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A new Oomycete species parasitic in nematodes, *Chlamydomyzium*
Dictyuchoides sp. nov.: developmental biology and phylogenetic studies.

Gordon W. Beakes¹, Sally L. Glockling² and Timothy Y. James³

¹School of Biology, Newcastle University, Newcastle upon Tyne, NE1 7RU, U.K.

² Department of Biological Sciences, Northern Illinois University, Dekalb, IL 60115
USA/Genome Damage and Stability Centre, University of Sussex, Falmer, Brighton, East
Sussex BN1 9RQ UK *¹

³ 830 North University, 1008 Kraus Natural Sciences, Dept. of Ecology and Evolutionary
Biology, University of Michigan, Ann Arbor, MI 48109 USA

*e-mail: gordon.beakes@ncl.ac.uk (corresponding author); sally@glockling.com ;
tyjames@umich.edu*

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¹ Present address 135 Brodrick Road, Eastbourne, BN22 9RA UK

Abstract

The genus *Chlamydomyrium* was a little studied holocarpic genus of aschelminth parasites, with uncertain phylogenetic and taxonomic position. A new holocarpic oomycete, *Chlamydomyrium dictyuchoides* with refractile cytoplasm and distinctive dictyuchoid pattern of spore release is described. The new species was observed infecting rhabditid nematodes from herbivore faeces and compost from diverse geographical locations. The maximum likelihood tree of sequences of the small ribosomal subunit (SSU) gene placed this isolate on the tree before the main saprolegnialean and peronosporalean lines diverge. A second undescribed *Chlamydomyrium* sp., which has direct spore release, also branches early in the oomycete phylogeny but is not closely related to *C. dictyuchoides*. Infection by *C. dictyuchoides* is initiated by the encystment on the host cuticle of biflagellate zoospores which germinate to form penetration hyphae. It produces sparsely branched, constricted, sausage-like thalli, which eventually occupy most of the body cavity of infected hosts. Walled primary cysts differentiate throughout the thallus in a characteristic dictyuchoid fashion and each cyst releases a single zoospore via an individual exit papillum. Thalli may form thick-walled, stellate, resting spores in uniseriate rows at later stages of infection, but without formation of antheridial compartments. The presence of an ooplast-like vacuole and thick multi-layered wall suggest these spores are parthenogenetically formed oospores. The features of this new species are compared with other *Chlamydomyrium* species, including an undescribed (but sequenced) isolate (SL02), originally isolated from Japan, as well as *C. anomalum* and *C. oviparasiticum*. *Chlamydomyrium* as currently constituted is a paraphyletic genus that is part of a group of phylogenetically problematic clades (which also include the Leptomitales and Rhipidiales) which makes it of phylogenetic interest.

Keywords: *Chlamydomyrium*, holocarpic, Leptomitales, parasite, nematode, oomycete, phylogeny, Rhipidiales, ultrastructure, SSU rDNA tree, zoosporogenesis

Highlights:

1. SSU phylogenetic analysis shows *Chlamydomyrium* species form closely related but paraphyletic clades towards the base of the saprolegnialean oomycete line, close to the Leptomitales s.lat. clade.
2. A new holocarpic oomycete parasite of rhabditid nematodes is described, which has a unique dictyuchoid pattern of spore formation and parthenogenetically formed oospore-like resting spores typical of the genus *Chlamydomyrium*.
3. This species was unusual in its dictyuchoid mode of spore release and its ability to completely degrade the nematode cuticle.
4. The widespread occurrence of holocarpic parasites of nematodes and other invertebrates within both basal oomycete clades and in early diverging clades within the main saprolegnialean and peronosporalean lines implies this life-style is evolutionarily deep rooted within the oomycetes.

Introduction

There are a number of holocarpic genera, such as *Chlamydomyzium*, *Gonimochaete*, *Haptoglossa* and *Myzocytiopsis* within the oomycetes, that are exclusively parasites of nematodes and rotifers (Aschelminths). Most of these genera appear to be obligate parasites in nature, with little or no saprotrophic ability, although a few have been artificially grown in liquid media (Glockling and Dick, 1997; Glockling and Beakes, 2006b). The genus *Chlamydomyzium* was erected by Dick (1997) and currently has six described species, all of which are parasites of nematodes or rotifers and their eggs (Dick 2001). The thick-walled resting spores, which form abundantly within the thalli, are the key morphological feature that defines this genus and which Dick (1997, 2001) considered to be pleurotic endospores (chlamydospores). Although Dick (2001) placed *Chlamydomyzium* in the Myzocytiopsidaceae along with the majority of other nematode parasites, he considered it to be an anamorphic genus that had morphological characteristics that were intermediate between his newly defined Myzocytiopsidaceae and Haptoglossaceae families.

There appears to be considerable variation within the genus *Chlamydomyzium* regarding the mode of asexual sporogenesis. In the type species *C. anomalum*, primary cysts (aplanospores) are formed within the sporangium and released via short, rounded, evacuation tubes. These primary cysts then release biflagellate zoospores which locate and infect nematodes (Barron, 1976; Glockling and Beakes, 2000a). *Chlamydomyzium internum* and *C. aplanosporum* also form aplanospores within the rotifer host body (Glockling and Dick, 1997). In contrast, the undescribed isolate SL02, which was isolated from rhabditid nematodes from Japan and temporarily brought into culture (Glockling and Beakes unpublished), discharges fully differentiated zoospores. *Chlamydomyzium oviparasiticum*, which was originally described from rotifer eggs by Barron (1989), has an achlyoid pattern of spore formation with primary spores encysting at the mouth of the sporangial exit tube immediately following their release

into a transient vesicle (Glockling and Beakes, 2006b). Secondary type zoospores later emerge and act as the infective agents. *Chlamydomyrium sphaericum* also appears to release a partially differentiated mass of zoospores directly from the sporangium as a slow moving bundle of spores (Glockling, 1994; Dick, 2001). Finally, the infective spores of *Chlamydomyrium* spp., in common with most other holocarpic nematode parasites, attach to their host cuticles either by means of their encysting zoospores or adhesive aplanospores (Glockling and Beakes, 2000a).

In this paper we describe a new *Chlamydomyrium* species with a distinctive dictyuchoid pattern of spore release, involving a true network of walled cysts. We compare the new species with mostly unpublished ultrastructural information we have gathered for other species in the genus. The placement of *Chlamydomyrium* spp. within the oomycetes is based upon phylogenetic analysis of 18S ribosomal subunit gene sequences, for which there is a dataset for a wide range of genera (Lara and Belbahri, 2011). We discuss the phylogenetic implications of this work in the context of the overall evolutionary phylogeny of the oomycetes.

