

## Fungal zoospores show contrasting swimming patterns specific to phylum and cytology

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### Abstract

Zoosporic fungi produce unicellular motile reproductive spores with swimming tails (zoospores). These fungi are key components of aquatic food webs, acting as parasites, saprotrophs and prey, with some species representing ecologically important infectious diseases (e.g., of amphibian hosts). However, little is known about the swimming behaviour of fungal zoospores, a crucial factor governing dispersal in aquatic environments and therefore the ecological function of these fungi. Here, we track the swimming patterns of zoospores representing six species from two different phyla: Chytridiomycota and Blastocladiomycota. We report phylum-specific patterns in which Chytridiomycota species swim in a circular fashion, while Blastocladiomycota species swim in a pattern more akin to a random walk (move-stop-redirect-move). Additionally, we performed fluorescence confocal analyses of zoospores for all six species confirming they possess variant phylum-specific zoospore ultrastructures, specifically the distribution of the main cytoskeletal proteins and the number and arrangement of lipid droplet organelles within the cell. Lastly, we hypothesize a possible link between cytology, sensing of environment and swimming behaviour for these zoosporic fungi. We suggest that comparative study of these two groups will enable new understanding

of the relationship between ultrastructure, cellular proteome, and how single celled eukaryotes direct flagellum-based movement.

## 1. Introduction

Zoosporic fungi are unicellular members of the fungal phylogenetic clade which, at some point in their life cycle, produce motile spores with swimming tails [1,2]. These fungi were originally placed into the phylum Chytridiomycota [sensu Barr (2001)[3]], and historically were assigned the term “chytrids”. Molecular phylogenetic methods have since shown that the chytrids are paraphyletic [4] with current studies demonstrating that zoosporic fungi comprise four-to-six phyla: Chytridiomycota (Neocallimastigomycota + Monoblepharomycota), Blastocladiomycota, Sanchytriomycota and Olpidiomycota [4–9].

Zoosporic fungi are ubiquitous in many environments, participating as saprotrophs [10] (digesters of dead biological matter) or as parasites that play important ecological roles, for example, amphibian species loss (e.g., [11]). Fungal zoospore forming species search and infect host cells of a variety of eukaryotic organisms or detrital matter, extracting nutrients and developing into mature sporangia that release numerous new zoospores [2,12]. Chytrids have been suggested to be an important component of aquatic food webs, where they act as both parasites, saprotrophs and prey (i.e. being grazed by zooplankton) effectively linking trophic nodes [13–16]. The overall function of zoosporic fungi within the wider food web is termed the mycoloop [15].

Most fungal zoospores are unflagellated, with only a few known multflagellated forms (e.g., *Neocallimastix* and *Piromyces* [17]) and lineages that form spores without flagella (i.e. due to the secondary loss of the flagellum in *Hyaloraphidium* [18]). Zoospores can move using two distinct mechanisms: swimming propelled by a flagellum [3,10] and amoeboid crawling using pseudopodia [8,19]. These different locomotive modes are suited towards specific behavioural goals; as such, we can find fungal species which are both swimmers and crawlers (e.g. *Paraphysoderma* [19]), species with swimming zoospores which under certain conditions will switch to crawlers (e.g. *Batrachochytrium dendrobatidis* [20]) or with zoospores that exclusively crawl (e.g. Sanchytriomycota [8]). Broadly speaking, crawling is better adapted for movement on short distances on liquid enclosed surfaces; however, swimming is ideal for short-medium range dispersal in larger volumes of water [21]. Thus, swimming likely plays an important role in facilitating the trophic life cycle of these fungi and therefore the mycoloop [13,15,22] and is often governed by chemo- or phototaxis function [23–26].

