The transcriptome of *Paraphelidium tribonemae* illuminates the ancestry of Fungi and Opisthosporidia

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SUMMARY

Aphelids constitute a group of diverse, yet poorly known, parasites of algae [1, 2]. Their life cycle and morphology resemble those of zoosporic fungi (chytrids) and rozellids (Cryptomycota/Rozellosporidia), another specious group of parasites of fungi and oomycetes [3, 4]. Unlike fungi, which are osmotrophs, aphelids and rozellids are phagotrophs, feeding on the host's cytoplasm. Combined RNA polymerase and rRNA gene trees [5] suggested that aphelids and rozellids relate to Microsporidia, extremely reduced parasites with remnant mitochondria [6]. Accordingly, aphelids, rozellids and Microsporidia were proposed to form a monophyletic clade, called Opisthosporidia, sister to Fungi [1]. Microsporidia would have subsequently lost the ancestral opisthosporidian phagotrophy. However, the limited phylogenetic signal of those genes combined with microsporidian fast-evolving sequences have resulted in incongruent tree topologies, showing either rozellids [5, 7] or aphelids [8] as the earliest-branching lineages of Opisthosporidia. We have generated the first transcriptome data for one aphelid species, *Paraphelidium tribonemae* [2]. In-depth multi-gene phylogenomic analyses using various protein datasets place aphelids in a deep, pivotal position as a sister group to Fungi, making Opisthosporidia paraphyletic. We infer a rich, free-living-like proteome for *P. tribonemae*, which includes cellulases likely involved in algal cell-wall penetration, enzymes involved in chitin biosynthesis and several metabolic pathways that were lost in the comparatively reduced *Rozella allomycis* genome [9]. Our results suggest that Fungi evolved from a complex phagotrophic opisthosporidian ancestor, likely a specialized endobiotic predator, which became osmotrophic at the fungal root and evolved towards phagotrophic parasitism in the rozellid/microsporidian line.

Keywords: aphelid; opisthokont; protist evolution; fungi; cell wall; chitin; phagotrophy; osmotrophy; parasitism; phylogenomics

RESULTS AND DISCUSSION

Aphelids Occupy a Deep Pivotal Position in Opisthosporidia and are Sister to Fungi

To improve the phylogenetic signal for an accurate placement of aphelids in the opisthokont branch containing fungi, nucleariids, rozellids and Microsporidia (usually referred to as Holomycota), we generated transcriptome data for the aphelid species *Paraphelidium tribonemae* [2]. Because *P. tribonemae* has a complex life cycle (Figure 1), we maximized transcript recovery by constructing two cDNA libraries corresponding to young and old enrichment cultures of its host, the yellow-green alga *Tribonema gayanum*, infected with the aphelid. Accordingly, transcripts corresponding to zoospores, infective cysts, trophonts, plasmodia, sporangia and sporocysts were represented [2]. After paired-end Illumina HiSeq2500 sequencing, we assembled a metatranscriptome of 68,130 contigs corresponding to the aphelid, its host and bacterial contaminants. After a process of supervised cleaning we obtained an initial set of 10,669 annotated polypeptides, which was further reduced to 10,439 protein sequences that were considered free of contaminants after comparison with a newly generated transcriptome of the algal host (see Supplementary Methods). The final predicted proteome (*Paraphelidium tribonemae* version 1.5; Table S1) was 91.4% complete according to BUSCO (eukaryote set) [10]. We found no stop codons interrupting coding sequences. Therefore, in contrast to *Amoeboaphelidium protococcarum*, for which the TAG and TAA stop triplets appear to encode glutamine [5], *P. tribonemae* has the canonical genetic code.

We incorporated *P. tribonemae* orthologs to a dataset of 93 single-copy protein domains (SCPD dataset) previously used for phylogenomic analysis of basal-branching opisthokonts [11], updating it with more microsporidian species including the early-branching *Mitosporidium daphniae* [12] and the metchnikovellid *Amphiamblys* sp. [8]. The concatenated matrix contained 22,207 conserved amino acid sites for 48 species including opisthokonts and close outgroups (see Supplementary Methods for details). Bayesian inference (BI) trees reconstructed under the CAT-Poisson mixture model [13] and Maximum Likelihood (ML) trees reconstructed under C60, the closest mixture model to CAT [14], yielded the same topology (Figure S1). We recovered the monophyly of all opisthokont lineages previously reported [11, 15]. In addition, we retrieved the monophyly of Opisthosporidia, as a sister group to Fungi in both, BI and ML analyses, as in previous analyses based on 18S rRNA and RNA polymerase genes [5, 7]. The aphelid always appeared as the earliest-branching lineage, *Rozella* being the sister group to Microsporidia with full statistical support. However, while Bayesian posterior probability provided full statistical support for the monophyly of Opisthosporidia, the support dropped in ML trees to 79% (ultra-fast bootstrapping [16]). To enrich the taxon sampling in the Opisthosporidia, we included data from the recently released genome of *Paramicrosporidium saccamoebae* [18]. The inclusion of this single

species to the dataset (SCPD49) changed the topology of the tree. Opisthosporidia appeared as paraphyletic and the aphelid was placed as sister to Fungi (Figure 1). However, neither the Bayesian posterior probability (0.66) nor the ML bootstrap support (ultrafast bootstrapping 96%) was maximal. To limit potential long-branch attraction artifacts derived from the inclusion of fast-evolving Microsporidia, we built a reduced dataset without the thirteen fastest-evolving microsporidian species (SCPD36, 36 species). Bayesian and ML trees obtained for this dataset reproduced the same topology (Figure S1E and F) with equally moderate support values (Figure 1).

