An ultrastructural study of *Paraphysoderma sedebokerense* (Blastocladiomycota), an epibiotic parasite of microalgae

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Abstract

Successful algal cultivation for biofuel production is one path in the transition to a renewable energy economy. The green alga *Scenedesmus dimorphus* is a candidate for biofuel production, but is subject to parasitism and subsequent population crash when cultivated in open ponds. From an open pond cultivating *S. dimorphus* for biofuel production in New Mexico, USA, an amoeboid parasite was isolated, designated as isolate FD61, and its rDNA operon sequenced. A BLAST search for nuc 18S rDNA (18S) sequence similarity identified the parasite as *Paraphysoderma sedebokerense* (Blastocladiomycota), which was initially observed as a parasite of *Haematococcus pluvialis* from Israel. Here, we examine the ultrastructure of *P. sedebokerense* for the first time and compare it with that of a sister taxon, *Physoderma maydis*. The parasite has inoperculate thin-walled vegetative sporangia and thick-walled resting sporangia. Our observations indicate that amoeboid swarmers are produced in the vegetative phase, while either amoeboid swarmers or zoospores are the product of meiosis in resting sporangia. Meiosis is confirmed by the presence of synaptonemal complexes in resting sporangia nuclei. Notably, *P. sedebokerense* has a Golgi apparatus with stacked cisternae, a feature reported for *P. maydis*, but

which is absent in all other examined taxa in Blastocladiomycota. This report furthers our knowledge of the life cycle of *P. sedebokerense* and extends the host and geographic ranges of the parasite.

Keywords: amoeboid swarmer, biofuel, Golgi apparatus, life cycle, synaptonemal complex, zoospore

1 Introduction

For much of its existence, the order Blastocladales was in phylum Chytridiomycota (Fungi), primarily because the asexual reproductive unit of described species was a posteriorly uniflagellate zoospore (James *et al.* 2014). Molecular phylogenetic analyses using DNA sequences from the ribosomal RNA operon (18S+5.8S+28S) revealed a divergence from Chytridiomycota and resulted in raising the order to a new phylum, Blastocladiomycota (Hibbett *et al.* 2007, James *et al.* 2006). To complement the molecular delineation of Blastocladiomycota from Chytridiomycota, life cycle and ultrastructural differences between Blastocladiomycota and Chytridiomycota were recognized (James *et al.* 2006).

Hoffman *et al.* (2008) isolated and morphologically characterized via light microscopy a unique organism in Blastocladiomycota that was parasitic on the green alga *Haematococcus pluvialis*. In an 18S molecular phylogeny (Hoffman *et al.* 2008), the organism (referred to as “*Haematococcus* parasite”) placed with three isolates of *Physoderma*, a genus of obligate parasites of pteridophytes and angiosperms (Sparrow 1960). Subsequently designated as *Paraphysoderma sedebokerense* Boussiba, Zarka & TY James (James *et al.* 2011; in Gutman *et al.* 2009, 2011 as *P. sedebokerensis*), placement of that isolate in Blastocladiomycota was confirmed in an expanded molecular phylogeny (Porter *et al.* 2011). A prominent and unique feature of *P. sedebokerense* was a non-flagellate and pseudopodiate spore (= amoeboid swarmer), rather than a flagellated spore (= zoospore), which is characteristic of Blastocladiomycota. Few instances of non-flagellate motile spores have been reported in Chytridiomycota (Sparrow 1960), and prior to *P. sedebokerense*, none in Blastocladiomycota. In a recent publication, Strittmatter *et al.* (2015) observed flagellated spores and conjugation of amoeboid swarmers.

In this study we report on an isolate recovered from an open pond of the green alga *Scenedesmus dimorphus* grown for biofuel production in New Mexico, USA. Our isolate was an

epibiotic parasite of *S. dimorphus*, and its genetic constitution and thallus morphology are similar to that of *P. sedebokerense*, such that we consider our isolate and *P. sedebokerense* to be conspecific. The importance of this organism is reflected in the issuance of a patent to prevent and treat contamination of *H. pluvialis* by *P. sedebokerense* (<https://www.google.com/patents/WO2013127280A1?cl=en>).

