Evolutionary and biological mechanisms underpinning chitin degradation in aquatic fungi.

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Keywords: Chytridiomycota, chytrids, evolution, saprotrophy, gene expansion, gene transfer.

Abstract

Fungal biology underpins major processes in ecosystems. The Chytridiomycota (chytrids) is a group of early-diverging fungi, many of which function in ecosystems as saprotrophs processing high molecular weight biopolymers, however the mechanisms underpinning chytrid saprotrophy are poorly understood. Genome sequences from representatives across the group and the use of model chytrids offers the potential to determine new insights into their evolution. In this study, we focused on the biology underpinning chitin saprotrophy, a major ecosystem function of aquatic chytrids. The genomes of chitinophilic chytrids have expanded inventories of glycoside hydrolase genes responsible for chitin processing, complemented with bacteria-like chitin-binding modules that are absent in other chytrids. In the model chitinophilic saprotroph *Rhizoclosmatium globosum* JEL800, the expanded repertoire of chitinase genes is diverse and almost half were detected as proteins in the secretome when grown with chitin. Predicted models of the secreted chitinases indicate a range of active site sizes, particularly exochitinases that cleave terminal ends of chitin polymers. We propose that high diversity of secreted chitinases is an adaptive strategy that facilitates chitin degradation in the complex heterologous organic matrix of the arthropod exoskeleton. Free swimming *R. globosum* JEL800 zoospores are chemotactic to the chitin monomer N-acetylglucosamine and accelerate development when grown with chitin. Our study sheds light on the underpinning biology and evolutionary mechanisms that have supported the saprotrophic niche expansion of some chytrids to utilise lucrative chitin-rich particles in aquatic ecosystems and is a demonstration of the adaptive capability of this successful fungal group.

Significance statement

The Chytridiomycota (chytrids) diverged early in fungal evolutionary history and maintain biological features of their last common ancestor with other fungi and animals. Unlike more familiar fungi, chytrid spores are often motile and can swim to find suitable substrates to grow on. Many chytrids in aquatic ecosystems grow attached to chitin-rich particles including insect remains. Our study has shown the biological mechanisms that underpin the evolution of chitin-based lifestyle in chytrids. The expansion of existing chitin degradation genes, probably adapted from cell wall remodelling functions, alongside novel gene transfer of complementary enzyme chitin-binding capability facilitated chitin utilisation evolution. Chytrid spores can also swim towards chitin along associated chemical gradients and accelerate their development in response to chitin.

Introduction

Fungi are leading drivers of ecosystem processes, including the degradation of high molecular weight, often recalcitrant, biogenic organic polymers. As high molecular weight material is too large to be directly absorbed across fungal cell walls, substrates must first be degraded in the extracellular environment. This process is carried out by enzymes secreted by the fungus into the environment before subsequent breakdown products are absorbed via osmotrophy. Our understanding of the biological mechanisms underpinning fungal saprotrophy is largely based on terrestrial Ascomycota and Basidiomycota (1), the evolution of which is synonymous with the successful expansion of the kingdom through exploitation of diverse ecological niches.

The Chytridiomycota (chytrids) is a group of early-diverging fungi that are typically unicellular, developing into a sporangium with substrate-attaching and feeding rhizoids (Figure 1). Within mature sporangia, multiple uniflagellate zoospores form that lack a cell wall and are usually motile when released into the environment. Once a suitable substrate is found by swimming zoospores they attach and encyst, developing into a new sporangium with rhizoids to repeat the life cycle (2). Chytrids broadly occupy two major functional roles in ecosystems, either as (i) parasites, mainly of algae with the exceptions of a few amphibian pathogens, or (ii) saprotrophs that degrade a range of recalcitrant organic biopolymers. A recent phylogenomic study has proposed that saprotrophy is a derived trait in chytrids and that the ancestral trait is algal parasitism (3). The biological mechanisms underpinning aquatic chytrid saprotrophy are currently poorly understood including in an evolutionary context (4).

Chitin-based particles such as arthropod exoskeleton remains (exuviae) are prominent aquatic chytrid habitats (5). Chitin is a polymer of N-acetylglucosamine (GlcNAc) and is the second most abundant polysaccharide on Earth after cellulose. Chitin degradation is carried out by chitinases that either remodel cellular chitin (e.g. in the fungal cell wall), or externally process chitin substrate (6). Fungal chitinases belong to the glycoside hydrolase family 18 (GH18) of carbohydrate-active enzymes (CAZymes) (7) and act by introducing random breaks into crystal chitin (endochitinases) or cleaving terminal ends of GlcNAc polymers (exochitinases). GH18 chitinases are classified into two main related groups (8). Group AC are typically exochitinases with a deep, tunnel shaped substrate-interacting groove whereas Group B are typically endochitinases with a shallow, open groove (6, 9, 10). In addition to GH18 functional domains, many GH18 domain-containing proteins include carbohydrate binding modules (CBMs) belonging to ChtBD3/ChiC_BD domain families, which increase substrate binding affinity (11). Following primary chitinolytic activity by GH18s, chitin-derived GlcNAc is further processed by GH20 family β-*N*-acetyl-D-hexosaminidases (12).

The aims of this study were to determine the biological mechanisms underpinning chytrid chitin saprotrophy by focusing on the following specific questions: (i) what is the underlying molecular machinery (i.e. genes, mRNA and proteins/enzymes) involved in chitin saprotrophy, and (ii) what chitin-associated behaviour is shown by swimming zoospores. Our approach was to compare the reference genome sequences from chitinophilic chytrids with representatives across the Chytridiomycota that occupy a range of different ecological niches, combined with the use of the model saprotroph *Rhizoclosmatium globosum* JEL800 to determine new insights into chytrid biology. *Rhizoclosmatium globosum* is an aquatic chytrid found prevalent in nature attached to chitin-rich arthropod exuviae. The strain *R. globosum* JEL800 (Figure 1) is an emerging model for biological studies of early diverging fungi (2, 13) that was isolated by chitin baiting and grows readily on chitin as a sole carbon source.