To better resolve the phylogenetic position of the aphelid, we analyzed two additional datasets previously used for phylogenomic analyses of Microsporidia with the same two sets of 49 and 36 species. The resulting datasets were called BMC49 and BMC36 for a set of 53 protein markers [19] which, after concatenation, yielded 15,744 and 16,840 amino acid positions, respectively; and GBE49 and GBE36 for datasets with 259 protein markers [8], which resulted in 87,045 and 96,029 amino acids, respectively. For all datasets, Bayesian and ML trees recovered the sister relation of *Paraphelidium* and Fungi (Figure S1) and, for the two datasets without fast-evolving Microsporidia, both posterior probabilities and ML bootstrap supports were maximal (Figure 1 and Table S2). In addition, we carried out alternative topology tests for the three datasets containing 49 species. All of them supported the aphelid relationship with Fungi (Table S2). Finally, to minimize systematic error due to the inclusion of fast-evolving sites in our protein datasets, we progressively removed the 5% fastest-evolving sites and reconstructed ML phylogenomic trees. The support for the monophyly of Fungi and the aphelid was very high until 50% (SCPD dataset) or up to 80% (GBE dataset) of sites were removed (Figure 1C).

Collectively, our phylogenomic analyses support the sister relation of aphelids and Fungi, although the branch joining the two groups is very short. Therefore, Opisthosporidia, united by their ancestral phagotrophy, appear as paraphyletic.

Enzymes Involved in Cell-Wall Synthesis and Degradation

Despite secondary losses in some fungi, the presence of chitin in cell walls was long considered a typical fungal trait [20]. However, chitin is also present in the cell wall of many other protists across the eukaryotic tree, implying that the machinery for chitin synthesis/remodeling originated prior to the radiation of Fungi and other eukaryotic lineages [20]. Microsporidia and *Rozella* are also able to synthesize chitin [9, 21] but, unlike fungi, which possess chitin cell walls during the vegetative stage, they produce chitin only in cysts or resting spores [22]. Staining with fluorescently-labeled Wheat Germ Agglutinin (WGA) showed that *Paraphelidium* also possesses chitin in the wall of infecting cysts, but not in zoospores or, unlike fungi, trophonts (Figure 2A-B). In agreement with this observation, we

identified homologs of chitin synthases, chitin deacetylases, chitinases and 1,3-beta glucan-synthases in the *Paraphelidum* transcriptome (Figure S2A-D). Specifically, we detected seven homologous sequences (including all alternative transcripts such alleles or splice variants) of division II chitin synthases [11, 21, 23, 24] corresponding to at least six distinct peptides (*Rozella* contains only four [9]). Three of them clustered with class IV chitin synthases, including Microsporidia and *Rozella* homologs (Figure S2A). The remaining four sequences branched within class V/VII enzymes [24], two of them (probably corresponding to a single polypeptide) forming a deep-branching group with fungal, mostly chytrid, sequences (Figure 2A). Class V enzymes include a myosin motor thought to intervene in polarized fungal hyphal growth that has been hypothesized to take part in the formation of the germ tube in aphelids and rozellids [12]. Class V chitin synthases were lost in Microsporidia (with the exception of *Mitosporidium*, still retaining one homolog, like *Rozella*), endowed instead with highly specialized polar tube extrusion mechanisms [12]. Neither spore wall nor polar tube proteins specific to Microsporidia [25] occurred in the *Paraphelidium* transcriptome. Therefore, our data (Figure S2A, Table S1 and-S4B) lend credit to the hypothesis that class V chitin synthases are involved in germ tube polar growth.

Among the rest of the chitin-related enzymes, we identified twelve sequences (at least five different homologs) of chitin deacetylase [26, 27] (Figure S2B). We detected at least three class II chitinase homologs (eight total sequences), which are ancestral in opisthokonts, containing the Glyco_hydro_18 (PF00704) domain, and a class I chitinase (CTSI) with a Glyco_hydro_19 (PF00182) domain [28]. The latter included the catalytic site, an N-terminal predicted signal peptide and a transmembrane region, suggesting an extracellular chitinase activity. CTSI has a peculiar phylogenetic distribution in eukaryotes, occurring only in Viridiplantae, Fungi, Opisthosporidia and Ecdysozoa (Figure S2C). *Rozella* contains two homologs and Microsporidia at least one; they have N-terminal signal peptides and are predicted to localize extracellularly but lack transmembrane domains. In our phylogenetic tree, opisthosporidian sequences appeared scattered within metazoan and fungal sequences. This might be the result of hidden paralogy and/or horizontal gene transfer (HGT) (Figure S2C). Regardless of its evolutionary origin, aphelid CTSI might be involved in the self-degradation of resting spore and cyst wall chitin. This might happen during their release from chitin-containing resting spores or at the tip of the germ tube during infection, as previously suggested for *Rozella* [9] (Figure 1).

Although not found in chytrids, 1,3-beta-glucan is also considered an idiosyncratic fungal cell-wall component [20]. Surprisingly, we identified two 1,3-beta-glucan synthase (FKS1) homologs (four sequences), with split Glucan_synthase (PF02364) and FKS1_dom1 (PF14288) domains (fused for the phylogenetic analysis) (Figure S2D). The presence of

FKS1, absent in *Rozella* and Microsporidia, in aphelids traces its origin back to the ancestor of Fungi and aphelids.

