ARTICLES

Reconstructing the early evolution of Fungi using a six-gene phylogeny

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The ancestors of fungi are believed to be simple aquatic forms with flagellated spores, similar to members of the extant phylum Chytridiomycota (chytrids). Current classifications assume that chytrids form an early-diverging clade within the kingdom Fungi and imply a single loss of the spore flagellum, leading to the diversification of terrestrial fungi. Here we develop phylogenetic hypotheses for Fungi using data from six gene regions and nearly 200 species. Our results indicate that there may have been at least four independent losses of the flagellum in the kingdom Fungi. These losses of swimming spores coincided with the evolution of new mechanisms of spore dispersal, such as aerial dispersal in mycelial groups and polar tube eversion in the microsporidia (unicellular forms that lack mitochondria). The enigmatic microsporidia seem to be derived from an endoparasitic chytrid ancestor similar to *Rozella allomycis*, on the earliest diverging branch of the fungal phylogenetic tree.

Fungi, Viridiplantae and Animalia are all large clades descended from unicellular, flagellated, aquatic forms that radiated extensively on land. For both plants and animals, biologists have developed unified hypotheses regarding the evolution of morphology and ecology from ancestral to highly derived traits. For example, among green plants, morphologically simple photosynthetic forms, such as unicellular green algae, gave rise to multicellular forms such as bryophytes, and were followed by a radiation of complex flowering forms with highly derived sexual mechanisms at the tips of the plant phylogeny[1,2]. Similarly, animals seem to have evolved increasingly complex tissue systems and development from a simple, flagellated, protist-like ancestor similar to extant Choanoflagellida[3].

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Currently, no accepted phylogenetic hypothesis exists for the evolution of form and nutritional mode for the earliest fungi. Traditional views of fungal phylogeny indicate that fungi with flagellated cells (Chytridiomycota) are the sister group of the remaining phyla of non-flagellated fungi (Zygomycota, Glomeromycota, Ascomycota and Basidiomycota), implying a single loss of the flagellum coincident with a shift to land. Key adaptations to the terrestrial habit in the fungi include the evolution of a filamentous growth form and the development of aerially dispersed spores. However, recent phylogenetic studies question the monophyly of the basal phyla Chytridiomycota and Zygomycota[4,5]. Resolving the phylogeny of the basal groups of the Fungi and their relationships to Ascomycota and Basidiomycota is necessary to understand the sequence of events leading to the colonization of land and the evolution of terrestrial ecosystems. Here we present a multilocus phylogeny of the kingdom Fungi, including representatives of all currently recognized phyla. This analysis provides a robust kingdom-level phylogeny and suggests that there were at least four independent losses of flagella during the early evolution of the Fungi.

We estimated the phylogeny of the Fungi using data from six gene regions: 18S rRNA, 28S rRNA, 5.8S rRNA, elongation factor 1-α (*EF1α*), and two RNA polymerase II subunits (*RPB1* and *RPB2*). Incongruence among gene regions was tested by maximum likelihood bootstrap (MLBS) analyses of each data partition. This strategy allowed us to identify potential contaminant sequences in addition to conflicting phylogenetic signal. Very little conflicting signal among genes was detected, allowing construction of one super-matrix combining the data for all six gene regions for 199 fungal taxa, 29 of which used data from genome sequencing projects (Supplementary Notes 1). Only 6% of the cells in the super-matrix were missing data, and the number of aligned nucleotides was 6,436. The data were analysed by bayesian methods using a heterogeneous amino-acid and nucleotide model (see Supplementary Notes 2 for a nucleotide-only analysis). Support was estimated at nodes by bayesian posterior probabilities (BPP), MLBS and analysis of individual gene partitions (Supplementary Notes 3).

Chytridiomycota is not monophyletic

The combined gene phylogeny of the Fungi supported monophyly of the Ascomycota, Basidiomycota and Glomeromycota (Fig. 1). The Ascomycota and Basidiomycota formed a clade of 'dikarya' (that is, fungi characterized by having a portion of their life cycle with paired nuclei). Phylogenetic analyses also supported, by BPP, a clade uniting the dikarya and Glomeromycota, in agreement with previously published 18S rRNA phylogenies[6,7]. The opisthokont clade (Fungi, Metazoa and Choanoflagellida) was also recovered, as has been reported in other studies[3,8,9]. Two unexpected results were the placements of the endoparasitic, spizellomycetalean chytrids *Olpidium brassicae* and *R. allomycis*. *Olpidium brassicae* grouped with the Zygomycota as sister taxon to *Basidiobolus ranarum*, and *R. allomycis* grouped with the microsporidia as the earliest diverging branch of the Fungi.

