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Chytrid rhizoid morphogenesis resembles hyphal development in multicellular fungi and is adaptive to resource availability

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Key to the ecological prominence of fungi is their distinctive cell biology, our understanding of which has been principally based on dikaryan hyphal and yeast forms. The early-diverging Chytridiomycota (chytrids) are ecologically important and a significant component of fungal diversity, yet their cell biology remains poorly understood. Unlike dikaryan hyphae, chytrids typically attach to substrates and feed osmotrophically via anucleate rhizoids. The evolution of fungal hyphae appears to have occurred from rhizoid-bearing lineages and it has been hypothesized that a rhizoid-like structure was the precursor to multicellular hyphae. Here, we show in a unicellular chytrid, Rhizoclosmatium globosum, that rhizoid development exhibits striking similarities with dikaryon hyphae and is adaptive to resource availability. Rhizoid morphogenesis exhibits analogous patterns to hyphal growth and is controlled by β-glucan-dependent cell wall synthesis and actin polymerization. Chytrid rhizoids growing from individual cells also demonstrate adaptive morphological plasticity in response to resource availability, developing a searching phenotype when carbon starved and spatial differentiation when interacting with particulate organic matter. We demonstrate that the adaptive cell biology and associated developmental plasticity considered characteristic of hyphal fungi are shared more widely across the Kingdom Fungi and therefore could be conserved from their most recent common ancestor.

1. Introduction

Hyphae are polarized, elongating and bifurcating cellular structures that many fungi use to forage and feed (figure 1a and b). The phylum Chytridiomycota (chytrids) diverged from other fungal lineages approximately 750 Mya and, with the Blastocladiomycota, formed a critical evolutionary transition in the Kingdom Fungi dedicated to osmotrophy and the establishment of the chitin-containing cell wall [2]. Chytrids produce filamentous hyphae-like, anucleate structures called rhizoids (figure 1a–c) [3], which are important in their ecological functions, in terms of both attachment to substrates and osmotrophic feeding [2]. 407-million-year-old fossils from the Devonian Rhynie Chert deposit show chytrids in freshwater aquatic ecosystems physically interacting with substrates via rhizoids in a comparative mode to extant taxa [4]. Yet surprisingly, given the importance of rhizoids in both contemporary and paleo-chytrid ecology, there remains a limited understanding of chytrid rhizoid biology, including possible similarities with functionally analogous hyphae in other fungi and the potential for substrate-dependent adaptations.

Character mapping of the presence of cellular growth plans against established phylogenies reveals the multicellular hyphal form to be a derived condition, whereas rhizoid feeding structures are the basal condition within the true fungi
Rhizoids are the basal feeding condition within the fungal kingdom and their morphogenesis is similar to hyphal development. (a–b) Correlating the major feeding types in fungi (a) to phylogeny (b) shows rhizoids to be the basal feeding condition in the true fungi (Eumycota). White circles indicate absence of a growth plan in a taxon and dark circles indicate widespread presence. Faded circles indicate a growth plan is present within a taxon, but not widespread. Rhizoids are the basal feeding condition within the fungal kingdom and their morphogenesis is similar to hyphal development. (Figure 1.)

(Eumycota) (figure 1a and b). Aseptate hyphae represent an intermediary condition and are not typically the dominant cell type in either unicellular or multicellular fungi. Hyphal cell types are sometimes observed outside of the Eumycota, such as within the Oomycota; however, the origin of fungal hyphae within the Eumycota was independent [5,6] and has not been reported in their closest relatives the Holozoans (animals, choanoflagellates, etc.). Comparative genomics has indicated that hyphae originated within the rhizoid-bearing Chytridiomycota–Blastocladiomycota–Zoosporangiophyta nodes of the fungal tree [6], which is supported by fossil Blastocladiomycota and extant Monoblepharidomycetes having hyphae [5,7]. This has led to the proposition that rhizoids, or rhizoid-like structures, were the evolutionary precursors of fungal hyphae [5,6,8]; however, investigation into such hypotheses have been hindered by a relative lack of understanding of rhizoid developmental biology.