The purposes of our study are to characterize our isolate of *P. sedebokerense* ultrastructurally and to compare its ultrastructural features with those that are characteristic of its sister taxa in *Physoderma*. Our results clarify events in the life cycle of this organism, localizing the site of meiosis and revealing the production of zoospores in the resting phase.

2 Materials and methods

2.1 Outdoor algae growth

Scenedesmus dimorphus (UTEX 1237, University of Texas Culture Collection of Algae, <http://web.biosci.texas.edu/utex/>) was grown in outdoor Oswald-style mini-pond raceways at 25 cm depth with a total operating volume of 350 L as described in Letcher *et al.* 2013.

2.2 Parasite isolation and laboratory culturing

Parasite isolation and laboratory culturing were as described in Letcher *et al.* 2013. When microscopic examination of samples collected from ponds of *S. dimorphus* revealed amoeboid-like organisms infecting algal cells, plaque plating was used to isolate the parasite, which was designated as isolate FD61. Organisms obtained from plaques were used to inoculate axenic uninfected algae, which were maintained as co-cultures. Axenic cultures of FD61 were also isolated and maintained on enriched medium similar to that used by Hoffman *et al.* (2008). To achieve this, a solid medium was prepared as per recipe for algae cultivation in McBride *et al.* 2014 (omitting the inorganic carbon and nitrogen) and supplemented with 0.25 g/L yeast extract, 0.25 g/L peptone, and 5 g/L glucose, plus 1.5 % w/v agar. Isolate FD61 has been deposited in the University of Alabama Chytrid Culture Collection (UACCC) and is available for non-commercial purposes upon request.

2.3 Parasite DNA extraction, purification, and amplification

Parasite DNA extraction, purification, and amplification of the ribosomal RNA operon was as in Letcher *et al.* 2013. Nuc 18S, 28S, and ITS rDNA sequence similarity and

identification was accomplished with a BLAST search of the NCBI nucleotide database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Candidate sequences were downloaded in FASTA format, automatically aligned in ClustalX (Thompson *et al.* 1997), and manually adjusted in BioEdit 7.1.9 (Hall 1999).

2.4 Phylogenetic analysis

The 18S sequences of 18 ingroup isolates (17 isolates representative of lineages in Blastocladiomycota [Porter *et al.* 2011] plus the 18S sequence of isolate FD61) and one outgroup isolate (*Nuclearia simplex*) were analyzed using Maximum Parsimony. Sequences were assembled and aligned as described in Vélez *et al.* (2011). Maximum parsimony trees were generated with PAUPRat (Sikes and Lewis 2001), and bootstrap values were generated as heuristic searches with 500 replicates, each with 10 random-addition replicates.

2.5 Parasite morphological identification via light microscopy

Parasite morphological identification via light microscopy was as in Letcher *et al.* (2013). By day 2 following inoculation, motile, multiple-pseudopodiate amoeboid organisms were observed and stages of the life cycle were recorded using phase contrast on an Olympus BX51 microscope equipped with an Olympus DP72 camera.

2.6 Parasite ultrastructure via transmission electron microscopy

Electron microscope preparation and observation followed protocols described in Letcher *et al.* (2013). Aliquots of a prepared co-culture of isolate FD61 on *S. dimorphus* were taken at 1, 2, 3, 4, and 5 d following inoculation. Aliquots were chemically fixed for transmission electron microscopy (TEM) in 2.5% glutaraldehyde in 0.1 M sym-collidine buffer, secondary fixed in 1% osmium tetroxide in 0.1 M buffer, and infused in molten agar. Small blocks of fixed material were stained with saturated aqueous uranyl acetate, dehydrated in a graded acetone series, infiltrated with EPON resin in a graded series, and polymerized. Embedded material was sectioned at 100 nm, collected on 300 mesh nickel grids, oxidized with 1 % periodic acid, and post-stained with (1) saturated uranyl acetate in 70% ethanol and (2) lead citrate (Reynolds 1963). Sections were observed at 60 kV on a Hitachi 7650 transmission electron microscope.