Materials and Methods

Isolation of C. dictyuchoides

A collection of horse dung taken from a farm in Rockford, Illinois, USA, in October, 2002, was diluted into a slurry with distilled water. Drops of the slurry were pipetted onto 3 Petri plates of cornmeal agar, allowed to dry and later irrigated with distilled water to cover the surface. The plates were regularly scanned using low magnification (x10 objective) of a Reichert Zetopan microscope. Rhabditid nematodes in the inoculum bred in the Petri plates and the infection became apparent after 2 weeks. Transferral of infected nematodes into fresh cornmeal agar plates prolonged the infection for several months. A second and third isolate

was made from horse dung from the same location in May and September 2003. Selected infected nematodes were removed from the plates for further microscopy studies. A very similar infection was found in June, 2009, infecting nematodes in rabbit and cow dung collected from a field at Chalk Farm, Willingdon, East Sussex. Infected specimens from this later UK collection were used for phylogenetic analysis. Other species of *Chlamydomyzium* (*C. anomalum*, *C. oviparasiticum* and an undescribed species from Japan) were isolated from samples of voided herbivore faeces collected at various locations in Japan and the United Kingdom between 1995 and 2002 (see Beakes and Glockling 1998, Beakes et al. 2006).

Molecular phylogenetic analysis of Chlamydomyzium dictyuchoides

The phylogenetic relationship of *C. dictyuchoides* to other oomycetes was determined by analysis of the 18S ribosomal RNA gene. DNA was extracted from ~20 infected nematodes in a 2X CTAB buffer containing 2% hexadecyltrimethylammonium bromide, 1.4 M NaCl, 20 mM Na₂EDTA, 100 mM Tris-HCl (pH 8.0). Cells were disrupted by grinding using a micropestle and vortexing the sample with 300 µm glass beads. The DNA was precipitated overnight with isopropanol at -20 °C and resuspended in 50 µl ddH₂O. Because the extracts contained contaminants and the *C. dictyuchoides* 18S RNA gene was long (>4,500bp) due to the presence of 5 introns, primers based on sequences from oomycetes were developed to specifically amplify *C. dictyuchoides* from the nematode extracts. An initial PCR was conducted using the universal forward primer SR1R (Vilgalys and Hester 1990) in combination with the oomycete-specific primer SRSt-1R (5'-AGTTTATGGTTAAGACTACGATG'3') and using the enzyme ExTaq (Takara) with the following PCR cycles: 94° C for 3 min; 35 cycles of 94° C for 30 s, 54°C for 30 s, 72°C for 1.5 min; and a final extension of 72°C for 7 min. Sequences for this region were used to develop primers specific for *C. dictyuchoides* (intron targeted), which were used to amplify

the remaining portion of the 18S gene. The following PCR primers were used to assemble the overlapping sequence: SR3.1-F (5' AGGGACAGTTGGGGGTATTC-3') + SR-Cd-R (5'-TTCCCTTTATGGCATCTTCG-3') and SR-Cd-F (5'-CAATTTGGGATACATTCTCATCA-3') + SR6.1 (Parrent and Vilgalys 2009). The amplifications were conducted with the following PCR cycles: 94° C for 3 min; 35X 94 ° C for 1 min, 50° C for 30 s, 72°C for 1 min; 72°C for 7 min. Amplicons were purified using ExoSAP-IT (USB Corporation) and sequenced on an ABI 3730 at the U. Michigan DNA Sequencing Core.

The *Chlamydomyrium* sequences were combined with 18S Oomycete and stramenopile sequences from GenBank. Sequences were manually aligned in MacClade 4 (Maddison and Maddison 2002). Ambiguously aligned regions and introns were excluded from the phylogenetic analysis, leaving 1,657 characters. The best fitting model of evolution was chosen using jModeltest (Posada 2008). The phylogeny was estimated using 100 heuristic searches to find the maximum likelihood tree using the GTR model with a proportion of invariant sites and gamma distributed rate heterogeneity implemented in Garli-2.0 (Zwickl 2006). Support for nodes was evaluated using 1,000 bootstrap pseudo-replicates.

Microscopy

Specimens were placed in a drop of water on a glass slide, covered with a cover slip and photographed using a Nikon Eclipse E600 microscope fitted with a Digital Eclipse DXM1200 camera. Photographs were taken using both phase contrast and differential interference contrast (DIC) optics. Video sequences were also made using a Zeiss Axiovert 135 inverted microscope fitted with a Dage MTI 72 digital camera and connected to a Sony SVO-9500MD video recorder. Image sequences were recorded using a Hamamatsu Orca 100 digital camera using Image Pro Plus capture software (Media Cybernetics Ltd). For transmission electron microscopy, specimens were fixed in glutaraldehyde and osmium

following the protocols described in Beakes and Glockling (1998) and Glockling and Beakes (2006a).

Results

Taxonomy

Chlamydomyzium dictyuchoides SL Glockling sp. nova

Etym.: sporulation being *Dictyuchus*-like

Typus: Slides deposited at BPI herbarium, USA, accession number: 842301.

Thallus elongate cylindrical, up to 500 μm x 8-15 (mostly 10-12) μm , unbranched or dichotomously branched, rounded at the ends. Primary cysts 5 μm in diameter, spherical to hexagonal, developing inside the sporangium. Nematode cuticle dissolved prior to sporulation. Zoospores 6.0-10 μm , ovoid, biflagellate, with laterally inserted flagella, emerging individually from the cysts through a short, narrow papillum, leaving a sporangial net; remaining outside the sporangium for several minutes before dispersing. Zoospores flattening against a nematode cuticle and encysting to initiate infection. Secondary cyst spherical, 6-8 μm diameter. Resting spores 10-14 μm diameter, very thick-walled with regular protrusions, formed in a row inside the nematode cuticle, germination not observed.

Molecular Phylogeny of Chlamydomyzium

A maximum likelihood phylogenetic analysis of 18S rRNA sequences for the new *Chlamydomyzium* species, *C. dictyuchoides*, and a second undescribed species from Japan (illustrated in Figs 52-58) is shown in Fig. 1 in relationship to a selection of other oomycete genera, which are representative of the main taxonomic clades. In particular, we have included a selection of sequence data for known aschelminth and crustacean parasites in this tree (asterisked Fig. 1), An un-named *Haptoglossa* isolate (SL20) is representative of the

monotypic order, the Haptoglossales (Dick 2001) and *Myzocytiopsis glutinospora* is representative of Dick's (1997) Myzocytiopsidaceae. As can be seen from Fig. 1, *Haptoglossa* forms the earliest diverging clade in the oomycete tree along with *Eurychasma*, although the two genera are separated by long branches. *Myzocytiopsis glutinospora* and the well known insect parasite *Lagenidium giganteum*, (which Dick 2001 considered to be the type and, in his treatment, the sole representative of his revised genus *Lagenidium*) both diverge early within the Peronosporales s.lat. (Fig. 1).

This analysis reveals that the two *Chlamydomyrium* isolates (*C. dictyuchoides* and SL02) do not form a monophyletic clade (Fig. 1). However, bootstrap support values (78%), suggest that *Chlamydomyrium* spp. share a common ancestor with most other non-marine oomycetes and that they are distantly related to two other holocarpic nematode infecting genera *Haptoglossa* and *Myzocytiopsis*, with which *Chlamydomyrium* was linked by Dick (2001). In the current analysis, the two *Chlamydomyrium* spp. and *Sapromyces* appear to diverge before both the main peronosporalean and saprolegnialean clades (Fig. 1). Together they form part of a non-monophyletic grade of taxa which include the Rhipidiales (as represented by *Sapromyces*), Leptomitales (as represented by *Apodachyla brachymena*) and the Atkinsiellales (as represented by *Atkinsiella dubia*) that are often problematic in their placement and therefore have been bracketed together in Fig. 1.

