Think again

No jacket required – new fungal lineage defies dress code

Recently described zoosporic fungi lack a cell wall during trophic phase

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Analyses of environmental DNAs have provided tantalizing evidence for ''rozellida'' or ''cryptomycota'', a clade of mostly undescribed and deeply diverging aquatic fungi. Here, we put cryptomycota into perspective through consideration of *Rozella*, the only clade member growing in culture. This is timely on account of the publication in *Nature* of the first images of uncultured cryptomycota from environmental filtrates, where molecular probes revealed non-motile cyst-like structures and motile spores, all lacking typical fungal chitinous cell walls. Current studies of *Rozella* can complement these fragmentary observations from environmental samples. *Rozella* has a fungal-specific chitin synthase and its resting sporangia have walls that appear to contain chitin. Cryptomycota, including *Rozella*, lack a cell wall when absorbing food but like some other fungi, they may have lost their ''dinner jacket'' through convergence. Rather than evolutionary intermediates, the cryptomycota may be strange, divergent fungi that evolved from an ancestor with a nearly complete suite of classical fungal-specific characters.

Keywords:
chitin synthase; cryptomycota; evolution; *Rozella*; rozellida

Introduction

Fungi lead hidden lives

Fungi lead cryptic lives by growing inside their food source, and if they emerge it is only to reproduce as mushrooms, cups, or other spore-producing structures. Although the reproductive structures form the traditional basis for detection and classification, they appear only briefly in the life of a fungus. Add to this that many groups of fungi are difficult or impossible to obtain in pure culture [1, 2], and the result is that by most estimates less than 10% of all fungi have been observed and formally described (100 thousand out of an estimated 1.5 million species). Ranging from unicellular organisms to some of the largest and most long-lived of all organisms [3, 4], the remaining fungi are hiding all around us and modern approaches to studying diversity and communities are beginning to reveal the true phylogenetic diversity of the group. After a full decade of progress in understanding fungal diversity using environmental DNA community studies, we now realize that most of the fungi in the environment do not actually match those specimens from herbarium cabinets and culture collections that were used to build the fungal tree of life [2, 5].

Most fungal sequences from environmental DNA studies can be assigned to a described class or even genus [6–8], but some represent unknown taxa on deeply diverging branches [9–12]. When lineages known exclusively from environmental DNA sequences cannot be assigned to a phylum, they challenge our understanding of the biodiversity and phylogeny (breadth and depth), and even characteristics of fungi. A recent breakthrough by Jones et al. [13] on the diversity and characteristics of one such enigmatic lineage, named ''cryptomycota'', raised the possibility that the lines dividing fungus from the protozoan soup from which they evolved [14] may be fuzzier than appreciated. Specifically, Jones et al. concluded that the widespread group cryptomycota were intermediate between fungi and ancestral protists. In their words:

''Co-staining with cell wall markers demonstrates that representatives from the

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clade do not produce a chitin-rich cell wall during any of the life cycle stages observed and therefore do not conform to the standard fungal body plan'' [13].

Our objectives here are to review the current state of knowledge of cryptomycota with a focus on its culturable species in *Rozella*. We provide evidence showing that, although the cryptomycota may have diverged early from other fungi, they have the capacity to make chitin-rich walls. While the cryptomycota do not conform to the ''standard fungal body plan'', derived adaptation to intracellular parasitism should be considered as an alternative explanation to retention of ancient, intermediate characters.

Cryptomycota: Unicellular bodies linked to environmental lineages

Cryptomycota were first detected as DNA sequences occurring in mesocosms of non-sterile water from Lake Ketelmeer, the Netherlands [15]. The cloned sequences were loosely related to fungi, but showed only a distant match to any of the known sequences at the time and the group was named after one of the clones, LKM11. After this discovery, the following decade of environmental DNA surveys uncovered sequences related to LKM11 numerous times. The sequences have variously been called ''fungi'', a ''novel clade'', or they have been inaccurately assigned to other phyla. LKM11-related sequences were recovered in essentially all environmental DNA surveys of freshwater aquatic ecosystems, such as the pH 2.0 Rio Tinto in Spain [16], anoxic sediments in lakes [17], and among picoeukaryotes (<5 μM) in lake water [18]. The LKM11 sequences were also described from terrestrial and marine systems, including the rhizosphere surrounding aspen roots [19], anoxic coastal sediments [20], and deep-sea sediments [21] (Fig. 1). These observations suggested that LKM11, like fungi in Chytridiomycota (chytrids), reproduce with motile spores and thus thrive in freshwater as well as in soil and marine ecosystems. By 2010 over 30 culture-independent environmental DNA studies documented the presence of LKM11 either as an early-diverging fungal clade or as a close relative to the fungi [21]. Because no member had ever been seen, the group remained an enigma.