The phylum Chytridiomycota consists of true fungi that produce flagellated spores (zoospores). On the basis of ultrastructural studies, the chytrid zoospore is homologous to that of non-fungal opisthokonts[10]. The ultrastructural complexity of the opisthokont zoospore suggests that it has evolved only once. Because the zoospore is an ancestral trait, Chytridiomycota is solely defined on a shared ancestral trait (symplesiomorphy) rather than a shared derived trait (synapomorphy). Our phylogeny indicates that the Chytridiomycota is polyphyletic (Fig. 1), consisting of early diverging lineages that have retained the zoospore. However, one large clade of Chytridiomycota uniting the orders Chytridiales, Monoblepharidales, Neocallimastigales and some Spizellomycetales (which we call the 'euchytrids') is recovered with high support values in the combined analysis as well as in multiple, single-gene-based analyses (Fig. 1 and Supplementary Notes 3).

In the present phylogeny (Fig. 1), six losses of the flagellum are inferred to have occurred during the evolution of the Fungi. Ancestral state reconstruction of the presence or absence of the flagellum along the phylogeny for each of the 58,611 credible trees demonstrated 4–6 losses (mean 5.86) of the flagellum within the Fungi. One well-supported loss took place along the branch leading to *Hyaloraphidium curvatum*, a unique fungus that grows superficially like a unicellular planktonic alga[11]. A second loss occurred in the lineage leading to the microsporidia and 2–4 losses occurred among Zygomycota. Variation in the number of losses of the flagellum is attributable, in part, to the uncertain placement of *O. brassicae* and members of the microsporidia. Rearrangement of the phylogenetic position of *O. brassicae* and microsporidia can create phylogenies requiring only two or three losses of the flagellum; however, each of these alternative phylogenies is rejected as statistically worse (in likelihood; $P < 0.05$) than that shown in Fig. 1.

Most molecular phylogenies of the Fungi based on 18S rDNA have placed the zygomycete *Basidiobolus* among Chytridiomycota[4,12]. This placement indicated that *Basidiobolus* might have made the transition recently from a zoosporic state, and that an independent loss of a flagellum occurred in this lineage[12]. This argument was strengthened by the presence in two *Basidiobolus* species of a ring-shaped spindle pole body that contains 11–12 singlet microtubules similar to a centriole, but lacks centriolar ninefold symmetry[13]. Our phylogeny is the first to place *Basidiobolus* close to Entomophthorales, the order within which it has been classified traditionally and to which it is ecologically and morphologically allied[14] (for additional phylogenetic support from a paralogous copy of *EF1α,* see Supplementary Notes 4). Unexpectedly, the phylogeny also suggests a relationship between *B. ranarum* and the chytrid *O. brassicae* (Fig. 1). A functional link between the two taxa is unclear: *O. brassicae* is an endoparasite of plant roots, whereas *Basidiobolus* is associated with insects, soil and amphibians.

Phylogenetic position of the microsporidia

Microsporidia are obligately endoparasitic, protist-like organisms with highly reduced morphology and genomes[15]. A defining characteristic of these parasites is the elaborate mechanism by which the spore contents are rapidly injected into the host's cytoplasm through a thin polar tube. Placement of microsporidia in the tree of life has been problematic owing to their extremely accelerated rate of sequence evolution. The earliest phylogenetic analyses of 18S rRNA placed the microsporidia among the earliest diverging lineages of eukaryotes[15]; however, these analyses now seem to have been an artefact of 'long branch attraction' of microsporidia to the base of the phylogeny[15]. More recent results using *RPB1*, α- and β-tubulin, and other genes, have suggested a fungal origin of the microsporidia[16–18], a placement consistent with their having the shared traits of closed mitosis and spores that contain chitin and trehalose [19]. Only one study has placed the microsporidia with a specific fungal lineage, in which a relationship was demonstrated between members of the Zygomycota and microsporidia by using tubulin proteins[18]. However, tubulin proteins seem to have evolved at different rates in flagellated and non-flagellated fungi[18,20].