Morphology of C. dictyuchoides.

Chlamydomyrium dictyuchoides was observed infecting several species of bacterivorous nematodes in the Rhabditidae (Figs 3, 9, 10). Infection started with the settling and encystment of secondary-type zoospores (with ventrally inserted flagella - Fig. 2) on the nematode cuticle (Figs 2, 3). Infection was often initiated in the vicinity of the nematode

mouth (Figs 3, 4) and anus. The cysts quickly germinated to form a germ tube. In some infections a prominent appressorium-like structure was observed (Fig. 5), but in others they appeared to directly penetrate the host cuticle (Figs 4, 6). The residual cyst cytoplasm became increasingly vacuolated as the germ tubes developed (Fig. 6). In germinating cysts (Fig. 6) and young thalli (Figs 7-8), these vacuoles often contained small granules of varying optical density, which were in Brownian motion.

The enlarging thalli were sparsely branched cylindrical structures, which showed regular constrictions which were never occluded (Figs 9, 12). Like many *Haptoglossa* spp., the thallus in this *Chlamydomyzium* appeared to have a homogeneously dense, granular, refractile and almost “glassy” looking cytoplasm (Figs 9, 10, 14, 15). As with *Haptoglossa*, it was not uncommon to observe multiple infections within the same nematode host (Figs 10, 11, 14). Mature thalli often occupied almost the entire host body cavity (see Fig. 14) as reported in other *Chlamydomyzium* species (Figs 59, 66). However, in contrast to most *Myzocytiopsis* spp. (Glockling and Beakes 2006a) and previously described *Chlamydomyzium* spp. (e.g. *C. anomalum*, the genus type - Fig. 59, and *C. oviparasiticum* - Fig. 66), the thalli of this new species were never compartmentalised by septa (Figs 9-21) even in thalli producing resting structures (Fig. 13).

As the thalli enlarged, a number of large, spherical, vacuoles began to appear in the cytoplasm (compare Figs 15 and 16). These vacuoles were initially concentrated towards the centre of the thallus (Fig. 16), but coalesced and expanded until they extended almost across the entire width of the thallus (Figs 11, 18-21). In young thalli these vacuoles frequently contained inclusion bodies, which had a glistening refractile appearance under DIC optics (Fig. 17) and which were not observed at later stages of development (Figs 20, 22). As the vacuoles enlarged they also became aligned in uniseriate, bead-like, rows along the axis of

the thallus (Figs 18-20, 21a, b). It is assumed this vacuole system eventually delimited the spore initials (Figs 21a, b, 22), which formed a network of polygonal primary aplanospores throughout the thallus following cleavage (Figs 12, 23). Under ordinary phase contrast optics, the optically translucent nuclei were also observed scattered amongst the somewhat similar vacuoles (Fig. 22). In early stages of thallus development the host nematode cuticle still remained intact (arrowed in Figs 14, 16, 20, 23) but, by the time spore formation was complete, it had often been totally (Fig. 12) or partially (Fig. 13) degraded. This is another unusual feature of this new species and obviates the need for discharge tubes that are found in most nematode infecting species, including some previously described *Chlamydomyzium* spp. (Figs 54, 59, 62, 66). Following aplanospore formation, the thalli acquire a scalloped margin which is accentuated by the unusual absence of host cuticle (Fig 12).

Mature aplanospores (Figs 23) released their contents individually (Figs 33, 34) via short exit papillae, well shown in Figs 37, 38, which rupture the original thallus wall (Figs 32-34, Fig. 38 arrowed). Thin section profiles also confirmed that the original host cuticle had completely disappeared by this stage (Fig. 38). Newly emerged “protozoospore” initials were more or less spherical in shape (Figs 33, 34) and, as in *Dictyuchus*, take several minutes to acquire flagella (Fig. 35). As the zoospores matured they became more ovoid or lens shape with a ventral nuclear spot (Fig. 39). Video microscopy also revealed that, just before the zoospores swam away, they started to rock gently as their newly assembled flagella began to beat (this stage is shown in Fig. 34). Zoospore emergence did not usually occur synchronously throughout the entire thallus but often began locally (Figs 32) and spread gradually throughout the sporangium, until all of the primary cysts had excysted (Figs. 33, 34, 36). The polygonal net-like array of residual cyst walls (Figs 34, 36, 38) gave the mature thalli their distinctive net-like (dictyuchoid) appearance (Figs 34, 36).

Ultrastructural features of morphogenesis in C. dictyuchoides

Transmission electron microscopy (TEM) of pre-spore thalli showed the dense cytoplasm contained well dispersed nuclei surrounded by a zone of perinuclear mitochondria, with small scattered vacuoles beyond (Fig. 24). The newly formed aplanospores had a centrally located nucleus, although there did not appear to be the well defined zonation of mitochondria and dense body vesicles (DBV) observed in *C. anomalum* (compare Figs 25, 27 with Fig. 64). The prominent nucleus-associated Golgi bodies were associated with large numbers of electron transparent vesicles, which accumulated in the cytoplasm adjacent to the distal cisternae (Figs. 25, 26). The Golgi were also surrounded by vesicles containing flimmer tubules (Figs 26, 28), which eventually decorate the anterior flagellum, and which, as the aplanospores mature, become peripherally concentrated (Figs 29, 31). Also, by the time the aplanospores had become fully mature, Golgi activity had reduced and the nucleus was associated with a prominent cluster of large ($< 1 \mu\text{m}$ diameter) vesicles with a moderately electron dense homogenous matrix (Figs 27, 29). These vesicles appear similar to K-bodies documented in many other oomycetes (Lehnen and Powell 1989). The differentiated aplanospores also contained scattered electron-dense lipid bodies and DBV (Figs 25-27), which had a characteristically reticulate in appearance in mature aplanospores (Fig. 27), but without any sign of the “finger print” lamellae inclusions that are associated with this vesicle fraction in many other oomycetes (Beakes 1989). The mature aplanospores also contained smaller ($< 150\text{-}300 \text{ nm}$) structured encystment vesicles (Fig. 28). The nucleus-associated kinetosomes appeared to be arranged more or less at right angles to each other (Fig. 30). They were associated with a strap of electron-dense material and microtubules, but an intervening striate fan was never observed in this species (Fig. 30). The complete rootlet structure was not determined, but we did observe an obliquely sectioned octet rootlet that has been reported in many other oomycetes (e.g. Beakes 1989). Mature cysts within the thallus often appeared

hexagonal in shape (Fig. 27) as a result of being closely packed. The thin (ca 100nm) uniformly dense walls of adjacent cysts (Figs 27, 29, 31) were often indistinguishable and the only space between them was at the corners of adjoining cysts (Fig 27).

