

Phylogenetic analysis identifies the invertebrate pathogen *Helicosporidium* sp. as a green alga (Chlorophyta)

Aurélien Tartar,¹ Drion G. Boucias,¹ Byron J. Adams¹ and James J. Becnel²

¹ Department of Entomology and Nematology, University of Florida, Gainesville, FL 32611, USA

² Center for Medical, Agricultural and Veterinary Entomology, USDA, ARS, Gainesville, FL 32604, USA

Author for correspondence: Aurélien Tartar. Tel: +1 352 392 1901 ext. 202. Fax: +1 352 392 0190. e-mail: aurelien@ufl.edu

Historically, the invertebrate pathogens of the genus *Helicosporidium* were considered to be either protozoa or fungi, but the taxonomic position of this group has not been considered since 1931. Recently, a *Helicosporidium* sp., isolated from the blackfly *Simulium jonesi* Stone & Snoddy (Diptera: Simuliidae), has been amplified in the heterologous host *Helicoverpa zea*. Genomic DNA has been extracted from gradient-purified cysts. The 18S, 28S and 5.8S regions of the *Helicosporidium* rDNA, as well as partial sequences of the actin and β -tubulin genes, were amplified by PCR and sequenced. Comparative analysis of these nucleotide sequences was performed using neighbour-joining and maximum-parsimony methods. All inferred phylogenetic trees placed *Helicosporidium* sp. among the green algae (Chlorophyta), and this association was supported by bootstrap and parsimony jackknife values. Phylogenetic analysis focused on the green algae depicted *Helicosporidium* sp. as a close relative of *Prototheca wickerhamii* and *Prototheca zopfii* (Chlorophyta, Trebouxiophyceae), two achlorophyllous, pathogenic green algae. On the basis of this phylogenetic analysis, *Helicosporidium* sp. is clearly neither a protist nor a fungus, but appears to be the first described algal invertebrate pathogen. These conclusions lead us to propose the transfer of the genus *Helicosporidium* to Chlorophyta, Trebouxiophyceae.

Keywords: *Helicosporidium*, phylogeny, invertebrate pathogen, entomopathogen, green algae

INTRODUCTION

The Helicosporidia are a unique group of pathogens found in diverse invertebrate hosts. Members of this group are characterized by the formation of a cyst stage that contains a core of three ovoid cells and a single filamentous cell (Fig. 1) (Kellen & Lindegren, 1974; Lindegren & Hoffman, 1976). The group is very poorly known and its taxonomic position has remained *incertae sedis*. This pathogen, initially detected in a

ceratopogonid (Diptera), was described and named *Helicosporidium parasiticum* by Keilin (1921) and was placed in a separate order, Helicosporidia, within Cnidospora (Protozoa) by Kudo (1931). Since then, additional helicosporidians have been detected in mites, cladocerans, trematodes, collembolans, scarabs, mosquitoes, simuliids and pond-water samples (Kellen & Lindegren, 1973; Fukuda *et al.*, 1976; Sayre & Clark, 1978; Purrini, 1984; Avery & Undeen, 1987). Weiser (1964, 1970) examined the type material and a new isolate of Helicosporidia from a hepialid larva and proposed that this organism should be transferred to the Ascomycetes, because of some analogies in pathways of infection. Additionally, Kellen & Lindegren (1974) isolated a *Helicosporidium* from infected larvae and adults of *Carpophilus mutilatus* (Coleoptera: Nitidulidae) and described its life cycle in a lepidopteran host, the navel orangeworm, *Paramyelois*

Abbreviations: LSU, large subunit; NJ, neighbour-joining; SSU, small subunit.

The GenBank accession numbers for the sequences of the 18S, 28S and ITS1–5.8S–ITS2 regions of the rDNA and the actin and β -tubulin genes of *Helicosporidium* sp. are respectively AF317893, AF317894, AF317895, AF317896 and AF317897.