Swimming patterns have been studied in several unicellular eukaryotes (e.g. [27–30]). Zoospore swimming patterns in Oomycota (e.g. *Phytophthora*) for example have been shown to contribute to their activity as plant pathogens. These findings have led to the suggestion that targeting motility performance may be a strategy for control of pathogenic zoosporic species [30]. However, little is known about the swimming behaviour of fungal zoospores, although this function is crucial for their dispersal in aquatic environments and pathogenetic function in some species. Here, we study the motility patterns of zoospores from six species of zoosporic fungi grouped in two different phyla, Chytridiomycota (*Chytrium confervae*, *Rhizoclosmatium globosum* and *Synchytrium microbalum*) and Blastocladiomycota (*Blastocladiella emersonii*, *Allomyces macrogynus* and *Allomyces reticulatus*). By tracking their swimming, we report phylum-specific movements patterns in which Chytridiomycota species swim in a circular fashion, and Blastocladiomycota species swim in a pattern more akin to a random walk (i.e., move-stop-redirect-move). Interestingly, using fluorescent confocal microscopy to study ultrastructure of the cellular cytoskeleton and lipid organelles confirms phylum-specific cell structural characteristics that correlate with these different swimming patterns. Specifically, Chytridiomycota possess actin as the main cytoskeletal protein and have only one large single-lipid droplet, while in Blastocladiomycota tubulin microtubules ‘ribs’ enclose the cell-body while multiple lipid globules are observed.

## 2. Material and methods

### (a) Culturing and Image acquisition

Cultures of *Blastocladiella emersonii* ATCC 22665 (ATCC; American Type Culture Collection), *Allomyces macrogynus* Australia\_3 (CZEUM; Collection of Zoosporic Eufungi at University of Michigan), *Chytrium confervae* CBS 675.73 (Westerdijk Fungal Biodiversity Institute) and *Rhizoclosmatium globosum* JEL800 (CZEUM) were grown vegetatively and induced to sporulate as described in Galindo et al. (2022) [31]. *Allomyces reticulatus* ATCC 42465 (ATCC) was similarly grown and induced to sporulate as described for *Allomyces macrogynus* Australia\_3 in Galindo et al. [31]. *Synchytrium microbalum* JEL517 (CZEUM) vegetative cells were grown on PYG agar plates (0.13% w/v peptone, 0.13% w/v yeast extract, 0.3% w/v glucose, and 1.5% w/v agar) and in PYG liquid media (0.13% w/v peptone, 0.13% w/v yeast extract, 0.3% w/v glucose). Zoospores for *S. microbalum* were obtained as described for *Chytrium confervae* CBS 675.73 in Galindo et al. [31]. Zoospores were then separated from sporangia using a 20 µm pluriStrainer (pluriSelect) in 50 ml falcon

tubes. Out of the six obtained liquid zoospore solutions three were PYG-based (*B. emersonii*, *C. confervae* and *S. microbalum*) and three were water-based (*A. macrogynus*, *A. reticulatus* and *R. globosum*). After sporulation, 7  $\mu$ l of zoospore suspension was placed on a slide and covered with a coverslip. Cells were imaged on an Olympus CKX53 inverted microscope with 10X, 20X, and 40X objectives according to the size of the zoospores and/or their swimming trajectories. Videos of 15 to 30 seconds were taken with a Std Chromyx HD camera mounted on a U-TV0.5XC-3-8 0.5x C-Mount Adapter. Exposures, focus and stage position were kept constant while recording for motility analyses. All videos were recorded on at the same condition with constant undirected light and no chemical or physical gradients evident on the slides. The number of trials  $n$  recorded for each species is shown in Figure 1b. For fluorescent confocal microscopy, zoospores were fixed, stained and imaged using the same protocol and equipment as described in Galindo et al.[31].

#### (b) Phylogenetic inference

We combined and curated three SSU+LSU rDNA alignments [31–33] adding new sequences from zoosporic fungal taxa. The new dataset of 117 sequences was aligned with MAFFT v7 [34] and trimmed with TrimAl [35] with the ‘automated1’ option for a total of 2578 nucleotide positions. For the maximum likelihood phylogenetic analyses, we selected the best-fitting model (GTR+F+R6) using IQ-TREE [36] TESTNEW algorithm with BIC. Topology support was evaluated using 1,000 ultrafast bootstrap replicates and 1,000 replicates of the SH-like approximate likelihood ratio test. Trees were drawn using FigTree [37].