Excysting spores show an accumulation of vesicles in the region of exit papillum formation (Fig. 37) where the thallus wall became ruptured. Some zoospores failed to disperse away from the host (Fig. 36) and encysted beside the empty sporangial net to which they still remain attached (Fig. 41 arrows). Initially, a thin electron-dense wall was secreted around the encysting zoospore (Fig. 40) which subsequently became thickened (Fig. 41). Pads of fibrillar material were observed between the encysted secondary spores and the outer thallus wall (Fig. 41 arrows). These secondary cysts quickly formed a large vacuole (Fig. 41) and the DBV reverted to their typical vegetative morphology, with predominantly electron opaque contents with an elliptical electron dense region (Figs 40 asterisked).

Resting spore formation and fine-structure in C. dictyuchoides

Thalli containing uniseriate chains of thick-walled resting spores (Figs 13, 43) were observed less frequently than those with aplanospores and the early stages of resting spore formation were only rarely observed (Fig. 42). Resting spores were more frequently observed as the infection began to decline and their formation could be enhanced by keeping cultures at 4°C for a few days. Unlike resting spore formation in *Myzocytiopsis spp.* (Glockling and Beakes 2006a), regular septation of the thallus did not appear to precede resting spore formation in this species. The onset of resting spore formation was indicated by the appearance of a chain of ovoid spore initials (<15 µm diameter) that formed internally within the cylindrical thallus (Figs 42, 43). Each spore initial was separated by a short intervening segment of cytoplasm (Fig 42 arrowed). Young resting spores had a less granular centre where the nucleus or nuclei may reside (Figs 44 asterisked, 35). As the resting spores

matured they became angular in shape and acquired a refractile central (or slightly eccentric) vacuole (Fig 14, 43 asterisked) and the intervening cytoplasm apparently disintegrated. In maturing resting spores the vacuoles progressively enlarged until they contained just a single eccentric “ooplast-like” globule surrounded by apparently homogenous “glassy” cytoplasm (compare Figs 46 and 49). The resting spore wall appeared papillate as it acquired its final angular/stellate form (Figs 46, 49).

Under the TEM these resting spores generally did not fix particularly well and their appearance proved rather variable (compare Figs 47 and 50). The cytoplasm contained lipid globules, and dense, but poorly preserved, ooplast vacuoles (Figs 47, 50, 51). In some spores the central region appeared almost free of organelles (Fig. 50), which may represent the location of the nucleus or nuclei (Fig. 50). The maturing resting spore walls were also somewhat variable in appearance (Figs 47, 48, 50, 51). In some spores an outer electron dense tripartite outer layer, similar to that described in many oospores, was visible (Fig. 48 arrowed). The thick papillate region of the wall was homogeneously granular except for pockets of dense granular material within the stellate projections (Fig. 48 asterisked). In other spores, the outer electron-dense layer was apparently absent and the inner wall appeared to have a more uniformly electron-dense appearance with a distinctly lamellate sub-structure (Figs 50, 51). However, the slightly more electron-dense homogeneous inclusions in the stellate projections could still be discerned at higher magnifications (Fig. 51 asterisked).

Morphology and ultrastructure of Japanese Chlamydomyzium isolate SL02

A Japanese isolate (SL02) of a nematode infecting species originally identified as a *Myzocytiopsis* spp. was brought into laboratory culture in the early 1990s using the methods described by Glockling and Dick (1997). Preliminary unpublished 18S gene sequence data

showed this isolate was more closely related to *Saprolegnia* rather than to *Pythium* and *Phytophthora*, where a lagenidiaceous *Myzocytiopsis* species would be expected to align. Subsequently, as more sequence data of holocarpic nematode infecting species was acquired, it was apparent that this species clustered quite separately in SSU trees from either *Myzocytiopsis* and *Lagenidium* or *Haptoglossa* (Fig. 1) and it was assigned to the genus *Chlamydomyziium sensu lato* (Beakes et al. 2006).

In order to observe the ultrastructural details of this isolate, *Rhabditis* nematodes were subsequently re-infected and some of the ultrastructural features are summarized in Figs 52-58. This species produced swollen segmented thalli (not shown but similar to the thalli illustrated in Fig. 66). In thin section the young thalli contained a prominent central vacuole system containing large electron-dense globules, which were often associated with the tonoplast membrane (Fig. 52). The nuclei were distributed around the thallus periphery (Fig. 53), together with abundant endoplasmic reticulum and various membrane bound vesicle fractions (Fig. 53 asterisked). As the thalli matured, the perinuclear distribution of mitochondria became more evident (Fig. 56) and electron-dense lipid bodies were more apparent. The maturing thalli formed slender exit papillae (not illustrated but similar to that shown in Fig. 66) which ruptured the still intact nematode cuticle (Fig. 54 arrowed). The apex of the developing papillae had very dense cytoplasm packed with large numbers of small vesicles (Fig. 55). Motile zoospores were eventually differentiated within the thallus and released directly into the environment through the dissolution of the papillum apex. These zoospores encysted but it was not established whether they release a secondary infective zoospore (as shown in 71) or whether they directly encyst on a new host and initiate a new infection cycle.

However, in addition to forming zoospores, some thalli formed a series of spherical smooth walled resting spores (ca 15-20 μm diameter), without any obvious formation of antheridial compartments (Fig. 57). These resting spores had dense lipid-filled contents and a thick smooth wall (Fig. 57). The main wall was moderately electron dense and delimited by a thin, more electron-dense outer layer (Fig 58 arrowed), which was partially surrounded by a layer of electron dense granular material (Figs 57, 58 asterisked), which had possibly been deposited on the spore from the surrounding cytoplasm.

Thallus development and ultrastructure of Chlamydomyzium anomalum

The general morphology and limited ultrastructural information on the aplanosporic type species of *Chlamydomyzium*, *C. anomalum*, is illustrated in Figs 59-64. In this species the individual sporangial compartments become delimited by septa and swell to produce a chain of ovoid thalli with characteristically broad, domed, exit papillae (Figs 59, 62, 63). The cytoplasm of pre-spore thalli contain peripherally located nuclei, which even at this stage are surrounded by an array of perinuclear mitochondria, beyond which are the lipid globules and DBV (Fig. 60). In maturing thalli, the uninucleate aplanospore initials become delimited by a vacuolar system (Fig. 61). Following cleavage the whole thallus becomes full of rounded, walled, uninucleate, aplanospores (Figs 62-63). Each walled aplanospore contained a single, more or less centrally located nucleus, which is still surrounded by perinuclear mitochondria and lipid globules, and then an outer zone of reticulate DBV (Fig. 64). The peripheral distribution of the DBV in the young aplanospores (Figs 63, 64) has not been noticed in other species. Barron (1976) reports that the aplanospores of this species can transform into secondary zoospores, although we did not observe packets of flimmer tubules, K-type or encystment type vesicles in the developing thalli of our isolate, all of which are specific zoospore marker organelles. This raises the possibility that, at least in some isolates,

aplanospores may be the primary infective spores. As noticed in *C. dictyuchoides*, there is still no evidence of finger-print lamellations associated with the DBV (Fig. 64). As infections progress, some thalli form large numbers of uniformly spherical resting spores, which in this species appear relatively thin walled (Fig. 65) but for which we have no ultrastructural information.