Results

Expansion of genes encoding chitin degradation enzymes in chitinophilic chytrids

The abundance of GH18 chitinase encoding genes was compared in the reference genomes of 36 chytrids across the Chytridiomycota, including Chytridiales (n=7), Caulochtriales (n=1), Cladochytriales (n=2), Polychytriales (n=1), Rhizophydiales (n=5), Spizellomycetales (n=6), *Blyttiomyces helicus incertae sedis* (n=1), as well as Monoblepharidomycota (n=2) and Neocallimastigomycota (n=11) that occupy a diverse range of ecological niches (Figure 2ai, Supplementary Figure 1, Supplementary File). All chytrids associated with arthropod exuviae in nature and isolated by chitin-baiting belong to the Chytridiales except *Polychytrium aggregatum* JEL109 from the sister order Polychytriales. All the chitinophilic chytrids i.e. *R. globosum* JEL800, *Obelidium mucronatum* JEL802, *Chytriomyces hyalinus* JEL632, *Chytriomyces* sp. MP 7 and *P. aggregatum* JEL109 showed the highest enrichment of GH18 encoding genes (Figure 2ai). All other chytrids, i.e. those occupying non-chitin associated niches, had relatively lower GH18 gene abundances with the plant-processing Neocallimastigomycetes found in herbivore guts and plant-baited *Cladochytrium replicatum* JEL0714 lowest (Figure 2ai). A similar pattern of increased gene frequency was observed for GH20 encoding genes, particularly for the chitinophilic *R. globosum* JEL800, *O. mucronatum* JEL802 and *P. aggregatum* JEL109 (Figure 2aii).

Bacteria-like chitin binding module genes in chitinophilic chytrids

The abundance of genes containing carbohydrate binding module 5 (CBM5) domains in the genomes of the chitinophilic chytrids was distinctive, with *R. globosum* JEL800 and *O. mucronatum* JEL802 particularly high (Figure 2aiii). Although GH18 and GH20 encoding genes were present but at lower abundances in the genomes of non-chitin associated

chytrids (Figure 2 ai-aii), genes containing CBM5s were only detected in the genomes of chitinophilic chytrids except for the Monoblepharidomycete *Hyaloraphidium curvatum* SAG235-1. In most cases, BLAST searches of the CBM5 domains from chitinophilic chytrids revealed similarity with equivalent bacterial ChtBD3/ChiC_BD domains (Supplementary File), many of which were found in bacteria with known chitin-degrading capacity (e.g. *Chitinilyticum aquatile*, *Paenibacillus chitinolyticus*, *Chitinolyticbacter albus*). Only three genes, one in *Chytriomyces* sp. MP71 (KAI8611252) and two in *Polychytrium aggregatum* JEL109 (XP_052967452 and XP_052967585), contained domains more similar to those found in non-chytrid fungi with matches to Agaricomycetes (Basidiomycota). In addition, our searches matched to a further three Chytridiales genomes not included with the reference Mycocosm genomes (*Podochytrium* sp. JEL0797, *Siphonaria sp.* JEL0065, and *Physocladia obscura* JEL0513), all of which were isolated from mayfly (Ephemeroptera) exuviae (Joyce Longcore, pers. comm.).

Rhizoclosmatium globosum GH18 and GH20 gene diversity and domain architecture

Forty-nine *R. globosum* JEL800 genes contained GH18 domains. Maximum-Likelihood phylogenetic analysis of the GH18 domains revealed two major clades that corresponded with either Group AC (predominantly exochitinases) or Group B (predominantly endochitinases) (Figure 2b, Supplementary Figure 2). Group AC contained domains for 24 proteins, with 21 forming a large sub-clade, with two domains appearing in ORY47749. Although ORY48020 fell within this clade, it was separated by a long branch with relatively low bootstrap support. ORY40370 and ORY40369 fell outside the main clade. Group B contained domains from 25 proteins. One clade contained domains from 16 proteins and a second clade contained a variety of GH18 domains from nine proteins with low bootstrap support for several branches within this clade.

Chitin binding domains were found on eight genes, all of which were CBM5-type (Figure 2b). Seven were associated with Group AC (ORY40369, ORY41001, ORY53850, ORY32764, ORY35945, ORY37254 and ORY34525) and one with Group B (ORY39595). Signal peptides associated with protein secretion were found in 17 proteins. Eleven of these were found in Group A proteins (45.8% of Group A) and six with Group B (24% of Group B). Fourteen *R. globosum* JEL800 genes were found to contain GH20 domains (Figure 2c), Maximum-Likelihood phylogenetic analysis of which revealed two well supported clades, each containing seven genes, nine of which had associated signal peptides.

GH18 and GH20 in the Rhizoclosmatium globosum secretome and transcriptome

A total of 23 proteins from GH18 domain-containing genes were detected in the *R. globosum* JEL800 secretome when grown on chitin as the sole source of carbon: 10 from Group AC

(exochitinases), 12 from Group B (endochitinases) and one other (Figure 3a), accounting for almost half (47%) of the GH18 genes detected in the genome (Figure 2a). Proteins for six of the GH20 domain-containing proteins were also detected in the secretome (Figure 3a). All GH18 and GH20 proteins detected in the secretome matched reciprocal mRNA sequences detected in the cellular transcriptome, with a positive correlation of secretome GH18 and GH20 protein abundance with mRNA fpkm (Figure 3b). Predicted structures of the proteins encoded from GH18 domain-containing genes that were detected as secretome proteins suggests that the Group AC (exochitinases) are relatively more variable in total size and substrate binding site size, while Group B (endochitinases) are smaller proteins, mostly with lower volume binding sites (Figure 3c and Supplementary Figures 3 and 4).

Rhizoclosmatium globosum zoospore behaviour indicates adaptations to chitinophilic lifestyle

Swimming *R. globosum* JEL800 zoospores are chemotactic towards the chitin monomer GlcNAc. Targeted swimming occurred with zoospores from cultures grown with GlcNAc as the sole carbon source (Figure 4ai) and when grown without GlcNAc in the complex medium PmTG (Figure 4aii). Even though *R. globosum* grows with glucose as an alternative sole carbon source (Supplementary Figure 5), chemotaxis did not occur towards glucose with either GlcNAc or PmTG grown cultures (Figure 4ai, Figure 4aii).