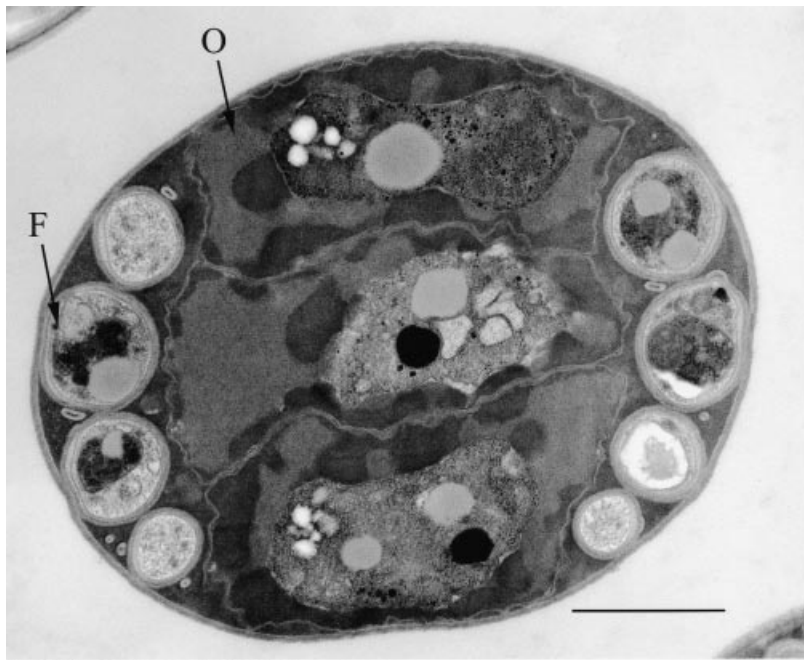


Fig. 1. Transmission electron micrograph of a cyst of the *Helicosporidium* sp. from *Simulium jonesi*. The mature cyst is composed of three central ovoid cells (O) and the peripherally located filamentous cell (F) contained within a multilayered cyst wall. Bar, 1 μ m.

transitella. They agreed that this organism is not a protozoan but remained uncertain about its taxonomic position. Later, Lindegren & Hoffman (1976) proposed that the developmental stages of this organism placed it closer to the Protozoa than to the Fungi. Because of this uncertain taxonomic status, the Helicosporidia have not appeared in classification systems of either the Protozoa or the Fungi (Cavalier-Smith, 1998; Tehler *et al.*, 2000).

Recently, a *Helicosporidium* sp. isolated from the blackfly *Simulium jonesi* Stone & Snoddy (Diptera: Simuliidae) has been shown to replicate in a heterologous host, *Helicoverpa zea* (Lepidoptera: Noctuidae), which provided a means to produce quantities sufficient for density-gradient extraction of the infectious cyst stage (Boucias *et al.*, 2001). In order to evaluate the taxonomic position of this *Helicosporidium* sp. within the eukaryotic tree, we extracted genomic DNA from the cyst preparation and amplified several targeted genes (5.8S, 28S and 18S rRNA regions and partial sequences of the actin and β -tubulin genes) by PCR. These genes were selected because they have been used extensively to infer deep eukaryotic phylogenies (Philippe & Adoutte, 1998). Amplified genes were sequenced and information from nucleotide sequences was subjected to comparative analysis.

METHODS

Cyst preparation and DNA extraction. *Helicosporidium* sp. was isolated originally from the blackfly *Simulium jonesi* Stone & Snoddy (Diptera: Simuliidae) and produced in *Helicoverpa zea*. Approximately 4×10^7 cysts suspended in 0.15 M NaCl were applied to a linear gradient of 1.00–

1.3003 g Ludox HS40 ml⁻¹ (DuPont). Helicosporidial cysts that banded at an estimated density of 1.17 g ml⁻¹ were collected, diluted in 10 vols deionized water and washed free of residual Ludox by repeated low-speed centrifugation steps. The pellet, resuspended in 50 μ l water, was extracted with the Masterpure Yeast DNA extraction kit (Epicentre Technologies), following the manufacturer's protocol. Examination of the cells before and after lysis treatment revealed the presence of numerous, highly refractile cysts before treatment and, after incubation in the lysis buffer at 50 °C, cysts appeared to dehisce, releasing the filamentous cells. However, no massive disruption of the ovoid cells or filamentous cells was observed in these preparations. Visible pellets were observed after RNase treatment, phenol/chloroform extraction and ethanol precipitation. The final pellet, suspended in molecular-biology-grade water, was frozen at -20 °C. The DNA preparations, electrophoresed on a 0.8% agarose gel and stained with ethidium bromide, produced a single, discrete ~20 kb band diagnostic for genomic DNA.

