FIG. 1, and measurements are given in Taxonomy. Zoospores were spherical when in motion and appeared, by light microscopy, to have one or sometimes two lipid globules (FIG. 1A). The rhizoidal axis arose from one site on the zoospore cyst, and a branched cluster of rhizoids developed at the distal end of the rhizoidal axis (FIG. 1B–D). The base of the rhizoidal axis became slightly swollen (FIG. 1E, arrowhead). Later-developing, sparsely branched rhizoids extended from near

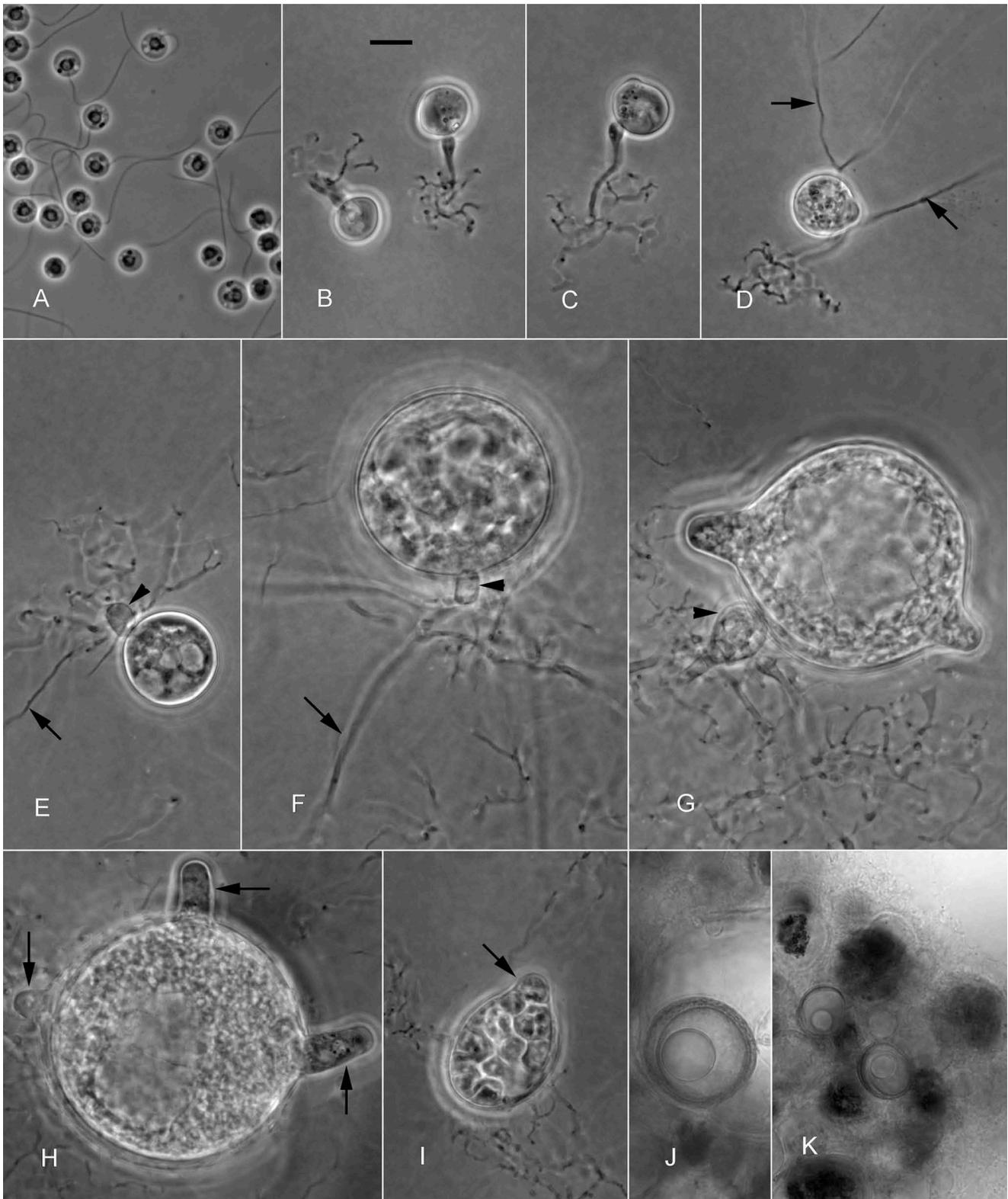


Figure 1. Morphology of *Queritorhiza haematococci* life cycle (isolate JEL0916) on PmT agar (A–I) or on *Haematococcus* cells (J, K) incubated at 30 C for 12 d. A. Spherical zoospores. B, C. One-day-old germlings with major rhizoidal axes terminated with a clump of branched rhizoids. D. One-day-old germling with rhizoidal axis terminated by branched rhizoids plus unbranched, “seeking” rhizoids (arrows) arising near the rhizoidal base. E. Three-day-old thallus with branched rhizoids (arrowhead) and “seeking” rhizoid (arrow) extending beyond clumped rhizoids. Arrowhead indicates apophysis. F. Zoosporangium with branched rhizoids plus less-branched, seeking rhizoid (arrow) extending beyond clumped rhizoids. Arrowhead indicates apophysis. G. Nearly mature zoosporangium with two discharge papillae; arrowhead indicates apophysis. H. Stacked images of nearly mature zoosporangium with three discharge papillae (arrows). I. Zoospores discharging from small zoosporangium with single, short discharge papilla (arrow). J. Large resting spore (26 µm diam) with single lipid globule and thick, crenulated wall. K. Smaller (12 µm diam) resting spores surrounded by dead host thalli. Bar in B = 10 µm for all parts of the figure.