3 Results

3.1 Parasite isolation and laboratory culturing

Mass cultures of the microalga *S. dimorphus* grown in outdoor ponds for biofuel production occasionally crash (McBride et al. 2014), resulting in a decimated population. Microscopic examination of crashed populations revealed the presence of a motile, amoeboid, organism and epibiotic sporangia on senesced algal cells. This organism was initially isolated via plaque plating and confirmed to be pathogenic by infecting axenic *S. dimorphus* cultures.

3.2 Parasite DNA extraction, purification, amplification, and molecular identification

The sequenced rDNA operon was 3943 bases in length and consisted of 18S (1771 bases), ITS1 (209 bases), 5.8S (156 bases), ITS2 (243 bases), and 28S (1564 bases), and was obtained from a pure culture. A BLAST query indicated that the 18S sequence of isolate FD61 (GenBank KJ563218) was 100% identical to that of isolate TJ-2007a *Paraphysoderma sedebokerense* (GenBank EF65163) and PS1 *P. sedebokerense* (GenBank KT270356); on that basis we consider the three isolates as conspecific.

3.3 Phylogenetic analysis

The 18S dataset had 1738 characters, with 399 parsimony informative sites. The alignment and tree have been deposited in TreeBASE (<http://purl.org/phylo/treebase/phyloids/study/TB2:S18080>). Of 1005 trees derived from PAUPRat, all were equally parsimonious (length [L] = 1034 steps, CI = 0.649, RI = 0.774) and were used to construct a majority rule consensus tree (>70% branch support). The MP phylogeny (Fig 1) is presented with bootstrap values indicated above branches. Isolate FD61 grouped with isolates TJ-2007a and PS1 *Paraphysoderma sedebokerense* with 100% support. Those three isolates were sister to a lineage composed of *Physoderma* and *Urophlyctis* isolates. Taxonomically, *Physoderma* and *Urophlyctis* (merged with *Physoderma* by Karling [1950]) are members of Physodermataceae Sparrow (1952), which supersedes Urophlyctidaceae Hadar (as '*Urophlyctaceae*' [1982]), while *Paraphysoderma* has been elevated to the family Paraphysodermataceae Doweld (2014).

3.4 Parasite morphological identification via light microscopy

Isolate FD61 was maintained as an axenic culture on solid media. Morphology via light microscopy of colonies propagated in this manner matched that reported in Figure 2 of Hoffman et al. 2008 (not shown). Within 24–48 hours following inoculation of axenic algal cultures with plaques containing the parasite, amoeboid swimmers were observed (Fig 2 A–C). The swimmers

were 1.5–2 μm in length, with multiple filose pseudopodia, one of which resembled the pseudocilium of *Amoeboaphelidium* (Karpov *et al.* 2013, Letcher *et al.* 2015). Swarmers encysted on host cells and produced a spherical, thin-walled sporangium with a rhizoid inside the host (Fig 2D, E) and thick-walled resting sporangia (Fig 2F).

3.5 Parasite ultrastructure via transmission electron microscopy

Results of ultrastructure analysis of FD61 are presented in Figs 3–8. What follows is a chronological description of parasite development which occurs generally, but may overlap, on sequential days.

At Days 1 and 2 following inoculation of axenic algal cultures with plaques containing the parasite, abundant amoeboid swarmers (1.7–2.3 μm diam) are apparent (Figs 3 A–H, 4). Within a swarmer is a single nucleus bordered with one or more layers of endoplasmic reticulum (Fig 3A), a large mitochondrion with flat cristae adjacent to the nucleus (Fig 3A, B, D–H), multiple lipid globules 0.25–0.3 μm diam (Fig 3A–D, F, G), microbodies adjacent to lipid globules (Fig 3G), ribosomes dispersed through the cytoplasm, and a pair of centrioles (Fig 6E, F). Pseudopodia are ~50–150 nm diam (Fig 3B–E, H). The swarmer is schematically depicted (Fig 4).

