**Understanding the evolution of parasitic oomycete and ‘fungal-like’ traits through genome analysis of the free-living pseudofungus *Hyphochytrium catenoides.***

**Comparative genomic analysis of the ‘psuedofungus’ *Hyphochytrium catenoides***

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**Abstract**

Eukaryotic microbes have three primary mechanisms for obtaining nutrients and energy: phagotrophy, photosynthesis and osmotrophy. Traits associated with the latter two functions arose independently multiple times in the eukaryotes. Fungi successfully coupled osmotrophy with filamentous growth, similar traits are also manifest in the Pseudofungi (oomycetes and hyphochytriomycetes). Both the Fungi and the Pseudofungi encompass a diversity of plant and animal parasites. Genome sequencing efforts have focused on host-associated microbes (mutualistic symbionts or parasites), providing limited comparisons with free-living relatives. Here we report the first draft genome sequence of a hyphochytriomycete ‘pseudofungus’; *Hyphochytrium catenoides*. Using phylogenomic approaches we identify genes of recent viral ancestry, with related viral derived genes also present on the genomes of oomycetes, suggesting a complex history of viral co-evolution and integration across the Pseudofungi. *H. catenoides* has a complex lifecycle involving diverse filamentous structures and a flagellated zoospore with a single anterior tinselate flagellum. We use genome comparisons, drug sensitivity analysis and high-throughput culture arrays to investigate the ancestry of oomycete/pseudofungal characteristics and metabolic traits, demonstrating that many of the genetic features associated with pathogenic traits evolved specifically within the oomycete radiation. Comparative genomics also identified differences in the repertoire of genes associated with filamentous growth between the Fungi and the Pseudofungi, including differences in; vesicle trafficking systems, cell wall synthesis pathways and motor protein repertoire, demonstrating that unique cellular systems underpinned the convergent evolution of filamentous osmotrophic growth in these two eukaryotic groups.

**Introduction**

Stramenopiles [1] (also known as heterokonts [2]) are a highly diverse branch of protists that encompass a multitude of biological forms including: huge multicellular kelps (“seaweeds”), abundant marine micro-algae, and a variety of microbial pathogens, some of which --oomycetes for example-- feed and grow like fungi and cause important diseases of animals, algae and plants [3, 4]. The stramenopiles are a phylogenetically robust group (e.g. [5]) defined by the presence of two motile flagella, a ‘standard’ smooth posterior flagellum and a ‘tinselate’ anterior flagellum with a tripartite rigid tubular mastigoneme (“hairs”) [2]. However, secondary flagellum loss has occurred during the radiation of this group, for example, in the hyphochytrids like *Hyphochytrium catenoides* [6], which have lost a smooth posterior flagellum but retained a tinselate anterior flagellum.

Environmental sequencing, specifically of marine environments (e.g. [7]), has increased the known phylogenetic diversity of the stramenopiles, suggesting that this group is one of the most diverse higher-level groups within the eukaryotes [8]. Representatives of these groups remain uncultured with little gene/genome sampling. Furthermore, genome-sequencing efforts in the stramenopiles have largely focused on photosynthetic algae (e.g. [9, 10]) or oomycete pathogens (e.g. [11, 12]), leaving the diversity of heterotrophic free-living stramenopiles undersampled. Here, we describe the sequencing and comparative genomic analysis of *Hyphochytrium catenoides* (ATCC 18719) originally isolated by D. J. Barr from pine tree pollen in Arizona USA (however, we note that there is no direct reference in ATCC that accompanies this original culture, see [13]). We propose this organism and associated genome data as a tool to investigate the evolution of stramenopile characteristics and for the purpose of comparing and contrasting the evolution of traits between free-living and parasitic pseudofungi.

*H. catenoides* is a free-living hyphochytrid protist that forms hyphal-like networks and spores with only a single anterior tinselate flagellum (Fig. 1a [6, 14]). The hyphochytrids are thought to branch sister to the oomycetes [4, 15] and both these groups grow as filamentous/polarized cells feeding osmotrophically by extracellular secretion of digestive enzymes coupled to nutrient uptake [4, 6, 14]. These characteristics mean that they ‘resemble’ fungi [4]. Here, we use genome sequence data to confirm the phylogenetic position of the hyphochytrids, investigate characters shared with oomycete parasites and identify the genes involved in cellular characteristics absent or shared with fungi that characterise filamentous/osmotrophic growth. We also use the genome data to investigate the protein repertoire putatively associated with loss of the posterior flagellum in the hyphochytrids. These data provide a unique genome sample of a free-living stramenopile in order to facilitate further evolutionary and cellular research.

**Results and Discussion**

**Genome assembly and gene model prediction**

Using a range of methods, we assembled and tested the completeness of the *H. catenoides* genome (see Materials & Methods). Comparisons measuring the fraction of transcriptome data that aligned to the genome with BLAST, along with CEGMA and BUSCO v1 demonstrated that the genome assembly was predicted to be respectively; 97.8%, 91.5%, and 52% complete in terms of gene sampling (for further analysis and discussion of genome ‘completeness’ analysis see Fig. S1). Both CEGMA and BUSCO (v1.2) are likely to underestimate the completeness of genomes, as the core gene list is derived from a subset of eukaryotic genomes that does not fully sample a diverse collection of eukaryotic genomes, (e.g. BUSCO version 1.2 only samples fungi and metazoan genomes) which inevitably gives a much lower estimation of completion. A full set of tRNA were identified in the *Hyphochytrium* genome, including an additional tRNA for selenocysteine. The >= 1 kbp scaffold assembly along with the predicted proteome has been submitted as a draft genome to the EMBL EBI (BioStudies: S-BSST46). Details comparing the assembly to other eukaryotic genome sequences are described in Fig. 1b. Analysis using RepeatMasker [16] determined that the >= 1 kbp genome assembly is comprised of 9.53% repeat regions of which 1.79% were assigned to transposable elements.

The protocol used for genome contamination assessment, genome assembly and identification of putative protein-coding genes and their predicted proteins are provided in the Materials and Methods. This approach identified 18,481 putative gene models (406 of these gene models demonstrated evidence of multiple splice forms according to MAKER [17]), a total gene count similar to the mean (15,946) for other sequenced stramenopiles (Fig. 1b). The number of introns and exons reported by the program Genome Annotation Generator (GAG) was 67,332 and 85,813 respectively with an average of 3.64 introns per gene and an average exon length of 228 and intron length of 208.

Using the genome assembly, we were able to identify and assemble a hypothetical circular mitochondrial chromosome (Fig. S2). Further analysis did not identify a candidate relic plastid genome (Fig. S3), while phylogenomic analysis identified only four genes that, under certain scenarios for gene ancestry, could represent genes acquired as part of the endosymbiosis that gave rise to the plastid organelle present in photosynthetic stramenopiles (Fig. S3).

**Genome size, ploidy and evidence of sexual reproduction**

K-mer counting [18] was used to predict a haploid genome size of between 54.1 Mbp and 68.6 Mbp with follow up analysis focusing specifically on the >= 1 kbp assembly suggesting a genome size of 65.7 Mbp across 4,758 scaffolds and a scaffold N50 size of 35.57 kbp (L50 of 399). The average sequencing coverage of the total assembly was estimated to be 312x, and the average coverage over the >= 1 kbp scaffolds is 610x. Extraction and purification of long strands of DNA was not achieved using multiple DNA extraction protocols preventing sequencing using a long-read technology and/or pulsed-field gel electrophoresis to estimate chromosome number. As such, we used a RT-PCR method for estimation of genome size [19] that indicated a haploid genome size of 46.9 Mb (S.E.M. = 1.5).

As mentioned in the methods, the N50 of the genome assembly was much improved by the use of Platanus - an assembly algorithm optimised for multi-ploidy genomes. To further investigate evidence of ploidy in our *H. catenoides* culture we mapped ~101 million reads to the 65.7 Mbp assembly identifying 1,393,505 single nucleotide polymorphisms (SNPs) with 1,332,610 (96%) of the SNPs identified consisting of a two-way nucleotide polymorphism (i.e. 58.8/41.2% mean character split). We also took all scaffolds and plotted SNP frequency against scaffold size. The majority of the scaffolds are clustered around a SNP frequency of ~0.0275 (Fig. S4), suggesting that this variation is consistent and not specific to a subset of chromosomes, for example, in the case of aneuploidy. Interestingly, this analysis showed two large scaffolds with very low SNP frequency compared to the rest of the assembly. These scaffolds contain a number of genes with high sequence identity to genes found on large DNA viruses, suggesting the presence of a viral genome or evidence of a recent introgression, discussed further below. K-mer mapping [18] showed two peaks in coverage frequency, which is consistent with the reads mapping to a diploid genome (Fig. S5) [6, 14].

Using reciprocal BLAST searches we confirmed that *H. catenoides* encodes and expresses putative homologues of all seven eukaryotic meiosis-specific gene families [20] in the culture conditions used to grow *H. catenoides* (see Table S1). To our knowledge sexual recombination has only been observed once in Hyphochytriomycota cultures, with Johnson (1957) identifying cellular forms suggestive of zygote production as a result of fusion in the resting spore development of *Anisolpidium ectocarpii* [21]. However, a range of different sexual reproductions systems have been identified in the oomycetes [22], collectively these data suggest meiosis is present in representative taxa across the wider Pseudofungi.