Chytrids are important aquatic fungi [9], feeding on a range of physically complex heterologous substrates, including algal cells [10], amphibian epidermis [11] and recalcitrant particulate organic matter (POM) such as chitin and pollen [12]. Appreciation for the ecological importance of chytrids as parasites, pathogens and saprotrophs in aquatic ecosystems is greatly expanding. For example, chytrids are well-established plankton parasites [10], responsible for the global-scale amphibian panzootic [13], and have recently emerged as important components of the marine mycobiome [9]. The chytrid rhizoid is critical in all ecological functions because it is the physical interface between the fungus and substrate or host, yet there remains a limited understanding of rhizoid functional biology in terms of substrate interaction.

Rhizoclosmatium globosum is a widespread aquatic saprotroph that is characterized by its association with chitin-rich insect exuviae and has an archetypal chytrid cell plan (figure 1c) and life cycle (figure 1d) [14]. With an available sequenced genome [15], easy laboratory culture and amenability to live cell imaging (this study), R. globosum JEL800 represents a promising model organism to investigate the cell and developmental biology of aquatic rhizoid-bearing, early-diverging fungi. To
study the developing rhizoid system for morphometric analysis, we established a live cell three-/four-dimensional confocal microscopy approach in combination with the application of neuron tracing software to three-dimensional reconstruct developing cells (figure 1c; electronic supplementary material, figures S3 and S4). We were subsequently able to generate a series of cell morphometrics to describe and quantify rhizoid development (figure 1f; electronic supplementary material, figure S5) under a range of experimental conditions with the aims of identifying potential similarities with hyphae in dikaryan fungi in terms of geometric organization, morphogenesis and underlying cellular control mechanisms. In addition, we set out to characterize substrate-dependent adaptations particularly in the ecological context of aquatic POM utilization.

2. Material and methods

Detailed materials and methods are provided as electronic supplementary material.

(a) Rhizoid tracing and reconstruction

Chytrid plasma membranes were labelled with 8.18 µM FM 1–43 and imaged using a Zeiss LSM 510 Meta confocal laser scanning microscope (Carl Zeiss). Z-stacks of rhizoids were imported into the neuron reconstruction software NeuronStudio [16,17] and semi-automatically traced with the ‘Build Neurite’ function. Rhizoids were morphometrically quantified using the btmorph2 library [18] run with Python 3.6.5 implemented in Jupyter Notebook 4.4.0. For visualization, reconstructed rhizoids were imported into Blender (2.79), smoothed using automatic default parameters and rendered for display only.

(b) Chemical characterization of the rhizoid

To label F-actin and the cell wall throughout the rhizoid system, cells were fixed for 1 h in 4% formaldehyde in 1× PBS (phosphate-buffered saline) and stained with 1 : 50 rhodamine phalloidin in PEM (100 mM PIPES (piperazine-N,N′-bis(2-ethanesulfonic acid)) buffer at pH 6.9, 1 mM EGTA (ethylene glycol tetraacetic acid), and 0.1 mM MgSO4) for 30 min, then with 5 µg ml−1 Texas Red-conjugated wheat germ agglutinin (WGA) in PEM for 30 min.

(c) Chemical inhibition of rhizoid growth

Caspofungin diacetate (working concentration 1–50 µM) was used to inhibit cell wall β-glucan synthesis and cytochalasin B (working concentration 0.1–10 µM) was used to inhibit actin filament formation. Cells were incubated for 6 h, which was found to be sufficient to observe phenotypic variation.