The first breakthrough on the placement of the LKM11 clade was the demonstration of a relationship to the aquatic genus *Rozella* with robust phylogenetic support [22]. *Rozella* is an internal parasite, primarily of water molds [23], that was once classified in the order Spizellomycetales (Chytridiomycota) [24] (Box 1, Fig. 2). However, James et al. [25] demonstrated clearly (with statistical support) that the older classification was wrong and *Rozella* diverged to form the primary (basal-most) branch on the fungal tree. Lara et al. [22] coined the name ''rozellida'' for the clade. Because the trophic phase of *Rozella* lacks a cell wall and may have retained the ability to phagocytose host cytoplasm, Lara et al. suggested that rozellida might be parasites positioned on the most deeply diverging branch of Kingdom Fungi that retained ancestral protistan characteristics.

Jones et al. [13] opened the door to a broader view of the environmental clade by adapting state-of-the-art cytological and nucleic acid probing techniques to directly observe cells [36]. They coined the name cryptomycota for the group, to highlight its cryptic nature and its characters, which they, like Lara et al., interpreted as intermediate between fungi and ancestral protists (Box 2). Jones et al. used tyramide signal amplification-fluorescent in situ hybridization [36] (TSA-FISH) to identify cells of cryptomycota in filtrates from multiple sources, including pond water from the campus of University of Exeter. The cryptomycota cells took three forms. Most stained with antibodies to α-tubulin, appearing similar to the uniflagellated zoospores of chytrids. Some lacked flagella, having either encysted or lost their flagellum during preparation. The third cell type appeared to be attached to other cells, sometimes to diatoms, and Jones et al. hypothesized that this was a parasitic or saprotrophic association. A key conclusion of the paper was that none of the many cryptomycota cells could be stained for the presence of chitin or cellulose with calcofluor white or wheat germ agglutinin. The wall-less cryptomycota and the genus *Rozella*, having drawn their origin from the primary node on the fungal tree of life, have prompted us to critically consider the characteristics that both define fungi and led to their dramatic success as dominant terrestrial forms.

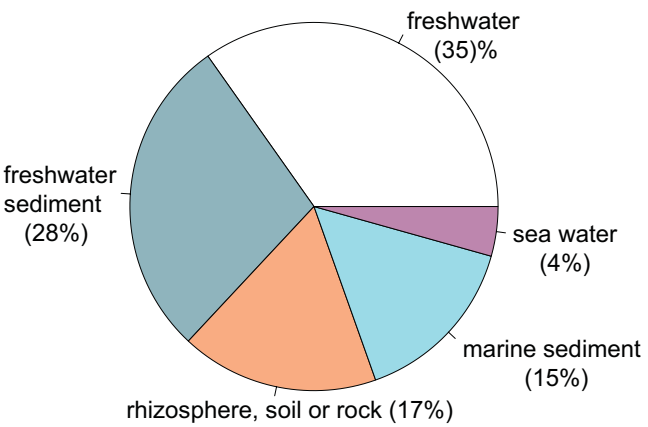

Figure 1. Proportion of habitats from 43 environmental DNA studies in which cryptomycota have been detected. Data from Table 1 of the supporting information of Jones et al. [13]. These data do not reflect the frequency of surveys reporting cryptomycota in the various habitat types, because cryptomycota are absent from many marine environmental DNA studies but are very common in freshwater studies.

Cryptomycota and the evolutionary origins of a chitinous wall

The cell wall of most fungi consists of β-1,3 glucans, chitin, mannans, and glycoproteins, with chitin microfibrils playing a major role in tensile strength and

Think again

Box 1

What are the *Rozella* parasites?