Mature cysts were also processed for electron microscopy according to previously published protocols (Boucias *et al.*, 2001).

Amplification, cloning and sequencing of extracted DNA.

The ITS1–5.8S–ITS2, 28S and 18S ribosomal regions of the helicosporidial DNA were amplified with a mixture of *Taq* DNA polymerase (Promega) and *Pfu* polymerase (Stratagene), using the primers TW81 and AB28 for the ITS–5.8S (Curran *et al.*, 1994) and NL-1 and NL-4 primers for the 28S (Kurtzman & Robnett, 1997). Two primer sets, designed from consensus regions of selected fungal, algal and protozoan sequences downloaded from GenBank, were used to amplify the 18S region. Several series of primers, also designed from consensus regions of selected fungal, algal and protozoan genes, were used to amplify partial sequences of the actin and β -tubulin genes by PCR. DNA was excised from agarose gels, extracted with the QiaxII gel extraction

kit (Qiagen) and sent to the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida for sequencing.

DNA sequence analysis. The helicosporidial 18S rDNA region sequence was aligned with 138 other sequences from representative eukaryotic taxa obtained from the Ribosomal Database Project (RDP; Maidak *et al.*, 2000). Downloaded sequences were pre-aligned based on the secondary structure of the rDNA. An additional 18S sequence from the pathogenic alga *Prototheca wickerhamii* was downloaded from GenBank (accession no. X56099) and incorporated in the small-subunit (SSU) RNA dataset. Additionally, 28S sequences were downloaded from GenBank and aligned with the helicosporidial 28S sequence using CLUSTAL X (Thompson *et al.*, 1997). Eventually, the SSU- and large subunit (LSU)-rDNA datasets were combined to infer one single ribosomal phylogeny. Both actin and β -tubulin sequences were aligned with homologous sequences downloaded from GenBank. Alignments were obtained using CLUSTAL X software with default parameters. All datasets were checked by eye before further analyses and are available upon request. They can also be obtained from TreeBase (Morell, 1996; <http://www.herbaria.harvard.edu/treebase>) with the study accession number S604. The 18S algal alignment was kindly provided by V. A. R. Huss (University of Erlangen, Germany).

Aligned datasets were subjected to a partition-homogeneity test using the program PAUP*, version 4.0b4a (Swofford, 2000), in order to assess the extent of character incongruence between the datasets (Farris *et al.*, 1994). Phylogenies were then reconstructed using neighbour-joining (NJ) as implemented in the PAUP* program version 4.0b4a. NJ analyses were based on the Paralinear/LogDet model of nucleotide substitution (Lockhart *et al.*, 1994). This method allows for non-stationary changes in base composition and has been shown to reduce support for spurious resolutions, such as long-branch attraction. Monophyly of groups was assessed with the bootstrap method (100 replicates). Additionally, maximum-parsimony analyses, including jackknifing (100000 replicates; Farris *et al.*, 1996), were also performed using PAUP*. We chose the latter, conservative approach for its ability to search rapidly a large amount of tree space and to estimate support for an unambiguously resolved group (Lipscomb *et al.*, 1998).

RESULTS

Five PCR-amplified gene fragments of the *Helicosporidium* sp. were sequenced. These sequences corresponded to the 18S rDNA, 28S rDNA, the ITS1–5.8S–ITS2 rDNA region and the actin and β -tubulin genes and were respectively 1558, 661, 844, 880 and 879 bases in length. All sequences, when examined by BLAST analysis (Altschul *et al.*, 1997), produced matches with extremely low expect (E) values. Sequences from two algal species, *Chlamydomonas reinhardtii* and *Volvox carteri*, were highly similar to all five sequences. Additionally, sequences from other algal genera, such as *Trebouxia*, *Scenedesmus* or *Chlorella*, were found to match recurrently with the helicosporidial sequences.