At Day 3, swarmers “dock” with the host, appearing in close proximity to the host cell surface, often with the swarmer surface adjacent to the host contoured with the host cell surface (Fig 3E–H). As a swarmer begins to encyst, a slight depression may occur in the wall of the host at the point of encystment, and the sporangial wall begins to form on the surface of the parasite where it is in contact the host (Fig 5A, B). The sporangial wall envelops the parasite plasma membrane, the developing sporangium assumes a spherical shape (Figs 5C, E–G, 6A, B, D), and a tubular rhizoid penetrates the host cell wall at the point of contact between the parasite and host and develops in the host cytoplasm (Fig 5B–F). The sporangium wall is continuous around the rhizoid (Fig 5C, D).

Two types of spherical sporangia develop simultaneously: (1) thin-walled vegetative sporangia 2.8–3.5 μm diam (Figs 5, 6) and (2) thick-walled resting sporangia 3.2–5.2 μm diam (Figs 7, 8). Multiple infections of a single host cell by both types of sporangia are frequent.

Regarding thin-walled vegetative sporangia (Figs 5, 6), the rhizoid is generally extensive, ramifying throughout the host cell, and may be lobed or branched (Fig 5B–F). Sporangial

development is rapid, in which the cyst nucleus, with a pair of centrioles at opposite nuclear poles (Fig 5F), undergoes mitosis to produce multiple smaller nuclei (Fig 5G). Golgi apparatus with stacked cisternae and vesicles occur in close proximity to nuclei (Fig 5H, I). At this point in development the contents of the host cell are significantly degraded, with primarily lipid globules remaining (Fig 5E–G). As the sporangium matures and becomes multinucleate, usually a single (occasionally two) slightly raised discharge papilla 0.5–1.0 μm wide (Figs 5G, J, 6E) appears apically or sub-apically on an inner wall, which is functionally an endosporangium (e.g., Sparrow 1964b, Figs. 1–5) (Fig 6A) and swarmer cleavage occurs, indicated by the presence of cleavage furrows (Fig 6A, C, E). When the sporangium is fully cleaved, swarmers with pseudopodia are evident (Fig 6G). In the cleaved swarmer, paired centrioles are adjacent to the single nucleus (Fig 6E, F). Fully developed swarmers (Fig 6G) are released through the discharge papillae (Fig 6G). Thin-walled sporangia remain attached to the host.

Thick-walled resting sporangia (Figs 7, 8) develop differently than thin-walled vegetative sporangia. The rhizoid of this type of sporangium is generally less developed than that of thin-walled vegetative sporangia (Figs 7A–C, 8E, F), most often being a swollen bulb at the distal end of the rhizoid. As with vegetative sporangia, inside the thick-walled resting sporangium an endosporangium occurs (Fig 7B–D). Within the thick-walled resting sporangium the nucleus (Fig 7B, D, E) undergoes meiosis, as evidenced by the occurrence of synaptonemal complexes (Fig 7E–G) characterized by a central element and two lateral elements (e.g., Lentz 1971, Page and Hawley 2004), producing additional nuclei (Fig 8A). Associated with each nucleus is a Golgi complex (Fig 8A, B) consisting of stacked cisternae and vesicles. Prior to cleavage, the apical portion of the resting sporangium wall dehisces (Figs 7D, 8A, C, E), allowing the endosporangium to protrude through the opening (Figs 7D, 8A, C). One or two discharge papillae occur in the endosporangium wall (Fig 8A), and cleavage produces either zoospores (Fig 8C, D), as evidenced by flagellar sections in the cleavage furrows, or amoeboid swarmers (Fig 8E), as evidenced by the absence of flagellar sections in the cleavage furrows. Indicative that mature resting sporangia may remain attached to the host or may detach from the host, empty thick-walled sporangia (Fig. 8F) are found both attached and detached from the host. Spores (whether swarmers or zoospores) are released through the discharge papilla.

At Days 4 and 5 most algal cells are degraded and swarmers are prevalent, often attaching to resting sporangia (Fig 7C). A presumptive life cycle is schematically illustrated (Fig 9).