The microsporidia and *R. allomycis* are intracellular parasites of primarily animals and fungi, respectively. A similarity between microsporidia and *R. allomycis* is the absence of a cell wall when invading host cells, such that the plasma membrane of the parasite makes direct contact with the cytoplasm of the host cell[19,21]. Although *R. allomycis* does not seem to occupy a long phylogenetic branch, we tested whether the placement of microsporidia with *R. allomycis* was due to long branch attraction. Two different methods suggested that the relationship between microsporidia and *R. allomycis* is not due to long branch attraction (see Supplementary Notes 5). We also tested whether alternative placements for the microsporidia could be statistically rejected from the maximum likelihood phylogeny shown in Fig. 1 using the approximately unbiased test[22]. Alternative placements of microsporidia with Fungi that have been suggested

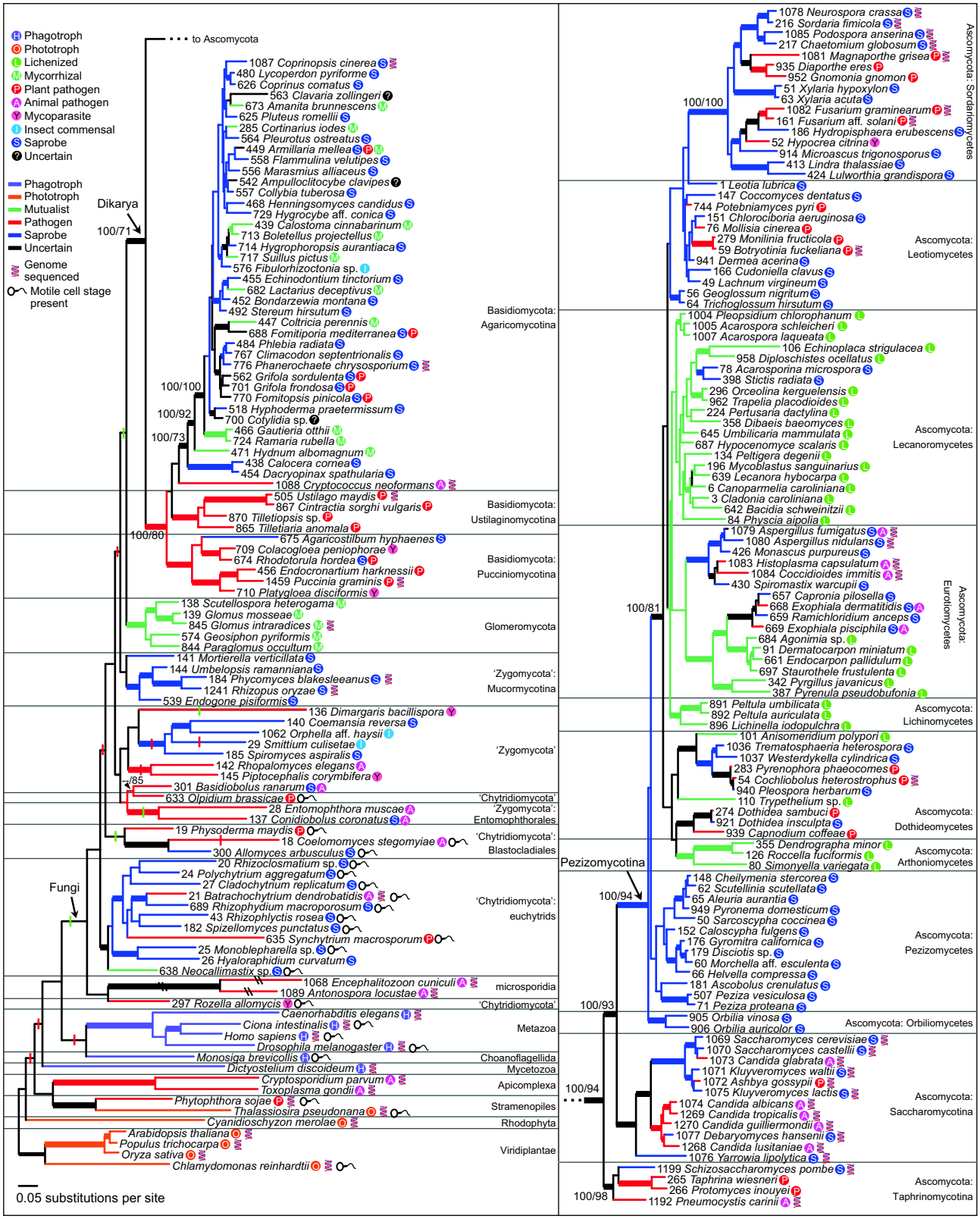

Figure 1 | Phylogeny of the kingdom Fungi using bayesian analysis of the combined, six-gene data set. Each fungal species begins with a unique 'Assembling the Fungal Tree of Life' identifier, followed by genus and species. Indicated for each terminal taxon are: nutritional mode, whether they produce flagellated cells and if there is a genome sequence for the taxon completed or underway. Thickened branches indicate those that are supported both by heterogeneous bayesian analysis (BPP ≥95%) and by MLBS (≥70%). Almost every branch was supported by BPP and thus values are not shown. Where indicated, support values (percentage of trees in agreement out of 58,611 trees) indicate BPP followed by MLBS. Branches are shaded according to reconstruction of nutritional mode. Microsporidia branches have been shortened three times (double black break) to increase readability. Red vertical ticks on branches indicate alternative placements of microsporidia that might be significantly rejected ($P < 0.05$) and green ticks indicate placements that cannot be rejected. Quotation marks indicate non-monophyly of the taxon. The name 'Mucormycotina' will be validated in a manuscript that is in preparation.

include: a sister relationship to the dikarya[23]; sister to the zygomycete order Entomophthorales[18]; and among the harpellid Trichomycetes[19], represented here by *Smittium culisetae*. We were able to reject ($P < 0.05$) nine alternative placements of the microsporidia (red vertical ticks in Fig. 1), including early divergences among eukaryotes. However, we were unable to reject a placement of microsporidia as sister to Entomophthorales, as sister to the blastocladialean chytrids, as sister to the zygomycete *Dimargaris*, as sister to dikarya and as sister to the Fungi (green vertical ticks in Fig. 1).