A preliminary partition-homogeneity test showed that the 18S, 28S and 5.8S sequences were highly con-

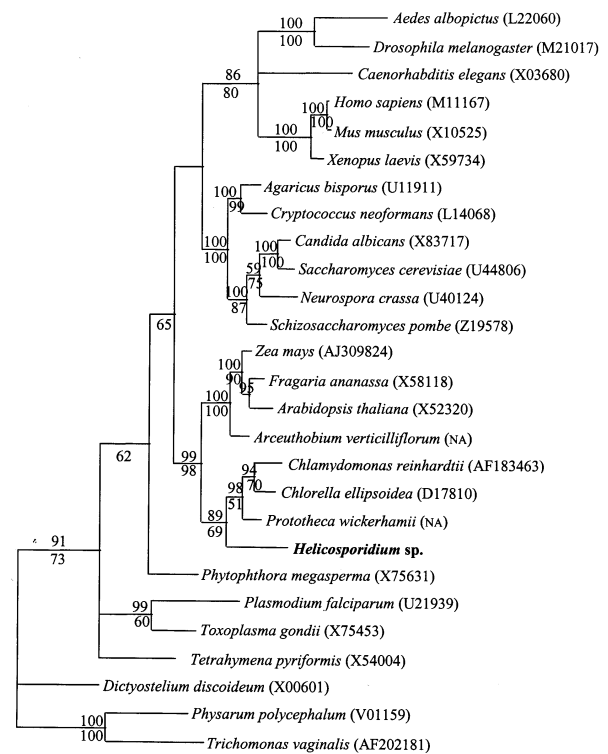


Fig. 2. Phylogram inferred from combined SSU-rDNA and LSU-rDNA nucleotide sequence alignment, showing that *Helicosporidium* sp. is grouped with the green algae. Numbers above nodes represent the results of bootstrap analyses (100 replicates) using the NJ method. Numbers below nodes are results of parsimony jackknife analyses (100000 replicates). Only values greater than 50% are shown. SSU-rDNA sequences were downloaded from the RDP Web site. LSU-rDNA sequences were downloaded from GenBank. Accession numbers for these sequences are indicated after each species name. NA, LSU rDNA sequence not available in GenBank.

cordant (data not shown). A first phylogenetic tree was inferred from the 18S sequence aligned with the 140 sequences downloaded from the RDP Web site. This tree placed *Helicosporidium* sp. as a member of the green algae, and this association was supported by significant bootstrap values (data not shown). The tree presented in Fig. 2 was inferred from a combined SSU + LSU rDNA dataset and is concordant with the preliminary result. This tree was rooted by using *Dictyostelium discoideum* as an outgroup (Fig. 2). Although the taxonomic position of *D. discoideum* is subject to debate (Baldauf *et al.*, 2000), it appears basal in conservative rDNA reconstruction (Lipscomb *et al.*, 1998). Our tree is fairly consistent with other previous molecular phylogenetic studies of eukaryotes (Drouin *et al.*, 1995; Lipscomb *et al.*, 1998; Baldauf *et al.*, 2000), showing that the animal and fungal lineages share a more recent common ancestor than either does with the plant lineage (Baldauf & Palmer, 1993) and that green algae and green plants form a monophyletic group. Due in part to limited sampling, the relationships between protists are not well resolved,