Thallus development and ultrastructure of Chlamydomyzium oviparasiticum

The development of this species has already been described (Glockling and Beakes 2006b) but relevant details will be included here to facilitate comparison with other *Chlamydomyzium* species. *Chlamydomyzium oviparasiticum* also produces extensive thalli, which become divided by septa into a large number of individually papillate sporangial segments (Fig. 66). The young thalli contain abundant small vesicles, with very large DBV inclusion bodies (Fig. 67). As the thalli mature, the perinuclear distribution of mitochondria again becomes apparent, as are the interspersed packets of flimmer tubules (Fig. 68). This species is characterised by the presence of a cap of fibrillar material in the apex of the discharge papillum (compare Fig 69 asterisked with Fig. 55). It has vesiculate release of its primary spores, which rapidly encyst and form achlyoid clusters (Fig. 70). These primary spores excyst (Fig. 71) in a similar way to that described here for *C. dictyuchoides*, in which the discharged protoplasts take several minutes to acquire their final bean shaped morphology and flagella (Fig. 71) before they swim off. As in other members of this genus, *C. oviparasiticum* also produces thick-walled resting spores (Fig. 72), without apparent involvement of antheridia. The resting spores are elongate to ovoid rather than spherical or stellate. The resting spore contents again fix rather poorly but contain DBV and lipid as described in other species (Fig. 73). The thick wall is again multi-layered, with an outer lamellate layer (Fig. 74 arrowed) and thicker homogeneous inner layer (Fig. 74 asterisked).

Discussion

Molecular Phylogeny

The refractile cylindrical thalli of this new species closely resembled that of another holocarpic aschelminth parasitic genus, *Haptoglossa*. However, the development of constricted and often branched thalli, together with the lack of gun cells, indicated it was a novel species. The subsequent finding of resting spores confirmed that this parasite was most likely an undescribed *Chlamydomyrium* spp. *Chlamydomyrium dictyuchoides* appears to be genetically quite divergent from another unidentified *Chlamydomyrium* isolate (SL02), which together with *Sapromyces* diverged just before the main saprolegniacean-peronosporalean species (Fig. 1). The undescribed isolate SL02 was originally isolated in Japan, and had initially been tentatively identified as a *Myzocytiopsis* species. It was maintained for a time in laboratory culture before being re-inoculated into new nematode hosts for subsequent TEM examination (described here for the first time) and sequencing (Beakes et al. 2006). In a later independent SSU rRNA analysis, Lara and Belbahri (2011) positioned our unidentified *Chlamydomyrium* SL02 isolate between *S. elongatus* and *Apodachlya brachynema* at the very base of the saprolegniacean lineage. A separate analysis (D and M Hudspeth unpublished sequence data) using the mitochondrial *cox2* gene for *C. oviparasiticum*, showed this species also clustered close to *Sapromyces*, but with this gene rooted at the base of the peronosporalean line. In another unpublished analysis (by Shigeki Inaba), which included isolates of the holocarpic keratinophilic and pollen infesting genus *Cornomyces* (which Dick 2001 tentatively placed in the Leptolegniaceae, with the Leptomitales), it was shown that this genus also formed a sister clade to the *Chlamydomyrium* SL02 isolate. An ongoing LSU study has also shown that *C. anamolium*, the genus type species, forms a clade close to *Haliphthoros*, outside of the main “crown oomycete” (as defined by Beakes and Sekimoto,

2009 and Beakes et al. 2012) clades (Spies, Lévesque and Glockling unpublished tree). From the current study and from these unpublished analyses, it is clear there is much diversity amongst the genera that appear to be basal to the main terrestrial lineages of oomycetes, and most of these will ultimately require taxonomic revision. However, until more genes have been included in the phylogenetic analyses and there has been far greater taxon sampling, the exact phylogenetic and taxonomic grouping of these clades remains problematic. For this reason we have identified this cluster of clades as problematic in Fig 1. However, there seems little doubt that these genera comprise some of the most primitive representatives of the main freshwater and terrestrial oomycete assemblages (Beakes et al. 2014). With the exception of the sewage moulds, such as *Apodachlya* and *Leptomitius* in the Leptomitales, most of the representatives of these early diverging “crown clades” are holocarpic and it is interesting that a significant proportion are parasites of either crustaceans (e.g. *Atkinsiella*) or invertebrates (such as *Chlamydomyzium* described here). The close morphological similarity between *C. dictyuchoides* described here and *Haptoglossa*, which forms the earliest branch of the oomycete tree, indicates this morphological pattern and life style may be representative of the earliest oomycetes.

However, as can be seen from Fig. 1, the genus *Chlamydomyzium* as currently recognised may be a paraphyletic genus and will need revision. However, it is also clear from molecular phylogenetic studies that the morphologically similar genus *Myzocytiopsis* s.str. (Dick 1997) is, as originally thought (Karling 1982), closely related to *Lagenidium* and *Pythium* in the Peronosporales (Fig 1). There is no support for the class Myzocytiopsidales and family Myzocytiopsidaceae erected by Dick (1997, 2001) to encompass these holocarpic parasites of aschelminths and this order and family have been rejected in a recent revised taxonomic revision of oomycetes (Beakes et al. 2014).

Morphology and cytology

Chlamydomyzium dictyuchoides is unique amongst holocarpic nematophagous oomycetes in the formation of aplanospores which subsequently released spores in a dictyuchoid fashion. Other unusual features are its sparsely constricted and branched coenocytic thalli, the formation of resting spores without septation and antheridial compartment formation and the dissolution of the host cuticle. Most previously described nematophagous genera, including other *Chlamydomyzium* spp., *Haptoglossa*, *Gonimochaete* and *Myzocytiopsis*, all form either aplanospores or zoospores that are discharged from the mature thallus via a short exit tubes or papilla (Karling 1981; Glockling and Beakes 2000a). There are exceptions, including the rotifer parasites *Chlamydomyzium internum* and *C. aplanosporum* (Glockling and Dick 1997), and the nematode parasite, *Haptoglossa erumpens* (Glockling and Beakes 2000b). However, in these previously described species the aplanospores burst out from the sporangium as a result of the rupturing of both the sporangial wall and the host cuticle. The ability of this new species to rapidly and completely digest the tough outer cuticle of the host nematode facilitates and enables dictyuchoid sporulation. Dissolution of the host cuticle is presumably brought about by the parasite releasing digestive enzymes including collagenases and suggests this species may possess a novel array of proteolytic enzymes.