As *Rozella* is the only member of the cryptomycota for which the morphology and life cycle have been described, we briefly review what is known about the genus. *Rozella* consists of obligately biotrophic endoparasites that can only be grown in dual culture with their hosts [23]. Hosts include aquatic molds in the Oomycota and Fungi. One species, *R. coleochaetis*, has been reported from the green alga *Coleochaete* [26]. Experimental inoculations have suggested that the host range of each species is limited, with most evidence pointing to either species- or genus-level host specificity [27–29]. The best-known species is *R. allomycis*, a parasite of the common ''model'' water mold genus *Allomyces* (Blastocladiomycota). *R. allomycis* is also relatively common, occurring on 2 of the 43 *Allomyces* isolates reported by Wolf [30]. The infection begins with posteriorly uniflagellate, wall-less zoospores of the parasite swimming to an uninfected host (Fig. 2A). The spores attach to the host, retract their flagellum, and form a cyst on the surface of the host cell (Fig. 2B). The cysts begin to develop a cell wall and form a penetration tube. A vacuole forms at the posterior end of the cyst (2C and D), and the parasite cytoplasm is injected into the host through a wall that is apparently weakened by the parasite [31]. Once inside the host, *R. allomycis* then grows as a wall-less form that feeds on the host cytoplasm [32]. A naked thallus may have the advantage of being able to proliferate through the host mycelium, allowing the parasite to squeeze through the occasional partial septa. The naked thallus may also facilitate phagocytosis of the host's cytoplasm, as suggested for *R. polyphagi* in which the host's mitochondria were inside a parasitic vacuole [33]. During reproduction of the parasite, the host displays hypertrophy and its transcriptome or proteome is somehow coopted into making the septa/cell walls that the parasite uses to produce zoosporangia (Fig. 2E). The septa are required to develop the pressure needed for forcible discharge of zoospores [23]. The walled segments of the host may also be converted into the parasite's pigmented and thick-walled resting sporangia (Fig. 2F).

The source and the chemical composition of the parasite's resting sporangial wall are unknown. We stained a culture of *R. allomycis* using methods similar to those used by Jones et al. [13]. As expected [34], calcofluor white stained the cell walls of *Allomyces*, the host, providing evidence for chitin (Fig. 2H). Mature resting spores of *Rozella* did not stain, indicating that the outer ornamentation lacked these polysaccharides (Fig. 2J). However, the immature, unpigmented resting spore walls in *Rozella* did contain chitin or cellulose judging from their strong staining with calcofluor white (Fig. 2J). In combination with the detection of a chitin synthase homolog in the *R. allomycis* genome, these data suggest that the parasite can make its own chitinous resting sporangium. Some parallels can be drawn between the injection of naked protoplasts of *Rozella* into the host through enlargement of a vacuole, and the mechanism by which the protoplasm of a microsporidian spore is rapidly ejected into a host cell through expansion of a posterior vacuole [35]. This mode of infection may reflect an evolutionarily conserved mechanism, determinable perhaps if the phylogenetic relationship between microsporidia and *Rozella*, demonstrated in at least one phylogenetic study [25], can be rigorously tested.

structural integrity [39]. Chitin synthases are widely distributed among opisthokonts. Several of the divergent opisthokont protists with recently sequenced genomes from the ''Origins of Multicellularity'' project [40] have chitin synthases (Fig. 3, Table 1). Insects have two chitin synthases (Table 1) [41]. Even humans have hyaluronan synthases that produce hyaluronic acid but are homologs to chitin synthases (Table 1) [42]. Like chitin, hyaluronic acid is secreted to the outside of cells, but it becomes a component of vitreous humor in the eye and synovial fluid in joints [43]. The wide phylogenetic distribution of chitin synthases together with evidence (Fig. 2J) of a wall in *Rozella*'s young spores suggest that cryptomycota, or their recent ancestors, have or had a chitinous wall at some life history stage.

Compared with walls from most other organisms, walls of fungi are distinctive in three ways. Their synthesis involves the combined action of an exceptionally large number of chitin synthases; they are continuously remodeled to permit active growth; and they surround fungal cells that are actively taking up nutrients [44–46]. Possibly related to the complexity and distinctive characteristics of the fungal wall, the gene duplications that gave rise to the oldest of the fungal chitin synthases are more ancient than the divergences of the fungi themselves [44] (Fig. 3).

While chitin alone cannot define fungi, the presence of division 2 chitin synthases, and especially chitin synthases with a myosin domain (Fig. 3), along with transport of chitosomes along the cytoskeleton, may be unifying characters for most of Kingdom Fungi. Most fungal genomes (except *Schizosaccharomyces*) encode at least one chitin synthase from each of two deeply diverging divisions (Table 1, Fig. 3). Further, the newly sequenced genomes of early diverging fungal phyla have twice the number of chitin synthases as the better-known Ascomycota (Table 1). In the fungal model systems in Ascomycota, where chitin synthases are best characterized, paralogous proteins differ in timing and location of activity [47]. Among the chitin synthases, the division 2 genes form a monophyletic group known only from fungi and microsporidia [48]. Class IV enzymes from division 2 have usually been found to synthesize the bulk of the chitin in walls [45, 47]. Microsporidia have only one chitin synthase, a division 2, class IV chitin synthase for spore wall production that is

