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Isolation and characterization of a novel chytrid species (phylum *Blastocladiomycota*), parasitic on the green alga *Haematococcus*

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ARTICLE INFO

Article history:
Received 21 February 2007
Received in revised form
17 June 2007
Accepted 11 September 2007
Corresponding Editor: Teun Boekhout

Keywords:
Algal pathogen
Amoeboid swarmer
Infection
Life-cycle
Parasite
Zoospore

ABSTRACT

A parasite was found in cultures of the green microalga *Haematococcus pluvialis* that grew epibiotically on algal cells and caused epidemics resulting in damage to the host cultures. The parasite was isolated into axenic culture on solid and liquid media. It was demonstrated to be the sole causative agent of the epidemics. According to its life cycle and phylogenetic analysis based on 18S ribosomal DNA sequences, the pathogen appears to represent a novel chytrid fungus closely related to the vascular plant pathogen *Physoderma* (*Blastocladiomycota*), although it differs from all other known chytrids by its infective stage, a wall-less propagule endowed with amoeboid motion and lacking the group's typical flagellum.

Introduction

Microalgae, both in natural habitats and in aquaculture are often plagued by harmful contaminants — viruses, bacteria, protists, fungi, and various grazers. Some of these parasites are 'chytrid fungi' or 'chytrids' belonging to the fungal phyla Chytridiomycota (Canter-Lund & Lund 1995) or *Blastocladiomycota* (James *et al.* 2006).

Chytrids are a diverse group of microscopic fungi (Barr 1980; Barr 2001; Kendrick 1992) that disperse in an aqueous environment by motile zoospores with a single posterior whiplash flagellum (Sparrow 1960). The chytrids have recently been divided into two phyla on the basis of various physical, chemical, genetic, and biological characteristics (James *et al.* 2006). All chytrids are microscopic, protist-like organisms but they have a distinct cell wall containing chitin, and they utilize the α-aminoadipic acid (AAA) lysine biosynthesis pathway — traits shared with other true fungi. Chytrids are widespread in aquatic habitats and in moist soil, where they live as saprophytes, or as facultative or obligate parasites (Powell

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0953-7562/$ – see front matter
doi:10.1016/j.mycres.2007.09.002

1993). Their range of hosts is quite impressive, including amphibians (Longcore *et al.* 1999), invertebrates (Johnson *et al.* 2006), rotifers (Glockling 1998), nematodes (Deacon & Saxena 1997), macroalgae (Nyvall *et al.* 1999), and vascular plants (Alexopoulos *et al.* 1996; Kendrick 1992, 2000). The potency of a chytrid infection and its timing are affected by several factors, such as water turbulence (van Donk & Ringelberg 1983), temperature, pH, light (Bruning 1991a; Bruning 1991b; Bruning 1991c; Sen 1987a; Sen 1987b), concentration of various ions in the medium (Abeliovich & Dikbuck 1977), as well as host density (Holfeld 2000).

In some natural ecosystems most algal species are used as hosts by at least one chytrid species (Holfeld 1998). These parasites can cause mortality of up to 90 % of the host population (Abeliovich & Dikbuck 1977), or even more (Canter-Lund & Lund 1995). Chytrid infection of green microalgae cultures have also been reported for *Scenedesmus* (Becker 1994) and *Chlamydomonas* (Shin *et al.* 2001). The green microalga *Haematococcus pluvialis* (*Chlorophyta*, *Volvocales*) is known for its ability to produce and store the red ketocarotenoid astaxanthin (Boussiba & Vonshak 1991), which has made it an attractive product for commercial growth (Olaizola 2000). The alga has a life cycle that starts with biflagellate motile cells enclosed in a soft gelatinous cell wall. A thick polysaccharide cell wall appears as the cell sheds its flagella and matures into a spherical palmelloid (Kobayashi *et al.* 1997). Under stress conditions the cells turn into red cysts (aplanospores). The cell wall of these cysts can be two to threefold thicker than that of the green cell, but retains its chemical nature, consisting mostly of cellulose microfibrils and a pectinaceous matrix (Montsant *et al.* 2001).

We report therein the first observation and description of epidemics in both green and red *Haematococcus* cultures caused by a chytrid-like organism originating from an unknown source. The parasite is monocentric and epibiotic, with its spore-bearing (sporangium) body on the host cell wall, and a bulbous rhizoid system in the host's cytoplasm. It seems to use nutrients that become available as it digests the host cell content, eventually killing the host cell in the process. The aim of the present study was to investigate the isolation of this parasite, its classification, life cycle, and physiology, and its association with the host.

Materials and methods

Algal strain and growth conditions

Haematococcus pluvialis Flotow 1844 K-0084 was obtained from the Scandinavian Culture Centre for Algae and Protozoa (SCCAP) at the University of Copenhagen, Denmark. Green monocultures of *Haematococcus* in modified BG11 (mBG11) medium (Wang *et al.* 2003), or alternatively nitrogen-starved red cultures used in all experiments were both obtained as described by Wang *et al.* (2003).

Isolation and culture of the parasite

Infected algal cells were smeared on agar plates as described by Chen & Chien (1995) and incubated at 32 °C. The medium contained the basal algal mBG11 components supplemented with 0.25 g l^{-1} yeast extract, 0.25 g l^{-1} peptone, 5 g l^{-1} glucose and 1.5 % agar; 0.15 g l^{-1} ampicillin and 0.15 g l^{-1} kanamycin were added to prevent bacterial growth. After several passes of plate inoculation, axenic cultures were obtained. For routine growth of the parasite on solid or liquid media, a chytrid growth medium (CGM) was devised, based on the algal growth medium. It contained the algal mBG11 medium salt components (omitting inorganic carbon and nitrogen), supplemented with 0.25 g l^{-1} yeast extract, 0.25 g l^{-1} peptone, and 5 g l^{-1} glucose. The liquid cultures were inoculated with homogenized chytrid suspensions (to yield ~ 0.01 OD^{600}) and further incubated at 32 °C on a shaker (125 rev min^{-1}) in Ehrlenmeyer flasks (100 ml), 24 or 96 well plates (3 or 0.15 ml) for a period of up to two weeks. The clumpy biomass was quantified by duplicate measurements of the turbidity (OD^{600}) of homogenized aliquots (1–5 ml), as well as their protein content (Bradford 1976) using ovalbumin as a standard.