The sporangial net is termed a “true net” in some species in the saprolegnian genus *Dictyuchus* because the spores encyst within the sporangium and planonts subsequently emerge individually from the cysts, leaving a network of empty cyst (Padgett and Seymour 1974; Dick 2001). A similar mode of release involving a true sporangial net formation is also reported in the monotypic keratinaceous genus *Aphanodictyon*, isolated from horse hoof (Huneycutt, 1948) which has been placed in the Leptolegniellaceae (order Leptomitales). Although the cysts of *C. anomalum* do not form a sporangial net, there was some similarity

between the ultrastructure of the fully cleaved sporangia of *C. anomalum* and *C. dictyuchoides* (compare Figs 25 and 64). In both species the cysts were packed closely and evenly within the sporangium. However, in *C. anomalum*, the discharge apex ruptures, allowing the aplanospores to spill out of the thallus en masse. Other species, such as the undescribed isolate SL02, form fully differentiated flagellate primary zoospores within the thallus, which swim away after discharge. However, *C. oviparasiticum* has *Achlya*-like sporulation, with the discharged aplanospores forming a cluster of encysted spores at the tip of the exit tube (Glockling and Beakes, 2006b). Therefore, as in other holocarpic genera such as *Haptoglossa* (Beakes and Glockling 1998; Hakariya et al. 2007; Glockling and Serpell 2010), different patterns of sporogenesis seem to occur in completely unrelated eucarpic and holocarpic families. This indicates that the same subtle morphogenetic changes that bring about these different patterns of sporogenesis have evolved several times within the oomycete lineage. In contrast, sporulation patterns in the Peronosporales order show much less variation, although zoospores may be formed either within the sporangium or within an extrasporangial vesicle (Beakes 1994). Interestingly, *C. oviparasiticum* also shows this characteristic vesiculate spore release (Glockling and Beakes 2006b)

However, the comparative ultrastructural details recorded in this paper for *C. anomalum*, *C. dictyuchoides*, and *C. oviparasiticum* have some similarities and these species all have some saprolegnialean traits or features which appear to unite them and affiliate them more closely with the saprolegnialean lineage of oomycetes. For instance, the diplanetic pattern of zoosporogenesis in *Chlamydomyrium* spp. is shared with *Atkinsiella* and most genera in the Leptomitales s. lat. (Karling 1981; Dick 2001). *Chlamydomyrium dictyuchoides* and other members of this genus also have structured encystment vesicles (Fig 28) and K-type bodies (Figs 29, 53; see Lehnert and Powell 1989), which are found throughout the saprolegnialean lineage, including both the Leptomitales and Saprolegniales

(Beakes 1989). However, we never observed lamellations (fingerprint-like striations – Beakes 1989) associated with the DBVs in any of the *Chlamydomyzium* species we have examined ultrastructurally, even though these structures are reported in *Myzocytiopsis* zoospores (Glockling and Beakes, 2006a) and all genera of Leptomitales, Peronosporales and Saprolegniales so far documented (Beakes 1989). In this respect *Chlamydomyzium* is similar to other early diverging oomycetes such as *Haptoglossa*, *Haliphthoros* and *Olpidiopsis* (Beakes and Sekimoto 2009; Beakes et al. 2012). The other interesting ultrastructural characteristic that is common to all *Chlamydomyzium* spp., as documented in this account, is the occurrence of a well-defined perinuclear zonation of mitochondria in differentiating thalli (Figs 24, 56, 60, 68). This is also a feature of developing spores in *Haptoglossa* and *Haliphthoros* (Beakes et al. 2012), but has not been described in members of the Saprolegniales and Peronosporales orders and therefore appears to be a good morphological marker for the more primitive oomycete genera.

The main morphological feature that has been used to define the genus *Chlamydomyzium* has been the formation of resting spores, without evidence of sexual reproduction taking place. Dick (1995; 2001) interpreted these resting spores as chlamydospores. However, the TEM profiles of resting spores of *C. anomalum* (Figs 72-74), *C. dictyuchoides* (Figs 46-51) and isolate SL02 (Figs 57-58) presented here, all suggest the resting spores in this genus are more likely to be parthenogenetically formed oospores. However, gametangial meiosis and nuclear fusion have yet to be demonstrated, so this conclusion needs to be confirmed. There is also no evidence of periplasmic oogenesis in any *Chlamydomyzium* species, which again suggests this genus is more closely allied to the saprolegnialean line than to the peronosporalean line, where one of the defining morphological features is periplasmic oogenesis.

Conclusions

Combined ultrastructural and molecular studies of morphologically similar holocarpic oomycete parasites of aschelminths and crustaceans have revealed they are much more phylogenetically diverse than originally thought. It is now clear that *Chlamydomyzium* may be an ill-defined group diverging early in the evolution of oomycetes and representing great phylogenetic and morphological diversity. It is part of a number of poorly studied clades that are probably basal to the main saprolegnialean line, although this has still not been completely resolved. Studies on these invertebrate parasites give clues as to the likely nature of primitive oomycetes.

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Figure Legends

Fig. 1. Maximum likelihood tree based on 18S rRNA sequences. Numbers above nodes indicate bootstrap support obtained from 1,000 pseudo-replicates. GenBank numbers: *A. bisexualis* (M32705.1), *A. brachynema* (AJ238663.1), *A. dubia* (AB284575.1), *C. dictyuchoides* (Bank it 1493779), *C. sp.* SL02 (EU271965.1), *E. dicksonii* (AY032607.2), *H. milfordensis* (AB178868.1), *H. sp.* DGb (EU271966.1), *H. parasitica* (AY742752.1), *H. catenoides* (AF163294.1), *L. giganteum* (M54939.1), *L. caudata* (AJ238659.1), *M. glutinospora* (EU271964.1), *O. porphyrae* (AB287418.1), *P. infestans* (AY742761.1), *P. viticola* (AY742754.1), *P. aphanidermatum* (AY742755.1), *P. insidiosum* (AF442497.1), *R. apophysatus* (AF163295.1), *S. ferax* (AJ238655.1), *S. elongatus* (AB548399.1). The main taxonomic clades are also indicated.

Plate 1. Figs 2-8. Light micrographs of *C. dictyuchoides* under ordinary phase contrast optics illustrating early stages of infection.

Fig. 2 Zoospore showing typical secondary type zoospore morphology with ventrally inserted flagella (arrowed). Scale bar = 5 μ m.

Fig. 3 Recently infected head of a rhabditid nematode showing typically hemispherical encysted zoospores (arrowed) attached to the nematode cuticle. Scale bar = 10 μ m.

Fig. 4 Cyst directly germinating into the mouth region of the nematode before penetrating the gut cuticle (arrowed). Scale bar = 10 μ m.

Fig. 5 Encysted zoospore penetrating through the nematode cuticle after forming what appears to be a small appressorium-like structure (arrowed). Scale bar = 10 μ m.

Fig. 6 More advanced germinating cyst, showing vacuole containing the optically dense granule (asterisked) and germ tube which has penetrated into the host body cavity (arrowed). Scale bar = 10 μ m.

Fig. 7 Infective cysts (black arrows) on a host which already contains a young thallus (white arrow). Scale bar = 20 μ m.