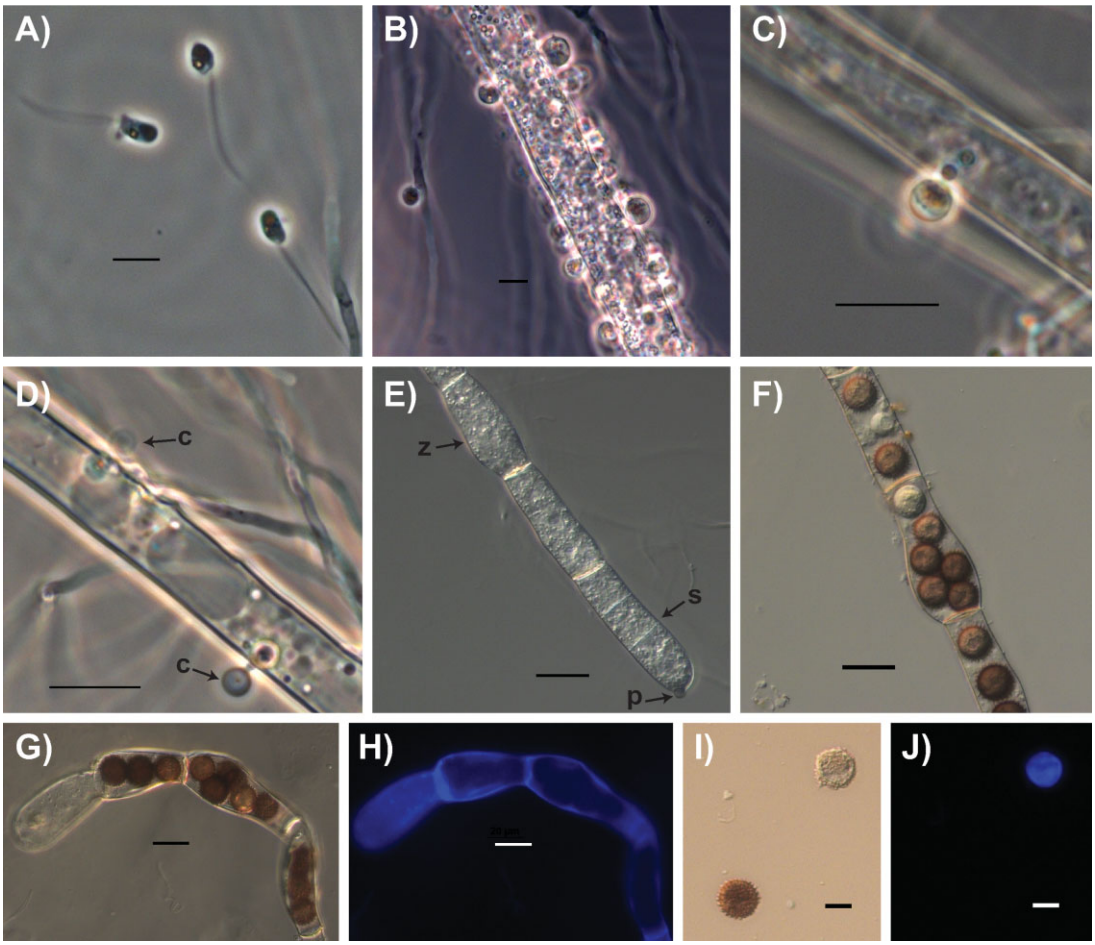

Figure 2. Life cycle of *Rozella allomycis*, a parasite of *Allomyces*. Photos are of strain CSF55 isolated from Hattiesburg, MS, USA. **A:** Posteriorly uniflagellate zoospores. Note the refractive lipid sac and slipper shape. **B:** Aggregation on host hyphae. **C:** Posterior vacuole observed at end of cyst as contents are injected into host cell. **D:** Empty cysts (c) on host hypha; note germ tube visible on one cyst and injected young amoeboid thallus appearing inside the host near cysts. **E:** Early stages of zoosporangium formation; note septa (s) produced by the host that separate the parasite's zoosporangia (z). Five zoosporangia are shown, and the terminal one displays a discharge papillum (p). **F:** Developing parasite resting sporangia delimited by host septa. Immature resting sporangia lack brown pigment. **G:** Mature, thick-walled resting sporangia. **H:** Resting sporangia in G stained with calcofluor white (marker for chitin and cellulose). Host hyphae and cross walls, but not parasite resting sporangia, stain. **I:** Resting sporangia removed from host cells; an immature and mature sporangium is shown. **J:** Immature wall of resting sporangium (from I) stains with calcofluor white, but the pigmented mature wall does not, possibly because final wall layers mask the inner polysaccharides. Scale bar = 5 μm in A and B; 10 μm in C, D, I, and J; 20 μm in E–H.

recognizable even though the highly reduced genomes of microsporidia evolved very quickly, erasing most traces of ancestral relationships [48] (Fig. 3). We predict that, at the least, a recognizable ortholog to the division 2 class IV fungal gene will be detected by sequencing a genome of *Rozella* or other cryptomycota. Preliminary results from draft genome sequencing of *R. allomycis* indeed have revealed a division 2 synthase (GenBank accession number JN646249), confirming that the parasite is capable of producing a chitinous wall at some stage of its life cycle.