Chytrid inoculation of algal cultures

Algal cultures (50 ml) of green cells or red cysts (0.5–1 × 10^6 cells ml^{-1}), in 250 ml Ehrlenmeyer flasks, were inoculated with 50 µl of 3-d-old chytrid-infected *Haematococcus* culture or with axenic chytrid culture. Chytrid colonies grown on agar or aliquots from axenic liquid culture were first homogenized in CGM, and samples (equivalent to 0.5 OD^{600} *ml) containing a few thousand sporangia were used. The inoculated cultures were further incubated at 32 °C on a shaker (125 rev min^{-1}) under continuous dim white light (15 mE μ^{-2} s^{-1}). The progress of infection was routinely monitored microscopically and quantified as the percentage of infected cells (i.e. algal cells with at least one chytrid sporangium attached). If no epidemic had developed within 14 d, the inoculum was considered non-infective.

Light microscopy

Cell suspensions mounted on microscope slides and covered were examined in bright-field (BF), dark-field (DF), or phase contrast (PC) using a Zeiss Axioscope microscope at magnifications of ×400 and ×1000. In some instances, the cells were stained for 10 min in 1.5 % methylene blue (MB) solution, washed, and resuspended in water before mounting. Agar colonies or well plates were examined at magnification of ×100. Microscope fields were photographed using an Olympus Camedia digital camera. Digital slide photographs had brightness and contrast optimized to enhance regions of interest.

Molecular taxonomy of the parasite

For DNA isolation, chytrid colonies were scraped from the surface of CGM agar and lyophilized. DNA was then extracted from ~10 mg of lyophilized material using a standard DNA extraction procedure with a CTAB buffer (Zolan & Pukkila 1986).

The 18S rDNA gene was amplified from DNA extracts of the parasite using the primer pairs SR1R (Vilgalys & Hester 1990) and NS8.1 (5′-CCG CAG GTT CAC CTA CG-3′). Amplification used RedHot Taq polymerase (ABgene, Rochester NY) and the

following protocol: an initial denaturation step at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 30 s, extension at 72 °C for 1 min, and a 7 min final extension at 72 °C. The amplification products were purified using a Qiaquick PCR Purification Kit (Qiagen, Valencia, CA) and sequenced using the amplification and internal primers [SR1.5 (James *et al.* 2000); BMB-BR (Lane *et al.* 1985); NS6 (White *et al.* 1990)] with a BigDye sequencing kit (Applied Biosystems, Foster City, CA). Sequences were analysed on an ABI3700 DNA sequencer (Applied Biosystems).

The phylogenetic placement of the parasite was established by adding the 18S rDNA sequence to a previously established alignment of sequences of *Chytridiomycetes* and other fungi (James *et al.* 2000). The total alignment length was 2003 bp, and 503 bp were eliminated from phylogenetic analyses due to ambiguity in alignment. Phylogenetic analyses used MP as implemented in the software package PAUP 4.0 (Swofford 2003) with the shortest trees found using 1 K heuristic searches, each started using random sequence addition. Statistical support was measured with 1 K BS pseudo-replicates with shortest trees of each replicate found by heuristic searching with ten random sequence addition replicates.

Results

Symptoms of epidemics

In infected green shaken algal cultures, the first macroscopic symptoms of an epidemic were usually flocculation, accompanied by a change of colour from green (Fig 1A, 0 h) to brown–green (Fig 1A, 48 h). Later the culture turned dark brown (Fig 1A, 72 h). If stirring was stopped even for a short period, big clumps formed which quickly sank to the bottom of the vessel. Although in shaken cultures 100 % of the algal cells were infected within 3 d, the infection in still cultures developed much slower. After 10 d of infection, less than 50 % of the host population was infected and only slowly expanding light-brown plaques were observed (Fig 1C).

In infected red cultures, a more vivid red colour was first observed, followed by gradual bleaching (data not shown). Microscopically, infected cultures exhibited a fast-growing number of *Haematococcus* cells with attached round, semitransparent 3–30 μm sporangia (Fig 1D), compared to uninfected green cultures. The algal cells hosting these sporangia were in varying stages of losing their contents, ending with a brown mass left at the centre of the otherwise empty cells. Young *Haematococcus* cells in their motile phase did not get infected, but were targeted as soon as they matured to non-motile green cells. Infected red cysts (Fig 1E) exhibited symptoms similar to those observed in green cells.

Isolation of the parasite

Placing infected *Haematococcus* cells on the surface of mBG11 agar plates resulted in the formation of microscopic colonies, composed of what looked like undeveloped parasite prosporangia around some infected cells. These colonies did not develop or grow beyond their initial size. Similar results were observed with other basic media (e.g. ¼ YPSS) (Barr 1987). Transfer of a one-week-old microcolony into clean algal cultures did not result in infection, apparently because the prosporangia had died without adequate nutrients to support further development. Only when the algal medium was supplemented with yeast extract, peptone, and glucose did macroscopic, infective colonies appear on the agar (Fig 2A). Recurrent picking and transferring colonies (or clusters of sporangia) to new plates with the enriched medium supplemented with antibiotics resulted in the isolation of clean colonies (Fig 2B) in axenic cultures of the chytrid. Liquid medium of similar constituency also supported growth of colonies (Fig 2C), with the younger sporangia growing on top of older sporangia. Upon maturation of both the solid and liquid cultures a characteristic dark pigmentation became apparent (see Fig 2K). A viable chytrid parasite could consistently be isolated from infected algal cultures and the inoculation of healthy *Haematococcus* cultures with fresh isolated chytrid samples was sufficient to result in the development of chytrid epidemics.

Nutrient requirements

A systematic study of media components helped to formulate a chytrid growth medium (CGM) yielding larger colonies on agar and faster growth in liquid. The inorganic salts (basically from mBG11) must include potassium, magnesium, phosphate, and sulphate, as macroelements, but also small amounts of calcium, iron, and microelements, missing in common yeast and fungal basal media. However, the growth ability on the latter was restored when tap water was used instead of double-distilled water, or when an excess of yeast extract (1 g l^{-1}) was supplied in the medium.

The chytrid thrived in the presence of 5 g l^{-1} glucose or sucrose, indicating the presence of functional glucose transporter and invertase activity. However, the growth was severely limited in the presence of 0.25 g l^{-1} peptone as the sole carbon source or in combination with 5 g l^{-1} of either bicarbonate or galactose. Finally, the nitrogen source in peptone could be replaced by 0.25 g l^{-1} glutamine or arginine, but not by nitrate or ammonia.