the rhizoidal base and grew beyond the first formed rhizoidal cluster (FIG. 1D–F). Mature zoosporangia differed in size and produced one to several (often three or four, depending on sporangial size) broad, long (FIG. 1G, H), or short (FIG. 1I), inoperculate, discharge papillae. Resting spores did not develop in pure culture but formed in 12-d-old inoculated algal cultures. As with the zoosporangia, resting spores varied in size depending on local conditions (FIG. 1J, K).

Inoculation trial.—JEL0916 infected clumps of host cells on the bottom of the plates, whereas motile stages of the alga seemed to remain uninfected. Three days after inoculation at 23–25 C in natural daylight, mature zoosporangia were present among clusters of stationary host cells, causing browning of cells (compare FIG. 2A with D). Although zoospores penetrated single host cells with germ tubes (FIG. 2B), rhizoids later extended to adjacent host cells within clumps and thalli frequently became larger than

individual host cells (FIG. 2C, D). Within 1 wk, most host cells were infected and, in crowded conditions, zoosporangia matured but remained small. At the macroscopic level, color of cultures was consistent with infection status. Algae in control dishes remained unchanged, and infected cultures turned greenish brown or, in the case of inoculated red cysts, colorless. Twelve days after inoculation, resting spores (FIG. 1J, K) were present in inoculated samples. Because host cells were disorganized at this stage, we could not determine the origin and development of resting spores.

Molecular analyses.—We generated an 18S rDNA sequence (MN586917) and a 28S rDNA sequence (MN587036) from JEL0916. Our analysis of the combined 18S and 28S rDNA data placed JEL0916 as sister to a lineage that contained Lobulomycetales, Mesochytriales, Gromochytriales, Polyphagales, and *Endocoenobium eudorinae* but did not group it within