A fungus-specific solution to targeting chitin synthesis to sites of active growth

Remaining to be determined is whether the *Rozella* genome has the distinctive fungal-specific chitin synthases with an N-terminal myosin head domain that are important in polar growth in other fungi [49–51]. Chitin synthases must be correctly targeted to the plasma membrane [52]. In filamentous fungi, chitin synthases, packed in vesicles called chitosomes, are transported along a cytoskeletal highway to the hyphal tip. In *Ustilago*, an analysis of mutants and of localization of fluorescently tagged proteins showed that the myosin domain is essential for exocytosis of its chitin synthase, and possibly also for its short range transport along the actin cytoskeleton to the hyphal tip [51]. The earliest diverging fungi are mostly like *Rozella*, with the main body growing isotropically to form a rounded globule, rather than elongating at a narrow hyphal tip. Surprisingly, even Chytridiomycota with globular bodies have several paralogs of chitin synthases with myosin domains (Fig. 3, Table 1). Like the Chytridiomycota, *Rozella* may require polar wall deposition for spatial orientation at specific life history stages, for example, when producing specialized apical exit papil-

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Box 2

What should we call the clade?

This essay revolves around a possible new fungal phylum that as yet lacks a formal name. It was first tagged as LKM11, the code name for a DNA sequence clone. Not intending formal naming, Jones et al. [13] proposed cryptomycota and Lara et al. [22] proposed rozellida as provisional names for the group. Here we use ''cryptomycota'' to emphasize that the clade is fungal but its diversity is largely cryptic. We could equally well have adopted the name rozellida to emphasize the connection with *Rozella*. In the spirit of rationalizing nomenclature, new higher level names for fungal taxa are, where possible, based on and typified by their first described genus. Newly rationalized names receive wide support [37] appearing in GenBank and in standard references such as the *Dictionary of Fungi* [38]. As a formal name, Cryptomycota would be unacceptable because *Cryptomyces* is already used for a genus in Ascomycota. So, if established as a new phylum, the group could be called ''Rozellomycota.''

lae for zoospore escape, or when forming germ tubes to penetrate host cells (Fig. 2D).

A strikingly different mechanism of localization of chitin synthases evolved in *Saprolegnia*, an oomycete (Straminopila). Superficially similar to Fungi, *Saprolegnia* is related instead to diatoms and brown algae (Fig. 3) and cellulose, not chitin, is the main constituent of its walls. Along with other Straminopila including diatoms, *Saprolegnia* nonetheless has genes sharing sequence motifs with fungal division 1 chitin synthases (Table 1). However, the lack of chitosomes in *Saprolegnia* suggests that it evolved an alternative way to shuttle chitin synthases to the hyphal tip [53]. Two of its six chitin synthase paralogs have microtubule interacting and sorting domains, which are not present in chitin synthases of any true fungus [54]. While the origin of chitin synthases is ancient, the mechanisms for subcellular localization appear to have evolved independently.

Evolution of fungus-specific chitin synthases: Loss is easy, gain was rare

The cryptomycota lack cell walls in some stages. Unlike most fungi, *Rozella* species lack walls when taking up nutrients and may use phagotrophy rather than absorptive nutrition across a cell wall. The lack of a wall during feeding may be a primitive character retained by the common ancestor of all fungi, but could also represent secondary loss of a wall, an adaptation to intracellular parasitism. Other pathogenic fungi in clades that normally produce chitinous walls can also grow inside a host cell as a wall-less trophic form. Wall-less trophic forms are found in Blastocladiomycota [55], Entomophthoromycotina [56], microsporidia [57], and *Beauvaria* (Ascomycota [58]). Chitin triggers a strong innate immune response from animals and plants [52], and its absence during intracellular parasitism suggests convergent adaptation to avoid host detection. These examples set the expectation that stage-specific suppression of chitin synthases is relatively easy.