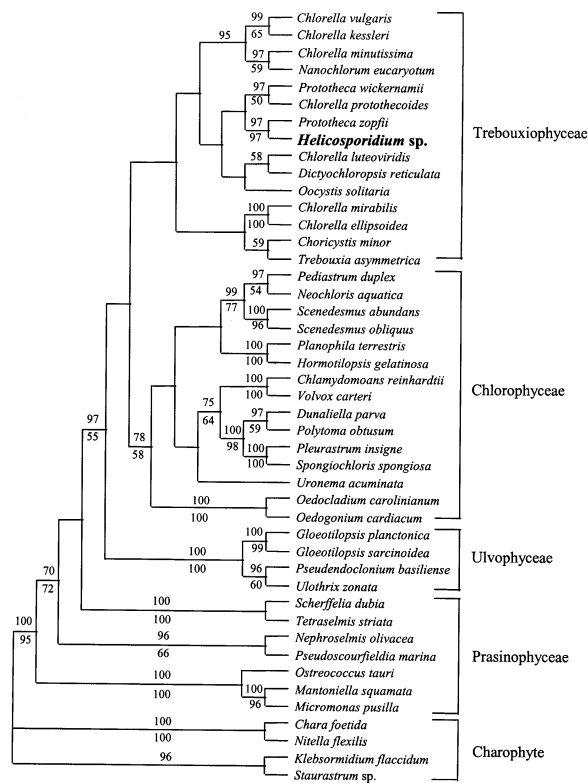


Fig. 3. SSU-rDNA phylogeny of the Chlorophyte green algae. *Helicosporidium* sp. appears as a member of the class Trebouxiophyceae, as a sister taxon to *P. zopfii*. Numbers above nodes represent the results of bootstrap analyses (100 replicates) using the NJ method. Numbers below nodes are results of jackknife analyses (100 000 replicates) using the maximum-parsimony method. Only values greater than 50% are shown. The tree is rooted with the Charophyte green algae.

but they all appear near the root of the tree. Importantly, the tree shows that *Helicosporidium* sp. clusters with the green algae (Chlorophyta), and this relationship is supported by both NJ (89%) and maximum-parsimony (69%) bootstrap/jackknife methods (Fig. 2).

The tree presented in Fig. 3 was inferred from an algal SSU-rDNA alignment and addresses the position of *Helicosporidium* sp. within the Chlorophyta. This tree is rooted with the branch leading to Charophyte algae and shows the four classes of Chlorophyta. As shown previously by Bhattacharya & Medlin (1998), the class Prasinophyceae is paraphyletic, whereas Ulvophyceae, Trebouxiophyceae and Chlorophyceae are monophyletic. In this tree, *Helicosporidium* sp. is depicted as a sister taxon to *Prototheca zopfii* (Trebouxiophyceae) by both distance and parsimony analyses (Fig. 3).

Preliminary alignments showed that neither the actin nor β -tubulin gene amplified from helicospiridial DNA possessed any introns. As a result, these sequences were aligned with homologous coding sequences (cDNA) downloaded from GenBank. The

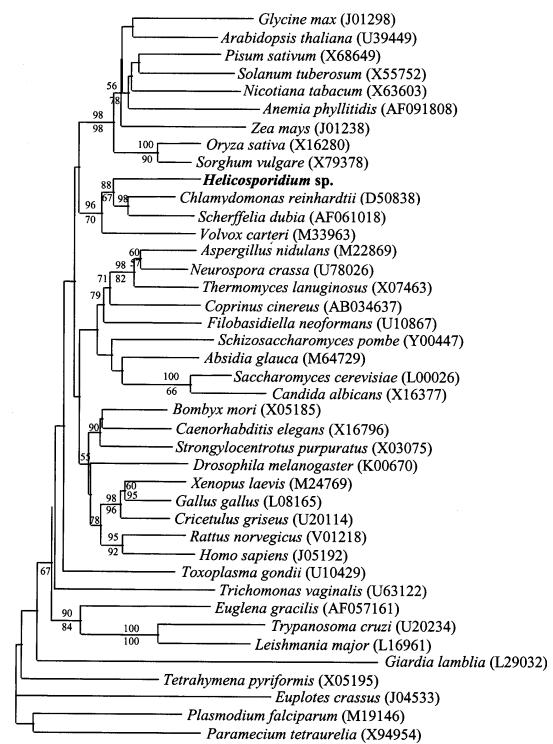


Fig. 4. Phylogenetic tree based on actin gene nucleotide sequences. The tree depicts *Helicosporidium* sp. as a member of the Chlorophyta. Numbers above nodes represent the results of bootstrap analyses (100 replicates) using the NJ method. Numbers below nodes are the results of jackknife analyses (100 000 replicates) using the maximum-parsimony method. Only values greater than 50% are shown. All but the helicospiridial sequence were downloaded from GenBank. Accession numbers for these sequences are indicated after the name of each species.