4 Discussion

4.1 Life cycle

Blastocladialean fungi have evolved a variety of life cycle strategies (James et al. 2014). Our understanding of the complexity of the life cycle of *Paraphysoderma sedebokerense* is increasing (Strittmatter et al. 2015), but details remain to be resolved. In first characterizing *P. sedebokerense*, Hoffman et al. (2008) illustrated thin-walled and thick-walled sporangia and reported only amoeboid swarmers. Significantly, Strittmatter et al. (2015) observed posteriorly uniflagellate zoospores as well as amoeboid swarmers and illustrated fusion between two amoebae; however, neither the origin of zoospores, the origin of conjugating amoeboid swarmers, nor the site of meiosis were resolved. As Hoffman *et al.* (2008) emphasized, electron microscopic studies of *P. sedebokerense* are needed for greater characterization of the life cycle. Using transmission electron microscopy, we provide new insights in the life cycle of *P. sedebokerense* as envisioned in Hoffman *et al.* (2008) and Strittmatter et al. (2015) and demonstrate the site of meiosis in the resting sporangium as well as the ability of the resting sporangium to produce zoospores as well as amoeboid swarmers. With our demonstration of meiosis in resting sporangia, recombination must occur at some point in the life cycle. The actual site of recombination is uncertain, but there are two possibilities. (1) Hoffman et al. (2008) and Strittmatter et al. (2015) hypothesize that cell fusion occurs between haploid amoeboid swarmers released from vegetative sporangia, with the resulting diploid zygote developing into a resistant sporangium (Fig. 9). This hypothesis predicts cells released from vegetative sporangia and resting sporangia are both haploid. (2) An alternate hypothesis is that haploid spores released from resistant sporangia recombine and the resulting diploid zygote develops into a diploid vegetative sporangium. This second hypothesis predicts cells released from resting sporangia are haploid and spores released from vegetative sporangia are diploid. Comparative measurements of DNA content in spores from resistant sporangia and vegetative sporangia could provide insights and support for one of these two hypotheses. For example, using this approach Horgan et al.

(1985) demonstrated that *Blastocladiella emersonii*, with a *Brachyallomyces* type life cycle (Emerson 1941, Karling 1973), released spores from vegetative sporangia with twice the DNA content of spores discharged from resistant sporangia. Thus, additional studies are needed to resolve *P. sedebokerense* life history.

Resting sporangium development occurs more quickly on the host than on complex-medium agar (Hoffman *et al.* 2008), suggesting that *P. sedebokerense* is a “reluctant” saprotroph. However, the resting sporangium appears to retain its infectivity after three to four weeks on agar (Hoffman *et al.* 2008). Thus, disarticulation of resting sporangia from a host indicates a resistant stage that can be dispersed by water and possibly wind, with survival over an extended period of time, until a suitable host is encountered. Additionally, production of a flagellated cell enables more rapid dispersal, especially if a host is not proximal.

4.2 Morphology and ultrastructure

Members of Blastocladiomycota have been distinguishable from Chytridiomycota on the basis of life cycles and morphologies of the reproductive spore (James *et al.* 2014). The flagellated zoospore characteristic of Blastocladiomycota has an anteriorly located membrane-bound ribosomal aggregation adjacent to the nucleus, known as a “ribosomal cap”, so prominent as to be observable via light microscopy. Many members of Chytridiomycota have aggregated ribosomes in the zoospore, but the aggregation is generally centrally located in the zoospore, and the nucleus may be variously located, being inside, inserted into, or outside the ribosomal aggregation (Letcher and Powell 2014). We observed paired centrioles associated with the nucleus in developing swarmers, as found in other blastoclads with flagellated spores (James *et al.* 2014). Less can be said regarding zoospore morphology of *P. sedebokerense*, because although we have observed cleavage of flagellated spores in the resting stage, we have not observed motile flagellated spores.