To feed on the algal cytoplasm, aphelids need to traverse the algal cell wall but, so far, the specific penetration mechanism, whether mechanical (germ-tube penetration at algal cell-wall junctures) or enzymatic (digestion of algal cell-wall components) was uncertain [1, 2]. Scanning electron microscopy (SEM) observations showed both, clear cases of mechanical penetration via junctures as well as infecting cysts scattered on the algal cell-wall surface far from junctures (Figure 2C). SEM images additionally confirmed WGA-epifluorescence observations of multiple parasitoid cysts co-infecting the same host cell (Figure 2A-C). Multiple infections open the intriguing possibility for aphelids to have sex within the algal host. *Tribonema* cell walls contain cellulose II based on 1,6-linked glucan (alkali soluble cellulose), 1,3 and 1,4-linked xylose, 1,3-linked rhamnose and, mostly, 1,3, 1,4 and 1,6-linked glucose [29]. We performed sequence similarity searches of known fungal cellulases [30] using the database mycoCLAP [31], which contains functionally characterized proteins, to look for these enzymes in aphelids, followed by phylogenetic analyses. In support of an enzymatic algal cell-wall penetration, we identified various cellulases in the *Paraphelidium* transcriptome belonging to glycoside-hydrolase families GH3 and GH5. We detected three homologs of the GH3 cellulase beta-glucosidase/xylosidase [32], which is not found in other opisthosporidians but is present in fungi, amoebozoans, several opisthokonts and other protists, as well as bacteria. Our phylogenetic analysis shows that the three aphelid sequences are most closely related to deep-branching opisthokont protists (respectively, *Capsaspora*, choanoflagellates, nucleariids) (Figure S2E). Additionally, we identified at least three GH5 cellulase [33] homologs (seven sequences in total) in *P. tribonemae*, which were related to the GH5 proteins that are widespread in fungi (GH5_11, GH5_12 and GH5_24) (Figure S2F). Collectively, these observations strongly suggest that these cellulases are involved in algal cell-wall penetration, but direct proof will only be obtained by purifying or heterologously expressing those cellulases and testing their activity *in vitro*.

In summary, *Paraphelidium* has a complex life cycle with chitin-containing infective cysts and a diversified set of enzymes involved in chitin metabolism and possibly cellulose hydrolysis.

Primary Metabolism Reminiscent of Free-Living Lifestyles

Analysis of the *Rozella allomycis* genome showed that, like microsporidian parasites, it has significantly reduced metabolic capabilities [9]. To comparatively assess the metabolic potential of aphelids, we investigated the presence of orthologous groups (OGs) related to eight primary metabolism categories (Gene Ontology) in the transcriptome of *Paraphelidium*, using eggNOG annotation [34]. We thus identified 1,172 OGs in *Paraphelidium* and a set of

41 eukaryotic species including representatives of the major fungal lineages, opisthokont protists and other eukaryotic parasites (Table S2). Based on their OG distribution, we built a dissimilarity matrix that was analyzed by Principal Coordinate Analysis (PCoA; see Supplementary Methods). The first axis clearly separated *Paraphelidium* from Microsporidia, *Mitosporidium*, *Paramicrosporidium* and *Rozella*, the latter two positioned near one another and having an intermediate position similar to other protist parasites (e.g. *Trypanosoma, Leishmania, Toxoplasma*) (Figures 3A and S3A). *Paraphelidium* was positioned at the same level as fungi, *Capsaspora, Corallochytrium* and *Parvularia*, along axis 1. However, axis 2 separated *Paraphelidium* and fungi from the rest of eukaryotes. These relationships were further visualized in cluster analysis of pairwise species comparisons (Figure S3B). The PCoA suggested that *Paraphelidium* had a rich metabolic gene complement, which was made evident by the OG presence/absence heatmap showing that aphelids have a metabolic potential fully comparable to that of (especially chytrid) fungi (Figure 3B).

The most distinctive categories when comparing *Paraphelidium* and other Opisthosporidia were “energy production and conversion” followed by “amino acid, nucleotide and lipid transport and metabolism”. In all metabolic categories, the aphelid clustered with fungi, and more specifically with chytrids, and sometimes with other free-living opisthokonts (e.g. nucleariids, *Capsaspora*). By contrast, *Rozella* always clustered with *Mitosporidium* and *Paramicrosporidium* either together with other Microsporidia or with other parasitic protists (Figures S3C-F). The only exception corresponded to *Paramicrosporidium* that, for “energy production and conversion”, clustered with nucleariids (Figure S3C), in agreement with their rich energy-related gene set [18].

To check whether these differences between *Paraphelidium* and *Rozella* affected particular metabolic pathways, we compared the annotated proteins in the two organisms based on KEGG annotation [35]. The comparison of the KEGG general metabolic pathway map showed that, even accounting for the possibility that we missed some genes in the *Paraphelidium*’s transcriptome (e.g. mitochondrial-encoded proteins), the aphelid map contained 200 more OGs than *Rozella* (548 vs 348 OGs) (Figure S3G). In agreement with previous observations, major differences were observed in “energy production and conversion”, and “amino acid, nucleotide and lipid transport and metabolism”. In particular, contrary to *Rozella*, which lacks most subunits of the mitochondrial electron transport chain complex I (NADH dehydrogenase; ETC-I) [9], *Paraphelidium* possesses a practically complete ETC-I as inferred from the nuclear encoded transcripts (the *P. tribonemae* transcriptome is biased against mitochondrial transcripts, which lack polyA) (Figure S3H). *Paraphelidium* also possesses wide capabilities related to nucleotide (e.g. purine, uridine and inosine biosynthesis) and amino acid (serine, threonine, methionine, lysine, ornithine, histidine, shikimate pathway) metabolism, which *Rozella* has lost (Figure S3I). Likewise, the

aphelid has also retained pathways for phosphatidylcholine, cholesterol and fatty acid biosynthesis that were subsequently lost in *Rozella* [9]. Most carbohydrate metabolic pathways were conserved in the two opisthosporidians, except for galactose synthesis and degradation, also lost in *Rozella* (Table S3).