Taken together, our results suggest that the relationship between the microsporidia and *R. allomycis* is a result of true phylogenetic signal. The present phylogeny provides an alternative hypothesis for the placement of microsporidia, specifically on the earliest diverging fungal branch with the chytrid *R. allomycis*. However, support for this relationship is derived only from the *RPB1* and *RPB2* gene partitions and is not supported by rDNA (see Supplementary Notes 3); alternative hypotheses in which the microsporidia diverge among early fungi cannot be rejected. The ultimate resolution of the placement of microsporidia will require sampling of additional genes from basal fungal taxa.

Dikarya

The majority (~98%) of described fungal species are members of the dikarya clade, which includes the two phyla Ascomycota and Basidiomycota. Ascomycota is the largest phylum within the Fungi and is characterized by the production of meiospores (ascospores) in specialized sac-shaped meiosporangia (asci), which may or may not be produced within a sporocarp (ascoma). Ascomycota is divided into three monophyletic subphyla: Taphrinomycotina, Saccharomycotina and Pezizomycotina (each of which is well supported as monophyletic in the phylogeny; Fig. 1). Taphrinomycotina is resolved as the earliest diverging clade; it includes a diverse group of species that exhibit yeast-like (for example, *Pneumocystis*) and dimorphic—that is, yeast-like and filamentous (for example, *Taphrina*)—growth forms. The subphylum Saccharomycotina consists of the 'true yeasts', including bakers' yeast (*Saccharomyces cerevisiae*) and *Candida albicans*, the most frequently encountered fungal pathogen of humans. Pezizomycotina is the largest subphylum of Ascomycota and includes the vast majority of filamentous, fruit-body-producing species. Data presented here resolved the Orbiliomycetes and Pezizomycetes as the early-diverging lineages of the Pezizomycotina, with the remaining seven classes sampled forming a well-supported crown clade. Reduced ascomatal morphologies, whereby asci are contained within fruit bodies that are enclosed partially (Dothideomycetes, Eurotiomycetes and some Sordariomycetes) or completely (Eurotiomycetes, Leotiomycetes and some Sordariomycetes), are restricted to the crown clade of Pezizomycotina.