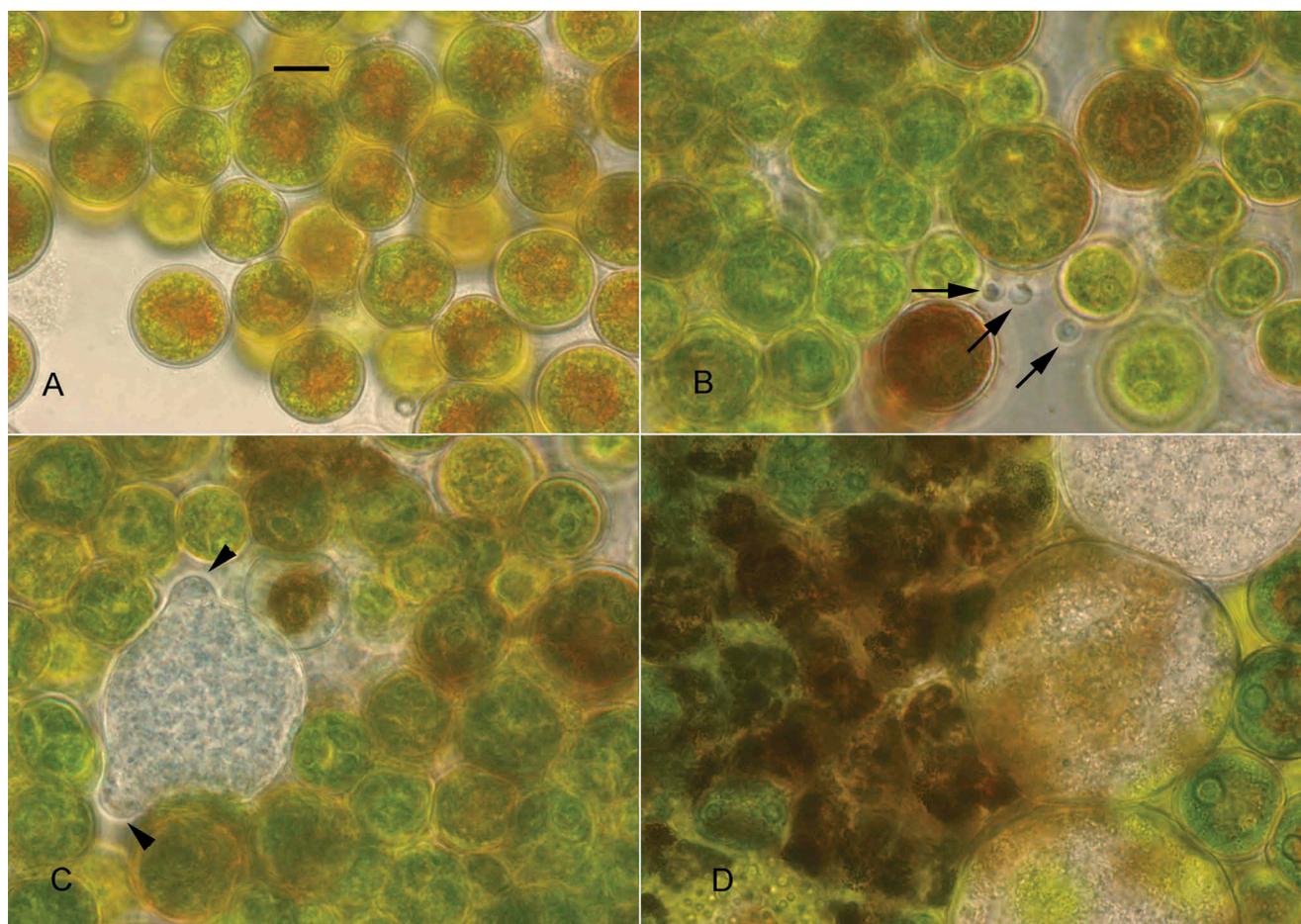


Figure 2. *Quakerithiza haematococci* (isolate JEL0916) on *Haematococcus pluvialis*. A. Cyst stage of uninoculated host. B. Germlings attached to host cells (arrows). C. Nearly mature zoosporangium with discharge papillae (arrowheads). D. Sporangia among remains of dead host cells. Bar in A = 10 μm for all parts of figure.

