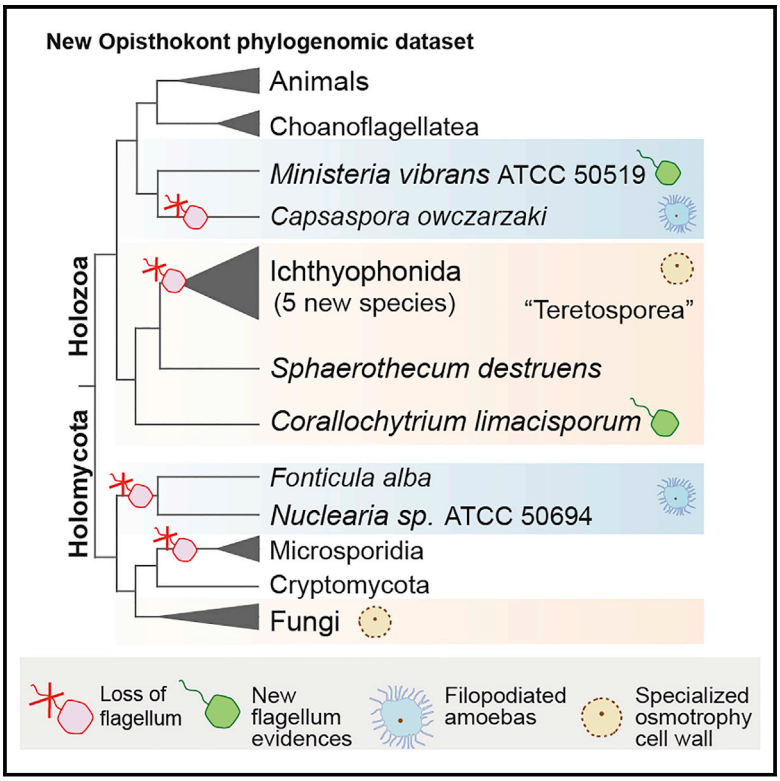
Report

Current Biology

Phylogenomics Reveals Convergent Evolution of Lifestyles in Close Relatives of Animals and Fungi

Graphical Abstract



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In Brief

Torruella et al. provide new molecular data from several protists and infer a novel phylogenomic framework for the opisthokonts that suggests rampant convergent evolution of several characters. Using comparative genomics, the authors show independent losses of the flagellum and delineate the evolutionary history of chitin synthases in this lineage.

Highlights

- Taxon-rich phylogenomics provides an evolutionary framework for the opisthokonts
- Specialized osmotrophy evolved independently in fungi and animal relatives
- Opisthokonts underwent independent secondary losses of the flagellum
- The last opisthokont common ancestor had a complex repertoire of chitin synthases

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Phylogenomics Reveals Convergent Evolution of Lifestyles in Close Relatives of Animals and Fungi

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SUMMARY

The Opisthokonta are a eukaryotic supergroup divided in two main lineages: animals and related protistan taxa, and fungi and their allies [1, 2]. There is a great diversity of lifestyles and morphologies among unicellular opisthokonts, from free-living phagotrophic flagellated bacterivores and filopodiated amoebas to cell-walled osmotrophic parasites and saprotrophs. However, these characteristics do not group into monophyletic assemblages, suggesting rampant convergent evolution within Opisthokonta. To test this hypothesis, we assembled a new phylogenomic dataset via sequencing 12 new strains of protists. Phylogenetic relationships among opisthokonts revealed independent origins of filopodiated amoebas in two lineages, one related to fungi and the other to animals. Moreover, we observed that specialized osmotrophic lifestyles evolved independently in fungi and protistan relatives of animals, indicating convergent evolution. We therefore analyzed the evolution of two key fungal characters in Opisthokonta, the flagellum and chitin synthases. Comparative analyses of the flagellar toolkit showed a previously unnoticed flagellar apparatus in two close relatives of animals, the filasterean *Ministeria vibrans* and *Corallochytrium limacisporum*. This implies that at least four different opisthokont lineages secondarily underwent flagellar simplification. Analysis of the evolutionary history of chitin synthases revealed significant expansions in both animals and fungi, and also in the Ichthyosporea and *C. limacisporum*, a group of cell-walled animal relatives. This indicates that the last opisthokont common ancestor had a complex toolkit of chitin synthases that was differentially retained in extant lineages. Thus, our data provide evidence for convergent evolution of specialized lifestyles in close relatives of animals and fungi from a generalist ancestor.