How much of the cryptomycota life cycle and ecology do we know

The life cycle stages detected in uncultured cryptomycota are also found in *Rozella* but the converse is not true. Zoosporangial and resting stages are known only from *Rozella*. An open question is whether Jones et al. captured a trophic phase among the motile, unattached cysts, or among the attached cells that they filtered from pond water. The unattached cysts may be able to phagocytose cells such as bacteria or picoplankton, or they may be a transitional, amoeboid crawling phase, as observed in members of Ichthyosporea (Mesomycetozoea) [59, 60]. Whether or not the attached cysts are parasitic requires further investigation, but if they are, they may – as in *Rozella* – inject their protoplasm into a host cell.

If they originated from the basal node of the Fungi, cryptomycota have had ample time for diversification. How successful have cryptomycota been over this time period, and are they as common as fungi in their preferred environments? Using an argument based on phylogenetic branch lengths, Jones et al. suggest that cryptomycota radiated to become nearly as diverse as all other fungi, although this estimate could be biased if the cryptomycota, like some other intracellular parasites, have unusually rapid rates of substitution [61]. The cryptomycota are primarily aquatic and are largely absent in studies of airborne fungal particles [62–64]. Phylogenetic analyses suggest that other fungal phyla originated on land or in freshwater [65]. Finding cryptomycota in a cold methane seep [66] and deep-sea sediments [21] justifies raising the possibility that they first diversified in the sea, like many animals and protists, but unlike most other fungi. In general though, most extant cryptomycota prefer freshwater habitats; they decreased in abundance along a salinity gradient in a salt marsh in Rhode Island [67], their frequency is low in the open oceans [13], and they are absent from some deep-sea surveys [68, 69]. Environmental DNA studies of picoeukaryote communities in lakes have documented ~2:1 to 3:1 (fungi: cryptomycota) [18, 70, 71]. However, in at least one study that utilized TSA-FISH rather than PCR-generated clone libraries to survey eukaryotic groups in French lakes, cryptomycota were found to be more abundant [72].

Such studies of fungi and cryptomycota in filtered water may be the aquatic analog of the many studies that have sampled airborne propagules of fungi [73]. These studies may provide a skewed picture of the community as number of propagules produced may reflect reproductive strategies and phenology more than population census size [63]. Resolution of the complete life cycle of cryptomycota should come from additional studies utilizing fractionation of environmental samples or direct sampling of each of the many

Table 1. All sequenced fungal genomes have numerous paralogous genes with motifs characteristic of chitin synthases, and copies of the deeply diverging division 1 and division 2 genes are present in all fungal phyla

Gene and source (phylum or higher group, genus, accession number[a] of example)	No. of paralogs	Any paralogs with motifs related to transport?[b]	Presumed functional motifs[c]				
			Substrate binding	Substrate binding	Catalytic base	Processivity	Processivity
Chitin synthase division 1 consensus			T(MY)NE	DXGT	LAEDRIL	QRRRW	(S/T)WG
Ascomycota, *Aspergillus*, ANID_04367	4	No	TYYNE	DAGT	LAEDRIL	QRRRW	SWG
Blastocladiomycota, *Allomyces*, AAMAG 10750.1	10	No	TMYNE	DVGT	LAEDRIL	QRRRW	SWG
Chytridiomycota, *Batrachochytrium* BDEG 08256.1	3	No	TMYNE	DVGT	LAEDRIL	QRRRW	SWG
Chytridiomycota, *Spizellomyces* SPPG 04845.2	6	No	TMYNE	DVGT	LAEDRIL	QRRRW	SWG
Zygomycota, *Rhizopus* RO3G_16230.3	6	No	TMYNE	DVGT	LAEDRIL	QRRRW	SWG
Microsporidia, *Encephalitozoon*	None	No					
Straminopila (not Fungi), *Saprolegnia* SPRG_02074.2	6	Yes; 3′ microtubule interacting and sorting domain *MIT* PF04212	TMYNE	DVGT	LAEDRIL	QRRRW	SWG
Chitin synthase division 2 consensus			(T/P)(A/C) Y(S/T)E	DADT	LGEDR(YFE)L	Q(R/G)RRW	(S/T)WG
Ascomycota, *Aspergillus*, ANID_06318.1	3	Yes	PAYTE	DADT	LGEDRYL	QRRRW	SWG
Blastocladiomycota, *Allomyces*, AMAG_07719.1	25	Yes; 5′ myosin cd00124; also note 5′ oligopeptide transporter protein in AMAG_15310.1	PCYTE	DSDT	LGEDRYL	QRRRW	SWG
Chytridiomycota *Batrachochytrium*, BDEG_03361.1	11	Yes; 5′ myosin cd00124	PCYTE	DADT	LGEDRYL	QRRRW	SWG
Chytridiomycota *Spizellomyces* SPPG 03441.2	11	Yes; 5′ myosin cd00124	PCYTE	DADT	LGEDRFL	QRRRW	SWG
''Cryptomycota'' *Rozella* GenBank JN646249	na	Unknown	TCYSE	DADT	LGEDRYL	QRRRW	SWG
Zygomycota *Rhizopus* RO3G_17187.3	20	Yes; 5′ myosin cd00124	PCYTE	DADT	LGEDRYL	QRRRW	SWG
Microsporidia, *Encephalitozoon* GenBank XP_965977	1	No	TCYSE	DADT	LGEDRYL	QRRRW	SWG
Straminopila (not Fungi), *Saprolegnia*	None	No					
Outgroups							
Choanoflagellata (unicellular opisthokont) *Salpingoeca* PTSG 01414.1	1	No	PCYNE	DCGT	LAEDRFL	QRRRW	TWG
Choanoflagellata *Salpingoeca* PTSG 01542.1	1	3′ SAM domain	TMYNE	DADI	MGEDRWL	QRRRW	SWG
Filasporea (unicellular opisthokont) *Capsaspora* CAOG 03353.2	1	No	ADFDN	DGDV	LGEVP-L	QRRRW	RWG
Apusozoa (unicellular) *Thecomonas* AMSG_12058.2	1	No	PNVTL	DGDT	MGEDRWL	QRKRW	SWG
Arthropoda, *Drosophila*, GenBank NP_730928	2	Yes; 3′ transmembrane amino acid transporter domain, pfam03845	TMWHE	DGDI	QGEDRWL	QRRRW	SWG
Chordata, *Homo*, hyaluronan synthase, GenBank AAH35837	4	No	SAYQE	DSDT	FGDDRHL	QQTRW	GWG