phylogenetic trees inferred from the analysis of actin and β -tubulin fragments are presented in Figs 4 and 5, respectively. The two trees are very similar: they are rooted with the branch leading to the ciliate *Euplotes crassus* and present branching patterns common to most eukaryotic phylogenies. All protists are clustered near the root of the trees and Metazoa, Fungi and Viridiplantae are all shown to be monophyletic. Both trees confirm that *Helicosporidium* sp. belongs to the green algae clade, even if the resolution within this clade is not very high. Once again, the nodes linking *Helicosporidium* sp. to the green algae are all supported, except for the parsimony jackknife of the β -tubulin tree (Fig. 5).

Additionally, further analyses led to the same conclusion, that *Helicosporidium* sp. groups with the green algae. Notably, realignments of the RDP SSU-rDNA dataset, modification of gap-penalty parameters or utilization of other distance methods available in PAUP* (such as HKY85 or maximum-likelihood distance) had no effect on the final position of *Helicosporidium* sp. within the eukaryotic tree.

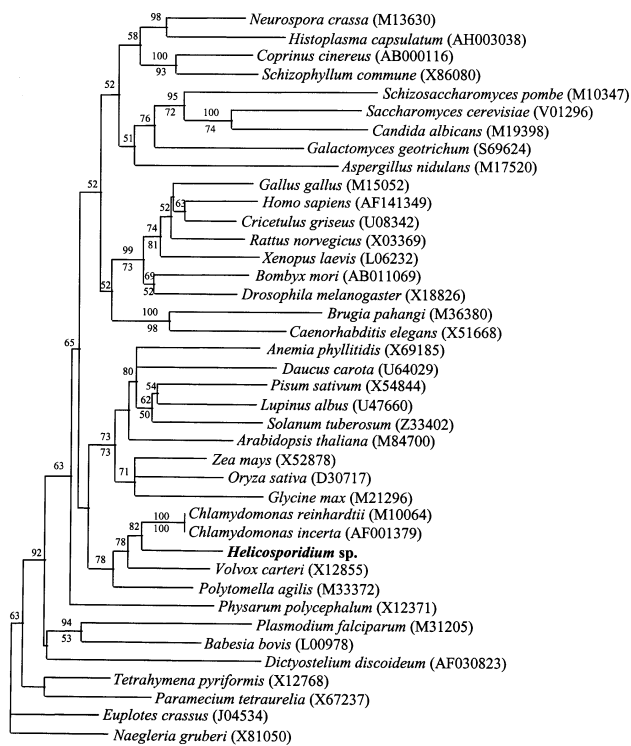


Fig. 5. Phylogenetic tree based on β -tubulin gene nucleotide sequences. In this tree, *Helicosporidium* sp. appears as a sister taxon to the genus *Chlamydomonas*. Numbers above nodes represent the results of bootstrap analyses (100 replicates) using the NJ method. Numbers below nodes are the results of jackknife analyses (100000 replicates) using the maximum-parsimony method. Only values greater than 50% are shown. All but the helicosporidial sequence were downloaded from GenBank. Accession numbers for these sequences are indicated after the name of each species.

DISCUSSION

All trees obtained in this phylogenetic study present a fairly reasonable branching pattern, with major divisions corresponding to conventional taxonomic classification (Kinetoplastida, Alveolata, Viridiplantae, Fungi and Metazoa). On the basis of these phylogenies, *Helicosporidium* sp. is clearly unrelated to any group of Protozoa (Philippe & Adoutte, 1998). This result suggests that the early attempt of Kudo (1931) to classify this organism within the Protozoa may have been wrong, but it is consistent with studies by Weiser (1970) and Kellen & Lindegren (1974), both of which proposed the removal of the Helicosporidia from the Protozoa. However, in the most recent study of which we are aware, Lindegren & Hoffman (1976) refused this suggestion and reaffirmed that the Helicosporidia have affinities with the Protozoa, based on the presence of well-defined Golgi bodies and mitotic division of the nucleus.