Fig. 8 Higher power view of young sausage-shaped thallus, showing vacuoles with prominent translucent granules (arrowed). Scale bar = 10 μ m.

Figs. 9-13 Phase contrast light micrographs, illustrating often multiple maturing thalli of *C. dictyuchoides* within infected nematodes.

Fig. 9 Single young branched thallus inside the partially digested, but still bounded by an intact nematode cuticle (black arrow). Note both the constricted region (arrowed) and single side branch. Scale bar = 20 μ m

Fig. 10 Infected nematode with still fully intact cuticle in which several developing (pre spore stage) thalli have almost completely filled the entire body cavity. Scale bar = 20 μ m.

Figs 11-13 Details of maturing thalli, clearly showing clearly differentiated spore initials as well as larger vacuoles (arrowed in 11), which span the entire thallus width. The host cuticle is completely (as in 12) or partially dissolved (13 arrowed). Although the mature thalli are sparsely branched and constricted (arrow 12) they remain coenocytic. Scale bars = 20 μ m.

Fig. 14 Mature thallus that has differentiated into a uniseriate row of thick walled resting spores, still contained within original thallus (not nematode) wall. Scale bar = 20 μ m.

Plate 2. Figs 15-23. Details of thalli of *C. dictyuchoides* showing changes in cytoplasm as spores develop. All ordinary phase contrast except for 17, 20 and 21b which were taken using DIC optics.

Fig. 15 Cytoplasm in a young thallus, showing uniformly granular refractile cytoplasm. Scale bar = 5 μ m.

Fig. 16 Detail of branching region in young thallus, showing axial disposition of small cytoplasmic vacuoles (arrowed). Scale bar = 10 μ m.

Fig. 17 Detail of young thallus, showing refractile/reflexive inclusion bodies (arrowed) observed in axial vacuoles under DIC optics. Scale bar = 10 μ m.

Figs 18-20 Young pre-spore initial thalli, showing development and appearance of the axial row of large vacuoles (asterisked), whose appearance precede spore initial formation. Scale bars = 10 μ m.

Fig. 21 Two adjacent pre-cleavage thalli with large vacuoles and peripherally distributed paler nuclei as observed under both phase contrast (a) and DIC (b) optics. Scale bar = 15 μ m.

Fig. 22 Detail of maturing thallus cytoplasm in which spore initials are beginning to become apparent. The rounded nuclei (arrowed) are evenly distributed throughout the cytoplasm. Scale bar = 10 μ m.

Fig. 23 Post cleavage sporangium still within nematode cuticle (arrowed), showing individual walled aplanospores, and loss of vacuoles. Scale bar = 10 μ m.

Plate 3 Figs 24-31. TEM micrographs illustrating ultrastructural details of cytoplasm in developing thalli and aplanospores.

Fig. 24 Cytoplasm of a young pre-cleavage thallus showing nucleus (N), with surrounded by zone of perinuclear mitochondria (m). Scale bar = 2.0 μm .

Fig. 25 Post cleavage immature aplanospore (cyst) inside mature thallus showing central nucleus with kinetosome (arrowed), and associated Golgi dictyosome (G). Lipid (L), droplets, dense body vesicles (DBV) and mitochondria (m) are scattered throughout the cytoplasm. Scale bar = 1.5 μm .

Fig. 26 Detail of an intensely active Golgi dictyosome in an immature showing associated small vesicles (gv) as well as surrounding DBV, relatively small lipid droplets (L) and packets of flimmer vesicles (fv). Scale bar = 1.5 μm .

Fig. 27 Profile of a fully matured aplanospore (cyst) showing central nucleus (N) and associated cluster of electron opaque K-bodies (Kb) vesicles and more peripherally distributed dense body vesicles (DBV), lipid (L) and mitochondria (m). Scale bar = 1.5 μm .

Fig. 28 Inset showing a cluster of peripheral encystment vesicles (EV) which in this species has rounded profiles and a dense peripheral layer and smaller central core. Scale bar = 0.5 μm .

Fig. 29 Detail of the conspicuous ovoid K-body vesicles (Kb) which in this species have a homogeneous and moderately electron dense appearance and lack the crystalline inclusions often associated with this organelle fraction. A transverse section of flimmer tubule vesicle (fb) is also shown. Scale bar = 0.5 μm .

Fig. 30 Detail of nucleus apex in a mature aplanospores showing pair of kinetosomes oriented at almost right angles to each other (seen in LS k1 and TS k2). An oblique section of the associated octet microtubular rootlet is asterisked. Scale bar = 0.5 μm .

Fig. 31 Detail of a flimmer hair vesicle (fb) containing tripartite tubular hairs (TTH) (arrowed). Scale bar = 0.5 μm .

Plate 4: Figs 32-36. Phase contrast light micrographs illustrating the typical dictyuchoid pattern of zoospore release which is characteristic of this species.

Fig. 32 Three excysting protozoospores (arrowed) emerging through papillae, which have ruptured the outer thallus wall. Scale bar = 5.0 μm .

Fig. 33 Newly excysted protozoospores (arrowed), which remain attached to their exit papillae until their flagella form. Note the high degree synchrony in spore discharge in this region of the thallus. Scale bar = 5.0 μm .

Fig. 34 Mature discharged thallus, in which all aplanospores are now just prior to the zoospores swimming off. An optically translucent nucleus is asterisked. Scale bar = 5.0 μm .

Fig. 35 Inset showing higher power detail of protozoospore showing nucleus (asterisked) and developing flagellum (arrowhead). Scale bar = 2.0 μm .

Fig. 36 Fully differentiated thallus in which all the aplanospore contents have released their protoplasts and the zoospores have swum away, leaving typical net of spore walls behind. A narrow exit papillum wall ghost is visible (arrowed). A single protozoospore remains attached to sporangium (asterisked). Scale bar = 2.0 μm .

Figs 37-38 TEM profiles of an excysting spore and a fully discharged empty cyst wall. Note the ruptured thallus wall (arrowed) and vesicles (wv) in the papillum. Scale bar = 2.0 μm .

Fig. 39 Phase contrast photograph of newly excysted secondary zoospore that has now acquired characteristic reniform shape. The ventral nucleus is asterisked. Scale bar = 5.0 μm .

Fig. 40 Encysting secondary zoospore with electron dense outer cyst wall (arrowed). Note lipid (L) and dense body vesicles (asterisked) with electron dense and electron opaque regions. Bar = 2.0 μm .

Fig. 41 Encysted secondary zoospores, attached by means of a pad of fibrillar material (arrowed) to the outer cuticle wall (asterisked) of a nematode host. Note the nucleus (N) and enlarge vacuole (V). Scale bar = 2.0 μm .

Plate 5: Resting spore development in *C. dictyuchoides*.

Figs 42-45. Light micrographs showing the differentiation of resting spores within thallus under phase and DIC (45) optics.

Fig. 42 Two neighbouring thalli, showing endogenous development of ovoid resting spore initials which are smooth walled at this stage and contain a more or less centrally located translucent vacuole (asterisked). Each spore is separated by a narrow isthmus of thallus cytoplasm (arrowed). The host nematode cuticle is still intact in this specimen. Scale bar = 10 μm .