Members of Blastocladiomycota also exhibit some ultrastructural features more common to higher filamentous fungi than to Chytridiomycota. In Blastocladiomycota, nuclear poles are closed during mitosis (Ichida and Fuller 1968, Olson 1984); in Chytridiomycota, nuclear poles are open (fenestrated) (Powell 1980). A distinct ultrastructural feature in Blastocladiomycota is the presence of Golgi equivalents (Bracker 1967, Feeney and Treimer 1979, Sewall *et al.* 1989) rather than a Golgi apparatus with stacked, smooth-membraned cisternae found in

Chytridiomycota. However, Lange and Olson (1980) reported a Golgi apparatus with stacked cisternae in *Physoderma maydis*, a member of a lineage in Blastocladiomycota (James *et al.* 2006, Porter *et al.* 2011). Other than here with *Paraphysoderma* and with *Physoderma*, all other members of Blastocladiomycota that have been examined ultrastructurally have Golgi equivalents (Bracker 1967). Additionally, Lange and Olson (1980) observed synaptonemal complexes (Westergaard and von Wettstein 1966) in nuclei of resting sporangia of *P. maydis*, as did Olson and Reichle (1978a, 1978b) in *Blastocladiella emersonii* and *Catenaria anguillulae*. We have made the same observation here in resting sporangium nuclei of *Paraphysoderma*. *Paraphysoderma* is sister of *Physoderma* (Hoffman *et al.* 2008, Porter *et al.* 2011). Thus, ultrastructural comparisons between the two taxa confirm their molecular phylogenetic relationship.

Our TEM analysis has revealed morphological and ultrastructural features that differentiate *Paraphysoderma* from *Physoderma*. Morphologically, the resting sporangium of *Paraphysoderma* is inoperculate, while the resting sporangium of species of *Physoderma* (except *P. menyanthi*; see Sparrow 1947) has a broad operculum that is dehiscence in a circumscissile fashion as the endosporangium protrudes from the body of the resting sporangium (Couch 1953, Sparrow 1964a, b, 1974, 1975, 1977, Sparrow and Griffin 1961, Sparrow *et al.* 1961, Sparrow and Johns 1970). Ultrastructurally, *Physoderma* has a posteriorly uniflagellate zoospore with ribosomes aggregated as a nuclear cap, typical of Blastocladiomycota (James *et al.* 2014). *Paraphysoderma* has a non-flagellated, amoeboid spore in which ribosomes are dispersed throughout the cytoplasm, in addition to the flagellated spore we report here.

4.3 Convergent evolution

Studies have revealed non-flagellated spores among diverse lineages typically characterized by flagellated spores. These include Chytridiomycetes (*Amoebochytrium rhizidioides* [Zopf 1884], *Sporophlyctis rostrata* [Serbinow 1907], and *Sporophlyctidium africanum* [Sparrow 1933, 1938]); Monoblepharidomycetes (*Hyaloraphidium curvatum* [Ustinova *et al.* 2000]); and Aphelida (*Amoeboaphelidium protococcarum* [Gromov and Mamkaeva 1968, 1970], [Letcher *et al.* 2013]; *Amoeboaphelidium occidentale* [Letcher *et al.* 2015]). The evolution of similar traits in independent lineages may be a result of having to adapt to similar environments or ecological niches. All the above mentioned organisms are from

aquatic environments, all are associated with algae, yet as there are innumerable organisms in aquatic environments and associated with algae that are flagellated, other environmental factors or host conditions may be contributing to this convergence. Interestingly, the chytrid *Irineochytrium*, a saprophyte of pollen and keratin found predominantly in soil, has been shown to produce both aplanospores and zoospores (Letcher *et al.* 2015).

4.4 Importance to biofuel industry to identify parasites and understand parasite life cycles

To be competitive with fossil fuels, production of biofuels from a renewable resource such as microalgae must be accomplished on a large scale, and with reliability. Open pond cultivation is inherently at risk of contamination from a variety of sources, and infection of or competition with the primary producer can quickly slow or halt production. Thus, identifying parasites is primary in strategies attempting to offset this situation, and understanding their life cycles may facilitate mitigation. *Scenedesmus dimorphus* is a candidate for biofuel production, but is subject to parasitic infection by a range of aphelids (Aphelida), principally strains of *Aphelidium* protococcarum, *Amoeboaphelidium* protococcarum, and *Amoeboaphelidium occidentale* (Gromov and Mamkaeva 1969a, b, Letcher *et al.* 2013, 2015). This is the first report of infection of *S. dimorphus* by a member of Blastocladiomycota.