RESULTS AND DISCUSSION

Broad Taxonomic Sampling Provides New Phylogenetic Insights into the Evolution of the Opisthokonta

Previous attempts to solve opisthokont phylogeny swayed between species-rich datasets with poor deep-node resolution based on small ribosomal subunit [1–3] and multigene supermatrices that included few taxa [4–6]. To improve upon our previously published phylogenomic dataset [6], we therefore sampled representative species in all described opisthokont lineages (see Table S1 and Supplemental Experimental Procedures). This included representatives of nucleariids, choanoflagellates, filastereans, and the two main lineages of Ichthyosporea (Dermocystidia and Ichthyophonida). In addition, we included two different strains of the enigmatic *Corallochytrium limacisporum*, a spherical free-living walled saprotroph found in coral reefs [7]. Originally classified as a thraustochytrid based on its morphology,

C. limacisporum has been unstably placed within the Opisthokonta in all molecular phylogenies to date because of the scarce molecular data available [8–11]. In order to improve the opisthokont outgroup, we also sampled the ancyromonad *Nutomonas longa* CCAP 1958/5 [12], which is putatively related to Apusomonadida [11]. Overall, we generated new transcriptomic data for 10 protistan taxa (11 strains in total, highlighted in bold in Figure 1), plus new genomic data from another strain (*Ichthyophonus hoferi*). This represents the broadest taxon sampling to date to infer the opisthokont phylogeny.

To investigate the phylogenetic relationships, we assembled two datasets comprising a total of 93 single-copy protein domains: one with 83 taxa and 18,218 aligned amino acid positions (S83), and the other with 70 taxa and 22,313 amino acid positions (S70). The latter dataset was constructed to maximize alignment length and to minimize topological artifacts by excluding putative problematic taxa with long branches (e.g., Microsporidia, Excavata) and high percentages of missing data (e.g., taxa with only expressed sequence tag data) (see Table S1). Both datasets were consistent in recovering the backbone of the eukaryotic phylogeny using both Bayesian inference (BI) (Figures 1 and S1C) and maximum likelihood (ML) (Figures S1A and S1B; see Supplemental Experimental Procedures for details).

As sister groups to Opisthokonta, we recovered Apusomonadida and Breviatea as recently reported [13], branching as independent lineages and not forming a monophyletic group or clustering with amoebozoans. Interestingly, the topology of the S83 dataset placed *Nutomonas longa* (Ancyromonadida) branching closer to the Excavata and not closely related to the Apusomonadida and Opisthokonta. This contrasts with previous analyses [11, 12] but is consistent with recent results based on multiple markers [14]. Within the Holomycota (which includes fungi and their protistan relatives), we recovered a clade formed by *Nuclearia* sp. and *Fonticula alba* (Discicristoidea) as the earliest-branching lineage [15]. This was followed by *Rozella allomycis* and Microsporidia [16] and the paraphyletic assemblage of Chytridiomycota (including Neocallimastigomycota) and Blastocladiomycota [17]. Finally, within the Holozoa we recovered Filasterea as the sister group to the clade formed by the Metazoa and Choanoflagellatea, as previously reported [5, 6].

Interestingly, we recovered *C. limacisporum* as a sister group to Ichthyosporea (including the two major groups Ichthyophonida and Dermocystida) [18] with both ML and BI methods. The S83 dataset recovered this position for *C. limacisporum* with weak support (56% ML bootstrap support [bs] and 0.8 BI posterior probability [pp]). However, support for this branch increased significantly (bs = 80%, pp = 0.84) when the long-branch taxa were excluded (see Figure 1 and Table S2). The position of the dermocystid *Sphaerothecum destruens* as sister group to the rest of ichthyosporeans was only moderately supported (S83: bs = 60%, pp = 0.97; S70: bs = 61%, pp = 0.87) but was consistently recovered in all analyses. Thus, the monophyletic group comprising Ichthyosporea and *C. limacisporum* appears to be the earliest-branching lineage in the Holozoa. We tentatively name this novel group "Teretosporea," meaning "rounded spores," through this study.