By contrast, compared to *Rozella*, and under the assumption that our transcriptome is rather complete, the aphelid seems to lack several enzymes involved in catecholamine biosynthesis (Table S3). However, some of these are also absent from free-living *Capsaspora, Monosiga, Salpingoeca* or *Spizellomyces*. These compounds are likely involved in cell-cell communication in microbes [36], e.g. parasite-host signaling, suggesting that they might have a role in rozellid parasitism. The aphelid seems to lack other parasite-specific proteins, such as crinkler, nucleoside H^+-symporters or ATP/ADP-antiporters, which occur in *Rozella* and/or Microsporidia [9].

These observations suggest that *Paraphelidium* has a complex metabolic profile, being functionally closer to free-living protists than to parasites and having affinities with fungi and, to a lesser extent, nucleariids and holozoan protists.

Distinct and Ancestral-Like Phagotrophy-Related Machinery

Like rozellids, aphelids are phagotrophs, but their global metabolism resembles that of osmotrophic fungi. What does their phagocytosis-related proteome look like? The core phagocytic molecular machinery, already present in the last eukaryotic common ancestor [37], is involved in multiple dynamic cell processes (endocytosis, exocytosis, autophagy, protein recycling, etc.). Structurally, the phagocytic machinery encompasses various endomembrane organelles (phagosomes, lysosomes, peroxisomes, endoplasmic reticulum, Golgi apparatus), and multiple membrane-trafficking components (signaling pathways, transporters, specific enzymes, cytoskeleton elements, motors, etc.) [38]. To look for phagotrophy-related genes in *Paraphelidium* and the 41 additional eukaryotes used for comparison, we built a presence/absence matrix of 695 KEGG orthologs (OGs) from 11 functional categories (5 KEGG maps and 6 BRITE categories) that aimed at including all necessary proteins to perform phagotrophy; i.e. phagolysosome biogenesis, membrane trafficking and the actin cytoskeleton [38] (see Supplementary Methods and Table S4A). A PCoA showed that *Paraphelidium* and *Rozella* are much closer to one another in terms of phagotrophy- than metabolism-related genes, grouping with fungi and far from Microsporidia (Figure 3C and S4A). This pattern was also evident in the presence/absence matrix (Figure 3D and S4B). In both, the two opisthosporidians clustered with early-branching fungi: chytridiomycetes (*Spizellomyces*, *Batrachochytrium*, *Gonapodya*) and blastocladiomycetes (*Allomyces*, *Catenaria*, *Blastocladiella*). Overall, *Paraphelidium* and *Rozella* have similar

phagosome, lysosome and endocytic proteins, thus arguing for a homologous ancestral phagocytosis (Figures S4C-E).

In order to gain more insights into the diversification of the actin cytoskeleton toolkit of fungi and opisthosporidians, we analyzed the evolution of myosin motor proteins. The myosin arsenal of *Paraphelidium* contains a mixture of classical fungal families and others previously identified in holozoans (animals and their unicellular relatives; Table S4B). We recovered diversified class I myosins in *Paraphelidium*, *Spizellomyces* and nucleariids (Figure S4G), with paralogs of Ic/h and Ik families, previously described only in holozoans [39]. *Paraphelidium,* nucleariids and *Gonapodya* also possess homologs of class XV myosins, formerly thought holozoan-specific [39]. In addition, the aphelid not only possesses homologs of the V/VII myosin-motor family associated to chitin-synthase (see above; Figure S2A), but also myosins of If (pan-eukaryotic), II (amorphean), and XXII (opisthokont) classes, which clustered with previously described fungal homologs (Table S4B; Figure S4F). Thus, compared with the ancestral opisthokont myosin complement [39], *Paraphelidium* retains all but one myosin class (VI), with homologs of all myosin families present in fungi (If, II, V, XVII - chitin synthase) plus four additional families (Ic/h, Ik, XV and XXII) that were lost in "eumycotan" fungi (i.e., fungi to the exclusion of chytrids). This suggests an independent step-wise simplification of the myosin complement in fungi, *Rozella* and Microsporidia, with *Paraphelidium,* nucleariids and chytrids retaining ancestral classes.

Recently, it has been proposed that WASP and SCAR/WAVE genes, encoding activators of branched actin assembly proteins [40], are essential to build actin-based pseudopodia (filopodia), which themselves are fundamental for the typical movement of filopodiated cells called "alpha-motility" [41] (Table S1). The aphelid zoospore motility (Video 1) resembles that of chytrid fungi [41] and its transcriptome contains homologs of WASP and SCAR/WAVE. Interestingly, although *Rozella* also contains WASP and SCAR/WAVE homologs, filopodia have not been described in its zoospores [42-44]. If *Rozella* truly lacks filopodia, this might call into question the proposed essential role of these genes for filopodial movement [41] and might instead suggest their potential involvement in phagotrophy in this organism.

Aphelids and the Complex Phagotrophic Ancestor of Fungi and Opisthosporidia

From an evolutionary perspective, the current situation in the holomycotan branch (including Fungi) of the eukaryotic super-group Opisthokonta mirrors that of the Holozoa (including Metazoa), where the discovery of deeply-branching unicellular protists that possess genes thought unique to animals continues to challenge previous evolutionary schemes about the emergence of Metazoa [15, 45]. Thus, the discovery that widely diverse environmental