We made several attempts to determine chemotaxis in zoospores from cultures grown with chitin but were unable to conduct the assay due to apparent reduction in zoospore motility. To explore this further, we compared general motility (i.e. swimming versus stationary) of zoospores harvested from cultures grown with chitin to the motility of zoospores from cultures grown with PmTG, showing that chitin-grown zoospores rapidly lose motility after release compared to zoospores grown with PmTG which remained more motile for longer (Figure 4b).

Discussion

Gene expansion and gene transfer underpin chitinophilic chytrid evolution

Here we provide genome-based evidence for two complementary mechanisms through which chitin saprotrophy has evolved in chytrids. Firstly, the abundance of genes encoding GH18 chitinases and GH20 β-N-acetyl-d-hexosaminidases responsible for the breakdown of chitin and subsequent processing of chitin-derived GlcNAc respectively were increased in chitinophilic chytrids relative to non-chitinolytic chytrids. Secondly, alongside GH18 gene expansion, bacteria-like chitin-binding CBM5 domains were abundant in the genomes of the chitinophilic chytrids, indicating that horizontal gene transfer (HGT) has taken place, potentially from chitinophilic bacteria to chitinophilic chytrids. The acquisition of chitin-binding

capability likely improved the functional efficiency of chytrid chitinases to degrade extracellular chitin-based substrates and therefore has clear fitness advantages.

Lineage-specific gene family expansion is an important mechanism of adaptive evolution generally in eukaryotes (14) and observed in fungi (15). Expansion of hydrolase gene families related to host/substrate specificity has been reported throughout the fungi. In non-chytrid fungi, necrotrophic Ascomycota and Basidiomycota exhibit expansion of GH28 family pectinases (16), and white rot fungi such as *Armillaria* spp. (Basidiomycota) have an increased complement of genes encoding cellulose- and xylan-degrading enzymes (17, 18). In chytrids, increased copy numbers of proteases have been associated with the evolution of amphibian pathogenicity in *Batrachochytrium dendrobatidis* by facilitating attachment and penetration of host cells (19, 20). Specifically, equivalent GH18 gene expansion has been shown within other fungal genomes (21, 22) where gene copy number is a strong indicator of chitin degradation capacity (22).

This increase in chitinase gene copy number in chitinophilic chytrids is in contrast to patterns seen in chytrids occupying non-chitin associated niches (e.g. the guts of herbivores (23, 24) or plant/pollen associated (25)) which have reduced abundances of chitinase encoding genes in their genomes. It is important to reiterate that GH18 chitinases are also used for chitin remodelling in the fungal cell wall and therefore necessary to some extent in all chytrids. Chitinase gene family expansion appears dynamic within chytrid lineages, with an apparent initial increase in chitinase gene copy number associated with the base of the Chytridiales lineage including the closely related chitinophilic *Polychytrium aggregatum* (Polychytriales) followed by subsequent gene reduction occurring in the likely plant-based saprotroph *Entophlyctis luteolus* (Chytridiales).

Here we show that the presence of chitin-binding CBM5 domains is a conserved and distinctive feature of chitinophilic chytrid genomes. In the model chitinophilic chytrid *R. globosum* JEL800, chitin-binding domains are present on multiple chitinases and are potentially derived from xenologs originating in chitinophilic bacteria. Horizontal gene transfer is well established in prokaryotes but has only relatively recently been determined in fungi including early-diverging fungi. Comparative assessment of herbivore gut anaerobic Neocallimastigomycota has shown the HGT has played a fundamental role in the evolution of the group (26). As we propose here for aquatic chitinophilic Chytridiomycota, anaerobic gut Neocallimastigomycota HGT occurred from functionally similar bacteria that likely co-occur in the same niche i.e. anaerobes that live in herbivore guts and also degrade plant-derived polysaccharides. Similarly, Neocallimastigomycota HGT is especially associated with CAZymes and extracellular polysaccharide degradation. Murphy *et al*. (26) proposed that HGT, including the acquired ability to degrade plant-derived polysaccharides, was a key feature of the expansion of the Neocallimastigomycota clade, as we potentially see here with

the evolution of the chitinophilic Chytridiales. Wider comparison between Chytridiomycota, Blastocladiomycota and Mucoromycota indicates that the early-diverging groups of aquatic fungi are more likely to undergo HGT compared to terrestrial taxa (27). Further work is needed using more sophisticated bioinformatic approaches to determine HGT in chitinophilic chytrids.

Diversity in chitinase gene architecture and modelled secretome chitinase structure

A key feature of gene family expansion in fungi is that it facilitates exploitation of specific niches by allowing either increased rates of enzyme production or functional novelty (28). The increased GH18 and GH20 inventory in *R. globosum* JEL800 and other chitinophilic chytrids may confer the ability to react quickly to substrate availability by transcribing multiple genes where necessary. Although cellular mRNA was detected for almost all the GH18 and GH20 genes encoded within the *R. globosum* JEL800 genome, not all transcribed genes were detected in the secretome. As well as accounting for cellular chitinases involved in cell wall processing, some of this untranslated mRNA may play an important biological role by maintaining a bank of mRNA for a variety of chitinases in the cytoplasm, *R. globosom* JEL800 may be able to remain 'primed' for rapid protein synthesis. At present we have a very limited understanding of cell wall associated processes in chytrids.

Relatively few crystal structures of fungal chitinases have been characterised (29) and prediction of specific chitinase functions is therefore difficult (8), but the overall sizes of substrate binding sites in each group were broadly consistent with what would be expected from the 'closed' or 'open' grooves determined from the crystal structure of bacterial exo- and endochitinases (30). The high levels of secretion of Group AC exochitinases compared to Group B endochitinases suggests that cleavage of terminal GlcNAc dimers from chitin is a particularly important characteristic of the chitinophilic lifestyle. Variation in binding site volume relative to overall protein volume in Group AC chitinases (Figure 3c) indicates considerable structural, and therefore functional, diversity which may be related to variation in substrate with different enzyme activities able to account for substrate complexity. In nature, chitinophilic chytrids are observed growing on arthropod exoskeleton remains (5) in which chitin is not available as a discrete molecule but instead packaged within a complex heterologous organic matrix. Even though exoskeleton matrices likely vary between taxa, the general structure is based around crystalline chitin nanofibrils in chitin-protein planes that are stacked in a twisted 'plywood' structure (31). We propose that the diversity of predicted chitinases, particularly exochitinases, as reported here have adaptive advantages for processing the heterologous organic matrix in which the chitin substrate is intertwined. The specific functional roles of the diverse chitin-active glycoside hydrolases in *R. globosusm* JEL800 remain undetermined (32). Further structural analysis of secreted proteins will

support determining the exact functions of the apparent range of chitinolytic enzymes in *R. globosum* JEL800.