(d) β-glucan quantification

*R. globosum* was processed for β-glucans using a commercial β-glucan assay (Yeast & Mushroom) (K-YBGL, Megazyme) following the manufacturer’s protocol. Briefly, samples were processed by acid hydrolysis then enzymatic break-down and β-glucans were quantified spectrophotometrically with a CLARIOstar Plus microplate reader (BMG Labtech), relative to a negative control and positive β-glucan standard. A sample of shop-bought baker’s yeast was used as an additional positive control.

(e) Identification of putative glucan synthase genes

All glycosyl transferase group 2 (GT2) domain-containing proteins within the *R. globosum* genome were identified using the JGI MycoCosm online portal. GT2 functional domains were identified using DELTA-BLAST [19] and aligned with MAFFT [20]. Maximum-likelihood phylogenies were calculated with RAxML [19] using the BLOSUM62 matrix and 100 bootstrap replicates.

(f) Carbon starvation and growth on chitin beads

For carbon starvation experiments, *R. globosum* cells were grown in either carbon-free Bold’s Basal Medium supplemented with 1.89 mM ammonium sulfate and 500 µl−1 f/2 vitamin solution [21] (BBM) or BBM with 10 mM N-acetyl-D-glucosamine as a carbon source. To investigate growth on POM, chitin microbeads (New England Biolabs) were suspended in carbon-free BBM at a working concentration of 1 : 1000 stock concentration. To understand rhizoid development in a starved cell that had encountered a chitin microbead, we imaged cells that contacted a chitin microbead following development along the glass bottom of the dish.

3. Results

(a) Chytrid rhizoid morphogenesis and development

During rhizoid development, we observed a continuous increase in rhizoid length (110.8 ± 24.4 µm h−1) (n = 5, ± s.d.) and the number of rhizoid tips (4.6 ± 1.2 tips h−1) (figure 1g; electronic supplementary material, table S1, movies S1–S5), with an increase in the total cell surface area (21.1 ± 5.2 µm2 h−1), rhizoid bifurcations (4.2 ± 1.0 bifurcations h−1), cover area (2,235 ± 170.8 µm2 h−1) and maximum Euclidean distance (5.4 ± 0.1 µm h−1) (electronic supplementary material, figure S6). The hyphal growth unit (HGU) has been used previously to describe hyphal development in dikaryan fungi and is defined as the distance between two hyphal bifurcations [22]. Adapting this metric for the chytrid rhizoid, the rhizoidal growth unit (RGU) (i.e. the distance between two rhizoid bifurcations; figure 1f) increased continuously during the first 6 h of the development period (i.e. cells became relatively less branched) before stabilizing during the later phase of growth (figure 1g). The local rhizoid bifurcation angle remained consistent at 81.4° ± 6.3° after approximately 2 h (electronic supplementary material, figure S6), and lateral branching was more frequent than apical branching during rhizoid development (figure 1h and i). Fractal analysis (fractal dimension = Df) of 24 h grown cells showed that rhizoids approximate a two-dimensional biological fractal (mean Df = 1.51 ± 0.24), with rhizoids relatively more fractal at the centre of the cell (max Df = 1.69–2.19) and less fractal towards the growing periphery (min Df = 0.69–1.49) (figure 1j; electronic supplementary material, figure S7).