sequences formed a deeply-branching clade that appeared sister to fungi and included the parasite *Rozella allomyces* (named rozellids [3], and subsequently Cryptomycota [4], Rozellomycota [22] or Rozellosporidia [47]), triggered the discussion of what Fungi actually are [20, 48]. A debate further nourished by the discovery that aphelids, another widely diverse group of parasites of algae [49], formed a large clade with rozellids and Microsporidia based on rRNA and RNA-polymerase genes [1, 5, 47, 50]. This seemingly monophyletic clade was named Opisthosporidia and branched as sister to classical Fungi (the monophyletic clade including from chytrids and their relatives to the Dikarya [48]) in phylogenetic trees [1]. Lately, many mycologists include the three opisthosporidian lineages, Aphelida, Rozellosporidia and Microsporidia, within Fungi [9, 20, 51, 52]. Some authors even incorporate as fungi the free-living phagotrophic chitin-lacking nucleariids, thus pushing the limits of Fungi to incorporate all holomycotan (possibly a confusing name) lineages, despite asserting that "...the kingdom Fungi is characterized by osmotrophic nutrition across a chitinous cell wall…" [53]. However, unlike fungi, the most basal-branching opisthosporidian lineages (aphelids and rozellids) are phagotrophs, lack a chitin cell wall at the vegetative stage and are endobiotic, having a unique mode of penetration into the host [1, 2, 48, 50]. Also, because *Rozella* has a very reduced genome, a typical trait of streamlined parasites, some authors inferred a parasitic nature for the ancestor of Fungi [9].

The study of the *Paraphelidium tribonemae* transcriptome sheds new light on this controversy. Our multi-gene phylogenetic analyses place the aphelids as a pivotal group branching very deeply in the Opisthosporidia/Fungi clade. However, contrary to previous phylogenetic analyses based on only a few genes, Opisthosporidia do not appear monophyletic, aphelids emerging as the sister group to Fungi (Figure 1). Our study strongly places aphelids in a very basal position and suggests that Fungi evolved from opisthosporidian ancestors that were related to, or potentially were, aphelids. Indeed, if the phylogenetic position of aphelids is confirmed by future analyses with increased taxon sampling, this would imply that Fungi are derived Opisthosporidia. Because of its deep pivotal position, the comparative study of the *Paraphelidium* transcriptome allows better inferring ancestral states for Opisthosporidia and Fungi (Figure 4). *Paraphelidium* has a complex metabolism resembling that of free-living chytrids but feeds by phagotrophy as free-living nucleariids and holozoan protists (Figure 3). This suggests that aphelids are 'borderline' parasites (or parasitoids, according to some definitions) that have not undergone the reduction process that characterizes *Rozella* and all members along the microsporidian branch [6, 8, 9]. Being more gene-rich and close to the root, aphelids may have retained more ancestral features. In this evolutionary scenario, the opisthosporidian ancestor was free-living-like in terms of metabolism and had a complex life cycle including chitin-containing resting cysts, amoeboflagellate zoospores and a phagotrophic amoeba stage possibly

specialized in endobiotic predation (Figure 4). By contrast, the fungal ancestor was a free-living osmotroph that had amoeboflagellate zoospores and chitin in the vegetative stage. From their aphelid-like opisthosporidian ancestor, the fungal lineage lost phagotrophy, acquiring its ecologically successful osmotrophy whereas, on the rozellid line, opisthosporidians shifted from endobiotic phagotrophic predation into obligate parasitism with more complex life cycles. The acquisition of additional genomes/transcriptomes for other aphelids, rozellids and deep-branching fungi should help establish a solid phylogenomic framework to validate and refine this evolutionary scenario

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures S1-S4, four tables S1 to S4, one video and one supplementary file (all_trees.txt).

AUTHOR CONTRIBUTIONS

G.T., D.M. and P.L-G. conceived and coordinated the study, and wrote the manuscript. G.T. performed culture cleaning, RNA extraction, *de novo* transcriptome assembly, phylogenomics and comparative genomic analyses. G.T. and X. G-B. cleaned the protein set from contamination. X. G-B. and A. S-BP. performed myosin comparative genomic analyses. X. G-B. carried out multivariate statistical analyses of metabolic genes. G.T. and J.A.B. carried out phagolysosome protein analyses. G.T. and S.K. maintained cultures and performed WGA staining and imaging. S.K. studied morphology and life-cycle aspects, E.V. performed SEM fixation and imaging. All authors commented on the manuscript.

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Figure legends

Figure 1. Phylogenomic Analyses and Cell Cycle of the Aphelid *Paraphelidium tribonemae*

(A) Bayesian phylogenetic tree based on single-copy protein domains for 49 species (SCPD49) inferred using a CAT-Poisson model. Statistical supports indicated at some crucial nodes correspond, from left to right, to PhyloBayes Bayesian posterior probabilities and IQ-TREE ML ultrafast-bootstrap support using the C60 model. Branches with maximum support values (BPP = 1 and UFBS = 100%) are indicated by black circles. The support for the monophyly of *Paraphelidium* and Fungi using other datasets (BMC [19], GBE [8]) and removing the fastest-evolving microsporidian sequences (36 instead of 49 species) is shown at the corresponding node. (B) Schematic cell cycle of *P. tribonemae*. Briefly, infecting cysts (red wall), deliver an amoeboid trophont to an algal filament cell via an infection tube; the trophont engulfs the algal cytoplasm by phagocytosis, leaving a growing residual body (dark red particle); after nuclear and cell division, a mature sporangium releases amoeboflagellated zoospores (occasionally seen as amoeboid only) that get out the algal cell wall and close the life cycle [2]. (C) Evolution of IQ-TREE ML UFBS support for the sisterhood of aphelids and Fungi (A+F) and the monophyly of Opisthosporidia (O) as a function of the proportion of fast-evolving sites removed from the dataset. All the phylogenomic trees can be seen in Figure S1.