Zoospore behaviours as adaptive traits for the chitinophilic lifestyle

Rhizoclosmatium globosum JEL800 zoospores are chemotactic to the chitin monomer GlcNAc independent of the growth conditions of the previous generation, a trait that has obvious advantages for targeting chitin-rich substrates such as arthropod exuviae in complex aquatic environments. Chemotaxis has been shown in non-saprotrophic chytrid zoospores, particularly species that are parasites. *Batrachochytrium dendrobatidis* zoospores are chemotactic towards amphibian-produced compounds including proteins (33) and algal parasites target cell extracts of stressed hosts (34). The biological mechanisms underpinning chemotaxis in aquatic chytrids are currently unknown (4). It is possible that response to environmental signals could be linked to the rumposome, an enigmatic structure connecting the zoospore cell surface to the flagellar machinery that is present in *R. globosum* JEL800 zoospores (2) and is likely a site of calcium-mediated signalling (35). Phototaxis has also been shown in chytrid zoospores, including with *R. globosum* JEL800, facilitated by a CyclOp-fusion protein based sensory system (36). Together, this suggests that via chemotaxis and phototaxis, swimming chytrid zoospores utilise sophisticated multi-signal mechanisms to navigate the aquatic environment.

The chemotactic response to GlcNAc shown here suggests that, in nature, chitinophilic chytrid zoospores respond to signals of ‘on-going’ chitin saprotophy that is releasing GlcNAc via degradation by other organisms. Chitin degradation in aquatic ecosystems is also performed by chitinophilic bacteria that share the same habitat and potential niche as chitinophilic chytrids such as *R. globosum* (37). It is likely that a range of chytrid-bacteria interactions are associated with chitin-rich particles, including particle colonisation, that involve the release of chemotactic signals from bacteria to chytrids and *vice versa* depending on which are the primary or secondary colonisers (37).

When grown with chitin, we observed faster loss of zoospore motility compared to non-chitin grown *R. globosum* which remained motile for longer. Accelerated transition from a non-feeding swimming zoospore to the next stage of the life cycle (i.e. encystment) is a possible trait that could facilitate the rapid colonisation and exploitation of ephemeral chitin-rich substrates such as mayfly exuviae in a dynamic resource-complex and competitive environment. Chytrid zoospore biology remains poorly understood, including the controls of cell cycle transition (4). Chytrid zoospores are metabolically active, yet are transcriptionally and translationally inactive, with ribosomes held dormant (38). As with other chytrids, in *R. globosum* JEL800 zoospores, ribosomes are maintained dormant in a distinct cluster that only dissipates once the cell transitions to the germling stage when transcription and

translation are initiated (2). Therefore, we propose the molecular mechanisms underpinning the zoospore behavioural traits that we report here that likely support chitin utilisation are maternally derived during zoosporogenesis, and combine independent (i.e. GlcNAc chemotaxis) and dependent (i.e. accelerated zoospore-germling transition) of maternal growth conditions (i.e. with or without chitin).

Conclusion

Our study has shown mechanisms that underpin and have likely supported the evolution of chitin-based saprotrophy in chytrids. We propose that the complementary expansion of existing GH18 and GH20 genes, probably from cell wall remodelling functions, alongside niche-specific novel gene transfer of GH18-associated chitin-binding CBM5 domains, potentially from functionally similar bacteria, facilitated chitin saprotrophy evolution. Expansion of GH18 genes, allowed the diversification of secreted chitinases, particularly exochitinases that may facilitate utilisation of chitin within the complex arthropod exoskeleton matrix and, along with substrate-dependent zoospore behavioural traits, determine the widespread and successful exploitation of the chitin-based particle niche in aquatic ecosystems.

Methods

Comparison of GH18, GH20 and CBM5 encoding genes across chytrid genomes

Counts of predicted GH18, GH20 and CBMs genes were retrieved from Chytridiomycete, Monoblepharidomycete and Neocallimastigomycete reference genomes from Mycocosm (https://mycocosm.jgi.doe.gov/, accessed 10/2023) (Supplementary File, Supplementary Figure 1). Conserved domains in *R. globosum* JEL800 GH18 and GH20 genes were identified using CD-search (39) and aligned separately in MAFFT (40) using E-INS-i before maximum likelihood phylogenetic analysis was carried out using the CIPRES (41) implementation of RaxML 8.2.10 (42) using the CAT model and the BLOSUM62 matrix for divergent protein sequences with automatic boot strapping. Domain trees were visualised using FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). GH18 domains were further subclassified by comparison with a phylum-wide sampling of fungal GH18 domains (43) (Supplementary Figure 2). Presence of signal peptides in each gene were predicted using the SignalP 4.1 webserver (44). Complete protein domains were plotted using genoplotR (45). Chitin binding domains (ChtBD3/ChiC_BD) in genes annotated as CBM5 in Mycocosm for all CBM5-containing lineages were identified using CD-search. Where domain homology was identified, domains were searched against GenBank using BLASTp (e-value cutoff = 0.00001) excluding hits for the chitinophilic chytrids in this study.