As an algal parasite, *P. sedebokerense* is here reported on a novel host (*S. dimorphus*), the first being the green alga *Haematococcus pluvialis*, thus extending the range of organisms infected. Other algal species have been shown to be susceptible to infection by *P. sedebokerense* including alternative species of *Scenedesmus*, although complete culture crashes were not observed (Gutman *et al.* 2009). Without being able to compare the same isolates under identical conditions, it is difficult to conclude if these observations result from host specificity or differences in experimental conditions. *Haematococcus pluvialis* is cultivated commercially for a variety of products, including biofuels (Brennan and Owende 2009, Razon and Tan 2011). The parasite was first reported from Israel (Hoffman *et al.* 2008), later in Portugal (Strittmatter *et al.* 2015), and with our isolate of *P. sedebokerense*, now is known to occur in the US. Thus, distribution of the parasite may be world-wide and a potential threat to microalgae culture and biofuel production in numerous geographical locations.

A factor revealed in our study of the life cycle of *P. sedebokerense* may impact dispersal of the parasite. Amoeboid swimmers often attach to resting sporangia, and resting sporangia often

disarticulate from the host. Floating free in the medium, these resting sporangia may transport the parasite to uninfected hosts.

4.4 Conclusions

Paraphysoderma is an interesting organism to research because of its potential as a worldwide biofuel pest, and because investigation of its life cycle stages reveal new insights about the biology of the organism. Here we demonstrated meiosis in the resting stage, through the observation of synaptonemal complexes in the resting sporangium nucleus. We also revealed the presence of flagellated spores in the resting stage, from observations of flagellar sections following spore cleavage. Further research may reveal the fate of these flagellated spores, as well as their ultrastructure.

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Figure legends

Abbreviations used in Figures 2–8: Ce, centriole; CE, central element of synaptonemal complex; CF, cleavage furrow; DP, discharge papilla; ER, endoplasmic reticulum; EsW, endosporangium wall; H, host; HCW, host cell wall; L, lipid globule; LE, lateral element of synaptonemal complex; M, mitochondrion; Mb, microbody; N, nucleus; NE, nuclear envelope; PM, plasma membrane; Ps, pseudopodium; R, ribosomes; Rh, rhizoid; RS, resting sporangium; RSW, resting sporangium wall; SC, stacked cisternae; Sw, amoeboid swarmer; V, vesicles; VS, vegetative sporangium; VSW, vegetative sporangium wall.

Figure 1. Maximum parsimony phylogeny derived from SSU rDNA sequences of 18 isolates in Blastocladiomycota (ingroup) and *Nuclearia simplex* (outgroup). Bootstrap values indicated at nodes. L= 1034 steps, CI = 0.645, RI = 0.774.

Figure 2. Light micrographs of *Paraphysoderma sedebokerense*. A, B. Sequential images of an amoeboid swarmer (arrows) approaching algal cells. C. Magnification of amoeba in B, showing three pseudopodia and possible pseudocilium (arrow). D. Thin-walled vegetative sporangium. E. Thin-walled vegetative sporangium with rhizoid inside host cell. F. Thick-walled resting sporangium on host cell. Scale bars in A, B, F = 2.5 μm ; in C = 1.25 μm ; in D, E = 5 μm .

Figure 3. Ultrastructure of *Paraphysoderma sedebokerense*, motile stage. A–D. Amoeboid swimmers, illustrating organelles and aplanospore features. E–H. Amoeboid swimmers “docking” with algal host. Scale bars = 0.5 μm .

Figure 4. Schematic of amoeboid swimmer. Swimmers have multiple filose pseudopodia (arrows) and contain a nucleus backed by layers of endoplasmic reticulum, centrioles, one or more mitochondria, multiple lipid globules, microbodies, and dispersed ribosomes.