[a] Sequences are from the Broad Institute http://www.broadinstitute.org/ unless otherwise noted.
[b] From comparisons with the Conserved Domain Database [79].
[c] Division-specific sequence motifs are from Choquer et al. [80]. We found fungal-specific chitin synthases with BLAST searches using as our queries the conserved amino acid domains from division 1 Chs2p gene (DVGTRL...HDVSWG, GenBank NP_009594.1) from *Saccharomyces cerevisiae* and division 2 class V gene from *Aspergillus nidulans* (HHHIRN...DDFSWG, GenBank AAB05797.1), with e^{-30} as a cutoff for fungi, e^{-9} for outgroup organisms. To find the motifs, we aligned sequences using MUSCLE [81]. Outside of fungi and animals, gene function is unknown and homology with the first substrate binding domain is uncertain.

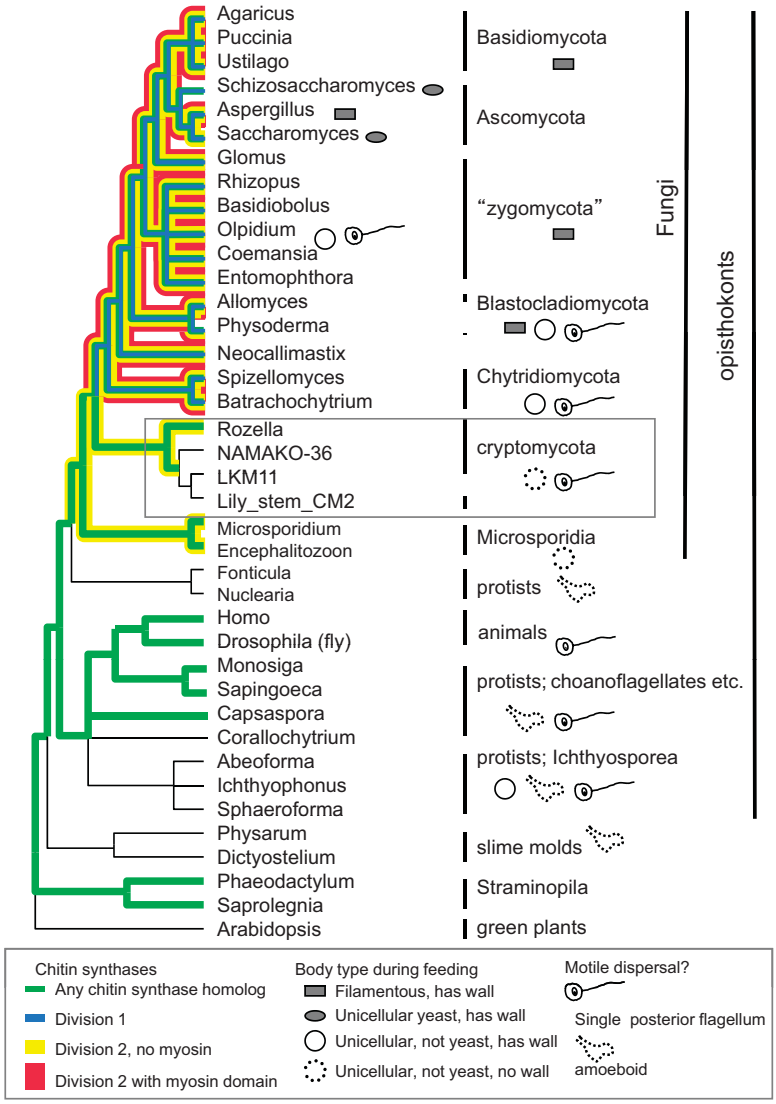

Figure 3. Diagrammatic tree of fungi showing that the evolutionary divergence of many chitin synthase paralogs predates all phyla of fungi, except, perhaps, the cryptomycota. We hypothesize that the ancestor of cryptomycota was able to make a chitinous cell wall because many animals and all other fungi have chitin, calcofluor white stains *Rozella*'s resting spores (Fig. 2J), and the genome of *Rozella* has at least one chitin synthase. This tree places taxa mentioned in this review in a commonly accepted phylogenetic context. We coded taxa for gene presence based on comparisons of genes from sequenced genomes (Table 1), and then predicted that taxa lacking sequenced genomes would share the chitin synthases of their closest relatives.

possible hosts that lurk in the sediments in which cryptomycota have been found using the developed nucleic acid probes [13, 72, 74].

Beware of error and uncertainty

Up until this point, we have accepted – based only on the ribosomal DNA locus – that the cryptomycota originated from the first divergence in the fungal tree of life. But is this phylogenetic position correct? The earlier analyses of James et al. [25] with the two available protein coding loci in addition to ribosomal DNA showed the position of *Rozella* to be generally consistent with Jones et al. [13]. However, tree topology tests did not rule out alternative positions of *Rozella* among the fungi [75]. This, along with the clustering of *Rozella* with the notoriously unstable microsporidia [25] raises the possibility that the basal position of the entire cryptomycota clade may be an artifact. Phylogenetic error can result from violations of standard molecular evolution models, such as heterotachy (variation in site-specific DNA substitution rates over time) [76]. A multilocus phylogeny using sequences from complete genomes of *Rozella*, and ideally other cryptomycota, would help to place these key taxa. No matter what its position, cryptomycota will undoubtedly remain a highly divergent group of fungi with characters that help illuminate early fungal evolution. However, the jury is still out about how early these fungi diverged from all others.