C. limacisporum is the only known free-living osmotroph in the Holozoa, whereas the ichthyosporeans thus far described are known to be associated with animal hosts as parasites or commensals [18], despite being frequently found in environmental surveys [3]. The life cycles of *C. limacisporum* and Ichthyosporeans [7, 18] are strikingly similar: both start as a single cell that grows as a coenocyte until it reaches maturation, when it undergoes schizogony. The dispersive amoeboid or flagellated progeny (merozoites) settle and close the cycle [18]. Chytrid fungi show a similar developmental mode, with both coenocytic growth and amoeboid or flagellated stages [19]. Similarly, fungi also evolved from phagotrophic ancestors (Discicristoidea, *Rozella*, and Aphelida [20]) to become saprotrophs and parasites. Moreover, some Ichthyosporea species (*A. parasiticum* and *I. hoferi*) present a mode of polar growth that clearly resembles fungal hyphae [21]. Thus, teretosporeans and fungi present tantalizing similarities regarding life style adaptations and morphologies.

The resulting opisthokont tree also confirms the convergent evolution of filose amoebas, Filasterea within the Holozoa and Discicristoidea within the Holomycota. Both lineages have evolved a similar cell morphology comprising long, actin-based filopodia [22], with some taxa going through an aggregative multicellular cell stage in their life cycles [23].

Independent Loss of the Flagellum within the Opisthokonta

A single posterior motile flagellum is a defining character of opisthokonts [2]. Our observation that both filose amoebas and fungal-like lineages evolved in independent branches within opisthokonts therefore predicts independent loss of the flagellum. To address this hypothesis, we analyzed the evolution of the flagellar toolkit [24, 25]. The molecules that comprise the flagellum include specialized tubulins (*epsilon*, *delta*) [26], the intraflagellar transport system (i.e., the IFT-A, IFT-B, and BBSome complexes [27]), and some motor molecules, mainly specialized subfamilies of dyneins and kinesins [24, 28] (Figure 2B). Large-scale genomic analyses have shown that the presence of these genes in a given genome correlates with the presence of a flagellum—revealing, in some cases, a previously unseen flagellar stage [28].

To clarify the evolution of the flagellum, we sought orthologs of a set of over 60 flagellum-specific proteins [24, 27, 28] in our taxon sampling (see Supplemental Experimental Procedures and Table S3). As expected, non-flagellated lineages such as Dikarya fungi, Discicristoidea, Ichthyophonida, and the filasterean *Capsaspora owczarzaki* yielded no significant hits (Figure 2A). This confirmed the recurrent secondary loss of the flagellum in at least four opisthokont lineages. In contrast, we found several proteins corresponding to key flagellar molecular components in the transcriptome of two taxa assumed not to be flagellated, the filasterean *M. vibrans* and the teretosporean *C. limacisporum*.

M. vibrans was originally described as a filose amoeba suspended in the water column by a stalk attached to the substrate. The stalk resembled a modified flagellum based on transmission electron microscopy (TEM) observations, which included structures resembling, according to the authors, doublet microtubules [2]. Interestingly, we observed the presence of axonemal dyneins, *epsilon* tubulin, and IFT-A/B complexes, clearly suggesting the presence of a flagellum in this species. Therefore, we tested whether the stalk is a modified flagellum by tubulin immunostaining on the original ATCC 50519 strain (see Supplemental Experimental Procedures). Confocal microscopy