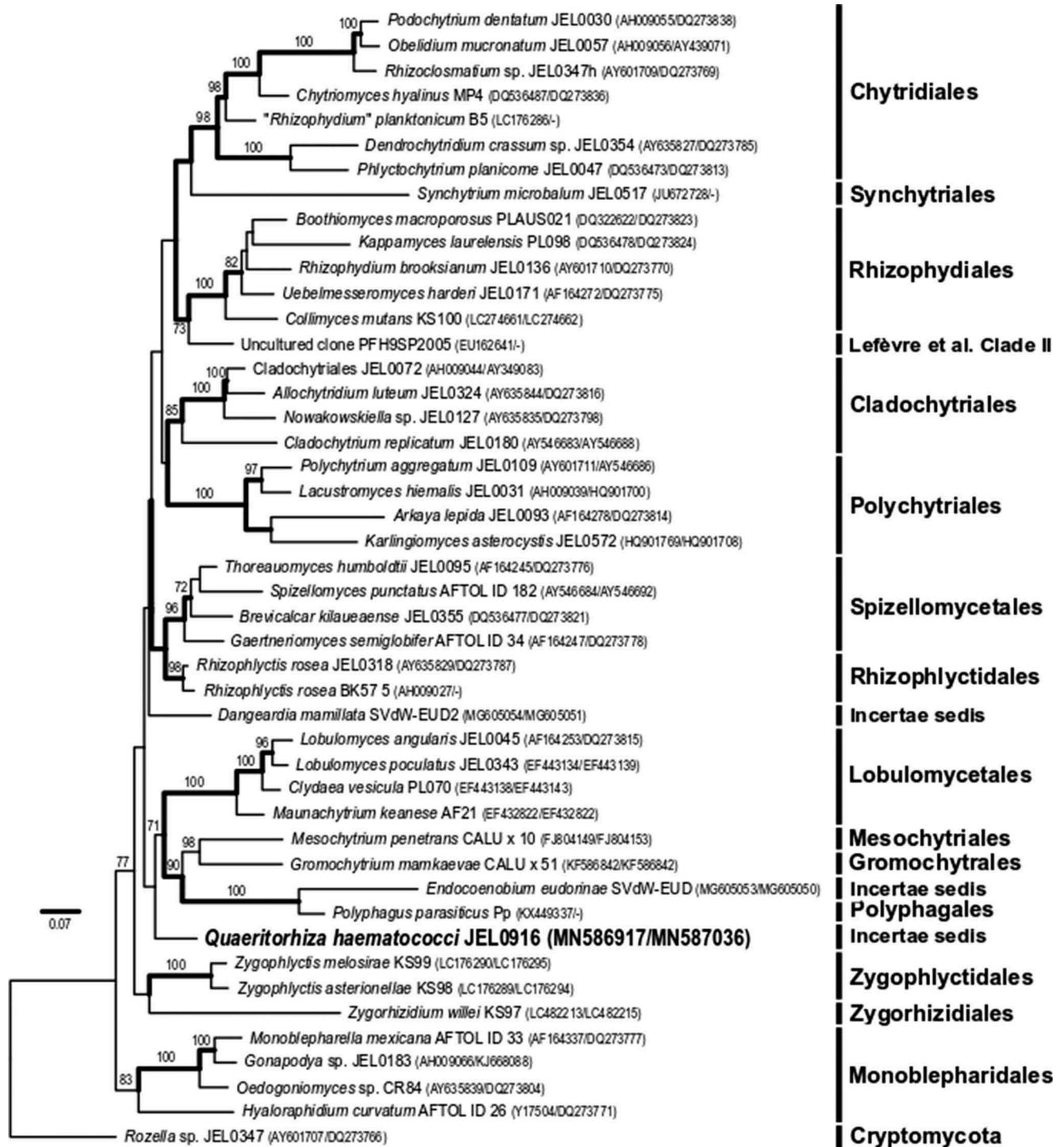


Figure 3. Phylogeny of *Queritorhiza haematococi* (isolate JEL0916) based on analyses of combined 18S and 28S with ordinal representatives of the Chytridiomycota. Support values are derived from 500 bootstrap replicates of RAXML analyses and reported when $\geq 70\%$. Branches in bold indicate Bayesian posterior probability support values ≥ 0.95 . *Rozella* sp. JEL0347 (Cryptomycota) was used as the outgroup.

any of the known orders of the Chytridiomycota (FIG. 3). Although most of these orders contain algal parasites, the grouping of JEL0916 with this lineage had weak support. A ML phylogeny of the taxa in FIG. 3 based only on 28S data produced a similar topology to

one based on the combined data set (SUPPLEMENTARY FIG. 1). A BLAST query of the 18S sequence on GenBank yielded a 98% match with several cultured members of the Spizellomycetales, and a ML phylogeny based only on 18S (SUPP

LEMENTARY FIG. 2) placed JEL0916 within the Spizellomycetales. In this analysis, however, all branches in the order, aside for one, had less than 50% ML bootstrap support, whereas when JEL0916 was removed from the analysis, ordinal and intraordinal support of the Spizellomycetales increased to 81% or above (not shown).

Zoospore structure.—Zoospores were consistently spherical when in motion, although they may be elongated when emerging from the zoosporangium. With the light microscope, the nucleus was seen surrounded by aggregated ribosomes with one or two lipid globules in the cytoplasm external to the aggregated ribosomes. Transmission electron microscopy (TEM) of the zoospores confirmed this arrangement of the nucleus and aggregated ribosomes (FIG. 4A), but what appeared as a single lipid globule was often more than one appressed lipid globule (FIG. 5A). Endoplasmic reticulum surrounded the ribosomal aggregation, and mitochondria were outside of the ribosomal aggregation (FIG. 5C). A simple rumposome (Fuller 1966; = fenestrated cisterna of Letcher and Powell 2014) surrounded a portion of the lipid globule(s), as did the microbody (FIGS. 4A, 5A, C). A small paracrystalline inclusion (PCI) occurred in the cytoplasm (FIG. 4A, 5B, C).

Vesicles and a Golgi apparatus (FIG. 4A, B) packed a hemispherical area around the kinetosome, and splayed microtubules extended from the kinetosome (FIG. 4B, C, E). In addition, a microtubule root extended from the kinetosome to the rumposome (FIG. 4A). The props that attached the flagellum to the zoospore membrane were interconnected (FIG. 4D). The nonflagellated centriole was nearly parallel to the kinetosome and attached to it by fibers (FIG. 4C, D). The flagellar transition zone contained a flagellar plug that was within the cirlet of microtubules but not external to them (FIG. 4B, E).