**Phylogenetic position of *Hyphochytrium***

*Hyphochytrium* has previously been shown to branch as a sister-group to the oomycetes in rRNA gene phylogenies (e.g. [3, 15]). Using suite of concatenated multiple amino acid sequence alignment approaches (supermatrix and per gene partitioned approaches) and a species tree from gene trees coalescence approach [Mirarab and Warnow 2015] established for investigating phylogenetic relationships throughout the eukaryotic tree (e.g. [23-25]) we investigated the phylogenetic branching position of *Hyphochytrium*. We generated a concatenated amino acid alignment of 325 orthologues (128 taxa and 90,230 amino acid sites) including a comprehensive sampling of eukaryotic taxa based on previously published papers [23]. We used this alignment to calculate a eukaryote wide phylogeny using maximum likelihood (ML) and 100 ‘real’ bootstrap replicates using the IQ-TREE software [26, 27] under the site heterogeneous model LG+G4+F+c60+PMSF [Wang et al. 2017] (Fig. 2) (Fig. S6A shows the wider tree topology). Additionally, we inferred a tree based on this supermatrix with per gene partitioned model in IQ-TREE with 1,000 ultrafast bootstraps replicates (Fig. 2). Furthermore, using a gene tree coalescence approach in ASTRAL [Mirarab and Warnow 2015] we inferred a species tree with 100 multilocus bootstrap replicates (Fig. 2). In order to examine the effect of orthologues selected for multi-gene tree analysis, we inferred the relative tree certainty for each of the 325 orthologues using RAxML (cite), using 100 rapid bootstrap replicates under the LG+G4 model of evolution. The orthologues were ranked and the top 50% with the highest relative tree consistency (RTC) scores were selected and multiple gene phylogeny conducted as above (Fig. S6B).

The resulting tree topology (Fig. 2) demonstrates that *H. catenoides* forms a sister-branch to the oomycete radiation with >=99% support from all methods used for both complete multi-gene analysis and the orthologues ranked in the top 50% with the highest RTCs (Fig. S7). The internode consistency scores showed moderate support for this relationship consistent with the possibility of mixed signal for this branching relationship in our ‘orthologue’ gene sets. Nonetheless, these results are consistent with the Pseudofungi hypothesis, i.e. the hyphochytriomycetes and the oomycetes are monophyletic and share a common evolutionary trend towards fungal-like osmotrophic feeding and polarized cell growth [3, 4].

Our tree places the Pseudofungi as a sister group to the photosynthetic stramenopiles (i.e. the Ochrophyta) plus *Developayella.* This has someconsistencies with previously published phylogenetic analysis based on three nuclear encoded genes [28] and wider phylogenomic analysis 23 and [29] and in contradiction to analyses of mitochondrial gene phylogenies (concatenation of ten genes, 7,479 positions), which have demonstrated that a separate stramenopile group the Labyrinthulida (i.e. Bigyra) form a sister-group to the oomycetes [30]. We note however, this phylogeny demonstrates a different branching relationship with *Developayella* which is now sister to the Ochrophyta, a relationship very weakly supported in the internode consistency analyses [IC/TC paper]. The tree recovered here has some similarities to that reported by Derelle *et al.* which uses a large phylogenomic dataset from different taxa. This work argues for monophyly of Bigyra (e.g. *Blastocystis + Aplanochytrium* and *Schizochytrium*) although, our tree shows that this group is paraphyletic, a relationship also shown in Noguchi *et al* [23]. Derelle *et al.* also recovered paraphyly of this group in a subset of their Bayesian analysis and in their ML analysis, but then went on to demonstrate that this relationship is likely due to a long branch attraction artefact (e.g. [31]) associated with the *Blastocystis* branch and which can lead to the misplacement of Opalozoa (e.g. *Blastocystis*). Interestingly, sisterhood of the Pseudofungi and Ochrophyta implies a minimum of two losses of photosynthesis [29] and independent specialization of ‘osmotrophic lifestyles’ in the Bigyra (e.g. *Aplanochytrium* and *Schizochytrium*) and the Pseudofungi (e.g. *Hyphochytrium* and *Phytophthora*)within the stramenopiles. However, this scenario implies that the stramenopile lineage was ancestrally photosynthetic [32] a subject of debate [33, 34] Fig S3.

**Shared derived traits across the Pseudofungi**

Given the placement of *H. catenoides* as a sister branch to the oomycetes, we were interested in investigating the conservation of cellular, biochemical and genetic traits shared across Pseudofungal taxa*.* Oomycete plant pathogens, for example *Phytophthora* spp., are sterol auxotrophs and appear to have lost the enzymes involved in sterol biosynthesis [35]. Sterol biosynthesis pathway has been predicted to function in *Saprolegnia* and a putative CYP51 sterol-demethylase encoding gene was identified from the *Saprolegnia parasitica* genome and transcriptome data [12, 36]. The protein encoded by this gene is a target of antimicrobial drugs such as clotrimazole and therefore has been suggested as therapeutic target for treatment of *Saprolegnia* infections of fish [37]. Reciprocal BLASTp searches and phylogenetic analyses demonstrated that *H. catenoides* also possesses a putative orthologue (Hypho2016\_00003038 - Fig. S8a) of the *S. parasitica* CYP51 sterol-demethylase, which appears to be lost in plant pathogenic oomycetes. To confirm that this is a viable drug target we grew *H. catenoides* in the presences of two azole ‘antifungals’ - clotrimazole and fluconazole – to assess effectiveness of these compounds in inhibiting *H. catenoides* growth. Both ‘antifungal’ agents were able to inhibit growth of *H. catenoides* (MIC100: clotrimazole 0.25 µg ml-1; fluconazole 4 µg ml-1. Fig. S8b), indicating that the *H. catenoides* is susceptible to azole compounds, consistent with *H. catenoides* having a functional and critical CYP51 enzyme.

There has been considerable effort to sequence a number of oomycete genomes, which has largely focused on parasitic taxa (e.g. [11, 12, 38-41]). This work has also in-part focused on identifying candidate effector proteins (secreted proteins that perturb host function for the benefit to the invading parasite [42] and which often contain N-terminal RxLR amino acid motifs [43-45]) or lectin proteins that bind host molecules. Searches of the *H. catenoides* genome demonstrate there is only one putative protein of unknown function with a candidate RxLR motif (Table 1). In addition, *H. catenoides* lacked several gene families linked with the evolution of plant pathogenic traits in the oomycetes, i.e.: NPP1 or NEP-like proteins (necrosis inducing *Phytophthora* protein [46, 47]), Elicitin proteins [48], Cutinase [49], pectin esterase and pectin lyase [50, 51]. The animal parasite *S. parasitica* was noted to show enrichment of Notch proteins and Ricin lectins, as well as presence of other galactose-binding lectins and the bacterial toxin-like gene family (haemolysin E) [12]. While the Notch protein and Ricin lectin gene families are present in *H. catenoides*,they show no evidence of enrichment comparable to *S. parasitica*. The galactose-binding lectin and haemolysin E gene families are absent. Protease gene families show no general enrichment in comparison to other stramenopiles (Table 1).

Comparative analysis of candidate secreted proteins defined by *in silico* identification of putative N-terminal secretion sequences demonstrated that*H. catenoides* contains a lower proportion of secreted proteins compared with many other stramenopiles, comparable with the paraphyletic obligate biotrophs *Albugo laibachii* and *Hyaloperonospora arabidopsidis* (Fig. 3)*.* The *H. catenoides* predicted proteome contains a moderate-to-low proportion of carbohydrate active enzymes relative to other stramenopiles [52]. Interestingly, *H. catenoides* has very few secreted carbohydrate active enzymes in comparison to other stramenopiles, suggesting that *H. catenoides* has a low diversity of extra-cellular carbohydrate processing functions and is therefore dependent on a limited subset of extracellular sources of fixed carbon (Fig. 3). To test this observation, we grew *H. catenoides* cultures in 190 different carbon sources using OmniLog PM1 and PM2 plates, which allows investigation of growth and respiration rate across a diversity of different carbon sources [53]. These data demonstrated (Fig S8a/b) a significant increase in respiration rate compared to the controls upon the addition of: α- or β- cyclodextrin (*p* value = 0.01 & 0.01), dextrin (*p* value = 0.02), Tween 40 or 80 (*p* value = 0.03 & 0.03), or melibionic acid (*p* value = 0.03). Of note, dextrin/ cyclodextrins are products of enzymatic activity upon starch, a typical component of *H. catenoides* growth medium (YpSs), and may be indicative of the environment in which this organism is typically found. The addition of Tween 40 or Tween 80 has been shown to improve yield in other organisms [54] and may result from direct accumulation of fatty acids, or altered membrane permeability affecting nutrient uptake. In contrast to many oomycetes (e.g. [55]), *H. catenoides* demonstrates a limited utilisation of diverse carbon sources. These data are consistent with the hypothesis that the evolution of a wide diversity of secreted carbohydrate active enzymes is associated with evolution of parasitic lifestyle within the oomycete lineages (e.g. [12, 56-58]), although this pattern could also be the product of secondary loss in the *H. catenoides* branch.