The Basidiomycota includes about 30,000 species of rusts, smuts, yeasts, and mushroom fungi[24]. Most are characterized by meiospores (basidiospores) on the exterior of typically club-shaped meiosporangia (basidia). Phylogenetic relationships among the three subphyla of Basidiomycota are uncertain. The subphylum Pucciniomycotina is primarily distinguished by containing the rust fungi (7,000 species), which are primarily pathogens of land plants. Cytological and biochemical data[25] are consistent with a sister group relationship between the subphyla Ustilaginomycotina and Agaricomycotina, as shown in Fig. 1. The Ustilaginomycotina includes 1,500 species of true smut fungi and yeasts, most of which cause systemic infections of angiosperm hosts. The Agaricomycotina includes almost two-thirds of known basidiomycetes, including the vast majority of mushroom-forming fungi. Much of the morphological diversity exemplified in mushroom fruiting bodies is the result of radiations of certain lineages within the Agaricomycotina, and recovering their relationships with confidence has proven difficult[26,27]. Early-diverging lineages in the Agaricomycotina, which are strongly supported in Fig. 1, also include parasitic and/or saprotrophic fungi capable of dimorphism or yeast-like phases. The mycorrhizal basidiomycetes seem to have multiple, independent evolutionary origins from saprotrophic ancestors as previously suggested[28].

Characteristics of early fungi

We reconstructed ancestral states for major nutritional modes in the Fungi using maximum likelihood (Fig. 1). Most of the ancestral character states of deep nodes are equivocal, with the exception of the common ancestor of members of the Basidiomycota, for which a parasitic ancestor is suggested. The phylogeny suggests that numerous transitions from a pathogenic to a saprophytic nutritional mode have occurred, as well as the reverse (Fig. 1). Although the nutritional mode of the common ancestor of Fungi is ambiguous, the earliest diverging branch in the Fungi contains parasitic species (*R. allomycis* and microsporidia). Recent studies[9,29] showed that the closest known relative to Fungi is the amoeboid protist *Nuclearia*, which grows phagotrophically on algae and bacteria. Amoeboid phases are also observed in basal fungi: *Rozella* seems to phagocytose the organelles of its host[30] and many chytrid zoospores undergo an amoeboid, motile phase before encysting. After the divergence of the *Rozella* and microsporidia lineage, the remaining fungi evolved filamentous growth (for example, hyphae and rhizoids), which aids in substrate attachment and absorptive nutrition involving extracellular digestion. Within the Basidiomycota and Ascomycota, a reversion to a unicellular, yeast-like growth form is observed among the earliest diverging lineages, perhaps implicating a prior advantage for this growth form in the early history of the Fungi.

It is unclear whether the common ancestor of Fungi was marine. Most zoosporic true fungi, including all of the chytrids sampled in this study, grow in freshwater or soil habitats. Therefore, the diversification of the major lineages (phyla) within the kingdom Fungi probably occurred in a terrestrial environment but before the emergence of land plants[31,32]. Mycorrhiza-like symbioses of the phylum Glomeromycota are suggested to have been crucial in the colonization of land by plants[33]. Extant members of the Glomeromycota live exclusively as obligate symbionts of photoautotrophs, including not only vascular plants and bryophytes, but also cyanobacteria. This raises the hypothesis that terrestrial members of the Glomeromycota living symbiotically with cyanobacteria or algae, in semi-aquatic and humid habitats later became the symbiotic partners of early land plants[34].

The present multilocus phylogeny explains the possible morphology and ecology of early fungi. The early-diverging lineages consist of a grade of zoosporic fungi, suggesting that the earliest fungi were primarily aquatic and lacked aerial spore dispersal. The loss of flagellated spores is inferred to have occurred at least four times. Each loss seems to have coincided with novel innovations in spore production and dispersal: microscopic wind-dispersed spores in terrestrial fungi; forcibly discharged conidia in the Entomophthorales; non-flagellated, mitotically produced spores in the planktonic *Hyaloraphidium curvatum*; and a complex polar tube apparatus in microsporidia. The sister kingdom to the Fungi (Animalia) evolved diverse body plans capable of feeding by ingestion, whereas the fungal branch developed a myriad of unicellular and filamentous forms optimized for absorptive nutrition. With a well-resolved phylogeny, fungal biologists can now study the evolution of complexity and multicellularity, and compare the evolution of these traits in fungi with their evolution in plants and animals.