TAXONOMY

Although molecular and TEM evidence is critical for placing JEL0916 in a new genus, the monocentric and interbiotic morphology also differed from that of other inoperculate genera that are parasites of algae. The interbiotic morphology of *Endocoenobium*, which is also a parasite of a volvoclean alga, is perhaps the most similar. However, the sporangium of JEL0916 developed simultaneously with the rhizoidal system, whereas the zoosporangium of *Endocoenobium* develops from an enlarged prosperangium (Ingold 1940). Because of evidence from DNA, TEM, and light

microscopy, we describe JEL0916 as a new genus and species; because it has no known close relatives, we place it in a new family, with incertae sedis ordinal placement.

Quaeritorhiza Longcore, D.R. Simmons & T.Y. James, gen. nov.

Mycobank MB833680

Type: Quaeritorhiza haematococci (described below).

Description: Based on light microscopy: Endogenous, monocentric sporangium with single major rhizoidal axis plus later-developing, interbiotic rhizoids; one to several broad, short or long, inoperculate discharge papillae. Zoospores spherical when in motion. Resting spores with single large lipid globule. Zoospore ultrastructure: Nucleus enclosed by aggregated ribosomes; mitochondria lie outside of ribosomal mass. One to several adjoining lipid globules; simple rumposome. Nonflagellated centriole nearly parallel and attached to the kinetosome from which extends a microtubule root to the rumposome and a splayed array of microtubules. Plug in flagellar transition zone limited to space interior to microtubule doublets. Small, striated inclusion in cytoplasm. Pathogen of green algae.

Etymology: *Quaeritorhiza* (Latin and Greek), seeking rhizoids, in reference to the secondary rhizoids that extend to additional host cells.

Quaeritorhiza haematococci Longcore, D.R. Simmons, T. Y. James & S. Qin, sp. nov. FIG. 1A–K

Mycobank MB833715

Typification: USA. TEXAS: Northeast Texas, host *Haematococcus pluvialis*, Jun 2017, S. Qin (**holotype** FIG. 1A–K). Ex-type culture JEL0916 (CZEUM-MICH). GenBank: 28S = MN587036; 18S = MN586917.

Description: Thallus monocentric, with one rhizoidal axis often swollen at the base; primary rhizoids clustered and repeatedly branched at the tip of the major axis, later-developing rhizoids extend farther into the medium and less frequently branched; becoming interbiotic on host. Generation time on PmT nutrient agar 3–7 d at 30 C. Zoospores release through 1–4 inoperculate discharge pores or broad tubes. Zoospores briefly elongate upon emergence, spherical when in motion, ~5–6 µm diam; flagellum ~ 25 µm in length. Grows well in groups on nutrient agar, but scattered zoospores rarely survive. Resting spores form in inoculated host culture. Parasitic on *Haematococcus pluvialis*.

Etymology: *haematococci* (Greek), in reference to the genus of the host.

Quaeritorhizaceae Longcore, D.R. Simmons & T.Y. James, fam. nov.