Effect of environmental factors on growth in liquid medium

The requirement for oxygen was qualitatively assessed in both freshly infected algal cultures and in pure chytrid cultures in CGM. Dissolved oxygen in the former was depleted by bubbling oxygen-free nitrogen gas for 30 min in the dark, and the tightly plugged culture vessels were incubated with shaking for 10 d in two parallel sets: one exposed to light and the other covered with aluminium foil (dark). The extent of infection (infected cells count) was inhibited in the dark culture, compared with the light exposed cultures, by more than 50 %. No effect of light was apparent when the infected cultures were exposed to ambient air. Similarly, axenic chytrid cultures in CGM failed to grow anaerobically, independently of their illumination status. Furthermore, on solid CGM medium, although extensive growth of inoculated chytrid was observed on slants (agar surface), it was essentially nonexistent in deep stabbed agar (>1 cm), both in the light or in the dark. These results suggest that the parasite is an obligate

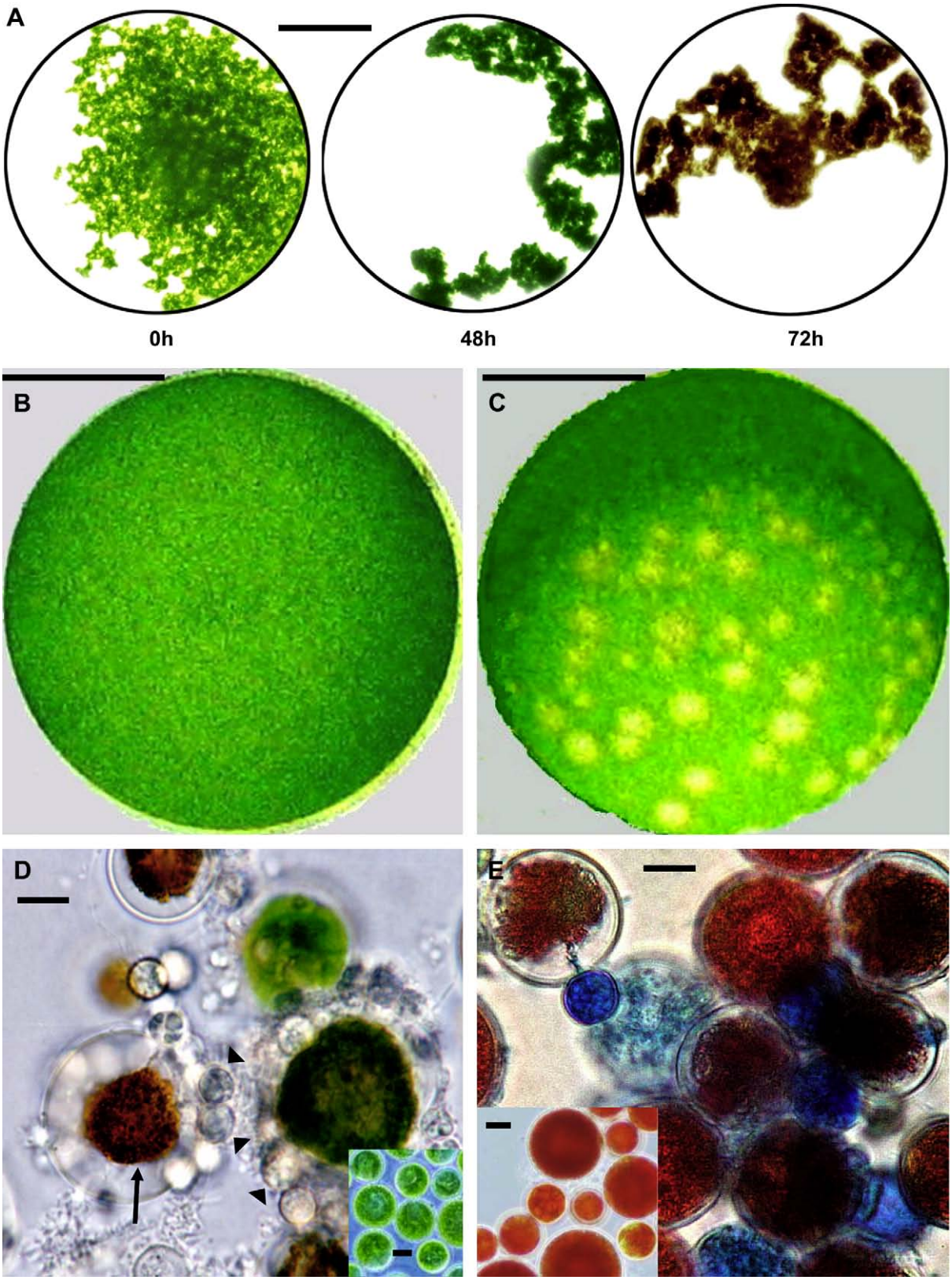

Fig 1 – Symptoms of epidemics caused by the chytrid parasite in cultures of *Haematococcus pluvialis*. Green (A–D) or red (E) *H. pluvialis* cultures (0.1 ml) were infected and incubated in shaken (A,D,E) or still (B,C) 96 wells plates as described in Materials and Methods. (A) macroscopic symptoms: microwells (bar = 2 mm). (B, C) Ten-day-old uninfected (B) or infected (~ 200 colonies forming units) still cultures (C; bar = 2 mm). (D) Green cells with attached parasite sporangia (arrowheads) and typical brown, depleted cell (arrow). (PC). (E) Red cysts with attached parasite sporangia (BF, MB). (D, E) Insets = healthy cells (bar = 10 μm).