Figure 2. Zoospores and Chitin-bearing Infective Cysts of *Paraphelidium tribonemae*

(A) Filament of *Tribonema gayanum* infected by *P. tribonemae* seen under optical microscopy and (B) the same filament stained with fluorescent wheat germ agglutinin (WGA) showing the presence of chitin in infective cysts under epifluorescence microscopy. (C) False-colored scanning-electron microscopy image of a filament infected by several *P. tribonemae* cysts (pedunculated rounded structures). The algal host filament is colored in green and parasite cysts in pink. Note that one cyst germ tube is penetrating the host cell by a cell-wall juncture (white arrowhead) and two cysts are broken (black arrowheads), showing the penetration channel. (D) Amoeboid zoospore (infrequent). (E) Amoeboflagellated zoospore. Scale bar: A = 5 µm, B-F = 1 µm. Phylogenetic trees related to chitin and other cell-wall synthesis and degradation-related enzymes are shown in Figure S2. Zoospore motility can be seen in Video 1.

Figure 3. Complexity of *Paraphelidum tribonemae* Metabolism and Cytoskeleton-Trafficking-Phagotrophy-related Proteome

(A) Principal Coordinate Analysis (PCoA) and (B) binary heat-map and species clustering based on the presence/absence of 1172 orthologous genes (OGs) belonging to 8 primary metabolism eggNOG categories across 41 eukaryotic genomes/transcriptomes (see Supplementary Methods). (C) PCoA and (D) binary heat-map and species clustering based on the presence/absence of 695 KEGG orthologs (OGs) related to cytoskeleton, membrane-trafficking and phagotrophy, which were selected from 11 KEGG categories (see Supplementary Methods). Species are color-coded according to their taxonomic assignment; particular members within Opisthosporidia are highlighted. See also Figures S3-S4 and Tables S3-S4.

Figure 4. Early Evolution of Fungi and Opisthosporidia

Schematic representation of evolutionary relationships between Fungi and Opisthosporidia, within the holomycotan branch of Opisthokonts. Inferred key ancestral features and life cycle stages are depicted at the corresponding ancestral nodes.

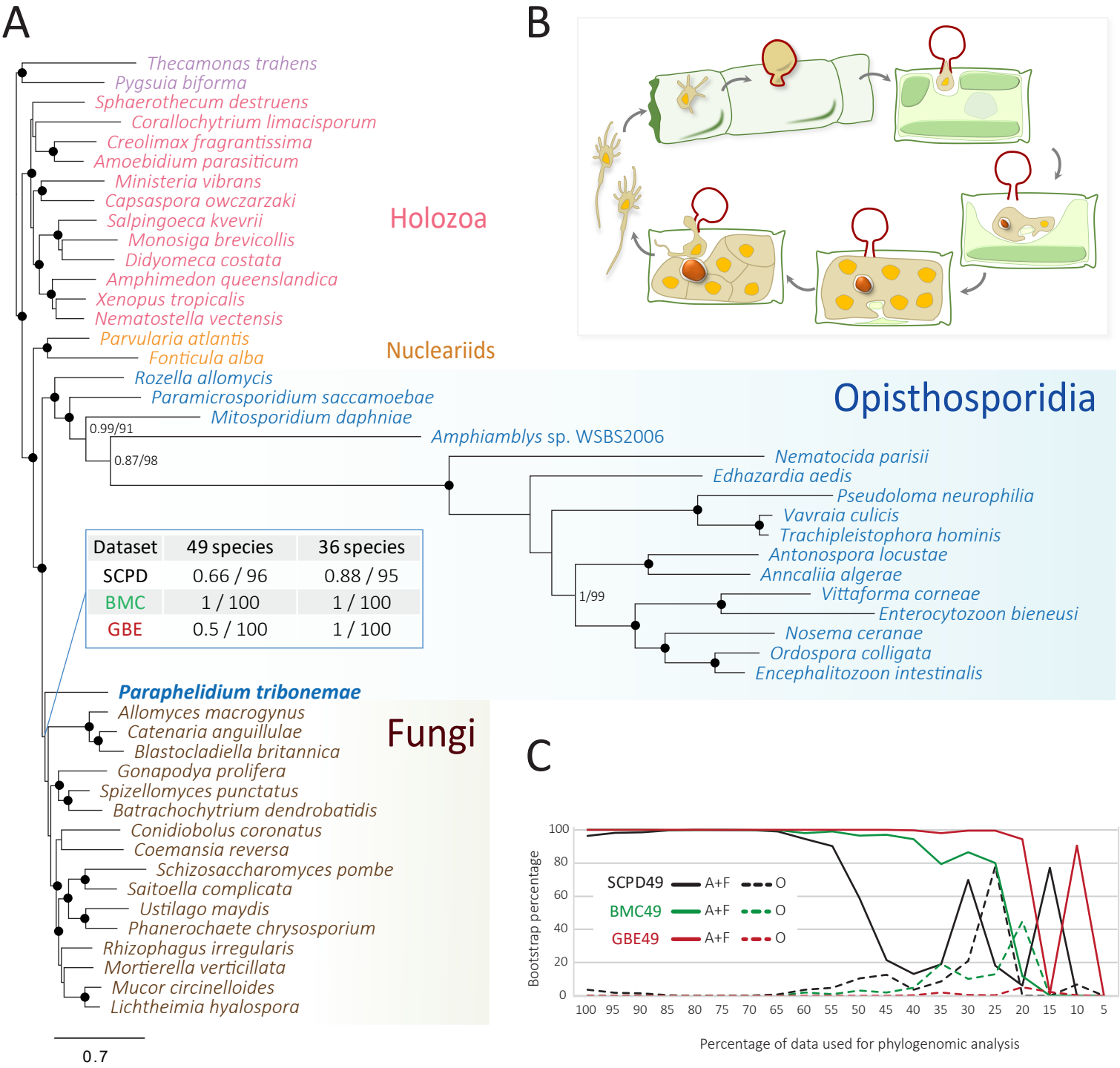
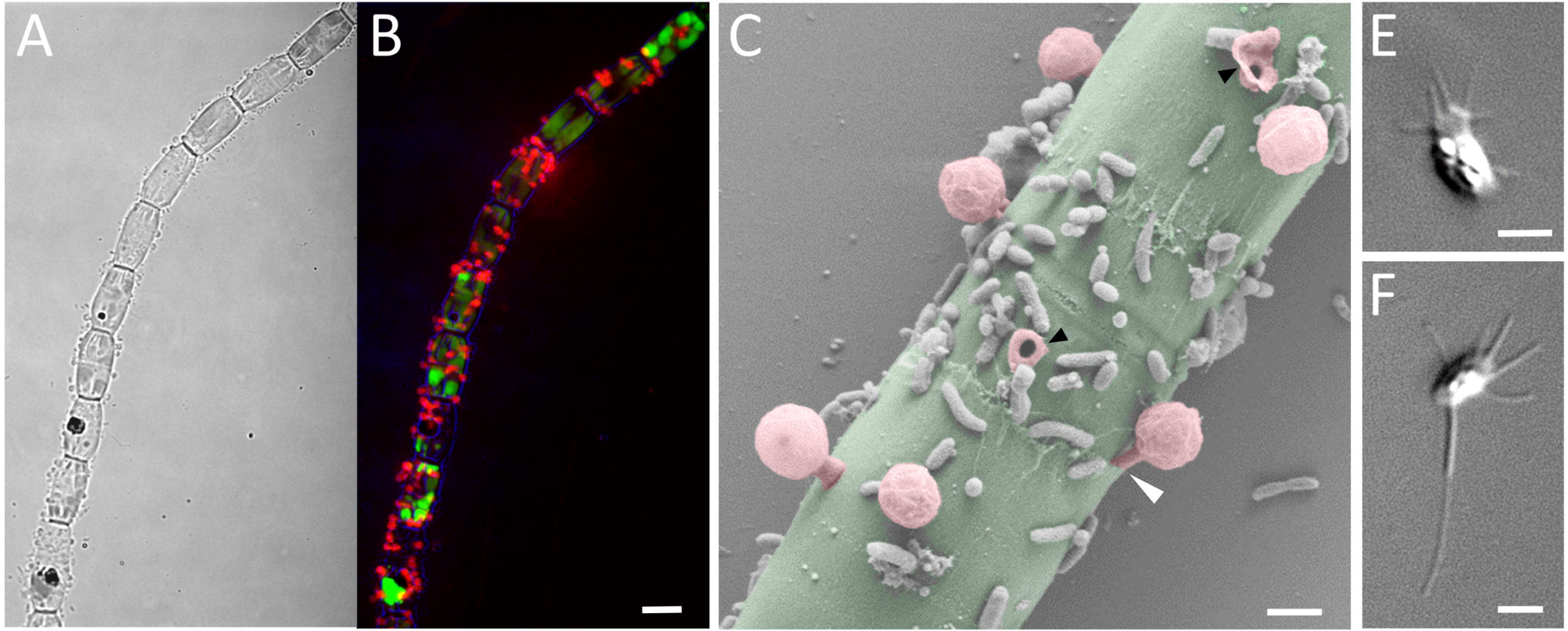