Figure 5. Ultrastructure of *Paraphysoderma sedebokerense*; thin-walled vegetative sporangium development. A. Attachment to host, and early encystment of a swimmer, with formation of sporangium cell wall (arrow) initiated. B. Sporangium cell wall is complete (arrowheads), and parasite has penetrated host cell wall with tubular rhizoid. C, D. Sporangium cell wall is continuous around rhizoid. E. Multiple infections of host by parasite. F. Encysted swimmer with a single nucleus with a pair of centrioles located at opposite nuclear poles (enlarged inset images). G. Sporangium with multiple nuclei, and development of a lateral discharge papilla. H. A nucleus with an adjacent Golgi apparatus having stack cisternae. I. Enlargement of Golgi apparatus. J. Discharge papilla. Scale bars in E–H = 1.0 μm ; in A–C, J = 0.5 μm ; in D, I = 0.25 μm .

Figure 6. Ultrastructure of *Paraphysoderma sedebokerense*; thin-walled vegetative sporangium development. A. Cleavage furrows indicate delineation of individual swimmers. B. Enlargement of the Golgi apparatus. C. Three cleaved swimmers. D. Enlargement of boxed area in C, illustrating mitochondrion and microbodies adjacent to lipid globules. E. Numerous cleaved swimmers; lower center swimmer contains a pair of centrioles (arrow). F. Enlargement of paired centrioles (arrow). G. Release of a mature swimmer through discharge papilla; arrow indicates a section through a filose pseudopodium. Scale bars in A, C, E, G = 0.25 μm ; in B, D, F = 0.5 μm .

Figure 7. Ultrastructure of *Paraphysoderma sedebokerense*; thick-walled resting sporangium development. A. Early development. B. Resting sporangium with a single nucleus; delineation of endosporangium wall evident. C. Resting sporangium with an attached swimmer. D. Resting

595 sporangium has dehisced, and endosporangium, containing a nucleus, protrudes through point of
 596 wall dehiscence. E. Resting sporangium nucleus containing multiple synaptonemal complexes;
 597 single arrows indicate longitudinal sections, double arrows indicate transverse sections. F.
 598 Longitudinal section through the central element of a synaptonemal complex. G. Transverse
 599 section through the two lateral elements and one central element of a synaptonemal complex.
 600 Scale bars in A–C = 1.0 μm ; in D, E = 0.5 μm ; in F, G = 0.2 μm .

601
 602 Figure 8. Ultrastructure of *Paraphysoderma sedebokerense*; thick-walled resting sporangium
 603 development. A. Endosporangium with two nuclei visible; lower nucleus with a Golgi apparatus
 604 with stacked cisternae adjacent to nucleus (arrow); a discharge papilla has formed on the wall of
 605 the endosporangium. B. Enlargement of Golgi apparatus. C. Fully cleaved zoospores (indicated
 606 with asterisks) within sporangium; flagellar cross-sections indicated with arrows. D.
 607 Enlargement of two flagellar cross-sections (arrows). E. Fully cleaved swarmers (indicated with
 608 asterisks) within sporangium; no flagellar sections apparent in cleavage furrows. F. Empty
 609 resting sporangium. Scale bars in A, C, E, F = 0.5 μm ; in B, D = 0.25 μm .

610
 611 Figure 9. A putative life cycle of *Paraphysoderma sedebokerense* based on the hypothesis of
 612 Hoffman et al. (2008) and Strittmatter et al. (2015). A. A haploid amoeboid swarmer contacts
 613 algal host. B. Swarmer encysts on host surface, producing a thin-walled vegetative sporangium
 614 and a rhizoid, which invades the host. C. Mitosis produces multiple nuclei in developing
 615 sporangium. D. Cytoplasmic cleavage into amoeboid swarmers. E. Amoeboid swarmers
 616 discharged from thin-walled vegetative sporangium and continue the asexual life cycle (A). F.
 617 Two amoeboid swarmers approach each other. G. Conjugation between amoeboid swarmers
 618 results in a diploid zygote. H. Zygote encysts on host surface. I. Zygote develops into a thick-
 619 walled resting sporangium, which invades the host with a rhizoid; meiosis occurs in the resting
 620 sporangium. J. Haploid nuclei as a product of meiosis. K. Cytoplasmic cleavage into either
 621 flagellated zoospores or amoeboid swarmers. L. Flagellated zoospores discharged from resting
 622 sporangium. M. Amoeboid swarmers discharged from resting sporangium.

Figure 1

Figure 2

Figure 3

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Figure 5

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Figure 8

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645 Figure 9