#### (c) Tracking and motility analysis

Movies of swimming zoospores were viewed in FIJI [38], and movement was tracked using a semi-automated protocol with the TrackMate plugin [39]. Before tracking, videos were pre-processed as follows: (1) the background was subtracted; (2) artefacts were detected and removed by subtracting the median image of the video from each frame. Cells were segmented using TrackMate's Difference of Gaussian detector; manual quality control by eye was performed on the first frame for each video. The center-of-mass of segmented images were joined to create tracks using a Linear Assignment Problem (LAP) tracker; maximum gap closing was set at 50 pixels and maximum frame gap at 10 frames. Final quality control of all tracks was performed using TrackScheme to ensure each track consisted of only one detected spot per frame. Plotting of tracks and quantitative motility metrics were performed from

exported COM data using in-house scripts written in Python. Full tracks for all species are shown in Figures S1-S6.

### 3. Results

The SSU-LSU rDNA phylogeny shows the phylogenetic distribution of the six species for which zoospore swimming patterns were analysed and confirms their taxonomic placement (Figure 1a). Although there was not a significant difference in instantaneous swimming speed among species (Figure S7), long-range motility patterns were phylum specific (Figure 1b). In particular, we found that all three zoospores from Chytridiomycota species swim following a circular pattern and all three Blastocladiomycota zoospores swim using a pattern more akin to a random walk (move-stop-redirect-move).

To gain insights into intracellular organization, we then performed confocal microscopy with antibodies/fluorescent to zoospores in all six species (Figure 2). We improved on previous analyses [31] by adding two extra species, one chytrid (*S. microbalum*) and one blastoclad (*A. reticulatus*) consistent with the six sets of swimming behaviour observed. Our results confirm that in Chytridiomycota actin is the main cytoskeletal protein in the cell body, creating actin patches in *C. confervae* and *R. globosum* but not in *S. microbalum*. No tubulin was found within the main body of Chytridiomycota cells, only in the flagellum. In contrast, for Blastocladiomycota, tubulin microtubules create complex cytoskeletal arrays or ‘ribs’ within the cell body which extend toward the flagellum; there is a minor contribution of actin to the cytoskeleton of the cell. As for the lipid organelle, we consistently observed one large lipid globule in Chytridiomycota ( $n \geq 15$  – for each of these three species) as part of the microbody-lipid globule complex organelle (MLC), and clusters of several lipid globules in Blastocladiomycota ( $n \geq 10$  – for each of these three species), usually located towards the flagellum as part of their side-body complex organelle (SBC). In the case of *A. reticulatus* the lipid droplet clusters are numerous and can run down the entire length of one side of the zoospore cell body (Figure 2a).

### 4. Discussion

Here, we report two distinct swimming patterns among fungal zoospores and show that these behaviours are phylum-specific (given current taxon sampling): Chytridiomycota zoospores swim in circular tracks ( $n = 3$  spp), while Blastocladiomycota zoospores adopt a random-walk-type movement ( $n = 3$  spp). To address whether these phylum-specific swimming patterns form

a consistent correlation with key cellular ultrastructure, we perform fluorescence confocal analyses in the zoospores of all six species. Our imaging confirms that two main systematic phylum-specific cytological differences are present. Specifically, in Chytridiomycota, we find actin to be the main cytoskeletal component while in Blastocladiomycota tubulin proteins are the major cytoskeleton component of the cell (Figure 2 - as observed in previous studies [20]). Furthermore, we find phylum-specific variability in the lipid organelles, structures typically known to be involved in both storage and provision of chemical energy for flagellum beating [40]. The lipid-filled organelle from Blastocladiomycota is known as the side-body complex (SBCs) and is composed of several lipid droplets (Figure 2), microbodies, mitochondria and membranes [1,41,42]. Chytrid zoospores possess a different prominent lipid organellar arrangement known as the microbody-lipid globule complex (MLCs), which are composed of microbodies, mitochondria, ribosomes and normally one large membrane-enclosed lipid globule (Figure 2) [2,40]. In addition to energetics, these organelles have been hypothesised to be involved in light perception [24,31]. Indeed, *Blastocladiella emersonii* and *Allomyces reticulatus* zoospores show positive phototaxis [24,25]. In particular, the SBC of *B. emersonii* was found to be in close spatial association with the CyclOp protein (a fusion protein composed of a type I microbial rhodopsin and guanylyl cyclase enzyme domain) which was shown to participate in a cGMP-mediated light-sensing pathway [24,43,44]. Recent analyses suggest that all the molecular elements necessary for a functional CyclOp light sensing pathway are present in the six fungal species analysed in this study [31].