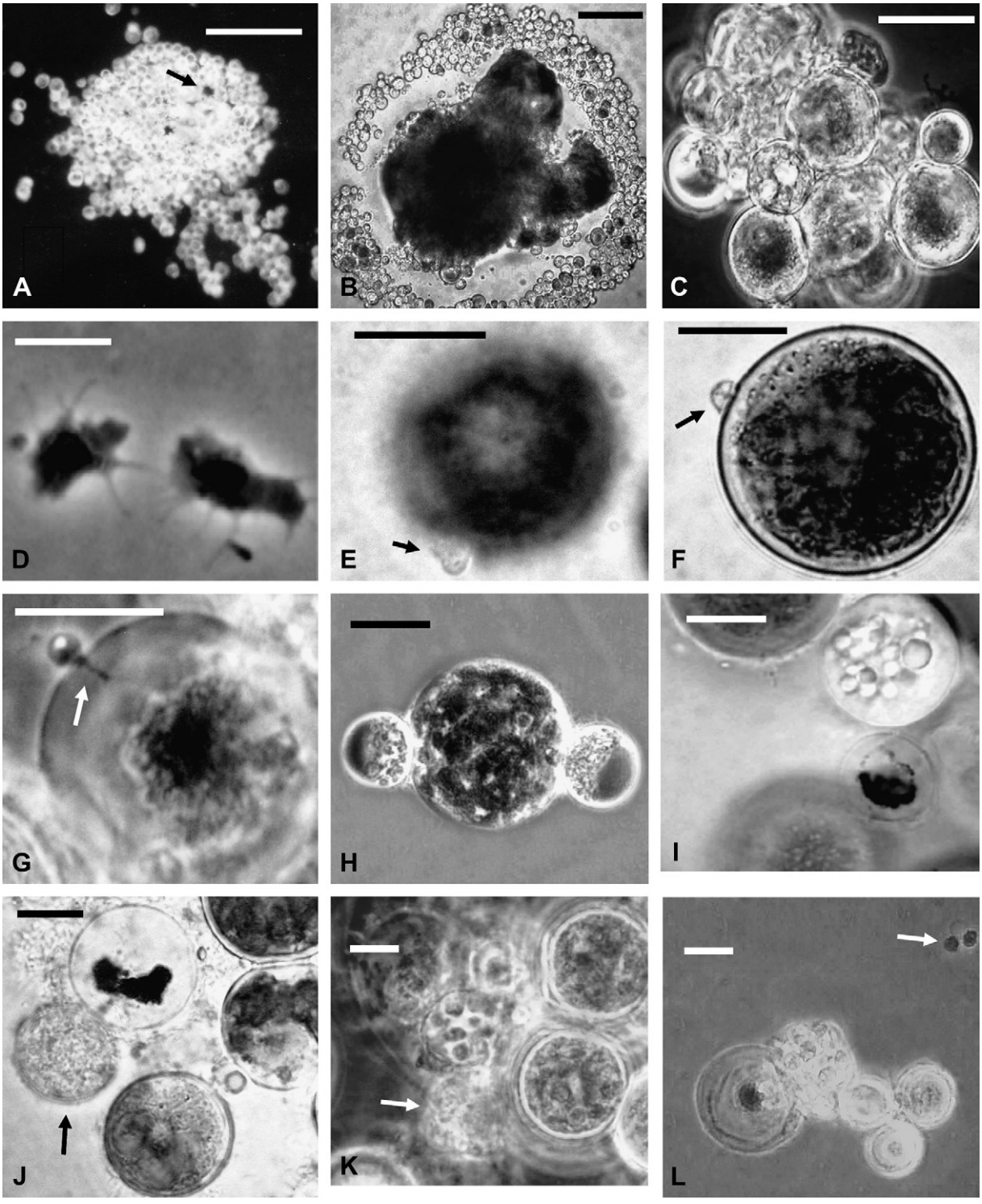

Fig 2 – Chytrid colonies growing in enriched medium (CGM). (A) Sporangia of first-generation chytrid colony growing on agar around remnants of an infected red *Haematococcus* cell (arrow) (DF; bar = 200 μm). (B) Isolated chytrid colony forming on CGM agar. Note the young sporangia in ring surrounding the older, pigmented mass in the centre (BF; bar = 100 μm). (C) A colony in liquid CGM (DF; bar = 40 μm). Stages in the parasite's life cycle; Vegetative phase: (D) free amoeboid swarmers magnified to show pseudopodia (PC; bar = 2 μm); (E) amoeboid swarmer (arrow) crawling on *Haematococcus* cell before settling (PC); (F) encysting swarmer (arrow; PC, MB); (G) penetration of a host cell by the germ tube (arrow) emanating from the cyst. Note the refractive lipid globule in the young cyst (PC, MB); (H) Two developing sporangia on a host cell (PC, MB); (I) a developing sporangium with many light-refractive bodies (PC); (J) a sporangium in transformation phase (arrow) on a typical brown, depleted cell (BF, MB); (K) a mature sporangium, full of amoeboid swarmers (arrow) (PC); (L) beginning of dehiscence. The new, free amoeboid swarmers (arrow) disperse in the water (PC). Bar (E–L) = 10 μm.

aerobic organism, and in infected algal cultures the effect of light can be partially attributed to the availability of oxygen and/or other secreted algal metabolites (serving as signals) derived from photosynthetic activity.

The time course of chytrid infection of a healthy *Haematococcus* culture is presented in Fig 3A. At optimal temperature (30 °C), the number of infected cells rises exponentially after a short lag, closely followed by a progressive depletion of algal chlorophyll. A similar but slower sequence was observed at 23 °C, whereas at 15 and 40 °C, no apparent infection has been observed after 15 d (not shown).

The growth of chytrid in liquid CGM was tested at various temperatures from 15–40 °C. At 15 and 40 °C there was no perceptible growth. The chytrid grew rapidly in the range 23–35 °C with the fastest rate observed at 32 °C. Typical growth kinetics of the chytrid in shaken liquid medium, as reflected by turbidity (OD^{600}) and the protein content at 32 °C is presented in Fig 3B. One may calculate an exponential asynchronous growth rate (μ) of about 0.77 d^{-1} for both the turbidity and protein content curves, indicating a near linear relation between the biomass and protein. However, the limiting factor causing the arrest of turbidity increase remains yet to be identified.