(b) Cell wall and actin dynamics are linked to rhizoid branching

The cell wall and actin structures were present throughout the chytrid rhizoid (figure 2a). Putative actin cables ran through the rhizoid system, punctuated by actin patches. Inhibition of cell wall β-1,3-glucan synthesis and actin proliferation with caspofungin and cytochalasin B, respectively, induced a concentration-dependent decrease in the RGU, with the development of atypical hyperbranched rhizoids (figure 2b–d; electronic supplementary material, table S2, movies S6–S7). As with *Batrachochytrium dendrobatidis* [23,24], we confirmed that *R. globosum* JEL800 lacks an apparent β-1,3-glucan synthase FKS1 gene homologue (electronic supplementary material, table S3). However, the quantification of glucans in
**Figure 2.** Cell wall synthesis and actin dynamics govern rhizoid branching. (a) Fluorescent labelling of cell wall and actin structures in 24 h *R. globosum* rhizoids. The cell wall and actin patches were found throughout the rhizoid. Arrowheads in the actin channel indicate putative actin cables. WGA = conjugated wheat germ agglutinin. Scale bar = 10 µm. (b) Representative three-dimensional reconstructions of 7 h *R. globosum* cells following treatment with caspofungin diacetate and cytochalasin B at stated concentrations to inhibit cell wall and actin filament biosynthesis respectively, relative to solvent only controls. Scale bar = 20 µm. (c) Application of caspofungin diacetate and cytochalasin B resulted in a concentration-dependent decrease in the rhizoidal growth unit, resulting in atypical hyperbranched rhizoids (n ~ 9). n.s > 0.05 (not significant), *p < 0.05, **p < 0.01, ***p < 0.001. This differential growth is diagrammatically summarized in (d). (e) β-glucan concentration of *R. globosum* (n = 10) relative to a baker’s yeast control (n = 2). (f) Maximum-likelihood phylogeny of GT2 domains (BcsA and WcaA domains) within the *R. globosum* genome (midpoint rooting). Full architecture of each protein is shown. Asterisk indicates the putative glucan synthesis protein ORY39038 containing a putative SKN1 domain.

*R. globosum* showed that they are present (figure 2c), with 58.3 ± 7.6% β-glucans and 41.6 ± 7.6% α-glucans of total glucans.

To identify alternative putative β-glucan synthesis genes in *R. globosum* JEL800, we surveyed the genome and focused on GT2 encoding genes, which include typical glucan synthases in fungi. A total of 28 GT2 domains were found within 27 genes (figure 2f). Of these genes, 20 contained putative chitin synthase domains and many contained additional domains involved in transcriptional regulation. Nine encode chitin synthase 2 family proteins and 11 encode chitin synthase 1 family proteins (with two GT2 domains in ORY48846). No obvious genes for β-1,3-glucan or β-1,6-glucan synthases were found within the genome. However, the chitin synthase 2 gene ORY39038 included a putative SKN1 domain (figure 2f), which has been implicated in β-1,6-glucan synthesis in the ascomycete yeasts *Saccharomyces cerevisiae* [25] and *Candida albicans* [26]. These results indicate a yet uncharacterized β-glucan-dependent cell wall production process in chytrids (also targeted by caspofungin) that is not currently apparent using gene/genome level assessment and warrants further study.

(c) Rhizoids undergo adaptive development in response to carbon starvation

To examine whether chytrids are capable of modifying rhizoid development in response to changes in resource availability, we exposed *R. globosum* to carbon starvation (i.e. development in the absence of exogenous carbon). When provided with 10 mM N-acetylglucosamine (NAG) as an exogenous carbon
source, the entire life cycle from zoospore to sporulation was completed and the rhizoids branched densely (electronic supplementary material, movie S8), indicative of a feeding phenotype. Carbon-starved cells did not produce zoospores and cell growth stopped after 14–16 h (electronic supplementary material, movie S9). Using only endogenous carbon (i.e. zoospore storage lipids), starved cells underwent differential rhizoid development compared to cells from the exogenous carbon-replete conditions to form an apparent adaptive searching phenotype (figure 3a,b); electronic supplementary material, figure S1). Under carbon starvation, R. globosum invested less in thallus growth than in carbon replete conditions and developed longer rhizoids with a greater maximum Euclidean distance and covered a larger area (figure 4d). Carbon-starved cells were also less branched, had wider bifurcation angles and subsequently covered a larger surface area. These morphological changes in response to exogenous carbon starvation suggest that individual chytrid cells are capable of differential reallocation of resources away from reproduction (i.e. the production of the zoosporangium) and towards an extended modified rhizoidal structure indicative of a resource searching phenotype.