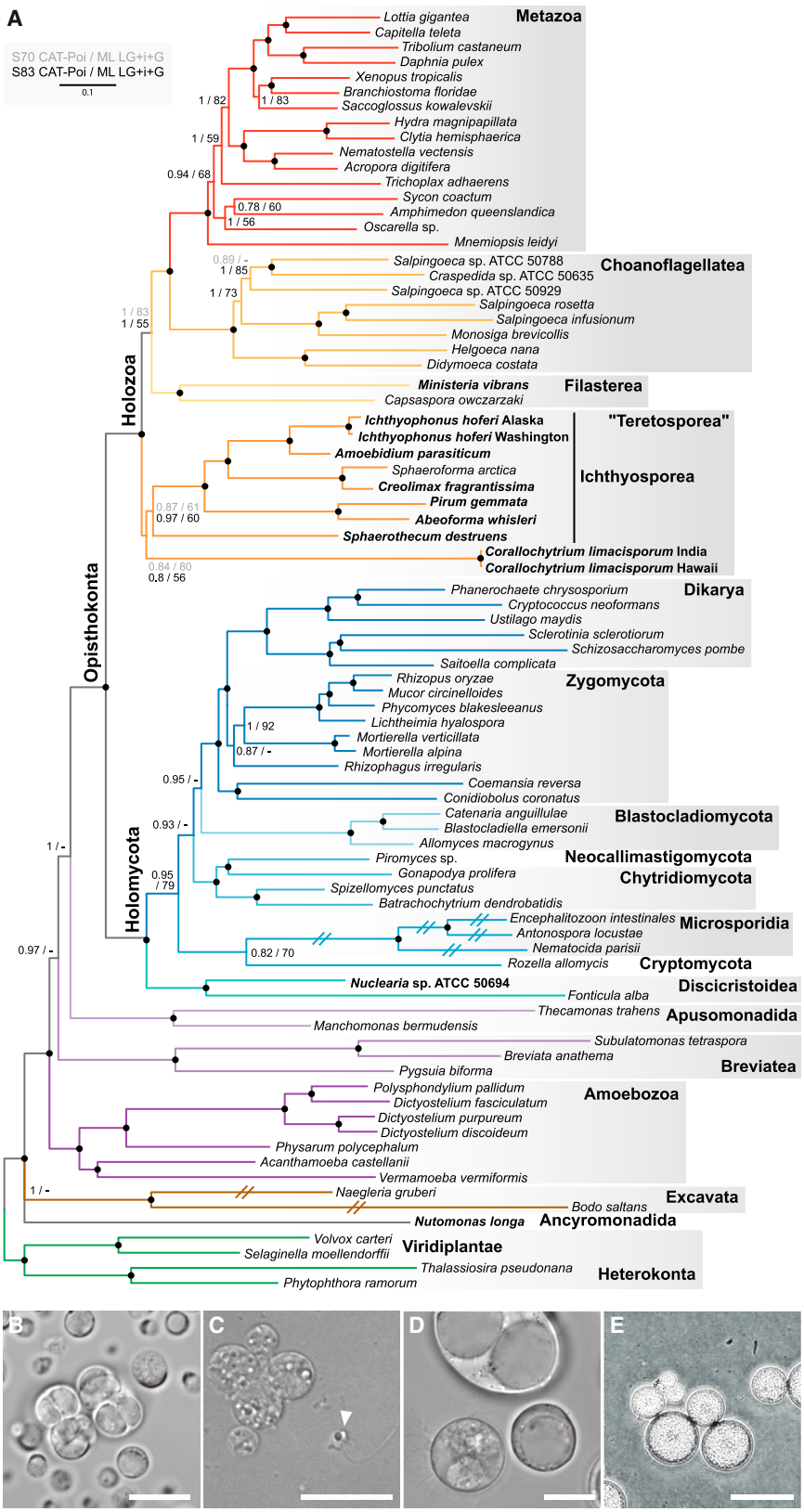

Figure 1. Phylogeny and Cell Biology of Opisthokonts

(A) Phylogenetic tree based on the 83-taxa matrix (see Tables S1 and S2 and Supplemental Experimental Procedures) and inferred by PhyloBayes under the CAT-Poisson model. Tree topology is the consensus of two Markov chain Monte Carlo chains run for 1,500 generations, saving every ten trees and after a burn-in of 25%. Split supports are posterior probabilities (pp) and nonparametric maximum likelihood (ML) bootstrap (bs) values obtained from 200 ML replicates using the LG+I+G model implemented in RAxML. Support values > 0.95 pp and > 95% bs are indicated by a bullet (•). The taxa sampled in this study are indicated in bold. For raw trees, see Figure S1.