Collectively, these data infer a link between zoospore cytoskeleton and ultrastructure type and the swimming behaviour of these cells. However, cell-protein-functional studies are required to explicitly test whether these cellular arrangements contribute to the motility patterns observed. Such studies would be dependent on finding element of the cell structural proteome that govern movement but for which genetic manipulation is not lethal to development of the zoospore. Genome sequences are available for many of the fungi compared here [33,45–47], however, we currently do not have any candidate gene families beyond the tubulin and actin structures identified. Genetic manipulation of such gene families, for example by gene deletion, is likely to be lethal. For this reason, we have focused on reporting this observation and how it correlates with ultrastructure while noting a long-term opportunity for studying the relationship between cellular-structure, proteome and single cell movement behaviour in ‘chytrid’ fungi.

Signalling-related swimming patterns have previously been observed in zoospores from species of oomycota (a clade of fungal-like stramenopiles) which swim in a circular corkscrew-



type fashion, similar to what is observed in Chytridiomycota [48,49]. As in other eukaryotes, it has been shown that the oomycotan zoospores swimming patterns (*Pythium* sp. and *Phytophthora* sp.) are strongly influenced by calcium [48–50]. The swimming behaviour of *Phytophthora infestans* was also shown to be regulated by G-protein signalling after observing the disruption of zoospore swimming patterns in PiGPA1-deficient mutants [51,52]. Collectively, these studies in oomycete zoospores demonstrate a direct coupling between extracellular sensing, downstream signalling and zoospore swimming behaviours [53]. It remains to be tested if a link between sensing, signalling and motility can also be found in zoospores of ‘chytrid’ fungi. However, we propose a hypothesis in which a light-sensing signalling pathways initiated at the SBCs of Blastocladiomycota and to the MLCs of Chytridiomycota may be involved in shaping these two different phylum-specific swimming patterns, a function alternatively controlled by the variant cellular cytoskeletons of these zoospores. Thus, future analyses should focus on studying these zoospores under different environmental conditions (e.g., light vs dark), and/or after the inhibition (e.g., drug trials) of the light sensing signalling pathway components, with the aim of detecting disruptions of their different swimming patterns. Our hope is that identification of these distinct movement patterns will provide a model for future study with the aim to understand the link between cytology, sensing and swimming behavior in single celled eukaryotes.

### **Data accessibility**

Plots of all tracks for each species are shown in Supplementary Material. All tracking data, detailed protocols, and analysis codes are provided at <http://github.com/jnirody/fungalzoospores>. Datasets, MSA files, sequence origin and trees from phylogenetic analyses can be found at: <https://doi.org/10.6084/m9.figshare.21866169.v1>

### **Authors' contributions**

L.G., T.A.R. and J.A.N.: conceived the study; L.G.: microscopy imaging, phylogenetic analyses, and manuscript writing; J.A.N.: imaging analyses and manuscript writing. All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

### **Conflict of interest declaration**

We declare we have no competing interests.

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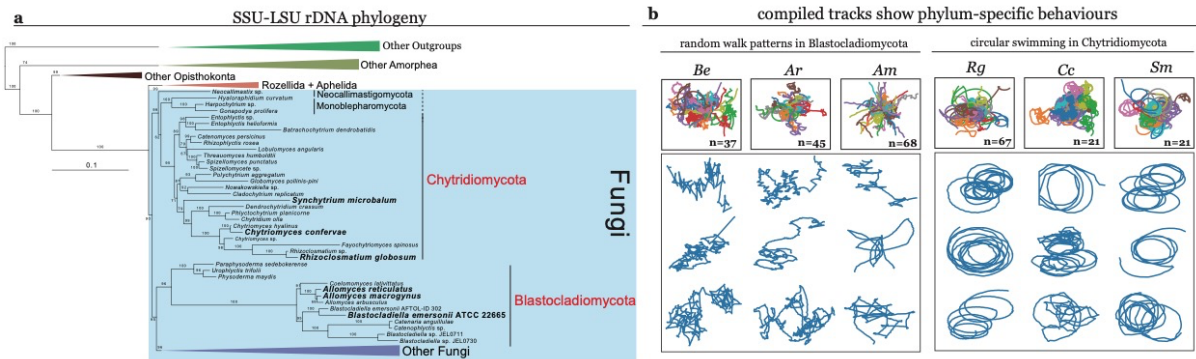