Rhizoclosmatium globosum JEL800 maintenance, growth and microscopy

Rhizoclosmatium globosum JEL800 was routinely maintained on peptidised milk, tryptone, and glucose (PmTG) agar plates (46) at 22°C in the dark. For experimental analysis, *R. globosum* JEL800 was also grown with modified Bold's Basal Medium (BBM) (47, 48) diluted 10-fold and with the addition of 0.25 g ammonium sulfate and 0.5 mL f/2 vitamin stock in 1 L (49) supplemented with either chitin, GlcNAc or glucose as carbon source. To produce samples for transcriptome and secretome analysis, *R. globosum* JEL800 was grown in 50 mL BBM with 10mM chitin in 250 mL Erlenmeyer flasks inoculated with ~14,000 zoospores and shaking at 70 rpm. Cultures were harvested after 7 days growth by transferring contents to a 50 mL centrifuge tube and centrifuging at 4,000 G for 10 min. Cell pellets were snap frozen with liquid nitrogen. The supernatant was removed and Halt™ Protease & Phosphate Inhibitor Cocktail (Thermo) added at 5 µL / 50 mL with mixing by inversion. Protease treated supernatant was concentrated to ~ 250 µL in 15 mL batches at 4,000 G for 20 min each (total ~ 1 hour) using 10 kDa Amicon® Ultra 15 mL Centrifugal Filters (Millipore). Concentrated supernatant was transferred to a 3 kDa Pierce™ Protein Concentrator PES 0.5 mL (Thermo) and centrifuged for 20 min at 15,000 G to a final volume of ~ 100 µL. All centrifuge steps were carried out at 4 °C and samples stored at -80 °C.

To screen a range of non-chitin carbon sources used by *R. globosum* JEL800 for growth, mature cultures on PmTG agar plates were sporulated using 4 mL dH_2O for ~ 2 hours at room temperature. Zoospores were then harvested and passed through a 10 µm sieved before being washed 3 times in carbon free BBM by centrifuging at 4000 G for 10 min at 4 °C and re-suspended in carbon free BBM. Biolog PM 1 and PM 2A Carbon Utilisation Assay plates were inoculated with 150 µL per well at a final density of 1×10^3 zoospores per well. Assay plates were incubated at 22 °C and absorbance at 405 nm (OD_{405}) was measured periodically using a plate reader (CLARIOstar). Growth rate was calculated by linear regression of the exponential phase (0-66 hour) from the growth curve of each substrate. For some substrates growth was minimal during the exponential phase due to poor growth, however sharp increases in OD_{405} were seen after 66 hours due to sporulation. To get an idea of which substrates induced a large release of zoospores linear regression was also performed on 66-120 hour growth.

To demonstrate *R. globosum* JEL800 growth on an arthropod exoskeleton, *Daphnia* were acquired from a local aquarium supplier and cleaned by incubation in 10% sodium dodecyl sulphate solution for 30 mins, followed by 100% ethanol for 1 hour and then autoclaved in ddH_2O. Carapaces were washed twice in sterile ddH_2O between each step. Culture dishes containing 3mL BBM and one carapace were inoculated with 500 µl zoospore suspension (1.03×10^6 zoospores.ml^{-1}) and incubated at 22°C. Chytrids growing on carapaces were imaged with DIC using a Leica Dmi8 microscope (Leica, Germany).

Transcriptome and secretome analysis

RNA was extracted from the cell pellets using the RNeasy Micro kit (Qiagen) according to the manufacturer's instructions. Pellets were thawed in 2 mL RLT lysis buffer (Qiagen) containing 10 µL mL^{-1} of 2-mercaptoethanol, incubated at room temperature for 5 minutes and mixed periodically using a vortex. Cell debris was removed by centrifuging at 4000 G for 1 min, the lysate was recovered through a QIA shredder (Qiagen). An equal volume of 100 % ethanol was added to the homogenised lysate before being transferred in batches to the RNeasy extraction column including on column DNase digestion step using the RNase-Free DNase set (Qiagen). RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo) and the RNA BR assay kit (Invitrogen) on the Qubit 4 fluorometer (Invitrogen). RNA quality was assessed using the RNA 6000 Nano kit total RNA assay (Agilent) run on the 2100 Bioanalyzer instrument (Agilent). Sequencing was carried out using Illumina NovaSeq 6000 technology and assembled with the Novogene WTS pipeline. Raw reads are deposited in SRA under BioProject PRNJxxxxx. GH18 and GH20 domain containing genes were identified in the assembled transcriptome by performing blastn searches for each gene identified in the *R. globosum* JEL800 genome (e-value cutoff = 0.00001). Concentrated supernatant samples (i.e. secretome) were sent to University of Bristol Proteomics Facility for comparative proteomics using tandem mass tagging (TMT). Identified peptides were cross referenced with predicted proteins from mRNA identified within the transcriptome.

Protein modelling from gene sequences

GH18 and GH20 models for all proteins detected in the secretome were predicted from reciprocal gene sequences using the Phyre2 webserver (50). Total model volumes were predicted using VADAR 1.8 (51) and binding site volumes were predicted using CASTp 3.0 (52) with default settings. Intensive models were generated for major representatives from GH18 groups A (ORY35945) and B (ORY34457). Proteins were visualised in UCSF Chimera (53) and structures were aligned with the Smith-Waterman algorithm using the BLOSUM-62 matrix with a gap extension penalty of one. RMSD values were calculated for the GH18 part of the alignment. Graphics were edited using Inkscape (http://inkscape.org).

Zoospore chemotaxis and motility

Prior to chemotaxis and motility experiments, *R. globosum* was maintained on agar plates made with PmTG, or BBM with 5mM GlcNAc or chitin for at least 3 generations. Mature plates were sporulated as described above and 1 mL zoospores spread onto fresh media and dried to create a lawn. Lawns were incubated at 22°C for 48 hours (PmTG and GlcNAc) and 24 hours (chitin) to allow sporangia to develop and mature. For chemotaxis

experiments, mature lawns were sporulated with 5mL sterile dH_2O, and after 30 min zoospores were harvested and passed through a 10 µm sieve, enumerated using a LUNA™ Automated Cell Counter and motility checked using a haemocytometer. Only zoospore suspensions with motility >80% and ~$1x10^7$ cells/ mL were used for experiments. 2mL zoospore suspension was placed in a 50 mL centrifuge tube and the top sealed with parafilm. 1 mL syringes with 21g needles containing either 150 µL 10mM GlcNAc, 10mM Glucose or H_2O (control) were held in the zoospore suspension and incubated at 22°C in the dark. After 1 hour, the contents of the syringe was emptied into a 1.5mL tube and fixed with glutaraldehyde (final concentration of 0.5%) and zoospores enumerated using a haemocytometer. For motility experiments, zoospore lawns were sporulated with 3mL sterile dH_2O, a 1 mL sample was taken immediately, a subsample of this was glutaraldehyde fixed to determine the starting concentration. Non-motile zoospores were enumerated using a haemocytometer every 5 min for 1 hour and % motility was calculated from the starting concentration.