Seidl et al. detected 53 domain architectures that were unique and conserved across the oomycetes *P. infestans, P. ramorum, P. sojae* and *H. arabidopsidis* [59]*.* Domain architectures are often recombined by a process of gene fusion and/or domain ‘shuffling’ [60]. As such gene fusion characters, although subject to sources of homoplasy (such as gene fission [61]), can represent synapomorphic traits useful for polarising phylogenetic relationships. We searched the *H. catenoides* genome for evidence of the 53 gene fusions previously identified in oomycetes [59] and found that 12 of these domain architectures were also present in *H. catenoides* (Table S2). Of note, we found a fusion gene of a putative β-glucan synthase enzyme domain and a putative membrane transporter gene (Table S2 & genbank ‘nr’ protein database) shared across the Pseudofungi, suggesting that domain fusion has led to a unique coupling of substrate transportation and enzymatic processing prior to the radiation of this group. Although theoretically, without proteomic data, we cannot exclude the possibility that this novel domain combination may be the product of a conserved operon-like gene structure.

Using OrthoMCL [62] combined with a custom pipeline we identified nine Pseudofungi-specific orthologues, with five of these orthologues representing additional Pseudofungi-specific domain combinations (Table S3). Of note, these combined results (Table S2 and S3) demonstrate a novel diversification of the Serine/Threonine Kinase gene families, consistent with an expansions of kinase encoding gene families present in oomycete genomes [12].

**Protein repertoire changes associated with loss of the posterior flagellum**

The stramenopiles (also known as Heterokonta, meaning possessing of two unequal flagella) were formally described as a phylum based on the presence of two motile flagella: a ‘standard’ smooth posterior flagellum and an anterior flagellum with tripartite rigid tubular mastigonemes (‘tinselate’) [2]. *Hyphochytrium* builds only a single, anterior tinselate flagellum [6] while the oomycetes build the stramenopile flagella pair. Therefore, the posterior smooth flagellum was lost in the ancestor of the hyphochytrids (Fig. 2). To explore the consequence of the loss of this organelle in *H. catenoides*, in terms of gene/protein repertoire, we used a comprehensive list of proteins putatively associated with flagellar function [63] to survey the *Hyphochytrium* genome. This list comprises 592 amino acid sequences, 355 are found in both the major eukaryotic phylogenetic groupings of Opimoda and Diphoda [64], suggesting they are universal flagellar proteins (UFPs - Table S4 & Fig. 4a). 330 of the 355 UFPs are also present in the predicted proteome of *H. catenoides*, suggesting that the majority (93%) of the UFPs have been retained and likely encode a function associated with the anterior tinselate flagellum.

Flagellum-specific proteomic analysis of the stramenopile brown alga *Colpomenia bullosa* identified fourteen proteins specific to the posterior flagellum and three specific to the anterior flagellum [63]. BLAST searches suggest that the three anterior flagellum proteins are also present in *H. catenoides,* as are twelve of the fourteen posterior flagellum proteins from *C. bullosa*. Conservation of these “posterior-specific” proteins suggests that they have functions associated with the anterior tinselate flagellum in *H. catenoides* (Fig. 4a)*.* One of the *C. bullosa* posterior specific flagellum proteins absent in *H. catenoides* and the oomycetes is the PAS/PAC sensor hybrid histidine kinase (also known as a Helmchrome, CBJ26132.1), a putative photo-sensor associated with a swelling in posterior flagellum of brown algae [63], discussed further below.

Twenty-nine of the UFPs (8%) were present in oomycetes and other eukaryotic groups but absent in *H. catenoides*. These may represent genuine gene losses, although absences in our draft genome may also be due to incomplete genome sequencing and assembly. If these are genuine losses, it suggests they represent UFP losses that correlate with loss of the posterior flagellum without the function of these UFPs being integrated into the anterior-tinselate flagellum (Fig. 4a)*.* These losses include a putative homologue of the dynein regulatory complex 1 (DRC1) protein, which regulates inner dynein motor activity in *Homo sapiens* and *Chlamydomonas reinhardtii* [65], and Radial Spoke protein 7 (RSP7), a protein that functions in flagellum structure and beating in *Chlamydomonas reinhardtii* [66].Further, analysis of the radial spoke protein repertoire encoded by *H. catenoides* identified a number of other components of the radial spoke complex which are putatively absent in *H. catenoides*. However, RSP7 was the only radial spoke proteome loss specific to the loss of the posterior flagellum in the *Hyphochytrium* lineage (Fig. 4a/b); this protein is putatively encoded in the oomycetes but has been separately lost within the Opisthokonta (e.g. *Homo sapiens*)*.* In *Chlamydomonas* [66], RSP11 and RSP7 have been shown to contain a RIIa domain [67]. Association between RIIa and AKAP domains and RSP3 at the spoke stalk are suggested to be important for flagellar function [66]. Interestingly, comparative analysis suggests that neither RSP7 nor RSP11 are conserved across flagellum-bearing eukaryotes with only *Chlamydomonas*, *Batrachochytrium* and *H. catenoides* retaining RSP11 in our comparative dataset (Fig. 4a/b)*.* Domain analysis [68] of the putative *H. catenoides* RSP3 and RSP11 confirmed these proteins contain an AKAP and a RIIa domain respectively suggesting that *H. catenoides* has retained only RSP3-RSP11 protein-protein interaction at the base of radial spoke, proximate to the outer doublet (Fig. 4b).

Phylogenomic analysis of motor protein repertoire, specifically Kinesins and Dyneins (Fig. 4c/d), confirmed that the *H. catenoides* genome assembly has retained many of the motor proteins associated with flagellum function. These include representatives of all seven axonemal dynein heavy chain families (plus their associated intermediate and light-intermediate chains) [69], both the retrograde (DYNC2) and anterograde (Kinesin-2) motors used in intraflagellar transport (IFT), and non-motor components of the IFT particles (Fig. 4c). Also identifiable are members of Kinesin-9 and -16 families, which are present in organisms which build motile flagella [70] (Fig. 4d). This motor repertoire is very similar to that seen in oomycetes and shows that the modified tinselate *H. catenoides* anterior flagellum has retained most functions associated with flagellar motors. Wickstead & Gull have also proposed that the Kinesin-17 family has a flagellar function based on its phylogenetic distribution [70]. Our analysis suggests that *H. catenoides* has lost Kinesin-17 (unlike in oomycetes). This may be associated with the loss of the posterior-smooth flagellum, but may also represent gaps in the draft assembly.

**Photoreceptors**

Stramenopile species have been shown to encode a range of photoreceptor proteins and to initiate a series of responses to light including phototaxis [71]. Specifically, the zoospores of some stramenopile algae can show positive and negative phototaxis [72] associated with a flavoprotein photoreceptor [73] putatively the ‘helmchrome’ located in the posterior flagellum [63] and associated with ‘flagellar swelling’ and a stigma [72]. Consistent with the loss of the anterior flagellum *H. catenoides* (Fig. 4/S9) also lacks a gene putatively encoding a helmchrome protein*.*

A number of additional putative photo-responsive proteins have also been reported from *Ectocarpus* [10]. Using these data and other seed sequences (e.g. [63, 74]) we searched the *H. catenoides* genome for putative homologues of photo-responsive proteins. Reciprocal BLAST searches demonstrated that the *H. catenoides* genome contained putative homologues of the flavoproteins; Cryptochrome (Hypho2016\_00016188), Cryptochrome DASH (Hypho2016\_00004514) and Photolyase (Hypho2016\_00002462) gene families (Fig. S10a)and transcriptome data demonstrate that these genes are transcribed. This analysis also identified three putative type I (microbial) rhodopsins (Hypho2016\_00006030, Hypho2016\_00006031 & Hypho2016\_00010050), the first putative representative of this gene family from a stramenopile (Fig. S10a/b). The three rhodopsins all contain a conserved 11-*cis*- retinal binding pocket, specifically the lysine residue site of the Schiff base where the retinal is covalently linked (Fig. S10a/b). Furthermore, reciprocal BLAST searches of both the genome and the transcriptome sequence datasets confirmed the presence of genes putatively encoding the latter two steps of the retinal biosynthesis pathway (e.g. a putative β-carotene-15, 15’-dioxygenase (Hypho2016\_00004122) and a putative retinol dehydrogenase (Hypho2016\_00000702). These genes encode the pathway steps that convert the vitamin β-carotene into 11-*cis*-retinal; the critical cofactor for rhodopsin to function as a light responsive protein.

**Gene families encoding hallmarks of fungal characteristics in the Pseudofungi**

One of the main purposes for the sequencing the *H. catenoides* genome was to investigate conservation and/or loss of genes that underpin the fungal/pseudofungal lifestyle. Many fungi grow as filamentous cells, reinforced by robust cell walls composed of polysaccharides such as chitin. These characters are not unique to fungi but are typical in many fungal lineages [75]. A suite of cellular systems allow fungi to grow as polarised cells, laying down cell wall and feeding on extracellular substrates by a combination of exocytosis of enzymes and cell wall material combined with endocytosis and transporter protein mediated uptake of target nutrients. Fungal filamentous structures such as hyphae grow almost exclusively from the tip of the hyphal structure [76] allowing fungi to ‘grow as they feed.’ This feature combined with a robust cell wall means they can generate high turgor pressures, ramify into recalcitrant material, feed osmotrophically and maximize metabolic rates [75, 77, 78]. Homologous cellular systems also drive bud growth in *S. cerevisiae*, allowing researchers to use *S. cerevisiae* to study proteome function involved in polarized growth (for review see [76, 79]). The proteins that are known to control this system are illustrated on Fig. 5a and involve key complexes, the Exocyst and the Polarisome. These systems are very important for establishing the temporal and spatial control of polarized cell growth in fungi [76, 79]. Comparative analyses show the Exocyst and Sec4 orthologues are conserved across a diversity of eukaryotes including *H. catenoides*, while the Polarisome and associated proteins are specific to fungi, given current taxon sampling. Comparative analysis demonstrates that specific elements of polarized cell growth control are not present in Pseudofungi, suggesting these filamentous microbes accomplish polarized growth using different proteome functions.