Figure 1. Torruella *et al.*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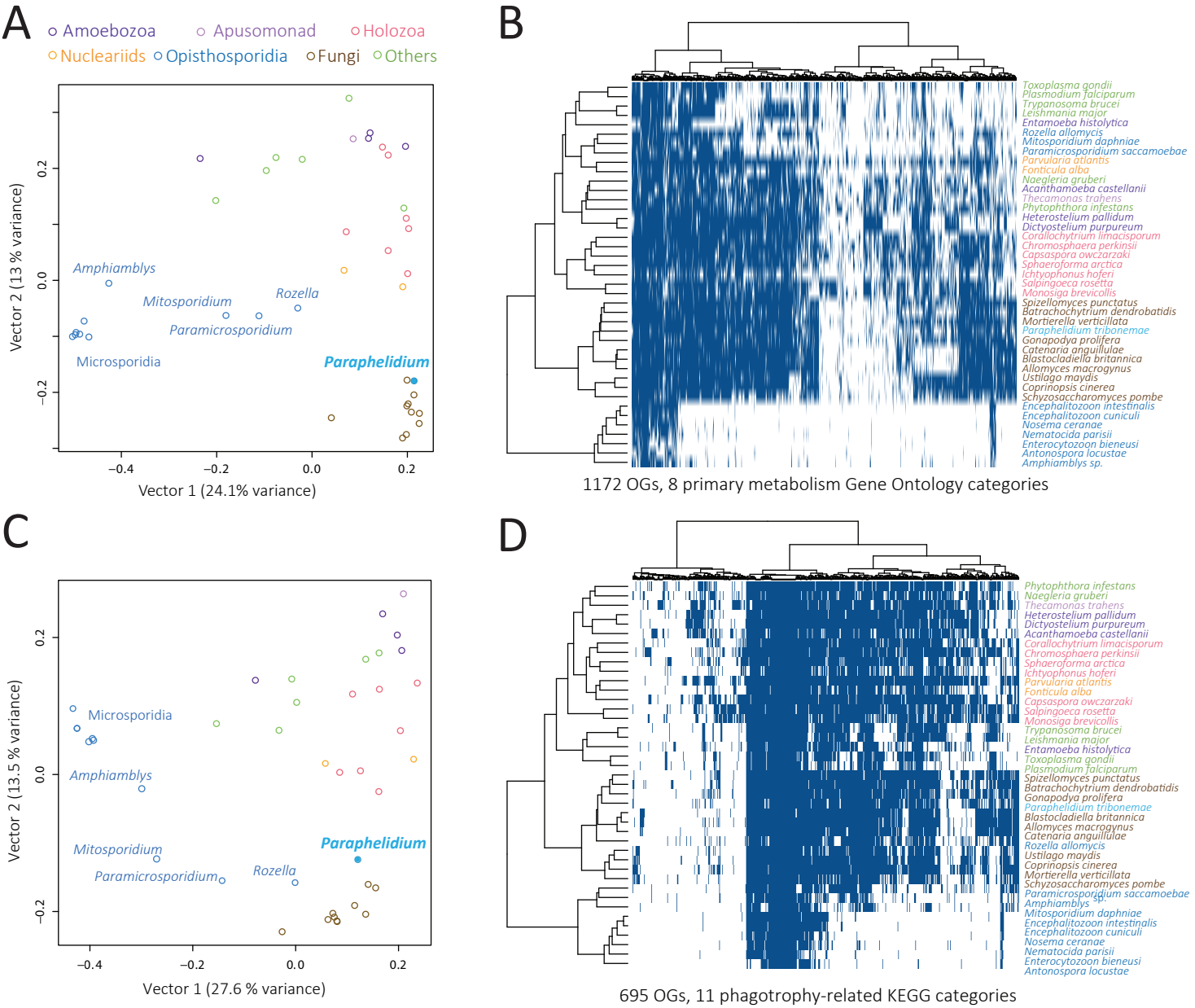
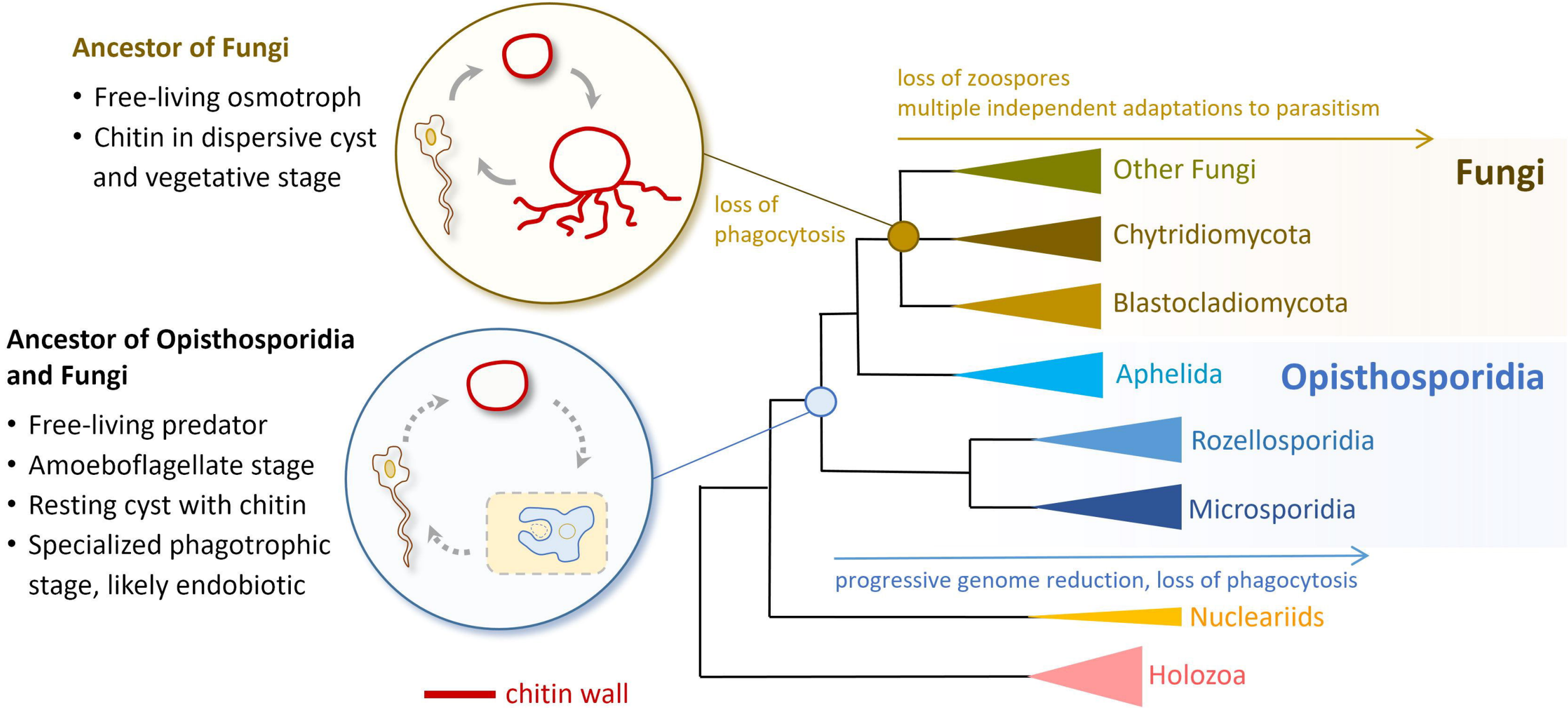


Figure 3. Torruella et al.


Ancestor of Fungi
• Free-living osmotroph
• Chitin in dispersive cyst and vegetative stage
Ancestor of Opisthosporidia and Fungi
• Free-living predator
• Amoeboflagellate stage
• Resting cyst with chitin
• Specialized phagotrophic stage, likely endobiotic
chitin wall
loss of phagocytosis
loss of zoospores
multiple independent adaptations to parasitism
Other Fungi
Chytridiomycota
Blastocladiomycota
Fungi
Aphelida
Opisthosporidia
Rozellosporidia
Microsporidia
progressive genome reduction, loss of phagocytosis
Nucleariids
Holozoa