(d) Rhizoids spatially differentiate in response to patchy resource environments
Rhizoid growth of single cells growing on chitin microbeads was quantified as experimental POM (figure 4a,b; electronic supplementary material, movie S1). Initially, rhizoids grew along the outer surface of the bead and were probably used primarily for anchorage to the substrate. Scanning electron microscopy (SEM) showed that the rhizoids growing externally on the chitin particle formed grooves on the bead parallel to the rhizoid axis (electronic supplementary material, figure S1f,g), suggesting extracellular enzymatic chitin degradation by the rhizoid on the outer surface. Penetration of the bead occurred during the later stages of particle colonization (figure 4d; electronic supplementary material, movie S12). Branching inside the bead emanated from ‘pioneer’ rhizoids that penetrated the particle (figure 4c).

Given the previous results of the searching rhizoid development in response to carbon starvation, a patchy resource environment was created using the chitin microbeads randomly distributed around individual developing cells in otherwise carbon-free media to investigate how encountering POM affected rhizoid morphology (figure 4d). We observed spatial differentiation of single-cell rhizoid systems in association with POM contact. Particle-associated rhizoids were shorter than rhizoids not in particle contact, were more branched (i.e. lower RGU), had a shorter maximum Euclidean distance and covered a smaller area (figure 4c). These rhizoid morphometrics closely resembled the feeding and searching modifications of the cells grown under carbon-replete and carbon-depleted conditions previously discussed (figures 4f and 3b) but instead are displayed simultaneously with spatial regulation in individual cells linked to POM-associated and non-associated rhizoids, respectively.

4. Discussion
Our results provide new insights into the developmental cell biology of chytrid fungi and highlight similarities between the organization of anucleate rhizoids and multicellular hyphae. The fundamental patterns of rhizoid morphogenesis...
that we report here for a unicellular non-hyphal fungus are comparable to those previously recorded for hyphal fungi (figure 5a) [22]. Trinci [22] assessed hyphal development in major fungal lineages (Ascomycota and Mucoromycota) and observed that the growth patterns of morphometric traits (HGU, total length and number of tips) were similar across the studied taxa. When the data from our study are directly compared to that of Trinci [22], we see that the hyphal growth pattern is also analogous to the rhizoids of the early-diverging unicellular Chytridiomycota (figure 5a).

Chytrid rhizoid development in this study is also comparable to the hyphal growth rates reported by Trinci [22] (figure 5b, c), as well as the elongation rates reported by López-Franco et al. [27] when scaled by filament diameter (figure 5d).

Such similarities also extend to rhizoid branching patterns, where lateral branching dominates over apical branching. This branching pattern is also the predominant mode of hyphal branching, where apical branching is suppressed by a phenomenon termed ‘apical dominance’ [28]. These findings suggest that a form of apical dominance at the growing edge rhizoid tips may suppress apical branching to maintain rhizoid network integrity as in dikaryon hyphae [28,29]. Chytrid rhizoids also become less fractal towards the growing edge in terms of their overall morphology, and similar patterns of fractal organization are also observed in hyphae-based mycelial colonies [30]. Taken together, these results show strong geometric analogies in the fundamental organization of unicellular chytrid rhizoid and multicellular hyphal morphogenesis.

Given the apparent hyphal-like properties of rhizoid development, we sought a greater understanding of the potential subcellular machinery underpinning rhizoid morphogenesis in R. globosum. Normal rhizoid branching was disrupted by inhibition of cell wall synthesis and actin polymerization, both of which are known to control branching and growth in hyphal biology [31–33]. These effects in R. globosum are similar to disruption of normal hyphal branching reported in Aspergillus fumigatus (Ascomycota) in the presence of caspofungin [34], and in Neurospora crassa (Ascomycota) in the presence of cytochalasins [35]. Recent studies have shown the presence of actin in the rhizoids of soil chytrids [36,37] and inhibition of actin in Chytridiomycetes hyalinus similarly disrupts normal rhizoid branching [36]. In this study, our quantitative characterization of cell wall and actin inhibited rhizoid paramorphs provides support that β-1,3-glucan-dependent cell wall synthesis and actin dynamics also govern branching in chytrid rhizoids as in multicellular hyphae.