METHODS

Molecular techniques. Sequence data were generated from 170 fungal species, primarily using pure cultures and herbarium material (Supplementary Notes 1). We used standard polymerase chain reaction (PCR) protocols[25] for amplification and sequencing of six gene regions: the 18S ribosomal RNA gene (nearly full length), the 28S ribosomal RNA gene (primers LR0R and LR7), the internal transcribed spacer (ITS) RNA gene region (full length), *EF1α* (mostly primers EF1-983F and EF1-2218R), RNA polymerase II largest subunit (*RPB1*, mostly primers RPB1-Af and RPB1-G2R) and RNA polymerase II second largest subunit (*RPB2*, primers RPB2-5F and RPB2-11bR). Information on the PCR primers can be found at http://www.aftol.org/primers.php. In a number of basal

fungal taxa, the *EF1α* gene was not detected, but a paralogous copy of the gene was recovered (*EFL*, or the *EF1α*-like gene[35]; see Supplementary Notes 4). We also obtained sequences from fungal and eukaryotic genomes by retrieving sequences from GenBank and genome servers. Although our data set contains both partial sequences and missing data points, in the case of only one taxon (the choanoflagellate *Monosiga brevicollis*) were fewer than four genes sampled.

Phylogenetic reconstruction. The data set consisted of 214 taxa, 199 of which were fungi. Sequences were aligned and ambiguous regions excluded in MacClade[36]. Conflict among the six genes was assessed by separate MLBS of each data partition using 250 bootstrap replicates in PHYML[37]. We ignored two conflicts, one including microsporidian 18S sequences (known to be subject to long branch attraction) and the other involving marginally conflicting signal of the Pyrenulales (Ascomycota). Data were combined into one matrix with *EF1α*, *RPB1* and *RPB2* translated into amino acids and 18S, 28S and 5.8S as nucleotides. We applied a heterogeneous maximum likelihood model to the data set with six unlinked partitions, one for each gene. The 18S and 28S genes were fitted to a general-time-reversible model with a proportion of invariant sites and gamma distributed rates (GTR+I+Γ), the 5.8S data used GTR+Γ and proteins used the JTT+I+Γ fixed rate model. The gamma distribution was approximated using four rate classes. We used MrBayes 3.1.1 (ref. 38) for phylogenetic estimation. Five independent runs were conducted (each with four chains) for 9.5×10^6 generations, sampling every 500 generations. Runs were discarded if they failed to reach the same likelihood plateau observed in other independent runs. We computed the consensus of the sampled trees, the posterior probabilities of clades, and average branch lengths from runs that converged to the same likelihood plateau (58,611 trees). For the analysis of the combined super-matrix we also tested for convergence of runs by analysing frequencies of splits using the software AWTY[39] and found that the consensus topology constructed using this criterion trivially differed from that based on log likelihood scores. We also assessed support for nodes on the nucleotide data (third codon positions excluded) by MLBS (500 replicates) using PHYML with a GTR+I+Γ model. Tests for statistical differences in likelihoods of alternative topologies were assessed using the approximately unbiased test[22] on the nucleotide data with site-wise, log-likelihood values calculated using TREE-PUZZLE v5.2 (ref. 40).

Ancestral character state reconstruction of nutritional mode was conducted using the maximum likelihood model Mk1 in Mesquite 1.0 (ref. 41). Taxa were assigned to ecological character states on the basis of published literature, resolving ambiguous assignments when possible. Reconstructions are reported for only those branches significantly assigned an unequivocal character state in a majority of 1,000 trees randomly drawn from the sample of credible trees. The number of losses of the flagellum within the Fungi was also estimated for all 58,611 credible trees using Dollo parsimony as implemented in MacClade.

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Author Information Data for this project have been deposited in GenBank (see Supplementary Notes 1 for accession numbers), and the alignments can be accessed on the Assembling the Fungal Tree of Life website at http://www.aftol.org/. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to T.Y.J. (tyj2@duke.edu) or R.V. (fungi@duke.edu).