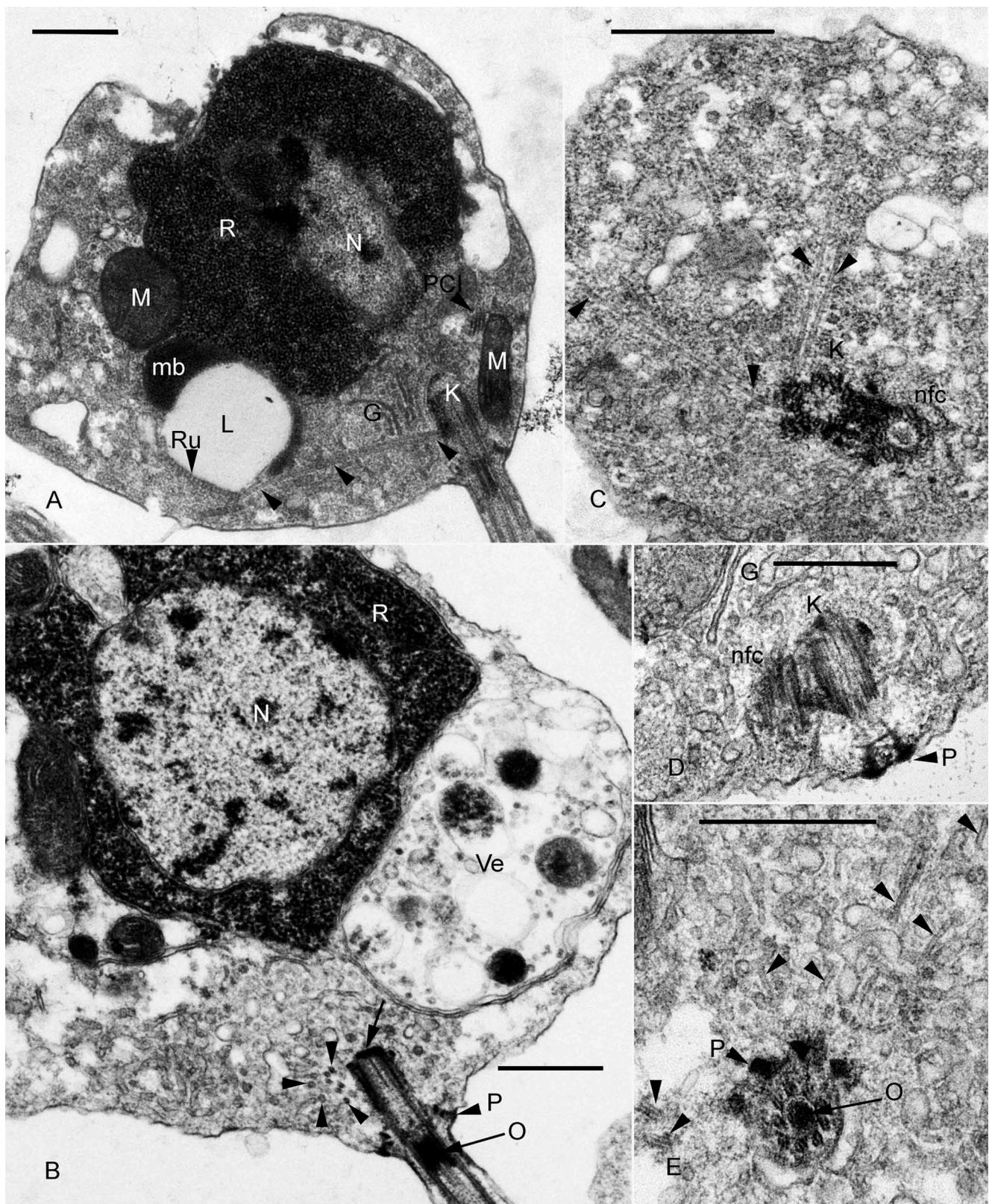


Figure 4. Ultrastructure of *Queritorhiza haematococi* (isolate JEL0916). A. Longitudinal section through zoospore showing kinetosome (K), aggregated ribosomes (R), nucleus (N), MLC consisting of microbody (mb), lipid globule (L), and rumpsome (Ru) connected to the kinetosome (K) by a microtubule root (arrowheads). A spur extends from the side of the kinetosome over its top and a Golgi apparatus (G) is present. A paracrystalline inclusion (PCI) lies adjacent to a mitochondrion (M). B. Longitudinal section through zoospore showing cap over the top of the kinetosome (arrow), a large vesicle (Ve), and endoplasmic reticulum (er)-rich area around the kinetosome with cross-sections of microtubules (arrowheads); plug (O) in flagellar transition zone. C. Cross-section of the kinetosome (K) attached to the nonfunctional centriole (nfc). Microtubules (arrowheads) extend from the kinetosome. D. Nearly longitudinal section of the kinetosome and nfc showing fibrous connection and interconnected props (P); G = Golgi apparatus. E. Cross-section of kinetosome showing dense plug in the center of the ring of microtubules. Microtubules (arrowheads) splay out from kinetosomal area; P = props. Bars: A–E = 0.5 μ m.