Figure 1. (a) SSU-LSU rDNA phylogeny of the Fungi showing the relative position of the zoosporic fungal species in this study, including eukaryotic outgroups. Fungi are highlighted in blue. (b) Movement tracks show phylum-specific behaviours, with Blastocladiomycota species swimming in a random walk pattern, while Chytridiomycota zoospores swim in circular movements. All tracks are shown compiled into a single plot with starting points (time = 0) centered at the origin are shown; three representative tracks are also shown for each species (*Blastocladiella emersonii*: *Be*, *Allomyces macrogynus*: *Am*, *Allomyces reticulatus*: *Ar*, *Synchytrium microbalum*: *Sm*, *Chytrium confervae*: *Cc* and *Rhizoclostium globosum*: *Rg*) to further illustrate observed motility patterns.

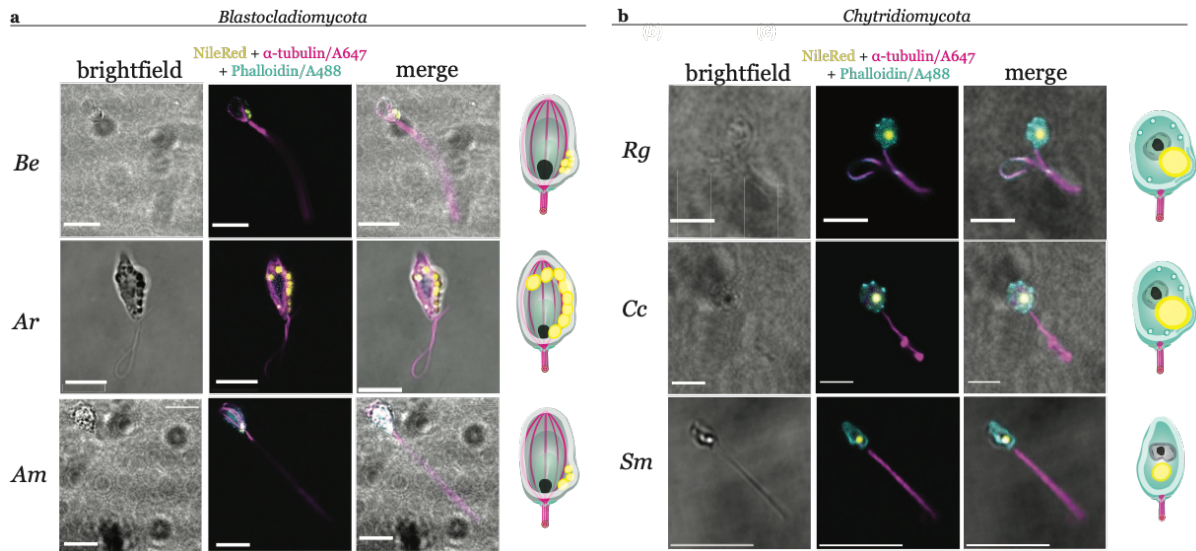


Figure 2. Imaging of ultrastructure in (a) Blastocladiomycota and (b) Chytridiomycota zoospores. First columns show brightfield phase contrast confocal microscopy images; second columns show fluorescent confocal microscopy micrograph of the zoospore; third columns show the merged images of brightfield and fluorescent channels. Microscopy images were taken and stained using the methods described in (Galindo, et al. 2022 [31]) with NileRed to stain lipid droplets,  $\alpha$ -tubulin DM1A + Alexa Fluor 647 to stain tubulin and Alexa Fluor 488 Phalloidin to stain actin. Fourth columns depict the candidate light-sensing organelle systems and cytoskeleton within the zoospore as per our confocal microscopy results and previous electron microscopy studies reviewed in Galindo et al. (2022) [31]. In both micrographs and schematic depictions, tubulin structures are stained magenta, actin stained cyan, and the lipid bodies proposed to function as a light-sensing eye-spot are stained yellow. Species key: *Blastocladiella emersonii* (*Be*), *Allomyces macrogynus* (*Am*), *Allomyces reticulatus* (*Ar*), *Synchytrium microbalum* (*Sm*), *Chytrium confervae* (*Cc*) and *Rhizoclosmatium globosum* (*Rg*).