Motor protein evolution has been suggested to be an important factor in the acquisition of filamentous growth phenotypes in the fungi, with a specific focus on myosin and kinesin genes that encode functions involved in polarised cell growth, vesicle-transit and chitin synthesis [80-82]. Phylogenomic analysis of the motor head domain of all three motor types (Fig. 4 c, d, and e) demonstrates no expansion in motor paralogues uniquely shared by fungi and pseudofungi. In addition, Pseudofungi lack the Myosin V and XVII shown to be important in fungal growth and chitin synthesis [81] (Fig. 4e). The lack of shared/unique motor repertoire between fungi and Pseudofungi, is consistent with the idea that these groups evolved filamentous polarized growth characteristic separately and based on different cellular systems. It has been noted that oomycetes contain a diverse complement of myosin paralogues. The analyses reported here demonstrates that elements of these oomycete motor protein gene family expansion are also present in *H. catenoides,* specifically; Myosin XXX and XXI and Kinesin 14 and 20 show high degrees of expansion by duplication specific to the Pseudofungi (Fig. 4 c, e, f), suggesting these motor proteins may be linked to unique filamentous polarized growth characteristic present in the Pseudofungi.

Like fungi [85] and many other eukaryotes [86-92], *H. catenoides* also produces chitin as cell wall material [93]. Oomycetes have also been shown to produce chitin in their cell walls [94]. This is consistent with previous data that suggest that chitin synthesis and deposition as a cell wall material predates the diversification of many major lineages of the eukaryotes [75, 93]. *H. catenoides* has a similar repertoire of chitin synthesis and digestion as found in the oomycetes (i.e. chitin synthase division I), while another group of stramenopiles, the diatoms, which also produce chitin [95], have a variant chitin gene repertoire, namely chitin synthase division II and a chitinase (GH19) not present in Pseudofungi (Fig. 6). This suggests that chitin production as a cell wall component is universal and anciently acquired in the eukaryotes, but the genes that control the synthesis and remodelling of this structural polysaccharide have been reconfigured numerous times. Specifically, Pseudofungi seem to lack all chitin synthase division II genes, which are numerous and diversified in fungi, suggesting another key difference, between the Fungi and Pseudofungi.

**Viral integration across the Pseudofungi**

The comparative genomic analysis of Pseudofungi demonstrated that *H. catenoides, Phytophthora cinnamomi, Phytophthora parasitica* and *Pythium ultimum* harbour genes putatively encoding viral major capsid proteins (MCP) (Table S5). These proteins have high sequence identity with each other and branch together with MCP proteins from African swine fever virus (Asfarviridae, lineage of the nucleocytoplasmic large DNA viruses - NCLDVs), but which are divergent when compared with other NCLDV MCP proteins (Fig. 7a+b). Exploring the *H. catenoides* genome assembly to determine the presence of viral-like genes, we identified 45 putative genes, 38 of which are present on two scaffolds which were shown to have very low SNP frequency in the assembly (Table S5). All of these 38 genes showed highest similarity to NCLDV families such as Mimiviridae, Marseilleviridae, Phycodnaviridae, Asfarviridae and Poxviridae (Table S5). The genome assembly in these regions was confirmed by nested PCR and sequencing from both the 5’ and 3’ end of the *polB*, *mcp*, *mg96* genes of viral ancestry (Table S6). The viral-like genes were found in linkage with genes of *H. catenoides*/pseudofungal ancestry. For example, the genome assembly demonstrated that the viral-like *mcp* gene was on the same DNA contig as a putatively native *H. catenoides* histone-encoding gene (Fig. S11). To confirm this assembly and linkage between ‘host’ and viral gene we conducted a bridging PCR resulting in an amplicon of 2,837 bp and sequenced this amplicon confirming that the *mcp* and histone genes are linked and on the same stretch of DNA (Table S6).

145 predicted genes were identified in the two contigs that contain a high number of viral genes. BLASTx analyses suggests that the two contigs contained 37 (26%) and 18 (12 %) genes of highest identity to genes of known viral genomes (Table S7). The BLASTx results for the remaining 235 putative genes showed a wide variation of top scoring hits including both prokaryotic- and eukaryotic-like genes. The frequency of putative exons for the two contigs was 1.62 and 1.49 respectively, a lower intron frequency than observed for the wider genome (3.64), thus suggesting that genes encoded on viral gene-containing contigs have introns. Indeed, multiple viral-like genes show evidence of introns suggesting these genes have been either: incorrectly modelled, subject to intronization, exon-like shuffling during integration, or these genes are undergoing pseudogenization and are therefore broken ORFs, which are being reported as intron/exon structures. However, we note that gene of viral provenance Hypho2016\_00000945-RA (scaffold 5419) contains multiple putative coding regions present in our transcriptome data. The low SNP frequency of these contigs suggest they represent a unique haploid portion of the genome, a viral genome captured in our assembly, or alternatively a site of viral introgression in the *H. catenoides* genome. We currently favour the hypothesis that this is a site of viral introgression due to the presence of putative introns in the contig and the low relative proportion of genes of clear viral provenance.

Products from *polB, mg96* and *rps3* were detected by RT-PCR in our culture conditions, suggesting that viral-like genes are transcriptionally active (Fig. 7b)*.* In contrast, a lack of transcript from the *mcp* gene suggests that a complete virus or a viral factory is not being manufactured in the culture conditions tested (Fig. 7b). Electron microscopy also failed to observe icosahedral structures typical of viral particles or an intracellular viral factory (69) (see Fig. S12).

These data combined with evidence of viral genes present in oomycete genome assemblies (Fig. 7a) [96] suggests a hitherto un-sampled diversity of large DNA viruses found infecting, or integrated within the genome of Pseudofungi. This is consistent with other data suggesting the Pseudofungi have been subject to viral transduction (70). It has also been shown that many different lineages of the stramenopiles have similarly retained fragments of viral genomes [97], suggesting a wider and undersampled diversity of stramenopile infecting large DNA viruses. It is tempting to speculate that this may be a mechanism driving HGT seen in the oomycetes (see [98]), given that NCLDVs have been shown to harbour host-derived and foreign genes [99, 100] and that fragments of large DNA viruses have now been shown to be present in fungi [96], a group shown to be a donor of HGT-genes to the oomycetes [58, 98]. Consistent, with this, we note that the two contigs containing the viral derived genes also contain two genes with top BLASTx hits to fungal genes (Table S7).

The Pseudofungi are thought to lack the capabilities to perform phagotrophy [4], a mechanism hypothesised to be important for HGT in eukaryotes [101]. However, there is evidence of gene transfer into the oomycetes from both fungi and prokaryotes [49, 58, 102-106]. The extent of ancient HGTs in eukaryotes has recently been questioned [107]. Yet, Ku et al. [107] also identified genes uniquely present in oomycetes and bacteria which are described as ‘recent lineage specific acquisitions’ (see *Fig. 1* in [107], marked as *b*). Evidence of viral introgression within the Pseudofungi therefore identifies a possible mechanism driving HGT in the Pseudofungi, which cannot perform phagotrophy. It is important to note that viral transduction as a vector for HGT in the eukaryotes would likely produce a very different profile of gene transfer compared to mechanisms such as phagocytosis (in eukaryotes), transformation (prokaryotes and eukaryotes), or conjugation (prokaryotes and eukaryotes). This is because gene transfer via a virus would likely transfer a lower number and lower diversity of gene families for two reasons; 1) genes carried by the virus would have been passaged by selection within the viral lineage, and, 2) the limited DNA carrying capacity of the viroid. Such a mechanism of HGT is therefore consistent with the results of Ku et al. [107], which suggests HGT is less frequent in eukaryotes compared to prokaryotes. However, this does not exclude the possibility that infrequent HGTs can lead to the acquisition of novel and positively selected traits.

**Conclusion**

The draft genome of the free-living stramenopile pseudofungus *Hyphochytrium catenoides* provides an important reference for comparative biology specifically with a view to understanding the evolution of filamentous growth and osmotrophic feeding. *H. catenoides* branches sister to the oomycetes that contains many important pathogenic groups. These data demonstrate that *H. catenoides* does not encode many of the gene families found in oomycetes that have been associated with pathogenic function, suggesting that these characteristics are more recent adaptation/acquisitions within the oomycetes (Table 1). Our data also demonstrates that *H. catenoides,* and the Pseudofungi more widely, possess the genes which encode a range of features associated with filamentous growth and osmotrophic feeding in fungi. These include the Exocyst vesicle trafficking system, sterol biosynthesis pathway, and a repertoire of chitin cell wall synthesis systems common to fungi. In contrast, Pseudofungi do not possess the genes encoding a Polarisome complex or chitinase I, chitin synthase II / Myosin V, Myosin XVII, identifying clear differences between these two filamentous osmotrophic groups. Figure 7 summarises how various features associated with filamentous growth and osmotrophic feeding arose relative to the branching position of the fungi and the Pseudofungi. We hope the *H. catenoides* draftgenome will provide a useful dataset for comparative biology within the Pseudofungi and across the eukaryotes especially with regards to understanding the evolution of filamentous osmotrophic characteristics.