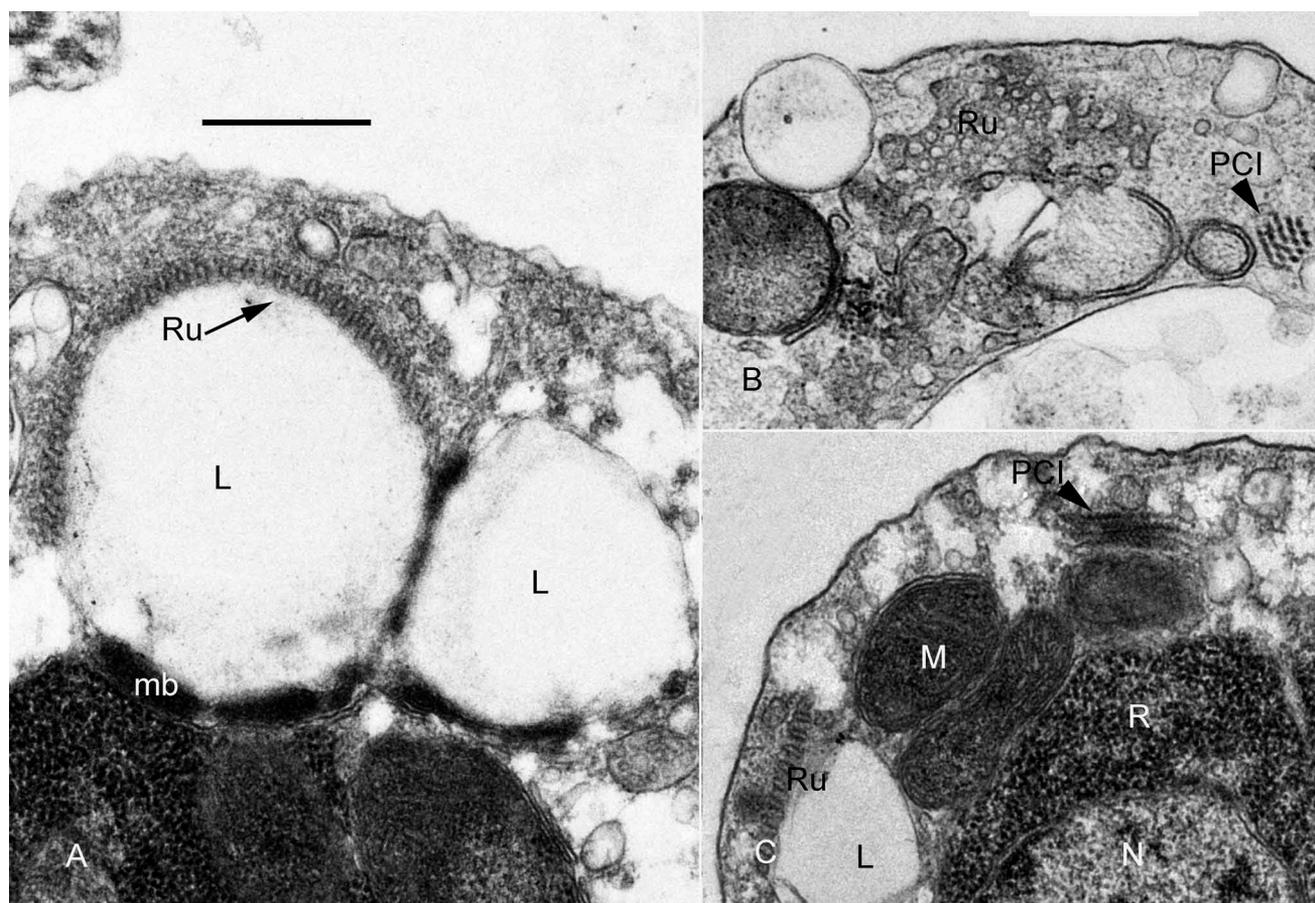


Figure 5. Ultrastructure of *Quakeritorhiza haematococi* (isolate JEL0916). A. Adjoining lipid globules with rumposome (Ru) on larger lipid globule (L) and microbody (mb) partially surrounding both. B. Surface view through rumposome and paracrystalline inclusion (PCI). C. Longitudinal view of PCI near mitochondrion exterior to aggregated ribosomes (R) surrounding nucleus (N). Lipid globule (L) with rumposome (Ru). Bar in A = 0.5 μm for all figure parts.

Mycobank MB833714

Type: *Quakeritorhiza* (described above). Description same as the genus.

DISCUSSION

Sparrow (1960) placed chytrids with monocentric inter-biotic development and lacking an operculum in the genus *Rhizidium*. However, he devoted a page to a discussion of the problematic nature of this genus, which was described by Braun (1856) based on morphology and without figures. Picard et al. (2009) further discussed the propriety of the genus in their paper describing a new species in the genus. Their concept of *Rhizidium* anchors the genus in the order Chytridiales. Consequently, although it could, based on morphology, be described as a species of *Rhizidium*, our new species, as determined by molecular analysis, does not fit into this genus nor in the Chytridiales.

Green algae historically have been reported as hosts of various chytrids. *Endocoenobium eudorinae* is a pathogen of the green alga *Eudorina*. Hosts of *E. eudorinae* and *Q. haematococi* are both in the Volvocales, and thalli of both genera produce some rhizoids that invade host cells and other rhizoids that are longer and extend to other host cells. Zoospores of both are spherical and are about 5 μm in diameter (Ingold 1940). The development of the new species differs, however. *Endocoenobium eudorinae* first forms a hyaline thallus, from which the zoosporangium enlarges, whereas the zoosporangium of *Q. haematococi* develops simultaneously with its subsporangial swelling. Fortunately, Van den Wyngaert et al. (2018) recently isolated *E. eudorinae* into culture with its host (*Yamagishiella uniccoca*; Volvocaceae) and sequenced its rDNA. Their molecular phylogeny, based on 18S and 28S, placed this algal pathogen as a sister to *Polyphagus parasiticus* (Polyphagales), which is also an algal parasite. Our phylogeny suggests that *Q. haematococi* is not closely related to *Endocoenobium*.