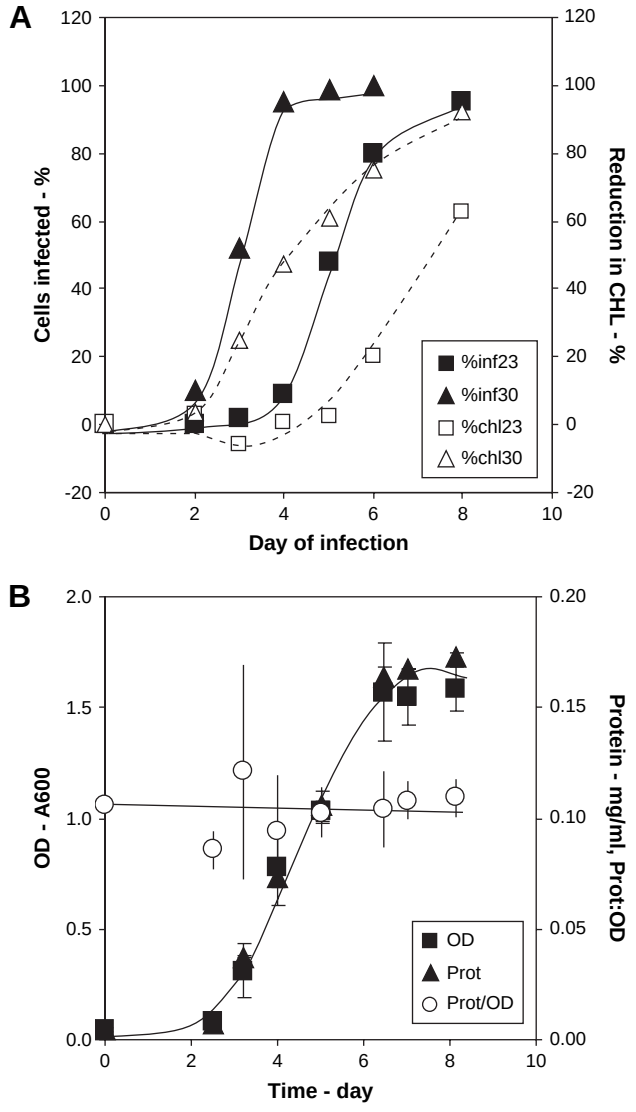

Fig 3 – Chytrid growth and algal infection progress. (A) Liquid CGM was inoculated with a chytrid suspension and further incubated as described in Materials and Methods. At the indicated times, samples were withdrawn and processed to determine turbidity and protein content. (B) An algal culture was inoculated with an axenic chytrid suspension and analysed for infection microscopically as described in Materials and Methods, at permissive temperatures of 23 and 30 °C, as indicated. In addition, the chlorophyll content of the algae was measured in hot DMSO extracts and plotted as the reduction of chlorophyll content as percentage of the initial concentration (100 % = 15 μg ml^{-1}).

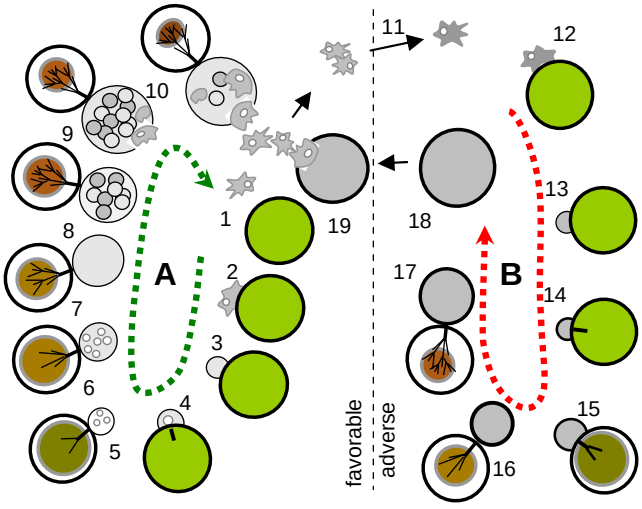

Fig 4 – Proposed life cycles of the parasitic chytrid in *Haematococcus* culture. (A) Vegetative cycle. An amoeboid swarmer arrives *via* water (1, Fig 2D), makes contact with a host cell (2, Fig 2E), settles and encysts (3, Fig 2F). The germ tube sprouts and crosses the cell wall into host cytoplasm (4, Fig 2G). Both the sporangium and the rhizoid system grow at expense of host cell (5, Fig 2H–J, Fig 5D). The fully-grown immature sporangium (6, Fig 2H) transforms (7, Fig 2J) into a mature sporangium with a thin cell wall full of new amoeboid swarmers (8, Fig 2I,K). Swarmers are released through a tear in the sporangial cell wall (9, Fig 5G,H), leave behind the empty sporangium and disperse in water (10, Fig 2L, Fig 5A,B). If they encounter a new host cell the cycle can repeat. (B) Resting phase. Dispersing amoeboid swarmers in stressful conditions conjugate and/or transform (11). A transformed (diploid?) amoeboid swarmer settles on the host cell (12) or possibly on another chytrid sporangium (see Fig 5J), and encysts (13). Germ tube crosses the cell wall into host cytoplasm (14), and a thick-walled resting sporangium grows (15–17, Fig 5E). A mature resting sporangium (18), now separated from host, disperses, perhaps *via* air or in soil. When a resting sporangium reaches favourable conditions, it begins to germinate (18), releasing new (haploid?) amoeboid swarmers (19). The vegetative cycle can resume as amoeboid swarmers find host cells.

Life cycle of the parasite in Haematococcus *cultures*

Based on numerous microscopic observations, the following is the proposed life cycle of the parasite (Fig 4). It begins as a 'zoospore' introduced into an uninfected culture. Unlike zoospores of other chytrids, those of the *Haematococcus* parasite are amoeboid spores, lacking the group's typical flagellum. For locomotion they use their many pseudopodia (Fig 2D). The amoeboid swarmers (3–6 μm) crawl until they find a suitable place to settle. Usually, but not always, it is a host cell to which they attach. Young, flagellated algal cells elude the swarmers; but as soon as they lose their flagella, they become susceptible to infection. The locus on the host cell at which the amoeboid swarmer settles and attaches appears to be arbitrary (Figs 1D, 2H). The amoeboid swarmer crawls on the cell surface for a while (Fig 2E) before it settles. Within minutes from settling, the swarmer cell encysts, acquiring a smooth, rounded morphology typical of walled cells (Fig 2F). At the point of attachment the cyst develops a peg-like germ tube that grows through the host's cell wall into the cytoplasm (Fig 2G), where it develops a rhizoid system (see also Fig 5D–F). The cyst at first appears as a tiny globe, *ca* 3 μm across. It is semi-transparent, and the only internal organelle that can easily be distinguished is a light refractive (probably lipid) globule that comprises *ca* 25 % of the cyst volume, while both the cell wall and the rhizoid are stained by methylene blue (Fig 2G, see also Fig 5D–E). Once the germ tube reaches the host cell cytoplasm, the cyst begins to grow rapidly and develops into a sporangium 20–35 μm diam, within 24–30 h (Fig 2H–L). Later, some internal differentiation becomes apparent as granular bodies within the growing sporangium (Fig 2I), while the rhizoid system grows in size, increasingly draining the host cell. Upon maturation, the white granules disappear and the sporangium becomes opaque for a few hours (Fig 2J). At the end of this stage, round and dark spores become visible within the now transparent sporangium (Fig 2K), and develop into amoeboid swarmers moving inside the sporangium. Release of the amoeboid swarmers' (or dehiscence) occurs gradually, and may last 30 min. The sporangial wall opens through an apparently random tear (see Fig 5G–H), and the amoeboid swarmers crawl out and disperse in the surrounding medium (Fig 2L), leaving an empty sporangium, still attached to the dead host cell by the degenerating rhizoid system (see also Fig 5B,E). The life-cycle can then be reinitiated from a single amoeboid swarmer. The number of swarmers in each sporangium depends on the host cell size and on the number of sporangia on the host cell. It is not uncommon to find host cells carrying dozens of tiny sporangia (see Fig 1D). The behaviour of the parasite when it settles on a red cyst is similar (Fig 5A–C), despite the differences in host cell appearance (colour and cell wall thickness).