Conclusions

The unveiling of cryptomycota over the last decade has revealed a prominent branch of ubiquitous and diverse organisms that straddle the divide between fungi and the opisthokont protozoa from which they evolved. Studies of these organisms from environmental samples will continue to be important in illuminating habitat and host relationships, while cultured isolates of *Rozella* serve as cryptomycota's most tractable representatives for experimental and genomic analysis. Our detection of the chitinous wall of *Rozella* required staining of immature resistant sporangia that have yet to be detected among environmental samples hybridizing to cryptomycota probes. Showing that *Rozella* produced one of the fungal-specific chitin synthases required genomic analysis, as will the reconstruction of its full suite of chitin synthases, now a work in progress. We predict that future analyses of environmental genomes will also reveal chitin synthases from other cryptomycota. The explosion of diversity of true fungi on land coincided with the evolution of polar tip growth facilitated by a semi-rigid cell wall. However, the semi-rigid wall predated terrestrial fungi and is a shared character of the cryptomycota and all other fungi.

Keeping in mind that their phylogenetic diversity rivals that of the rest of the fungi, the varied characteristics and ecological capabilities of the cryptomy-

cota will offer surprises. Major additions to the tree of life are far from unique to mycology, and the vastness of protistan diversity is only recently becoming widely appreciated [77]. New lineages are sprouting across the tree, such as the uncultured rappemond algae [78], demonstrating that our understanding of global biodiversity has been skewed towards organisms that can be cultured or that are macroscopic. Because these are the early days of research into uncultured cryptomycota, Lara et al. [22] and Jones et al. [13] are extrapolating from fragmentary evidence. However, by opening a window on uncultured cryptomycota, their work is leading to stimulating new hypotheses that challenge classical concepts of fungal life cycles, ecological niches, and evolutionary trajectories.

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