Do subcellular features suggest an order for *Q. haematococci*?—We examined subcellular features under TEM of *Q. haematococci* zoospores and found that the suite of subcellular features is not specific for any described order. A nucleus enclosed by an aggregation of ribosomes, parallel and connected nonflagellated centriole and kinetosome, and a rumposome and a microbody associated with the oil globule(s) are common subcellular features within many chytridiomycete orders, but they differ significantly from those in spizellomycetalean zoospores. Notably, zoospores of the Spizellomycetales have scattered rather than aggregated ribosomes and no members have a rumposome, whereas a rumposome is present in *Q. haematococci* and in 9 of the 13 chytridiomycete orders.

The presence of a small paracrystalline inclusion (PCI) is a character shared with members of the Chytridiales, the only other chytrid order with a PCI (Letcher and Powell 2014). However, we do not infer a close relationship with the Chytridiales based on the common possession of a PCI. A PCI is also present in zoospores of *Coelomomyces punctatus*, which is in the Blastocladiomycota (Martin 1971). The function of the PCI is unknown, and it may be plesiomorphic.

Based on subcellular characters, *Q. haematococci* could be inferred to belong in the Polychytriales, primarily because genera in the Polychytriales display a large diversity of zoospore ultrastructural characters. Shared with the Polychytriales are the similar interlaced props and the splayed microtubules plus the microtubule root extending to the microbody-lipid globule complex (MLC; Longcore and Simmons 2012). The flagellar plug that occupies only the area inside the microtubule doublets of *Q. haematococci* has not been reported for other orders, but its presence in a member of the Polychytriales would not be surprising because some members of that order have a flagellar plug, whereas in others it is lacking. Development and substrate, however, vary vastly between *Q. haematococci* and members of the Polychytriales. In contrast to the algal pathogen, all of the known members of the Polychytriales are saprobes of chitin.

Is any described order appropriate for *Q. haematococci*?—With the advent of molecular tools, additional pathogens of algae have been described and categorized, leading to the description of the orders Mesochytriales (Karpov et al. 2014), Gromochytriales (Karpov et al. 2014), Zygorhizidales (Seto et al. 2020), and Zygothyridiales (Seto et al. 2020). In our molecular analysis, *Q. haematococci* did not fit into any of these orders but did group most closely, although without convincing

support, with a clade that contains three of these orders plus the Polyphagales and Lobulomycetales. When considered separately, the conserved 18S gene and more phylogenetically informative 28S gene differed on the placement of *Q. haematococci*, and no support values can be interpreted to definitively place the new taxon. Based on the phylogenetic fluidity of *Q. haematococci*, and its ultrastructural character suite, we contend that this species is not closely related to any currently molecularly characterized chytrid taxon. We believe that assignment of this new species to an order should remain in abeyance until additional algal parasites are examined and a more resolved phylogeny can be obtained, perhaps with the inclusion of additional genetic loci. Clearly, the diversity of chytrid parasites of algae is still a productive area of research.

Quaeritorhiza haematococci came to our attention because of its effect on commercial production of the green alga *Haematococcus*. In recent years, increased attention has been paid to algal parasites, especially those that affect algae of commercial interest (Carney and Lane 2014; Smith et al. 2015; Carney et al. 2016; Ding et al. 2018). In spite of the widespread distribution of the *Haematococcus* pathogens *Paraphysoderma sedebokerense* (which also parasitizes the green alga *Scenedesmus dimorphus* grown for biofuel production; Letcher et al. 2016) and *Aquamyces chlorogonii* (Rhizophydiales), this is the first time this new species has been recognized.

We detected resting spores in old cultures of *Q. haematococci* growing on its host. The presence of a thick-walled resting stage suggests that this *Haematococcus* pathogen can be spread unintentionally via dust or equipment. In our preliminary test of pathogenicity, the death of inoculated algal cells, especially those in the transitional palmelloid stage (intermediate between motile green and red cyst stage) indicates that this parasite from the American Southwest needs to be considered by commercial-scale growers when developing pathogen monitoring and control plans.

ACKNOWLEDGMENTS

We thank Shaun Pennycook for his nomenclatural advice and Jerry Longcore for his editorial comments. We also thank two anonymous reviewers and an editor for their time and improvement of the manuscript.

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