Formation and characteristics of resting sporangia

Throughout the epidemics, some of the cysts develop into resting sporangia. This process becomes prominent in the later stages of the epidemics. The resting sporangia can be recognized mainly by their dark (grey), opaque look. Compared with vegetative sporangia (Fig 5D), they have a thicker cell wall (Fig 5E). By the end of the epidemics, only resting sporangia remain, attached to dead host cells. A similar phenomenon can also be observed in isolated chytrid cultures in the stationary phase, indicating this may represent a response to stress. Resting sporangia can sometimes grow as parasites on vegetative sporangia. When added to a *Haematococcus* culture, resting sporangia develop and release amoeboid swarmers that look and behave like those emanating from vegetative sporangia, and can cause epidemics.

A major characteristic of these resting sporangia is their resistance to drought, i.e. they have retained their infectivity to *Haematococcus* even after three weeks in an evacuated desiccator. It is not clear whether the resting sporangia are formed as a result of sexual reproduction. Although amoeboid swarmers in agar colonies have been observed to engage in apparent conjugation when facing stressful conditions, such behaviour has not yet been confirmed in infected algal cultures.

Life-cycle and development on agar

The life-cycle of the parasite as observed on CGM agar seemed similar to its life-cycle in *Haematococcus* cultures. Endogenously produced sporangia bearing a small rhizoid system were produced on agar media (Fig 5F). At maturation the sporangium developed a tear in its cell wall and the spores crawled out (Fig 5G). The amoeboid spores used pseudopodia to crawl over the surface of the agar (because of their small diameter and transparency, the pseudopodia cannot always be seen in the photographs). The amoeboid spores contained many tiny (< 1 μm) light refractive globules. The released spores crawled around the sporangium for about 1–2 h and then stopped moving and became round. Within minutes from settling, a distinct cell wall formed around the spore, and the tiny refractive globules coalesced into two to three bigger globules. As encystment was completed the cyst sprouted a germ tube, polarizing the thallus into a rhizoid and prosporangium (Fig 5I). Colony formation started as the settled sporangia matured and released more amoeboid swarmers that settled and formed a ring around the original ones (Fig 5J). Additional rounds of sporulation generated thalli over the whole surface of the agar and also on top of older sporangia (Fig 5J), thus forming a colony. The colony continued to grow reaching a diameter of 1.5–3 mm. The colour of the colony was creamy at first (see Fig 2A), becoming darker as the colony aged (Fig 5K, see also Fig 2B). At the age of three to four weeks it stopped growing and turned black as resting sporangia took over. Both young and older colonies, if placed in *Haematococcus* cultures, proved to be highly infective.

Phylogenetic assignment of the parasite

The 18S rRNA gene was amplified from DNA extracted from an axenic culture of the parasite. A parsimony analysis of 18S sequences from fungi with *Chlorophyta* as outgroup (Fig 6) placed the parasite with three sequences from *Physoderma* spp. (*Blastocladiales*: *Blastocladiomycota*). Statistical support for this relationship as assessed by BS analysis provided 100 % support for this close relationship between the *Haematococcus* parasite