Acknowledgements

We thank Joyce E. Longcore (University of Maine) for providing *R. globosum* JEL800, now held at the Collection of Zoosporic Eufungi at the University of Michigan (CZEUM), and information on the isolation of *Podochytrium* sp. JEL0797, *Siphonaria* sp. JEL0065 and *Physocladia obscura* JEL0513. NC, KB and MC were supported by the European Research Council (ERC) (MYCO-CARB project; ERC grant agreement no. 772584). DL was supported by an EnvEast Doctoral Training Partnership (DTP) PhD studentship funded from the UKRI Natural Environment Research Council (NERC grant no. NE/L002582/1).

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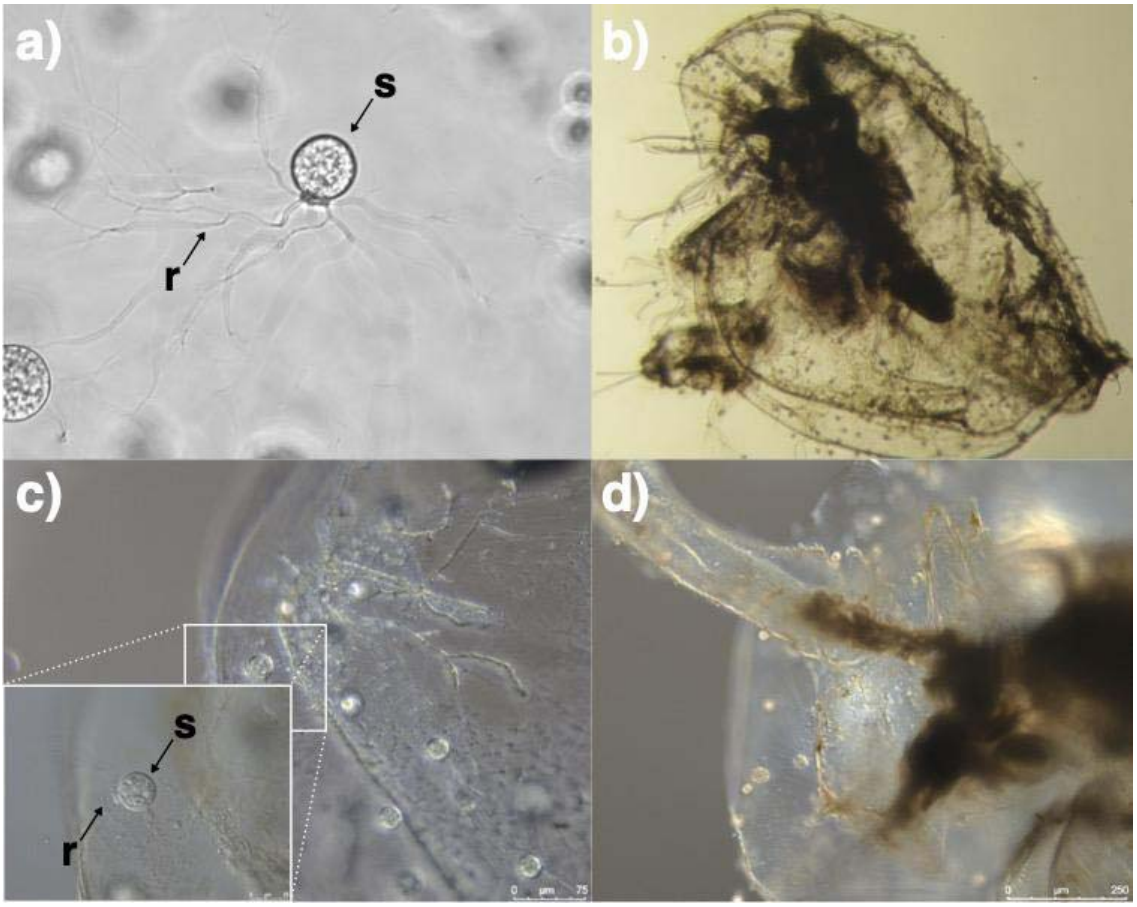

Figure 1 a) A *Rhizoclosmatium globosum* JEL800 cell with branching rhizoids (r) used for attaching to substrates and sporangium (s) inside which develop multiple zoospores. b-d) *Rhizoclosmatium globosum* JEL800 attached to the exoskeleton of the freshwater crustacean *Daphnia* across various levels of magnification. Note rhizoids (r) penetrating the exoskeleton surface in c.