(B–E) Light micrographs showing the coenocytic stage of representative species of the tentatively named "Terestosporea" (*Corallochytrium* + Ichthyosporea) sequenced in this study, including *Corallochytrium limacisporum* (B), *Sphaerothecum destruens* (C; arrowhead indicates flagellated zoospore), *Abeoforma whisleri* (D), and *Ichthyophonus hoferi* (E). Scale bar represents 10 μm in (B)–(D) and 100 μm in (E).

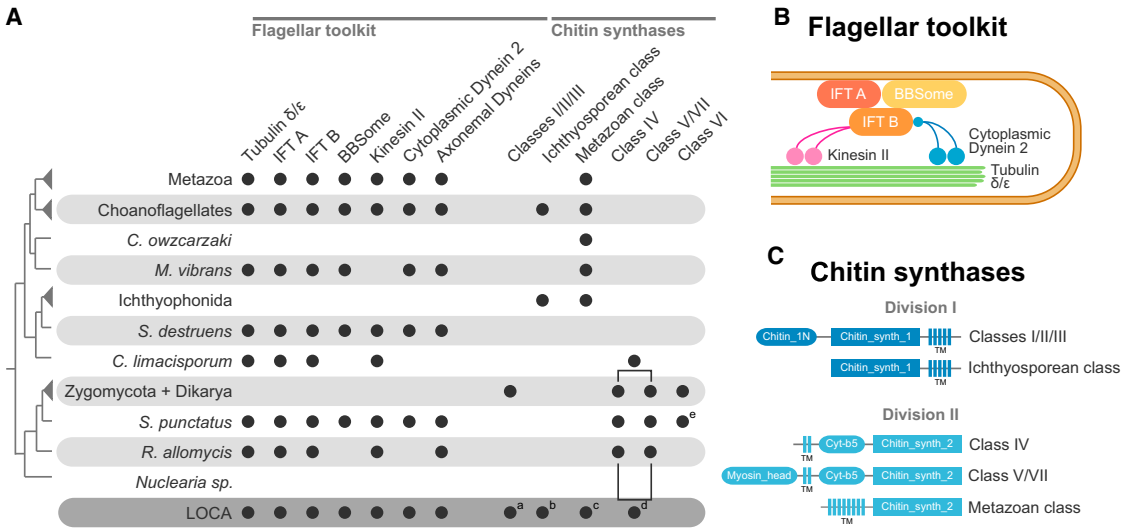

Figure 2. Multiple Independent Losses of the Flagellar Toolkit and CHS Genes in Opisthokonta

(A) Presence versus absence of key molecular components of the flagellar apparatus and chitin synthases (CHS) in distinct Opisthokonta lineages and taxa. [a]present in oomycetes, *Chlorella variabilis*, and *Paramecium tetraurelia*; [b]present in *Acanthamoeba castellanii*; [c]present in *Entamoeba histolytica* and *Thecamonas trahens*; [d]present in *Thalassiosira pseudonana*; [e]present in the chytrid *Batrachochytrium dendrobatidis*.

(B) Components of the flagellar apparatus and names of the molecular complexes. Adapted from [24]. See flagellar gene distribution in Table S3.

(C) Main chitin synthase classes and their canonical protein domain architectures (see CHS phylogeny in Figure S2).

revealed a tubulin protrusion branching from the cell body, which was specifically stained with α-tubulin (Figure 3A) and acetylated tubulin antibodies (Figures 3B and S3). Moreover, our own TEM observations revealed a putative dense basal body and a flagellar section with nine outer ring structures and central microtubules (Figure 3C). Our transcriptomic data and experimental analysis thus revealed a flagellar structure in *M. vibrans*. Consequently, the ancestral filasterean must have had a flagellum, which was secondarily lost from *C. owczarzaki*.