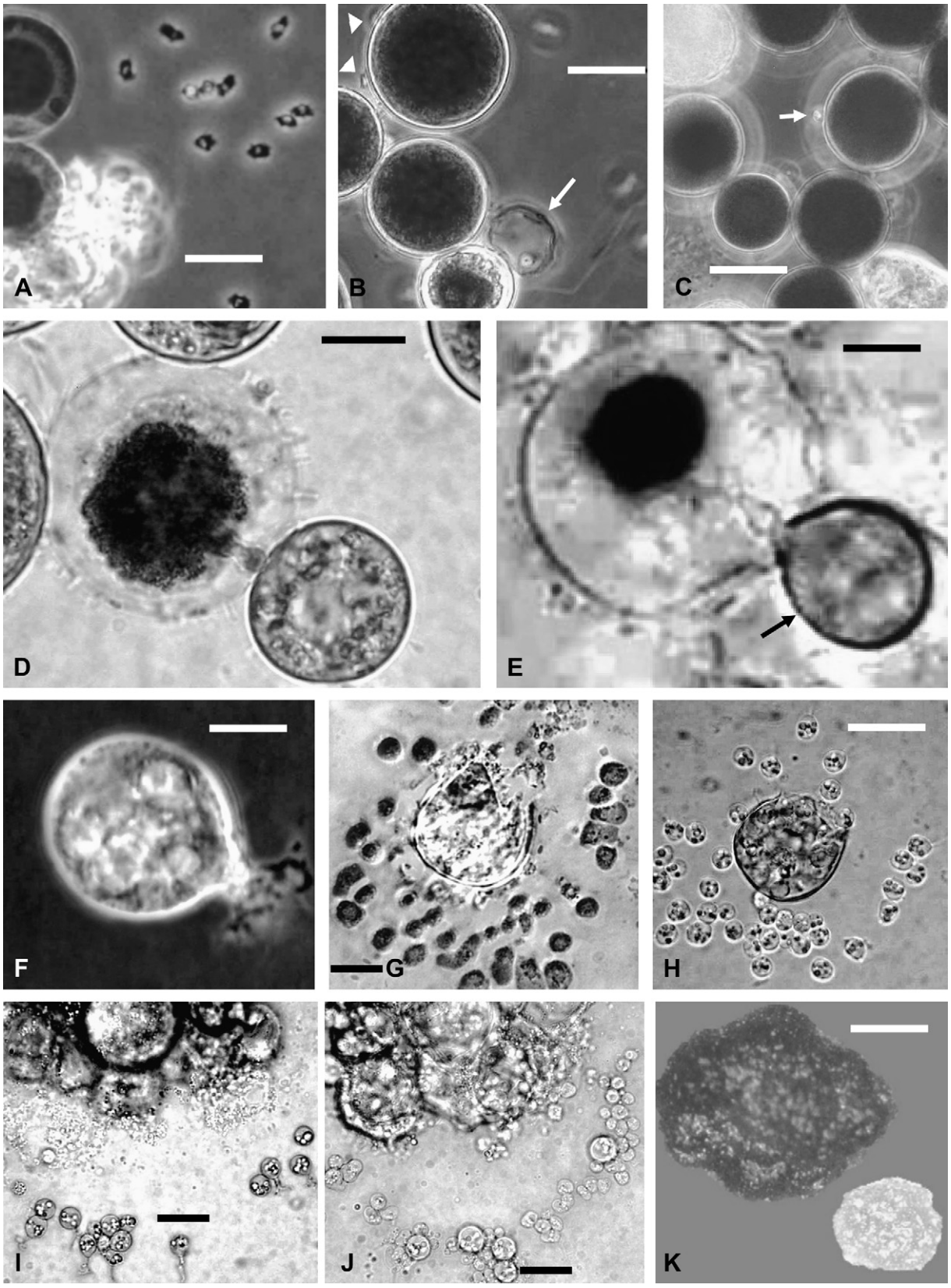

Fig 5 – Dehiscence and settling in red culture. (A) Dispersing amoeboid swarmers (sporangium out of focus). (B) One amoeboid swarmer left in the otherwise empty sporangium (arrow), as the released swarmers settle on host cells (arrowheads). (C) A new cyst (arrow); PC. Formation of resting sporangia (green culture). (D) Vegetative sporangium. (E) Resting sporangium with a thick cell wall (arrow); BF, MB. Colony formation on enriched agar plates. (F) A developing sporangium (notice short rhizoids). (G) Amoeboid swarmers emerge from an opening in the wall of the sporangium. (H) Encysting spores and germinating cysts. (I) Germinating cysts at the edge of a growing colony. (J) New amoeboid swarmers spread and encyst beside and beyond young sporangia, expanding the colony. (K) Two colonies; a young (bright) and an older one (dark). Bars (A–J) = 10 μm; (K) = 50 μm.

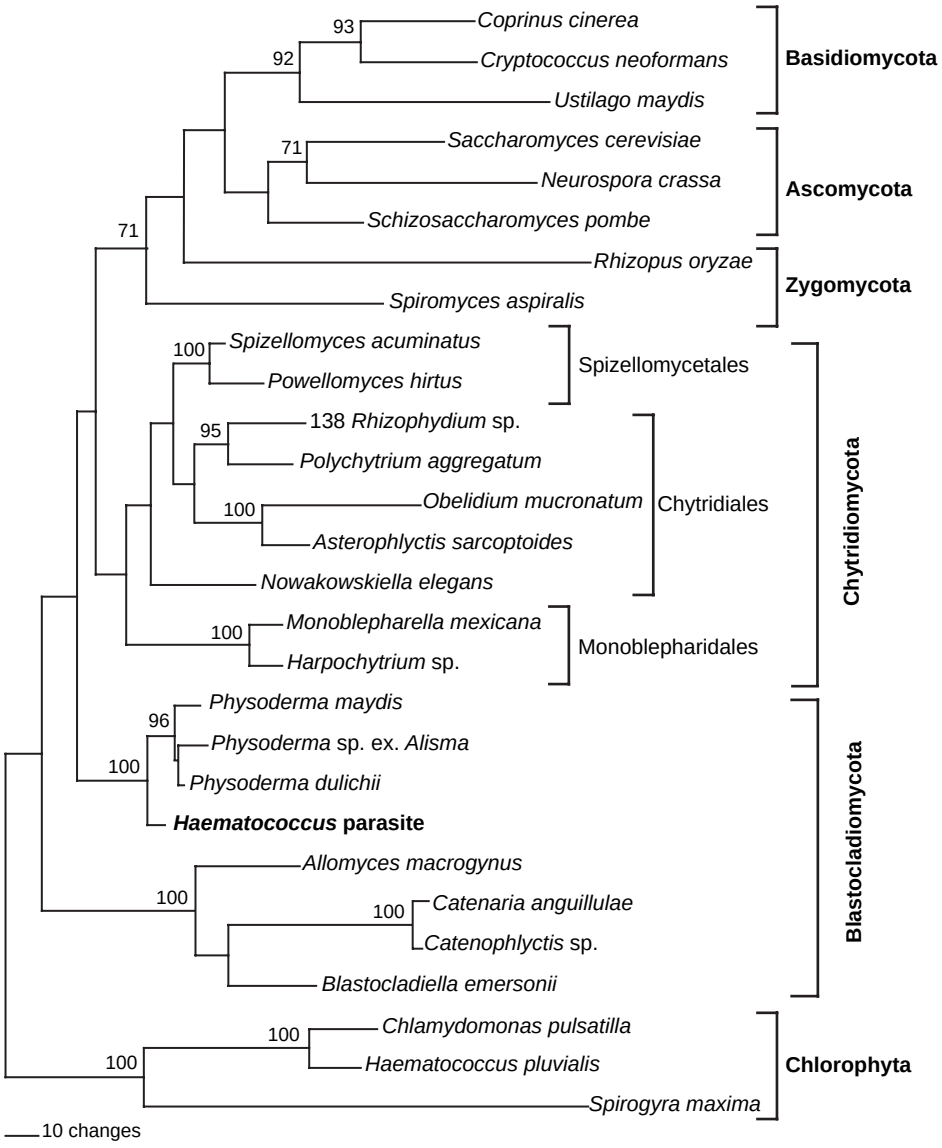

Fig 6 – Phylogeny of the *Haematococcus* parasite based on DNA sequences of the 18S rRNA gene. Shown is one of six most parsimonious trees. Values above nodes indicate percent BS support; only values greater than 70 % are displayed. GenBank accession numbers for the sequences used in the phylogeny are as follows: *Spizellomyces acuminatus* (M59759), *Powellomyces hirtus* (AH009022), 138 *Rhizophydium* sp. (AH009034), *Polychytrium aggregatum* (AY601711), *Nowakowskiella elegans* (AH009042), *Obelidium mucronatum* (AH009056), *Asterophlyctis sarcoptoides* (AH009060), *Monoblepharella mexicana* (AF164337), *Harpochytrium* sp. (AH009067), *Allomyces macrogynus* (U23936), *Catenaria anguillulae* (AH009068), *Catenophlyctis* sp. (AY635822), *Physoderma maydis* (AY601708), *Physoderma* sp. ex. *Alisma* (T. James, unpublished), *Physoderma dulichii* (T. James, unpublished), *Blastocladiella emersonii* (X54264), *Saccharomyces cerevisiae* (V01335), *Schizosaccharomyces pombe* (AY251633), *Neurospora crassa* (X04971), *Coprinus cinerea* (M92991), *Ustilago maydis* (X62396), *Cryptococcus neoformans* (X62396), *Rhizopus oryzae* (AF113440), *Spiromyces aspiralis* (AF007543), *Haematococcus* parasite (EF565163), *Chlamydomonas pulsatilla* (AF514404), *Haematococcus pluvialis* (AF159369), *Spirogyra maxima* (AF408236).

and *Physoderma* spp. Neither the *Chytridiomycota* nor the *Blastocladiomycota* appear monophyletic in these analyses.