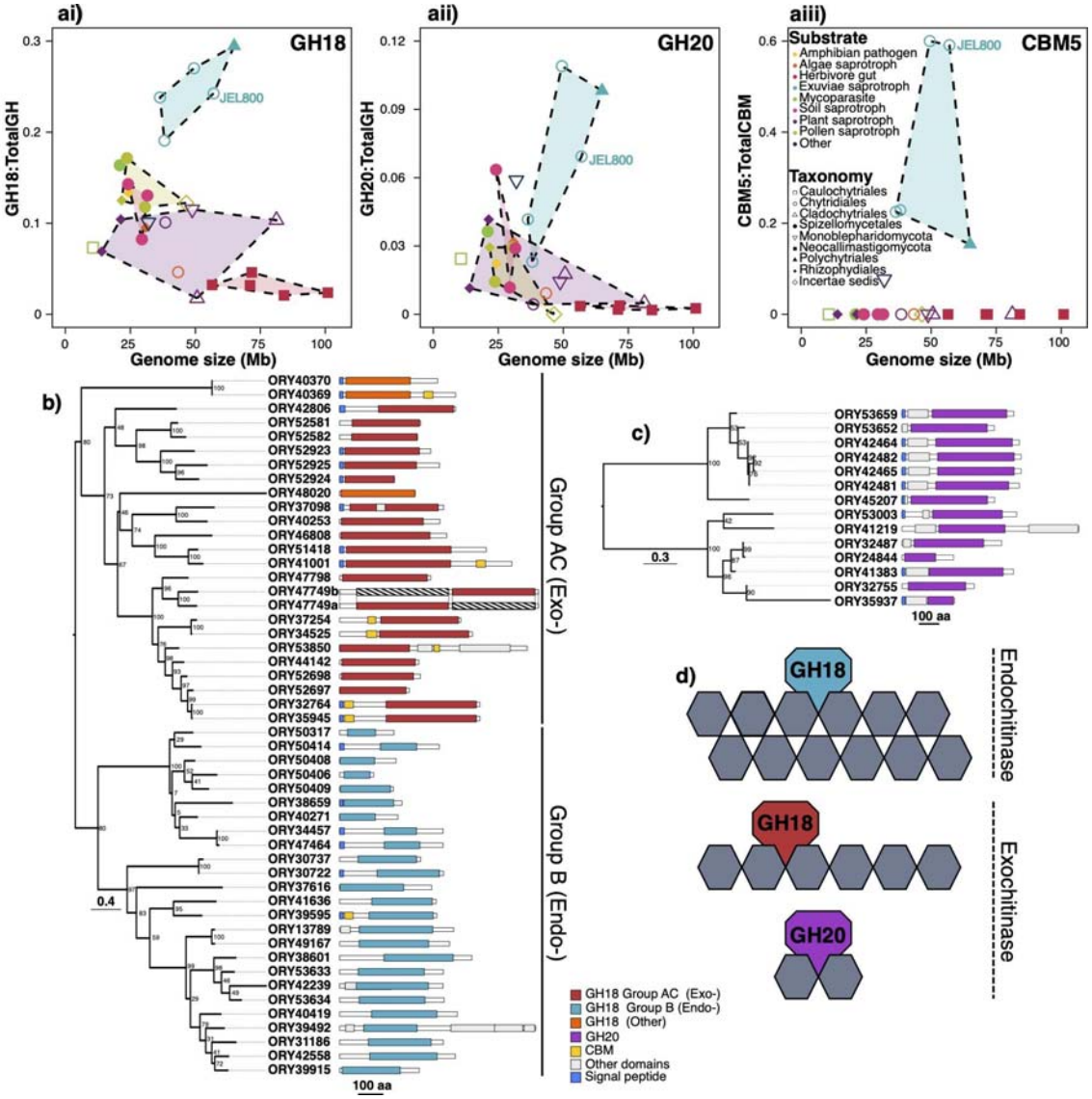

Figure 2 ai) Comparison of ratios of glycoside hydrolases GH18 chitinases and aii) GH20 β-*N*-acetyl-D-hexosaminidases with total GH encoding genes, and aiii) carbohydrate binding module CBM5 genes with total CBM encoding genes and genome sizes of 36 members of the Chytridiomycota that occupy a diverse ecological niches and utilise a range of substrates. Point colours indicate the substrate strains were isolated from, convex hulls link strains isolated from the same substrate; point shapes indicate order. Maximum likelihood phylogenies of *Rhizoclosmatium globosum* JEL800 b) GH18 and c) GH20 domains showing architecture of GH domain-containing genes. Genes encode for proteins with either d) endo- (GH18) or exochitinase (GH18, GH20) activity.