**Materials and Methods**

**Cell Culture in Preparation for Sequencing**

*Hyphocytrium catenoides* (ATCC 18719) was inoculated onto Emerson YpSs agar. Cell mass was prepared for DNA and RNA extraction as described previously [58]. DNA samples were checked for contamination using an environmental DNA SSU PCR approach [58] using both eukaryotic 18S PCR primers 1F (CTGGTTGATCCTGCCAG) and 1520R (CTGCAGGTTCACCTA) (e.g. [108]) producing a clean chromatogram of a *Hyphochytrium* 18S sequence and prokaryotic 16S PCR primers PA (AGAGTTTGATCCTGGCTCAG) and PH (AAGGAGGTCATCCAGCCGCA) which were negative (e.g. [109]).

**Genome and Transcriptome Sequencing, Assembly and Validation and ORF Calling**

One lane of paired-end (100 bp) Illumina HiSeq data was generated along with two lanes of paired-end (76bp) Illumina GAiix at the Exeter Sequencing Service producing 2x 212,760,559 HiSeq reads along with 2x 15,266,599 and 2x 16,274,715 GAiix reads. After trimming and cleaning (using TagCleaner [110] and PRINSEQ [111]) of the data, we subsequently digitally normalised it with KHMER [112] in order to discard redundant data, sampling variation and remove errors. This reduced the number of reads to 415,241,668 HiSeq along with 28,964,302 and 30,961,514 GAiix; a reduction of 13,436,262 reads in total. The raw reads are deposited in NCBI and EBI with accessions as: Illumina GAiix = SRX033129 and Illumina HiSeq = ERS1151585 respectively. An initial assembly, using the program Ray v2.2.1 [113] was generated (see tps://github.com/guyleonard/hyphochytrium/tree/master/manuscript/data for details of commands used), and produced 29,448 scaffolds, with a total of 107,387,882bp, and an N50 of 8,746bp.

Next, we investigated the possibility that *H. catenoides* was a diploid using an assembly program that allows for multiple ploidy. The program Platanus v1.2.1 was used to produce an assembly with 53,358 scaffolds incorporating 68,330,525bp, and an N50 of 29,450bp (see tps://github.com/guyleonard/hyphochytrium/tree/master/manuscript/data for details of commands used).

The Platanus assembly was subsequently filtered into 4 datasets; all scaffolds, scaffolds >= 10 kbp, scaffolds >=5 kbp and scaffolds >=1 kbp in order to test the effects of the N50 statistic and gene recovery rate by removing short and erroneous scaffolds/contigs (Fig. S1). We determined that the set of scaffolds >= 1kbp did not affect our predicted proteome complement and increased the N50. The filtered >=1 kbp Platanus assembly, along with the mitochondrial genome assembly, are deposited in EBI with the accessions; Study ID: PRJEB13950, Scaffolds: FLMG01000001-FLMG01004758 and Mitochondria: LT578416. The full assembly and other filtered datasets, can be accessed here: <https://github.com/guyleonard/hyphochytrium> or <https://www.ebi.ac.uk/biostudies/studies/S-BSST46>.

K-mer counting analysis was conducted using Jellyfish along with two publically available scripts (estimate\_genome\_size.pl and the website GenoScope, see https://github.com/josephryan/estimate\_genome\_size.pl and [114]). The average sequencing coverage of this assembly was estimated using the ‘estimate\_genome\_size.pl’ tool for the total assembly and using the ‘genomeCoverageBed’ from BEDTOOLS [115] for the >=1kbp subset of scaffolds.

Gene prediction was conducted by using CEGMA to predict which of the 246 core genes are present in our *Hyphochytrium* >=1kbp scaffolds, these predicted CEGs are then used in the training step of the program SNAP (see http://korflab.ucdavis.edu/software.html) to generate a set of *ab initio* gene models. The program GeneMark-ES [116] was also run independently on the >=1kbp scaffold data, which produced another set of gene models. Both these sets of gene models are in the form of a hidden Markov model (HMM). A first pass of the pipeline MAKER is then run with the default settings, incorporating the gene models from SNAP & GeneMark-ES whilst also deriving alignment statistics from the 454-transcriptome assembly with tBLASTn, RepeatMasker [117] and exonerate [118]. The output is a set of gene models in GFF3 format. A second round of SNAP is then performed with the new predictions (after the GFF3 has been converted to a HMM) and the program AUGUSTUS [119] is run in *ab initio* mode using the MAKER first pass predictions (i.e. AUGUSTUS default gene models were not used as they are generated from distantly related taxa). Both outputs of SNAP (run 2) and AUGUSTUS are then fed back into MAKER for a second run with stricter settings (gene predictions are available here: <https://github.com/guyleonard/hyphochytrium/tree/master/gene_predictions>). The final output is a GFF3 file, transcripts and protein FASTA files. The resulting gene predictions were then BLAST searched against the SwissProt database along with InterproScan to assign putative annotations. The results were then used with the program ANNIE [120] to provide the correct format of annotation information to the program GAG [121] for database deposition. The resulting genome data is submitted as an update of a prior BioProject sequence submission [58], to do this we used the ‘gff3toembl’ program from PROKKA [122].

Previously we had sequenced a transcriptome from the same culture strain of *Hyphochytrium* [58] using 454 FLX sequencing of cDNA reads and assembled it with Newbler 2.5 [123] using the default cDNA settings. We removed 70 sequences from this assembly of less than 100 bp in length (excluding the polyA regions) and/or contigs that consisted of predominately repeat motifs. This resulted in 6,202 transcript sequences assembled in Newbler 2.5 using the standard settings for cDNA. The reads were also assembled in Trinity but resulted in significantly more [nearly double] contigs.

**Assessment of contamination of the genome sequence**

To identify any prokaryotic contamination in the >=1 kbp scaffold assembly we first conducted BLASTn searches of the assembly using prokaryotic SSU and LSU rDNA sequences as search seeds (*Escherichia coli* taken from [CP012802] and *Sulfolobus acidocaldarius* [NR\_043400 & NR\_076363]). This analysis only returned sequences of similarity to the *H. catenoides* mitochondria genome assembly, suggesting that no, or very limited, prokaryotic sequence contamination was present. To support this, we subjected all 4,758 genome scaffolds to a BLASTx analysis against a database of 65 eukaryotic and 164 representative prokaryotic complete predicted proteomes (Table S8) with a gathering threshold of 1e-10. This approach did not identify any scaffolds that did not have at least one top hit to a eukaryotic genome for a subsection of the scaffold. Indeed, only 87 of the scaffolds had > 50% of the subsections with a top BLAST hit to a prokaryotic genome and only 20 of the scaffolds had > 70% of their top BLAST hits to a prokaryotic genome. These 20 scaffolds were inspected manually, 11 of these showed the presence of putative spliceosomal introns and/or other genes more similar to other eukaryotic genes. For the remaining nine scaffolds (totalling 31.8 kbp) we could not exclude them as possible prokaryotic contamination (listed in Table S9).

Comparisons of GC content versus read coverage coupled with BLASTn analysis to identify likely aberrant genomic affiliation of assembly scaffolds (e.g. “blobology” [124]) has emerged as useful tool for identifying contamination of genome sequencing projects [125]. We undertook this approach on both the >= 1 kbp scaffold assembly and the total assembly, and the graphs did not identify any suspect traces of contamination, however, they do show the presence of the mitochondrial genome as an aberrant cluster of ‘blobs’, i.e. with lower than average GC content (Fig. S131a-d).

A fourth round of checks for contamination were conducted by using tetramer counting of the >=1 kbp scaffold dataset for the building of Emergent Self Organising Maps (ESOMs) [126]. These use similarities in the 4-mer frequencies to build, by way of an artificial neural network, an emergent ‘map’ of the input space properties of the data. Two runs of the software developed in [126] were completed (see Fig. S14a+b): a) the *Hyphochytrium* scaffolds only, and b) the *Hyphochytrium* scaffolds along with the scaffolds from eight other ‘small’ genomes which were added to the tetramer frequency dataset, (Bacteria (blue): *E. coli, Mycobacterium tuberculosis;* Archaea (grey): *Methanococcus vanniellii, Sulfolobus solfataricus;* Fungi (purple): *Encephalitozoon intestinalis, Saccharomyces cerevisiae;* Archaeplastida *Ostreococcus tauri;* Protist (red): *Cryptosporidium hominis*). The maps produced in Fig. S14 show no indication of overlap or features indicative of contamination.