Discussion

The present investigation was initiated by the discovery of a chytrid-like parasite in a culture of the green alga *Haematococcus pluvialis*. The parasite appeared to cause destructive epidemics in the host culture in which infection rates can reach 100 %. The parasite was isolated from infected algal cultures on semi-synthetic media and its life cycle was studied both in host cultures and isolated on solid or liquid medium. In numerous experiments this isolate proved to be the sole causative agent of the epidemics, thus satisfying Koch's postulates.

The nutrient requirements supporting the growth of the isolated chytrid indicate a general fastidious metabolism. Indeed, the stringent requirement for vitamins (supplied in yeast extract) and the narrow profile of effective carbon and nitrogen sources point to a lack of a broad spectrum of inductive pathways enabling the chytrid to grow as an opportunistic saprophyte. Nevertheless, the preliminarily observed positive results for sucrose, peptone, and selected amino acids suggest some ability to secrete lytic enzymes and/or use basal transporters. Finally, all organic nutrients requirements seem to be fulfilled by the host, as expected from a true parasite. In contrast, *Physoderma* and all other chytrid pathogens of vascular plants, such as *Olpidium* and *Synchytrium*, have never been isolated into axenic culture, whereas the nutrient requirement of the facultative aerobes *Rhizophydium* and *Phlyctochytrium* have been described as somewhat different than in our study (Goldstein 1960).

Like many other chytrids (Ibelings *et al.* 2004; Kagami *et al.* 2007), the *Haematococcus* parasite develops an epibiotic sporangium on the host and an endobiotic rhizoid system, which supports the sporangium by transferring nutrients from the host. The mature sporangium releases partially water-dependent, unwalled amoeboid swarmers. Like some other chytrids, it can also develop thick-walled resting sporangia, which release unwalled spores, when placed in an appropriate environment. However, unlike other chytrid fungi, the spores released by both sporangial forms lack the characteristic posterior whiplash flagellum, used as a locomotive means for dispersal. Only very few chytrids disperse by other means, such as wind or insects (Powell 1993).

After numerous observations, and under various conditions, both on the host and on solid medium, the only spores this organism produced were non-flagellated and amoeboid, which produce either vegetative or resting sporangia. Only one other chytrid, *Hyaloraphidium curvatum*, has been reported to produce only amoeboid swarmers or aplanospores (Ustinova *et al.* 2000). However, some chytrids produce aplanospores as secondary spores, whereas others produce zoospores that can enter into an amoeboid phase (Sparrow 1960). Spores of *Spizellomycetales* are irregular in shape and may undergo amoeboid movement while swimming (Barr 2001). Another example, *Mycromycopsis* spp., produces two generations of spores. One is composed of amoeboid spores that attach themselves to the host, encyst, and develop a sporangium that produces a second generation, composed of uniflagellated zoospores (Subramanian 1974). The amoeboid properties of fungal spores are likely an ancestral characteristic shared with the related protist group, order *Ichthyophonida*, class *Mesomycetozoa* (Mendoza *et al.* 2002; Ustinova *et al.* 2000), some of which can produce amoeba-like, but not flagellated infective spores.

Sequencing of the 18S rRNA gene of the parasite strongly suggests that it is a member of the chytrid phylum *Blastocladiomycota*, closely related to the genus *Physoderma*, a group of obligate parasites of pteridophytes and angiosperms, primarily in marsh and aquatic habitats. Although *Physoderma* spp. and the *Haematococcus* parasite do not form a clade with the remaining *Blastocladiomycota*, DNA sequences of the rDNA genes (James *et al.* 2006) suggest that *Physoderma* and the *Haematococcus* parasite are truly members of the phylum *Blastocladiomycota*. *Physoderma* differs from most members of the *Blastocladiomycota* in having a Golgi apparatus (Lange & Olson 1980) rather than Golgi equivalents (Bracker 1967). Furthermore, *Physoderma* and the *Haematococcus* parasite differ from other *Blastocladiomycota* in having a pathogenic relationship with plants rather than animals as most other parasitic genera of *Blastocladiomycota*, e.g. *Catenaria*, *Coelomomyces*, and *Polycarum* (Johnson *et al.* 2006).

There are both similarities and differences between the *Haematococcus* pathogen and *Physoderma*. Zoospores of *Physoderma maydis*, a parasite of corn, shed their flagella and go through an amoeboid phase before they penetrate the host tissue (Lange & Olson 1980). The *Haematococcus* pathogen could thus represent a descendant from a *Physoderma*-like ancestor that has forgone the flagellated phase. *Physoderma* is different from the *Haematococcus* pathogen in having both an epibiotic sporangial stage and endobiotic sporangial stage. Resting spores are produced epibiotically in the *Haematococcus* pathogen *versus* endobiotically in *Physoderma*. Thus, the resting spores of the *Haematococcus* pathogen may not be homologous to those of *Physoderma*, which have the distinctive thick-walled, ovoid shape of most *Blastocladiomycota*, as well as a flattened side (Sparrow 1960). Another major characteristic of the *Blastocladiomycota* is the nuclear cap of ribosomes in the zoospore. Whether this nuclear cap exists in the amoeboid swarmers of the parasite remains to be determined, as do many other morphological details (vestigial flagellum, cell wall, *etc.*) available only at electron microscope resolution. Nevertheless, the *Haematococcus* pathogen closely resembles *Physoderma* in terms of sporangial morphology (monocentric, epibiotic), and in having a limited rhizoid emanating from a single point on the sporangium.

The question of how this *Physoderma*-related parasite ever found its way into our algal cultures is unresolved, considering that no other member of this clade has been reported as a parasite of microalgae. Its desiccation-resistant resting sporangia, not uncommon among chytrids (Gleason *et al.* 2007), may allow it to spread *via* air. The microalgal host *Haematococcus*, often found in small or temporary water ponds, is remarkably resistant to desiccation, and may resume vegetative growth upon rehydration after long periods of drought. It is likely that our chytrid, a parasite of other algal or even non-algal species, has adapted to the life cycle of *Haematococcus* to enable colonization of this new ecological niche. There are many reports regarding the ability of chytrids to infect and live on more than one microalgal host. Some of the chytrids were found to be species-specific (Holfeld 1998), whereas others seem rather promiscuous (Gromov *et al.* 1999). The *Haematococcus* parasite, when placed in cultures of various strains of *Haematococcus*, readily infected every one of them. The issues of specificity, regarding different species of microalgae, and other growth and development features of this new isolate are currently under investigation.

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