We also show that rhizoid development is plastic to resource availability, with chytrid cells displaying an adaptive searching phenotype under carbon starvation. Adaptive foraging strategies are well described in multicellular hyphae [38,39], and our data support the existence of analogous strategies in
rhizoidal fungi. Dense branching zones in dikaryan mycelia are known to improve colonization of trophic substrates and feeding by increasing surface area for osmotrophy, while more linear ‘exploring’ zones cover greater area and search for new resources [39]. Similar morphometrics are displayed by R. globosum exhibiting feeding and searching phenotypes, respectively. In addition, exogenous carbon starvation has also been shown to be associated with a decrease in branching in the multicellular dikaryan fungus Aspergillus oryzae (Ascomycota) [40]. Overall, these results highlight that adaptive search strategies are more widely spread than previously known in the Kingdom Fungi.

Finally, we report the spatial and functional differentiation of feeding and searching sections of anucleate rhizoid systems from individual cells. The simultaneous display of both rhizoid types in the same cell indicates a controlled spatial regulation of branching and differentiation of labour within single chytrid rhizoid networks. Functional division of labour is prevalently seen in multicellular mycelial fungi [38,39] including the development of specialized branching structures for increased surface area and nutrient uptake, as in the plant symbiont mycorrhiza (Glomeromycota) [41]. Our observation of similarly complex development in a unicellular chytrid suggests that multicellularity is not a prerequisite for adaptive spatial differentiation in fungi.

5. Conclusion

The improved understanding of chytrid rhizoid biology related to substrate attachment and feeding we present here opens the door to a greater insight into the functional ecology of chytrids and their environmental potency. Our approach of combining live cell confocal microscopy with three-dimensional rhizoid reconstruction provides a powerful toolkit for morphometric quantification of chytrid cell development and could shed light on the biology underpinning chytrid ecological prevalence. In the future, the application of this approach to different systems could provide a detailed understanding of chytrid parasitism and host interaction, development under different nutrient regimes and degradation of diverse carbon sources.

From an evolutionary perspective, the early-diverging fungi are a critical component of the eukaryotic tree of life [42,43], including an origin of multicellularity and the establishment of the archetypal fungal hyphal form, which is responsible in part for the subsequent colonization of land by fungi, diversity expansion and interaction with plants [2]. Our cell biology focused approach advances this developing paradigm by showing that a representative unicellular, rhizoid-bearing (i.e. non-hyphal) chytrid displays hyphal-like morphogenesis, with evidence that the cell structuring mechanisms (e.g. apical dominance) underpinning chytrid rhizoid development are equivalent to reciprocal mechanisms in dikaryan fungi.

Perhaps our key discovery is that the anucleate chytrid rhizoid shows considerable developmental plasticity. R. globosum is able to control rhizoid morphogenesis to produce a searching form in response to carbon starvation and, from an individual cell, is capable of spatial differentiation in adaptation to patchy substrate availability indicating functional division of labour. The potential for convergent evolution aside, we propose by parsimony from the presence of analogous complex cell developmental features in an extant representative chytrid.
and dikaryan fungi that adaptive rhizoids are a shared feature of their most recent common ancestor.

Data accessibility. All data that support the findings of this study are included in the electronic supplementary material of this paper.

Authors’ contributions. D.L. and M.C. conceived the study. D.L. conducted the laboratory work and data analysis. N.C. analysed the R. globosum JEL800 genome. G.W. provided support with microscopy. M.C. secured the funding. D.L. and M.C. critically assessed and interpreted the findings. D.L. and M.C. wrote the manuscript, with the help of N.C. and G.W.

Competing interests. The authors declare no competing interests.

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