None of our phylogenetic trees depicted this *Helicosporidium* sp. as a member of the kingdom Protozoa (as defined by Cavalier-Smith, 1993). Instead, they

consistently and stably grouped *Helicosporidium* sp. among members of Chlorophyta, suggesting that this invertebrate pathogen is a green alga. Considering the fact that comparative sequence analysis is a robust method that provides resolving power for clade identification, we think that the appropriate place for *Helicosporidium* is within the Chlorophyta. Furthermore, an 18S rDNA-based phylogeny of the Chlorophyta depicted *Helicosporidium* sp. as a member of the class Trebouxiophyceae and as a very close relative of the genus *Prototheca*. Although *Helicosporidium* sp. always clusters with *P. zopfii*, this is probably due to a long-branch-attraction phenomenon, as *Helicosporidium* sp. is believed to have a very fast evolutionary clock (V. A. R. Huss, personal communication). The nucleotide sequences are sufficiently different to warrant the conclusion that *Helicosporidium* represents a separate genus within the Trebouxiophyceae.

It may be argued that our helicosporidial sequences, because they were amplified with universal primers, may be from a potential algal contaminant. However, it should be noted that our *Helicosporidium* sp. was purified carefully by gradient centrifugation after propagation in *Helicoverpa zea*. Furthermore, Boucias *et al.* (2001) also propagated *Helicosporidium* sp. *in vitro* and extracted DNA from both *in vitro* and *in vivo* sources. An RFLP analysis of the 18S rRNA gene amplified from these two sources produced identical digest patterns, demonstrating the integrity of the extracted helicosporidial genomic DNA used in this study (Boucias *et al.*, 2001). Also, DNA has been extracted from a second strain of *Helicosporidium* sp. and SSU-rRNA gene sequences from the two strains are highly similar (data not shown).

The association of *Helicosporidium* sp. with the genus *Prototheca* is interesting from a biological perspective. Members of both genera are achlorophyllous and animal pathogens. To date, *Helicosporidium* spp. have been identified as invertebrate pathogens, whereas *Prototheca* spp. are known to be pathogenic to vertebrates, including humans (Galan *et al.*, 1997; Mohabeer *et al.*, 1997). Mohabeer *et al.* (1997) reported that *P. wickerhamii*, although being primarily skin-infectious, can invade several human tissues, including liver, spleen, small intestine, lymph nodes, central nervous system and blood. *P. zopfii* is also reported to be a human pathogen (Galan *et al.*, 1997). Morphologically, the vegetative cells of the *Helicosporidium* sp. produced under *in vitro* and *in vivo* conditions are reminiscent of those reported for the genus *Prototheca*. We noticed that, as protothecans, the vegetative cells of *Helicosporidium* sp. undergo one or two cell divisions within a pellicle. This pellicle eventually splits open or dehisces, releasing either two or four daughter cells from the parent cell wall or pellicle (Boucias *et al.*, 2001). However, to our knowledge, it has not been reported that protothecans produce a mature cyst containing the filamentous cell, which is the unique morphological feature that charac-

terizes the genus *Helicosporidium* (Fig. 1). We are confident in the fact that deeper analysis may provide more precision with regard to the relationship between the genera *Helicosporidium* and *Prototheca*. Notably, comparative analysis of mitochondrial genomes has been shown to be a very powerful tool for classification of green algae (Nedelcu *et al.*, 2000). Similarly, observations on ultrastructure and cell biology may provide additional data to confirm our conclusions (Taylor, 1999).

Both morphological and molecular evidence suggest that the appropriate place for the group Helicosporidia is within the green algae. Therefore, we propose that the genus *Helicosporidium* represents the first reported algal entomopathogen and should be placed among the Chlorophyta, Trebouxiophyceae.

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