***H. catenoides* genome qPCR size estimation**

The haploid genome size of *H. catenoides* was estimated using a qPCR based method (30). 50 ml of a *H. catenoides* culture, grown in YpSs for 7 days at 25°C, was centrifuged for 3 mins at 3200 x g. The supernatant was removed and genomic DNA was extracted from the remaining cells using a PowerSoil DNA isolation kit (MO BIO Laboratories). An rps3 PCR standard was amplified using primers Hcat\_rps3\_F (CGAGGGCTACATGGTCAAGA) and Hcat\_rps3\_R CCTTTGGCTCGATGATGGTG). Each 25 μl reaction consisted of 0.5 U Phusion polymerase (New England Biolabs), 1x HF buffer, 400 μM dNTPs, 2 μM each primer and 1 μl *H. catenoides* genomic DNA (11.6 ng μl-1). Cycling conditions consisted of an initial denaturation of 5 mins at 98°C, followed by 30 cycles of 10 s at 98°C, 30 s at 61.0°C and 30 s at 72°C, then a final extension of 5 mins at 72°C. The 185 bp PCR product was purified by gel extraction (Thermo Scientific GeneJET Gel Extraction kit) and eluted using elution buffer. Concentration of the purified product was determined using a Qubit dsDNA HS assay kit (Thermo Fisher Scientific). Real-time PCR was used to quantify the number of copies of rps3 present in each genomic DNA sample. Quantitative PCR was performed in a StepOnePlus Real-Time PCR system (Thermo Fisher Scientific). Reaction conditions were optimised using a gradient PCR and a standard curve was determined using dilutions of *H. catenoides* genomic DNA and analysed using StepOne Software v2.3 (slope: -3.367; y-intercept: 33.841; efficiency: 98.15%). Each 20 μl PCR contained 10 μl PowerUp SYBR Green Master Mix (Thermo Fisher Scientific), 500 nM each primer (Hcat\_rps3\_F and Hcat\_rps3\_R, sequences as above) and 1 μl template DNA. Template was either *H. catenoides* genomic DNA or the PCR standard. Standards were diluted (10-1 to 10-7) from an initial concentration of 24.7 ng μl-1 and performed in triplicate, while three independent genomic DNA samples were run in quintuplicate. Cycling conditions were as follows: UDG activation for 2 mins at 50°C and DNA polymerase activation for 2 mins at 95°C, followed by 40 cycles of 15 secs at 95°C and 1 min at 60°C. ROX was used as a reference dye for analysis of CT values. Each reaction was followed by melt-curve analysis, with a temperature gradient of 60°C to 95°C at 0.3°C s-1, to ensure presence of only a single amplicon. The PCR standards were used to create a calibration curve (y = 8x1010 x e-0.67x; R2 = 0.99992); CT values from amplifications of genomic DNA templates were then applied to this curve and the ‘mass’ of the haploid genome was calculated (30). This value was then used to calculate the haploid genome size, using 615.8771 g mol-1 as the mean molar mass of a base pair [127].

**Mitochondrial genome assembly**

Contigs of putative mitochondrial origin, from both assemblies, were identified by BLAST searches against the mitochondrial genome of *Phytophthora infestans* (NC\_002387.1). The contigs from the genome assemblies were visualized, linked and edited using the program SEQUENCHER (<https://www.genecodes.com>) resulting in two contigs. However, we were unable to circularise the genome using these two fragments. Therefore, regions spanning the gaps in the mtDNA super-contigs were amplified by polymerase chain reaction (PCR) with primers specific to the flanking sequences. Purified PCR products were sequenced using Sanger chemistry (externally at Eurofins Genomics, Ebersberg). This allowed the two contigs to be joined, resulting in a linear genome flanked on one end with rpl16 and atp8 on the other. These genes were identical to the other rpl16 and atp8 genes found in the assembled mitochondrial genome; we therefore inferred that these represented the beginning and end of a 19 kb inverted repeat (Fig S8). Mitochondrial genes were identified and annotated using mfannot (<http://megasun.bch.umontreal.ca/cgi-bin/mfannot/mfannotInterface.pl> last accessed 20 June, 2017) followed by manual inspection. The putatively circular genome was visualized using CGView [128] (Fig. S2). Results and discussion of the mitochondrial data can be found in Fig. S2.

**Search for *H. catenoides* representatives of key oomycete gene families**

Using Pfam searches (Pfam release 29.0) with default defined e-value cut-offs we searched the *H. catenoides* predicted proteome for: NPP1-like proteins (Pfam domain: PF05630), elicitin (PF00964), cutinase (PF01083), pectin methyl esterases (PF01095), pectate lyase (PF03211), polygalacturonase (PF00295) PAN lectin (PF00024), Ricin lectin (PF00652), Jacalin lectin (PF01419) Galactose binding lectin (PF00337), Legume lectin (PF00139), Legume-like lectin (PF03388), ABC transporters (PF00005), Protein kinase (PF00069 & PF07714), Notch protein (PF00066) and Haemolysin E (PF06109). In addition, the *H. catenoides* predicted proteome was searched against the MEROPS database <https://merops.sanger.ac.uk/> to identify putative protease inhibitors and proteases and the CAZymes analysis [129] toolkit (using Pfam) at <http://mothra.ornl.gov/cgi-bin/cat/cat.cgi?tab=PFAM1> to identify putative carbohydrate interacting proteins**.** Predicted proteins containing putative RxLR motifs and Crinkler domains were identified using the pipelines described in the following publications [130, 131].

**Secretome analysis**

Putatively secreted proteins were predicted using a custom pipeline (<https://github.com/fmaguire/predict_secretome/tree/refactor>) which identifies sequences predicted to have a signal peptide (via SignalP 4.1 [132]), no TM domains in their mature peptide (via TMHMM 2.0c [133, 134]), a signal peptide that targets for secretion (via TargetP [135]) and belonging to the extracellular ‘compartment’ (as predicted by WoLFPSort 0.2 [136]). The CAZY database [137] was downloaded, converted into a BLAST-DB and searched using the predicted proteome and secretomes using BLASTp with an expectation of 1e-5. Hit tallies were then summed, proportions calculated and data plotted in Python via the Pandas and Seaborn packages (Fig. 3).

**Phylogenetic analysis of individual gene families**

Unless otherwise stated in the figure legends all phylogenetic analyses were conducted using the following protocols. Using BLASTp we used the seed sequence to identify putative homologues across a locally maintained database of eukaryotic and prokaryotic genome-derived protein datasets (Table. S10) with a gather threshold of 1e-10. The Multiple Sequence Comparison by Log-Expectation (MUSCLE) program (v3.8.31) [138] was used to produce a multiple sequence alignment for each set of proteins. Alignments were then manually corrected and masked in SeaView (version 4.2.4) [139]. Sequences that required a high level of site exclusion (due to the sequence not aligning or not masking well) or where they formed long-branches in preliminary analysis were removed. The phylogenies were calculated using parallelised-PTHREADS RAxML (version 7.7 -Pfeiffer and Stamatakis 2010) with 1,000 (non-rapid) bootstrap replicates and using the substitution matrix and gamma distribution identified using ProtTest3 (version 3.2.1). In some cases, the invariant sites parameter was also included in the model (if indicated in the ProtTest3 analysis).

To identify putative orthologues that arose at the base of the Pseudofungi, gene clusters identified from 74 genomes (Table S11) were mapped onto the species phylogeny (e.g. Fig. 2) using a pipeline described here (https://github.com/guyleonard/orthomcl\_tools & <http://dx.doi.org/10.5281/zenodo.51349)>. Putative pseudofungal specific orthologues were individually tested by conducting gene phylogeny as described above combined with additional BLAST searches of NCBI and JGI databases to test and improve taxon sampling. See Table S3 for the resulting set of pseudofungal specific orthologues.

**Multi-gene concatenated phylogenetic analysis to identify the branching position of *H. catenoides***

Using the methods established in [[24](and Kang et al. 2017)] we built a concatenated amino acid alignment of 325 orthologues resulting in a masked data matrix of 128 taxa consisting of 90,203 amino acid sites constructed from the seed alignments used in [24].This dataset encompassed a wide sampling of eukaryotes as well as the most broad sampling of Stramenopiles available in public databases (e.g. [23, 24]). Single gene trees were inferred in RAxML under the PROTCATLG model with 100 rapid bootstraps. To examine the effect of the genes used in our phylogenomic analyses we estimated the relative tree certainty (RTC), i.e., the average of all internode confidence (IC) values for a given single gene tree given the bootstrap replicates (Salichos et al. 2014; Kobert et al. 2016). These were calculated in RAxML v 8.2.6 by comparing the best tree bipartitions to those in the bootstrap trees. The average relative tree certainty value for all single gene trees was 0.263, from the RTC values of all single gene trees the top 50% (162 genes, ranging in RTC values from 0.608 to 0.260) were extracted and further analysed (162-50RTC) (Fig. S6B). The 50RTC genes were concatenated into a supermatrix (128 taxa, 60,059 amino acids) as above and analysed also in a partitioned and coalescence framework, as was the whole 325 gene dataset.

Using these alignments (325 gene and 162 gene (162-50RTC) datasets), we calculated a maximum likelihood (ML) and 100 real bootstrap replicates using the IQ-TREE software [26, 27] using the site heterogenous model of evolution LG+G4+C60+F+PMSF (posterior mean site frequencies) substitution model [Wang et al. 2017,140, 141]. The full phylogeny for each are shown on Fig. S6A and B. Partitioned phylogenomic species trees were inferred using IQTree v1.5.5 allowing each partition to have its own model and evolutionary rates. Each partition was independently analysed under the LG+G4 model of evolution. This analysis was bootstrapped with 1000 ultrafast bootstrap replicates. For summary-coalescent species tree estimation, we employed ASTRAL with default settings and with species tree topology and node support estimated with ASTRAL multilocus bootstrapping (100 replicates). For this coalescence tree, ASTRAL was given all single gene RAxML (PROTCATLF) best ML phylogenies and 100 rapid bootstrap replicates for each single gene alignment. Internode certainty (IC) was calculated for the IQ-tree supermatrix ML tree (LG+G4+C60+F+PMSF) for both datasets (325 and the 162-50RTC). These were calculated in RAxML v 8.2.6 by comparing the overall ML bipartitions to those in the best individual ML gene trees. These IC values are mapped upon the branches on Fig S7A and B

**Identification of genes of plastid ancestry**

We constructed a database of taxonomically diverse representative genomes (Table S11) and clustered the respective proteomes into putative orthologue groups using OrthoMCL [62], retaining only the groups containing *H. catenoides* genes. Next we resampled sequences from a wider database of 1205 taxa (Table S10) using BLASTp searches [142] to recover up to three sequences from each genome using a gathering threshold of 1e-10. We then filtered these clusters identifying only those containing both a *H. catenoides* gene and genes from photosynthetic or ancestrally-photosynthetic eukaryotic taxa. These sequences were then aligned using MAFFT [143], masked using TRIMAL [144] and a phylogeny was calculated from the data matrix using FASTTREE2 [145]. The resulting phylogenies were manually inspected for a phylogeny that showed *H. catenoides*/Pseudofungal/Stramenopile genes which: a) branched within the Archaeplastida radiation, b) branched with genes of photosynthetic eukaryotes and within a bacterial radiation, or c) branched with cyanobacterial genes. This process required re-running of the phylogenetic pipeline for many gene clusters either reducing gene sampling or removing long-branch sequences. A subset of 101 gene cluster phylogenies putatively showed a phylogenetic relationship consistent with criteria *a-c* described above. The alignments from these clusters were then manually refined, the taxon sampling checked using manual BLAST searches of the NCBI nr database and phylogenies recalculated using the RAxML approach described above. The results of this analysis identified four candidate plastid endosymbiosis acquired genes, these are presented and discussed in Fig. S3.