The transcriptome of *C. limacisporum* was found to contain *delta/epsilon* tubulins, IFT-A and IFT-B components, and the retrograde motor kinesin-II (Figure 2A). Although this organism does possess an ortholog of HEATR2 recently linked to motile cilia [29], we did not find evidence of flagellar motility components, such as cytoplasmic dynein 2 or any of the axonemal dyneins (heavy, light, and intermediate chains; Table S3). Consistent with the original description of *C. limacisporum* [7], we did not observe a flagellum using light and TEM microscopy, at least under the culturing conditions employed. Therefore, our data suggest that *C. limacisporum* has a cryptic flagellated stage in its life cycle, as has been inferred for other eukaryotes (i.e., *Aureococcus* and *Ostreococcus*) based on their genome sequences [28]. Consequently, within the Teretosporea, a flagellated stage would be a feature shared by *C. limacisporum* and Dermocystida that was secondarily lost from the Ichthyophonida (Figure 4). This confirms the recurrent loss of the flagellum in both filose amoeboid lineages (Discicristoidea and Filasterea) and specialized osmotrophic lineages (Fungi and Teretosporea).

At Least Four Chitin Synthases in the Last Opisthokonta Common Ancestor

Given the apparent similarities in the evolution of the Fungi and Teretosporea, we investigated the evolutionary history of another feature of fungal evolution, the cell wall. Chitin is a key biopolymer present in some fungal cell walls and animal cuticles [30], synthesized by chitin synthases (CHS), a large and complex multigene family. Several CHS classes have been described in fungi (classes I/II/III from division I and classes IV/V/VI/VII from division II) [31], with three ancestral classes known in animals [32]. Some fungal CHS classes are held as molecular synapomorphies of fungi (classes IV/V/VI/VII from division II), as they have been found exclusively in the genomes of fungi, including *R. allomycis* and microsporidian genomes [33]. Moreover, CHS homologs with uncertain classification have been found in other eukaryotes, including the oomycete *Saprolegnia monoica* [34], diatoms [35], and unicellular holozoans [18, 36].

To investigate which CHS classes are present in Teretosporea and to clarify their phylogenetic relationships with those in fungi and animals, we gathered CHS sequences from all eukaryotic supergroups and built a tree based on the chitin synthase domain (see Supplemental Experimental Procedures and Figure S2). This revealed three genes in *C. limacisporum* that belong to division II CHS and branch within the clade that comprises fungal classes IV/V/VII. These sequences consistently present the canonical functional motifs of fungal sequences (see Table S4). Interestingly, two of the genes encode an N-terminal myosin head domain, resembling genes from fungal classes V/VII [36] (Figure 2C). The myosin head of *C. limacisporum* CHS is sister group to fungal V/VII CHS, forming the myosin class XVII [37]. We thus propose that the CHS class IV/V/VII containing a myosin domain is an ancestral state in the Opisthokonta.

We also found that the Ichthyophonida contain CHS from both division I and division II clades. Ichthyophonida homologs from division I form a new clade with various eukaryotic sequences, including diatoms, choanoflagellates, and amoebozoans (Figures 2A and S2), revealing it also to be an ancestral class in