Fig 43 As resting spore development progresses the spores become more angular in profile, with a translucent central vacuole (asterisked). The host cuticle is still intact (white arrow). Scale bar = 10 μm .

Fig. 44 A higher power view of an immature (still smooth) resting spore central optically leucic structures (asterisked), which are possibly nuclei, surrounded by optically dense granules (white arrow). The scalloped nematode host cuticle is indicated by the black arrows. Bar = 2.0 μm .

Fig. 45 DIC image of similar aged resting spore, showing central structure (asterisked) surrounded by granules (arrowed). Bar = 2.0 μm .

Figs 46-51. Approximately corresponding DIC images and TEM of maturing and fully mature spores of *C. dictyuchoides*.

Fig. 46 DIC of maturing resting spore showing acquisition of stellate wall ornamentations (asterisked) and large ooplast vacuoles and lipid droplets in cytoplasm. Bar = 5.0 μm .

Fig. 47 Section through a maturing resting spore, showing large lipid droplets (L) and enlarging (but poorly preserved ooplast vacuoles (OPV) and pronounced wall ornamentations (asterisked), probably accentuated by shrinkage of the cytoplasm. Bar = 2.0 μm

Fig. 48 Detail of maturing resting spore wall, showing outer electron dense layer (white arrow) as found in oospore walls. The rounded spine ornamentations contain a pocket of more electron dense inclusion material (asterisked). Scale bar = 1.0 μm .

Fig. 49. DIC of fully mature resting spore showing stellate wall profile and large eccentric ooplast vacuole (OPV) surrounded by structureless glassy looking cytoplasm. Scale bar = 5.0 μm .

Fig. 50 TEM profile of not quite fully mature resting spore, with fully differentiated wall. The centre of this spore is poorly preserved nucleus and is surrounded by ooplast vacuoles (OPV), interspersed amongst lipid globules. Scale bar = 1.0 μm .

Fig. 51 Detail of maturing resting spore wall with thick striate wall layers (arrows) traversing the denser inclusion region (asterisked) of the rounded wall spine. A possible mitochondrion (m) and part of a densebody vesicle is shown in the peripheral cytoplasm. Scale bar = 1.0 μm .

Plate 6 Figs 52-58. Ultrastructural cytology of *Chlamydomyrium* isolate SL02.

Fig. 52 Section through a segment of young thallus, showing peripherally distributed nuclei (N) and large central vacuole system (V) containing large electron dense inclusions (DB). Scale bar = 2.0 μm .

Fig. 53 Detail of nucleus in a young pre spore initial thallus, showing perinuclear array of mitochondria (m) surrounding the nucleus, and droplets of lipid (L). Scale bar = 1.0 μm .

Fig. 54. Low power TEM of a developing thallus, showing developing discharge tube, which has ruptured the host cuticle (arrowed). Scale bar = 10 μm .

Fig. 55 High power detail of some of the densely packed uniform vesicles (ve) which pack the apex of the developing discharge tube. Scale bar = 0.5 μm .

Fig. 56 Detail of nucleus in a maturing thallus, showing perinuclear cisternae of endoplasmic reticulum and large structure vesicles, which may be early stage K-bodies (asterisked) as well as smaller structured encystment vesicles (white arrow). Scale bar = 2.0 μm .

Fig. 57 Part profile of a mature resting spore, showing completely smooth thick wall (asterisked) and dense, lipid (L) packed cytoplasm. Scale bar = 2.0 μm .

Fig. 58 Detail of mature resting oospore wall, showing tripartite layer (arrowed) separated by a partial outer fuzzy coat (white asterisk) from the more uniform inner endospore layer (black asterisk). Scale bar = 0.5 μm .

Plate 7. Figs 59-64. Ultrastructural cytology of *Chlamydomyzium anomalum*.

- Fig. 59 Phase contrast light micrograph (taken from a video sequence), showing mature thalli, which have become compartmentalized into individual sporangia, which are characterized by their ovoid profiles and broad domed discharge papillae, which rupture the outer nematode cuticle. Scale bar = 50 μm .
- Fig. 60 TEM showing cytoplasm of a young pre-spore initial thallus, showing nucleus (N) with perinuclear mitochondria surrounded by a zone packed with lipid (L) and dense body vesicles (DBV). Scale bar = 2 μm .
- Fig. 61 TEM of thallus, in which uninucleate aplanospore initials are being delimited by vacuoles (V). Scale bar = 5 μm .
- Fig. 62 DIC light micrograph (taken from a video sequence) of a fully mature thallus, showing delimited aplanospores just before their discharge. Note how the broad discharge tube has ruptured the nematode cuticle. Scale bar = 25 μm .
- Fig. 63 TEM section through longitudinal thallus, in region of the discharge papillum, showing fully differentiated walled uninucleate (N) aplanospores. Scale bar = 5 μm .
- Fig. 64 Profile of a fully differentiated aplanospore, showing central nucleus, and retention of the zoned organelles, with a perinuclear layer of mitochondria and asymmetric outer zones of lipid (L) and dense body vesicles (DB). Scale bar = 2 μm .
- Fig. 65 Rounded resting spores, which lack the thick walls observed in most species. Scale bar = 10 μm .

Plate 8. Figs 66-74. Ultrastructural cytology of *Chlamydomyrium oviparasiticum*.

Adapted in part from Glockling and Beakes (2006b).

- Fig. 66 Phase contrast light micrograph, showing young thalli, which have become compartmentalized and are beginning to differentiate discharge tubes (arrowed). Scale bar = 50 μm .
- Fig. 67 Low power TEM of a young pre-spore initial thallus, showing cytoplasm densely packed with vacuoles containing electron dense inclusions (DBV). Scale bar = 5 μm .
- Fig. 68 Detail of cytoplasm in pre-spore thallus, showing nucleus (N) with perinuclear array of mitochondria (m). Packetes of flimmer tubules (fv) can also be seen. Scale bar = 2 μm .
- Fig. 69 Apex of developing discharge tube, showing a cap of fibrillar material (asterisked which gives rise to the vesicle into which zoospores are discharged). Scale bar = 1.0 μm .
- Fig. 70 Phase contrast light micrograph of discharging thallus, showing primary zoospore initials still restrained by the discharge vesicle (arrowed). Scale bar = 20 μm .
- Fig. 71 High power light micrograph of an excysting secondary cyst, releasing a reniform secondary type zoospore with laterally inserted flagella (arrowed). Scale bar = 5 μm .
- Fig. 72 Phase contrast micrograph of ovoid, thick-walled resting spore showing glassy cytoplasmic contents. Scale bar = 5 μm .
- Fig. 73 TEM of a resting spore, showing rather collapsed profile and contentes composed of lipid globules and larger ooplast vacuole (OPV). Scale bar = 5 μm .
- Fig. 74 TEM detail of mature oospore wall, showing tripartite layer (arrowed) as well as outer exospore layer and thicker inner endospore layer (asterisked). Scale bar = 1 μm .