**Testing for CYP51 sterol-demethylase drug sensitivity**

Azole susceptibility was assessed using a modified protocol [37]. Briefly, fluconazole and clotrimazole were dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 25.6 mg ml-1. Dilutions were then made with DMSO to prepare 100x stock solutions. These stocks were diluted in PYG medium to a final volume of 5 ml, each containing 100 µl of *H. catenoides* liquid culture (grown in YpSs at 25°C shaking for 7 days) to achieve final azole concentrations of 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, and 0.25 μg ml-1 and with control samples containing 1% (v/v) DMSO. Cultures were incubated, in triplicate, for 7 days at 25°C with 200 rpm shaking and MIC100 was scored manually by assessing for presence/absence of hyphal growth. See Fig. S8 for the results of the CYP51 and drug treatment analysis.

**OmniLog ‘Phenotype Microarrays’**

100 ml *H. catenoides* cultures were grown in PYG (1.25 g L-1 peptone, 1.25 g L-1 yeast extract, 3 g L-1 glucose) in baffled flasks, 25°C 170 rpm shaking, to minimise aggregation. Cells were recovered by centrifugation at

3200 x *g*, washed twice with water and re-suspended in PY-G (as above, no carbon-source) to a final concentration of approximately 1.5 x 103 cells ml-1. Cells were allowed to recover at 25°C with shaking for 30 minutes before Dye mix D (Biolog) was added to a 1x final concentration. 100 µl of cells was inoculated into each well of PM1 and PM2 carbon-source plates and incubated for 7 days at 25°C. Each growth assay was performed in triplicate from independent cultures.

OmniLog Phenotype Microarray outputs were analysed using OPM [146]. Data were aggregated using the ‘opm-fast’ method, analysed using the A parameter (maximum value of OmniLog units reached) and tested by t-test. Significant p-values were extracted if they resulted in increased respiration rate in comparison to the negative control well A01. See Fig. S9 for the results of the Omnilog analysis.

**Confirmation of viral genes in the *H. catenoides* assembly and reverse transcriptase PCR of viral genes**

To confirm that the viral genes were assembled correctly and were resident in the *H. catenoides* genome, PCRs across the 3’ and 5’ junctions of the putative viral open reading frame for three of the viral genes *polB*, *MCP*, and *mg96* were performed. 25 µl PCR reactions (1x Phusion HF buffer, 400 µM dNTP mix, 200 nM each primer, 0.5 U Phusion polymerase) were performed with the following cycling conditions: initial denaturation of 5 mins at 98°C, followed by 30 cycles of 10 s at 98°C, 30 s at 56-64°C and 1 min at 72°C, then a final extension of 5 mins at 72°C. These were purified using a GeneJET PCR Purification Kit or GeneJET Gel Extraction kit (Thermo Scientific) and sequenced to confirm that each product matched the expected amplicon. To confirm that the *mcp* gene was on the same contig as the histone H3 gene, we performed a PCR across these two genes (expected amplicon of 2837 bp) using the same conditions as above, except with an annealing temperature of 64 °C and with a 3-min extension. The PCR product was purified and A-tailed using *Taq* polymerase; then cloned using the StrataClone PCR Cloning Kit (Agilent Technologies). The resulting vector was sequenced using T3/T7 primers, with primer-walking to confirm the entire 2.8 kb sequence.

To investigate if the viral derived genes are actively transcribed in our culture conditions, we conducted RT-PCRof the *polB, mcp*, *mg96* and *rps3* virus confirming *polB, mg96 and rps3* are expressed in our culture conditions and suggesting that the viral like genes are transcriptionally active. RNA was extracted from *H. catenoides* using RNA PowerSoil Total RNA Isolation (MoBio). Residual genomic DNA was removed using RQ1 RNase-Free DNase (Promega) and Taq PCR was performed to confirm absence of DNA. Reverse-transcriptase PCR was then performed using a Qiagen OneStep kit according to the manufacturer’s instructions, alongside genomic DNA positive and no-template controls. The following cycling conditions were used: reverse-transcriptase of 30 mins at 50°C and initial denaturation 15 minutes at 94°C, followed by 32 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, then a final extension of 10 mins at 72°C. Samples were then analysed on a 2% (w/v) agarose gel.

**WGA staining**

*H. catenoides* was grown for 7 days at 25°C and 100 µl of mycelial growth was removed and suspended in 1 ml PBS. 5 µg ml-1 calcofluor white (Fluka) and 10 µg ml-1 WGA, Alexa Fluor 488 conjugate (Invitrogen) were added and cells were incubated for 30 minutes in the dark. Cells were washed twice in PBS and imaged using an Olympus IX73 microscope on a 40x objective. Unstained cells were also checked to confirm the absence of autofluorescence.

**Data Accessibility:** All relevant data generated for this project can be found at <https://www.ebi.ac.uk/biostudies/studies/S-BSST46> or <https://github.com/guyleonard/hyphochytrium>. Sequencing data can be accessed via the EMBL accessions SRX033129 (Illumina GAiix) and ERS1151585 (Illumina HiSeq). Genomic assemblies of the nuclear and mitochondrial genomes are available under the EMBL accessions; Study ID: PRJEB13950, Scaffolds: FLMG01000001-FLMG01004758 and Mitochondria: LT578416.

**Competing interests:** The authors declare no conflicts of interests.

**Authors Contribution:** GL conducted genome assembly and validation, proteome prediction and automatic genome annotation. DSM conducted drug treatment studies, high throughput culture assays and conducted various gene, mitochondrial and expression RT-PCR and PCR validation experiments. SS conducted the electron microscopy. All authors contributed post proteome bioinformatic analysis and contributed towards the primary text of the paper. GL & TAR were the main drafting authors of the paper.

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Figure 1. **Developmental characteristics of *H. catenoides,* genome statistics of representative stramenopiles, and a concatenated multi gene phylogeny**. Sketches of a subset of different stages of *H. catenoides* lifecycle, adapted and redrawn from [14][6] showing: (i - iii) different views of zoospores, (iv) germination stage of large spore, (v) primary enlargement or primary sporangium, (vi + vii) thallus development on substrate, (viii) unusual extensive branched thallus, which consists of separated sporangia at different stages of maturity (e.g. xii & xiv), connected by long, tubular, septate, hyaline and empty hyphae (x & xi), sometimes with enlargements without sporangia (e.g. ix). Zoospores may fail to swim coming to rest near exit tube (xiii). **(B)** Table of genome statistics for a range of different stramenopiles. \* Indicates k-mer estimation of genomes size. All numbers are from the respective genome datasets (see table XX). Numbers in italics (contigs) are inferred from the scaffolded data. Cegma: C = Complete, P = Partial recovered gene models. BUSCO: C = Complete, D = Duplicated, F = Fragmented, M = Missing gene models. **Figure 2.** Subsection of a 245 orthologous multi-gene concatenated phylogeny (85 taxa and 71,862 amino acid sites) demonstrating the branching position of Hyphochytrium within the stramenopiles calculated using Maximum Likelihood methods. See <https://github.com/guyleonard/hyphochytrium/blob/master/manuscript/data/data_s1_fig_1a_hyphochytrium_phylogeny.tree> for the full tree file. Support values at nodes are Maximum Likelihood bootstrap values. Dots on the branches of the phylogenetic tree indicate 100% bootstrap support, values below 50% have been removed. Cartoons of cells indicate change in only stramenopile flagellum morphology over the tree. Key indicates generalized lifestyle of the taxa sampled.

Figure 3. **Comparison of secreted proteome and putative carbohydrate active proteins across the Pseudofungi including photosynthetic stramenopile taxa as an outgroup**. The schematic phylogeny at the top indicates the relationship between different oomycete species with the ‘lifestyle’ of each species indicated by text colour; green (*Phytophthora* species) indicates plant hemibiotroph, blue (*Hyaloperonospora* and *Albugo*) obligate plant biotroph, teal (*Pythium*) plant necrotroph, orange (*Saprolegnia*) animal saprotroph/necrotroph, and black indicates putatively free living (e.g. *Hyphochytrium, Ectocarpus* and *Thalassiosira*). The first heat map in white/purple indicates the proportion of proteome of each organism which was identified as belonging to a particular CAZY ([www.cazy.org](http://www.cazy.org/)) category using BLASTp with an expectation of 1e-5. The number listed is the proportion, and the colour relates to magnitude of the listed number (as shown by scale bar). The second heat map, in blue/yellow, indicates the proportion of the secretome (predicted via a custom pipeline <https://github.com/fmaguire/predict_secretome/tree/refactor>) that is identified as belonging to each of these CAZY categories. The bar chart at the bottom shows the proportion of the proteome for each organism which is predicted to be secreted. Auxiliary Activities (AA) cover redox enzymes that act in conjunction with CAZymes.