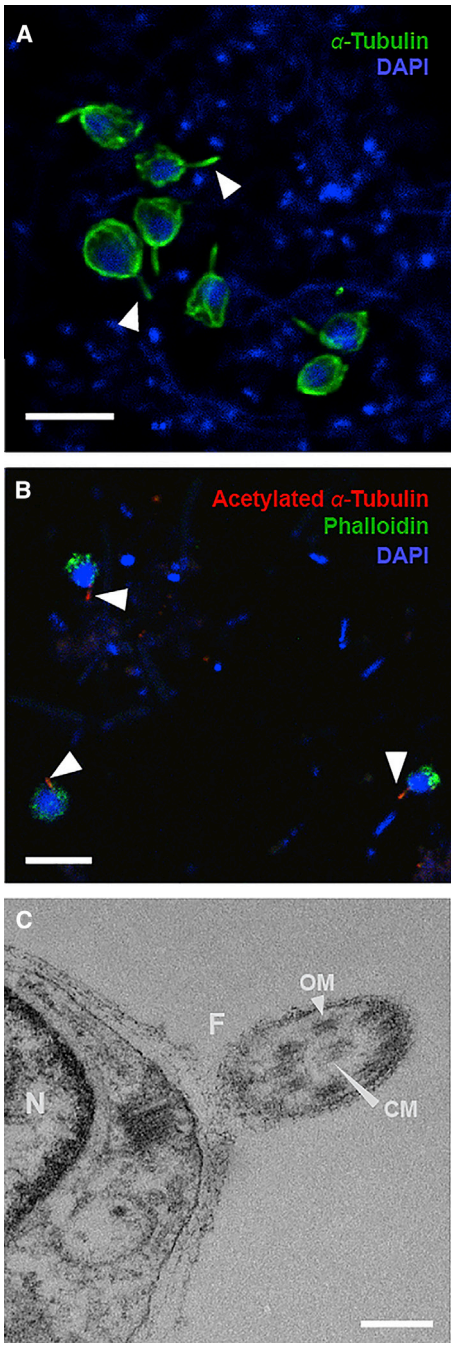

Figure 3. Confocal and Electron Microscopy of *Ministeria vibrans* Flagellum

(A and B) Confocal microscopy showing *Ministeria vibrans* ATCC 50519 stained with DAPI (blue) and anti-α-tubulin antibody 12G10 (Developmental Studies Hybridoma Bank) (green) (A) or with DAPI (blue), anti-acetylated-tubulin antibody T7451 (Sigma) (red), and phalloidin (green) (B). Arrowheads indicate the flagellar structure. Whereas the flagellar structure is specifically stained with cilia marker (acetylated tubulin) in (B), the cytoplasmic tubulin cytoskeleton is stained only with general anti-tubulin antibody in (A). *M. vibrans* feeds on bacteria, seen here as DAPI-stained bodies outside the cell. Scale bar represents 5 μm. See also Figure S3.

(C) TEM micrograph showing a transverse section of the flagellar structure of *M. vibrans*. N, nucleus; F, flagellar structure; OM, outer microtubules; CM, central microtubules. Scale bar represents 200 nm.

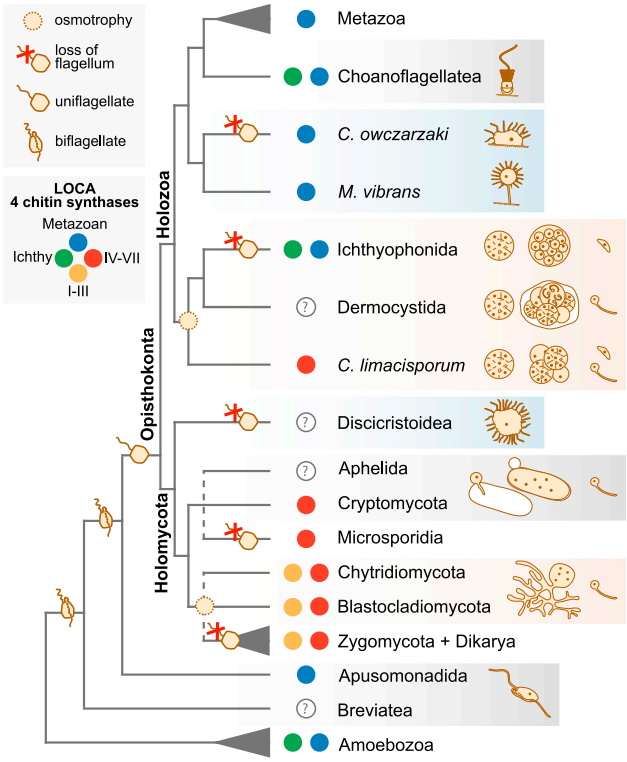

Figure 4. Evolution of Lifestyles and Some Cell Features of the Opisthokonts

Opisthokonta cladogram displaying lifestyle characteristics such as feeding mode, flagellated stage, CHS repertoire, and developmental mode (see Figure S4 for wheat germ agglutinin [WGA] staining) and ancestral state reconstruction of the last opisthokont common ancestor (LOCA). Choanoflagellate image is adapted from http://www.dayel.com/ (CC BY-SA 3.0).