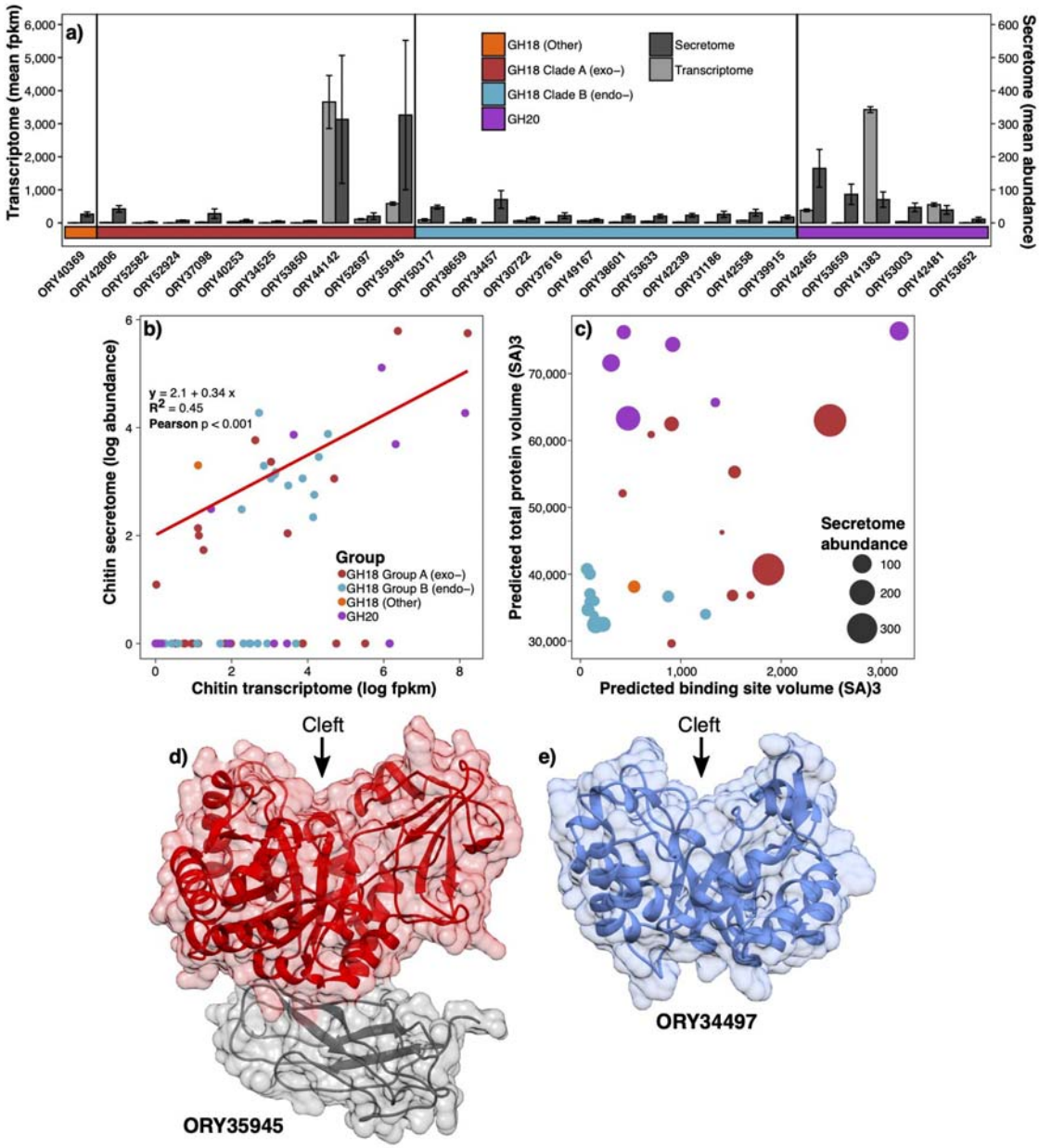

Figure 3 a) mRNA (fpkm) and secretome abundance for all *Rhizoclosmatium globosum* JEL800 glycoside hydrolases present in the secretome. b) Relationship of abundance of proteins in the secretome to equivalent mRNA in the transcriptome c) Relationship of substrate binding site volume to total protein volume. GH18 Clade A have a broad range of protein and binding site volume. GH18 Clade B are typically small proteins with small binding sites. GH20s were typically large proteins with small binding sites (with the exception of ORY53659 which had an uncharacteristically large binding site. d) Phyre2 model of ORY35945 based on crystal structure of chitinase ChiW from *Paenibacillus* sp. str. fpu-72 (Itoh et al 2016) (chitin-binding domain not shown). e) Phyre2 model of ORY34457 based on the crystal structure of a GH18 chitinase from *Pseudoalteromonas aurantia*.

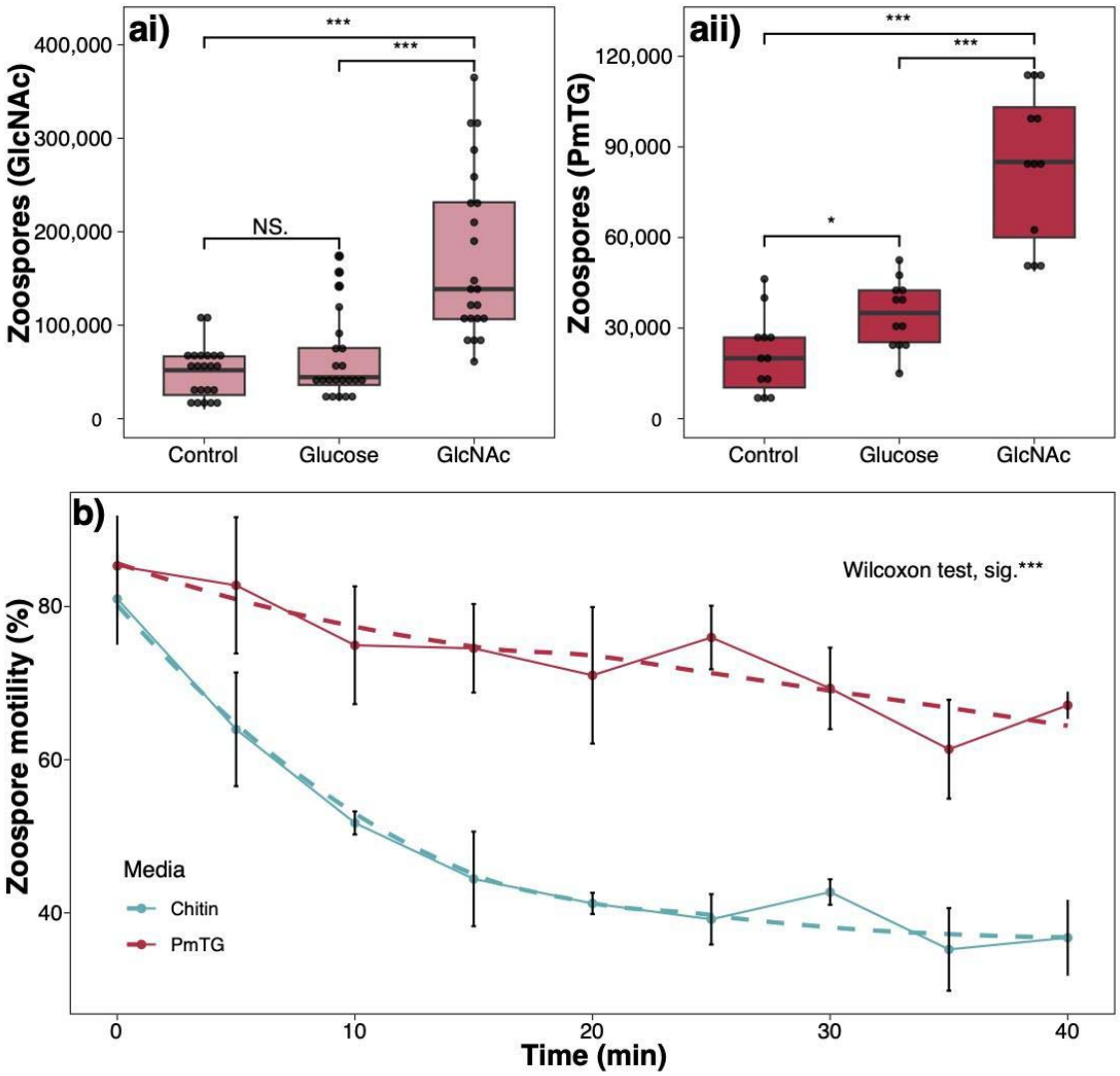

Figure 4 ai-ii) Chemotactic response of *Rhizoclosmatium globosum* JEL800 zoospores to the chitin monomer N-acetylglucosamine (GlcNAc) compared to glucose and no-attractant control from cultures grown with GlcNAc as the sole carbon source and in complex GlcNAc-free medium PmTG. Significance values (pairwise Wilcoxon test) are indicated as *** = $p < 0.001$, * = $p < 0.05$, NS. = not significant. Points are binned at 1/30 range of the data. b) Loss of motility in *R. globosum* JEL800 zoospores from cultures grown with chitin as the sole carbon source compared to the complex medium PmTG.