Figure 4. **Comparative genomic analysis of *H. catenoides* flagellum proteome and motor protein repertoire**. **(A)** Heat map showing sequence identity profiles for flagella proteins with putative homologues present across the eukaryotes (see, Table S8 for full dataset). The heat map identifies 29 proteins present in the oomycetes but absent in *H. catenoides*, suggesting that this gene had been lost at the same proximate point to the loss of the posterior flagellum. The analysis also shows 12 proteins (marked\*) identified as posterior flagellum specific in *C. bullosa* that are retained in *H. catenoides* putatively function in the anterior flagellum. Three *C. bullosa* anterior flagellum specific proteins are also retained in *H. catenoides*. The putative radial spoke proteome also shows numerous losses similar to *Homo sapiens* (\*\*), this includes the loss of RSP7 (\*\*\*). Only changes in flagella cytology relevant to the evolution of the stramenopiles are sketched on the top tree. **(B)** Shows a cartoon of the radial spoke protein complex identified in *Chlamydomonas* with each shape number referring to the RPS number [66]. Black shapes illustrate proteins of the spoke complex conserved across the eukaryotes sampled, grey are non-conserved proteins (showing evidence of mosaic loss), while the white complex refers to RPS7 which although absent in *Homo sapiens* and other eukaryotes has been lost separately and is consistent with the loss of the posterior flagellum in the ancestor of *H. catenoides*. **(C)** Distribution of major kinesin paralogue families. Kinesin-2, -9, -16 & -17 have been suggested to have function associated with the flagellum [70]. **(D)** Distribution of major dynein paralogue families, plus presence of components of the IFT-A and IFT-B functioning in intraflagellar transport. Paralogues are grouped according to the class of component: Dynein Heavy Chain (DHC), Intermediate Chain (IC), Light Intermediate Chain (LIC) and Intra-flagellar Transport (IFT), and coloured according to function. **(E)** Distribution of major myosin paralogue families focusing on variation between Fungi and Pseudofungi. **(F)** Different myosin domain architectures identified in the stramenopiles including *H. catenoides*.

Figure 5. **Comparative genomic analysis of gene families that function in polarised filamentous growth in the Fungi**. **(A)** Cartoon outlining proteins and complexes involved in polarised growth in *Saccharomyces cerevisiae* (this is a variation of a figure shown in [75]).Vesicles are delivered from the Golgi (Fig. 5A-i) along cytoskeleton tracks to predetermined sites on the plasma membrane. Cdc42p is activated by Cdc24p (Fig. 5A-ii) promoting [79] assembly of the Polarisome complex (Fig. 5A-iii) resulting in the formin Bni1p radiating actin cables [147, 148]. Msb3p and Msb4p interact with Spa2 in the Polarisome (Fig. 5A-iv) which is thought to recruit Cdc42 from the cytosol at the site of tip growth [149]. Post-Golgi secretory vesicles are transported along actin cables using a type V myosin motor protein [150, 151] (Fig. 5A-v), to dock with the Exocyst complex in a process dependent on Sec4 and its GEF Sec2 [152, 153](Fig. 5A-vi) and so the vesicle is guided to its target site on the plasma membrane [154]. Cdc42p and Rho1 are required for localisation of Sec3p, which together form a spatial marker for the Exocyst (Fig. 5A-vii) and Rho3p and Cdc42p mediate vesicle docking (Fig. 5A-viii). Cdc42p plays a key role in regulating these processes in *S. cerevisiae* but in Pezizomycotina and basidiomycete fungi equivalent functions are performed by Rac1p [155, 156]. **(B)** The domain architecture of the 17 proteins associated with polarised growth in fungi. **(C)** The taxon distribution of putative homologues of polarised growth proteins across a representative set of taxa including the Pseudofungi. ‘P’ indicates a putative paralogue relationship as identified using phylogenetic analysis.

Figure 6 **Comparative genomic analysis of gene families that function in cell wall synthesis**. **(A)** Micrographs showing the Wheat Germ Agglutinin fluorescent staining of a chitin cell wall on *Hyphochytrium* structures. **(B)** The domain architecture of eight proteins that function in cell wall synthesis. **(C)** The taxon distribution of putative gene families associated with cell wall synthesis across a representative set of taxa including the Pseudofungi.

Figure 7. **Phylogeny of viral MCP proteins indicating the branching position of the Pseudofungal genes and evidence of transcription of viral derived genes in *H. catenoides***. **(A)** Homologous sequences were identified using three psi-BLAST iterations with *H. catenoides* putative MCP as query; to remove sequence redundancies, retrieved sequences were clustered at 90% amino-acid identity with cd-hit v4.6. Sequences were then aligned using MAFFT v7 iterative, global homology mode (G-INS-i); alignment sites retained for subsequent phylogenetic analysis were selected using trimAL [144] gap distribution mode. Final MCP multiple sequence alignment was composed of 386 sites. ML tree was inferred using iq-tree v1.3 and LG + I + Γ4 + F model (determined as the best-fitting model by Bayesian information criterion). Node supports were evaluated with 100 non-parametric bootstrap replicates. The Mimiviridae clade was used to root the ML tree (unrooted version displayed on the lower left part). **(B)** Reverse transcriptase (RT) PCR showing expression of polB and mg96 viral genes alongside an rps3 positive control. No expression of the mcp gene was detected. RT-PCR was performed on *H. catenoides* RNA alongside genomic DNA (+) and no template (-) controls, with PCR products run on an agarose gel alongside a 1kb ladder (Promega; 250 bp shown).

Figure 8. **Schematic Phylogenetic tree summarising the evolution of cell and genomic characters relevant to the evolution of the Pseudofungi**. Only changes in flagella complement relevant to the evolution of the stramenopiles are sketched.

­**Table 1.** Comparison of pseudofungal genes with generalised function.

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Gene families** | *Hyphochytrium catenoides* | *Albugo laibachii* | *Hyaloperonospora arabidopsidis* | *Phytophthora infestans* | *Phytophthora ramorum* | *Phytophthora sojae* | *Pythium ultimum* | *Saprolegnia parasitica* | *Ectocarpus siliculosus* | *Thalassiosira pseudonana* |
| RXLR | 1 | 0 | 23 | 317 | 102 | 106 | 0 | 0 | 0 | 0 |
| NPP1-like proteins | 0 | 0 | 21 | 27 | 62 | 74 | 7 | 0 | 0 | 0 |
| Elicitin | 0 | 9 | 14 | 43 | 47 | 53 | 44 | 25 | 0 | 0 |
| **Plant Cell wall**  **degrading:** |  |  |  |  |  |  |  |  |  |  |
| Cutinase | 0 | 3 | 2 | 4 | 4 | 16 | 0 | 0 | 0 | 0 |
| Glycosyl hydrolases | 357 | 384 | 242 | 533 | 838 | 1208 | 436 | 415 | 282 | 264 |
| Pectin methyl esterases | 0 | 0 | 4 | 11 | 13 | 19 | 0 | 0 | 0 | 0 |
| Pectate lyase | 0 | 0 | 8 | 36 | 25 | 24 | 16 | 0 | 0 | 0 |
| Polygalacturonase | 0 | 3 | 3 | 24 | 17 | 25 | 6 | 3 | 0 | 0 |
| **Lectins** |  |  |  |  |  |  |  |  |  |  |
| PAN lectin | 4 | 3 | 2 | 5 | 8 | 5 | 11 | 6 | 1 | 0 |
| Ricin lectin | 1 | 1 | 3 | 5 | 9 | 10 | 5 | 57 | 0 | 1 |
| Jacalin lectin | 2 | 0 | 8 | 15 | 23 | 15 | 3 | 4 | 1 | 0 |
| Galactose lectin | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Leguminous lectin | 2 | 1 | 0 | 2 | 1 | 1 | 2 | 0 | 2 | 1 |
| Legume-like lectin | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 0 |
| **Protease functions** |  |  |  |  |  |  |  |  |  |  |
| Protease inhibitors | 13 | 11 | 14 | 51 | 35 | 46 | 30 | 28 | 15 | 15 |
| Proteases, all | 428 | 379 | 324 | 450 | 541 | 602 | 482 | 630 | 361 | 367 |
| Serine proteases | 166 | 84 | 106 | 170 | 182 | 189 | 200 | 248 | 112 | 140 |
| Metalloproteases | 92 | 91 | 80 | 98 | 100 | 91 | 107 | 129 | 88 | 101 |
| Cysteine proteases | 115 | 124 | 92 | 140 | 116 | 113 | 121 | 208 | 117 | 85 |
| **Others** |  |  |  |  |  |  |  |  |  |  |
| ABC transporters | 81 | 36 | 49 | 148 | 171 | 175 | 158 | 138 | 70 | 58 |
| Protein kinases | 243 | 305 | 217 | 423 | 398 | 430 | 232 | 690 | 330 | 160 |
| Notch protein | 3 | 0 | 1 | 1 | 1 | 1 | 1 | 18 | 11 | 2 |
| Haemolysin E | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 0 | 0 |

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