the eukaryotes. Ichthyosporean division II CHS homologs belong to the Metazoan class, which is also present in other unicellular holozoans, apusomonads, and amoebozoans but is secondarily lost in fungi. Finally, fungal class I/II/III is found in several bikonts, including oomycetes and chlorophytes, suggesting an ancestral origin and secondary loss from the Holozoa. In summary, at least four ancestral paralogs of structurally different CHS (Figure 2C) were found in the last opisthokont common ancestor (LOCA), and secondary loss appears to have been common in descendant lineages (Figure 4). The presence of a complex CHS repertoire in the ancestor of all Opisthokonta, and the retention of rich CHS repertoires in the cell-walled lineages, suggests that the presence of chitin in the cell wall was an ancestral feature and not a fungal synapomorphy [33]. Consistent with this suggestion, Ichthyosporeans encoding a complex CHS repertoire showed chitin staining in the cell wall (Figure S4), and therefore only CHS VI class and the diversification of CHS IV/V/VII class into paralogous groups could be still considered fungal molecular synapomorphies.

A New Phylogenetic Framework for the Opisthokonts

By obtaining the transcriptomes of 10 new protist taxa (11 strains), plus the genome of an additional strain (12 strains in total), we have improved the previously biased representation

of genomic information for unicellular Opisthokonta. This allowed us to reassess the phylogenetic relationships among the opisthokonts through an unprecedented gene- and taxon-rich approach. Our dataset, with few missing data (Table S1), includes representatives from all opisthokont lineages, providing a stronger phylogenetic framework for internal relationships. Our phylogenetic analyses reveal a new clade: [Ichthyosporea + *C. limacisporum*], which we tentatively call Teretosporea, and which represents the earliest holozoan divergence (Figure 1).

Our data reveal that convergent evolution explains similarities in the lifestyles of the Fungi and Teretosporea as well as in Filasterea and Discicristoidea (Figure 4). The ancestral LOCA was most likely a filopodiated and flagellated generalist bacterivore [38]. Consequently, the specialized osmotrophic feeding mode, cell wall, and transition from saprotrophic to parasitic lifestyles in Fungi and Teretosporea occurred independently. This is not rare in eukaryotes, since similar adaptations are also found in stramenopiles such as the oomycetes and the thraustochytrids [39, 40]. However, our data provide the first example of such a process occurring in a close relative of animals. Through analysis of secondary loss of the flagellum and differential retention of ancestral CHS paralogs in opisthokonts, we have also provided molecular evidence to explain these lifestyle adaptations. Therefore, this study provides a striking example of convergent evolution through differential retention of ancestral genomic characters in the unicellular relatives of animals and fungi.

ACCESSION NUMBERS

The accession numbers for new data reported in this study are NCBI Sequence Read Archive: SRS502375, SRS502376, SRS721318, SRS725979, SRS725801, SRS726091, SRS724896, SRS725006, SRX179384; and NCBI BioProject: PRJNA290639.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.07.053.

AUTHOR CONTRIBUTIONS

G.T., A.d.M., and I.R.-T. designed and coordinated the study. *C. limacisporum* isolation was performed by M.A.C. and S.D. *I. hoferi* genomic data was performed by K.M.N. and C.M.W. *S. destruens* RNA data was performed by R.P. Other strain cultivation and RNA extractions were performed by G.T., J.d.C., M.A., G.P.-C., and X.G.-B. Phylogenomics was performed by G.T., L.E., and A.J.R. Flagellum and CHS comparative genomics were performed by A.d.M. WGA staining was performed by A.d.M. and M.A. Immunostaining and TEM were performed by X.G.-B., M.A., A.S.-B., and G.T. Figures were assembled by X.G.-B., G.T., and A.d.M. G.T., A.d.M., and I.R.-T. wrote the manuscript. All authors commented on the manuscript.

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