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Evolutionary Microbiology

The search for the fungal tree of life

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The *Fungi* comprise a diverse kingdom of eukaryotes that are characterized by a typically filamentous but sometimes unicellular vegetative form, and heterotrophic, absorptive nutrition. Their simple morphologies and variable ecological strategies have confounded efforts to elucidate their limits, phylogenetic relationships, and diversity. Here we review progress in developing a phylogenetic classification of *Fungi* since Darwin's *On the Origin of Species*. Knowledge of phylogenetic relationships has been driven by the available characters that have ranged from morphological and ultrastructural to biochemical and genomic. With the availability of multiple gene phylogenies a well-corroborated phylogenetic classification has now begun to emerge. In the process some fungus-like heterotrophs have been shown to belong elsewhere, and several groups of enigmatic eukaryotic microbes have been added to the *Fungi*.

Fungal diversity and antiquity

Fungi make up a remarkably diverse kingdom whose species interact with a broad array of other organisms. Their compact genomes have been completely sequenced in more than 70 species. Nevertheless, the phylogenetic relationships of the *Fungi* remain incompletely known because of the challenges presented by the antiquity of fungal lineages and the incomplete documentation of extant species. Improved sequencing methods, expanded datasets and sophisticated phylogenetic algorithms, coupled with community-wide collaborations, are now contributing to the emergence of a well-supported phylogeny and classification for the kingdom *Fungi*.

Roles and antiquity of *Fungi*

Fungi interact extensively with plants, animals, bacteria and other organisms. Their heterotrophic, absorptive nutrition, aided by their filamentous and occasionally unicellular growth forms, allows them to play major roles as decomposers, mutualists and parasites [1]. They form symbioses with cyanobacteria and algae in lichens, and with the roots and aerial parts of most plants as mycorrhizae and endophytes, respectively. In animals these mutualisms may be external and aerobic, as in ant-fungal gardens, internal and aerobic in insect gut, or anaerobic in the rumen or caecum of herbivorous mammals. Parasitism of both plants and animals has a significant impact on humans and ecosystems.

The ages of fungal clades have been estimated from fossils and molecular sequence data. The fossil record is very incomplete but the data suggest that most fungal phyla were present at least 400 to 500 mya although their actual ages might be much greater [1,2].

Numbers of *Fungi*

The number of extant species of *Fungi* is unknown. The most widely cited estimate of 1.5 million [3] has been supported by the data of Schmit and Mueller [4] that suggest about 700,000 species as a conservative lower limit. This estimate is based primarily on the ratio of

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Glossary

Ascomycetes: *Fungi* that produce filaments or yeasts, and reproduce sexually with spores formed internally in an ascus.
Basidiomycetes: *Fungi* that produce filaments or yeasts, and reproduce sexually with spores formed externally on a basidium.
Chytrids: an informal term for *Fungi* with flagellated cells at some point in the life cycle.
Flagellar apparatus: the region of a zoospore comprised of the kinetosome, transition zone and flagellum.
Homology: two genes are said to be homologs if they derive from a single gene in a common ancestor.
Monophyletic group: a group of species that includes an ancestor and all of its descendants, a clade.
Ontologies: controlled structured vocabularies.
Ortholog identification: a method to detect a homologous gene among species.
Orthologous genes: homologous gene copies in two or more species that arose by speciation.
Paralogous genes: homologous gene copies in one or more genomes that arose by gene duplication.
Paraphyletic group: a group of species that includes the most recent common ancestor and some of its descendants.
Phylogenomics: phylogenetic analysis using whole genomes of species.
Polytomy: unresolved branching in a phylogenetic tree resulting in multiple branches arising at a branch point reflecting uncertainty about the order of cladogenesis.
Synapomorphy: a shared derived character that unites species in a monophyletic group.
Septal pore: opening in the cross wall between adjacent cells of a filament.
Spindle pole body: a structure that forms spindle and astral microtubules in *Fungi* that lack flagella.
Spitzenkörper: a fungal-specific hyphal tip organization.
Supermatrix: multigene phylogenetic dataset in which not all taxa are represented by the same genes.
Water molds: filamentous, fungal-like species that produce biflagellate cells; relatives of the brown and golden algae.
Zygomycetous fungi, or zygomycetes: coenocytic, filamentous species that lack complex fruiting bodies.

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Fungi to plant species for several ecologically defined groups from different regions of the world. There are approximately 100,000 described species and this number is increasing at about 1.2% per year [5]. Knowing the number of species of *Fungi*, and their phylogenetic distribution, is important for the understanding of the pattern and tempo of fungal diversification, as well as the complexity of ecosystems. Moreover, species-rich phylogenies assist in taxon identification in molecular ecology studies [6–10]. These phylogenies have practical application in ecosystem management, agriculture, drug discovery and medicine.

Search for the missing *Fungi*

Like other microorganisms, *Fungi* still harbor many undescribed and undiscovered lineages. Many of these represent species that have never been cultured or collected previously by fungal taxonomists. The number of unidentified fungal sequences of environmental origin in public databases has grown significantly in the past 10 years [8–12], suggesting that a large number of fungal lineages remain undiscovered [13,14]. Many of the undescribed species of *Fungi* are probably inconspicuous or microscopic forms that do not produce fruiting bodies, such as yeasts, molds, endophytic or arbuscular mycorrhizal (AM) fungi, and particularly those that live in obscure, poorly-explored habitats. For example, new yeast species obtained from beetle digestive tracts have increased the number of known yeasts by more than 20% [4,15]. However, common habitats such as plant leaves (phylosphere) are known to host a hyperdiversity of unknown fungal species [8–10,12–14]. Molecular environmental studies have revealed unknown major clades of *Fungi*, some of whose species are winter active and grow beneath the snow at high elevations [7]. One of these clades, known only from molecular sequences, is a basal clade in the Ascomycota and is thus important in understanding the evolution of the phylum; this clade is distributed on three continents and might require metagenomic analysis to understand its role in ecosystems [16]. In addition, multiple lineages of undescribed *Fungi* have been encountered repeatedly within taxa previously thought to contain only a single species. Examples of such cryptic diversity have been found in a wide variety of fungal groups, including chytrids (*Rhizophydium*) [17], molds (*Trichoderma*), animal pathogens (*Pneumocystis*), and mushrooms (*Armillaria, Cantharellus*) [18].

In this review we trace how the relationships among *Fungi* have been viewed since Darwin's *On the Origin of Species*, the current state of fungal systematics, and future prospects for reconstructing the Fungal Tree of Life (FToL). Highlights in the development of a phylogenetic classification of the *Fungi* will be presented.

Evolving knowledge of fungal phylogeny and classification

First century following Darwin's On the Origin of Species: mid-19th to mid-20th century

The publication of Darwin's *On the Origin of Species* in 1859 resulted in the rapid introduction of evolutionary thought into the study of fungi. Anton de Bary in his 1866 textbook was the first to introduce evolution into fungal classification [19]. He based his classification of the basal fungi on similarities in morphology between certain algae and aquatic and zygomycetous fungi, and considered other fungal groups – ascomycetes and basidiomycetes – to be more derived. By the second edition of the textbook in 1884 his tentative classification resembled that used until the second half of the 20th century (Figure 1, Box 1). In this period the characters used for phylogenies were morphological, anatomical and chemical.
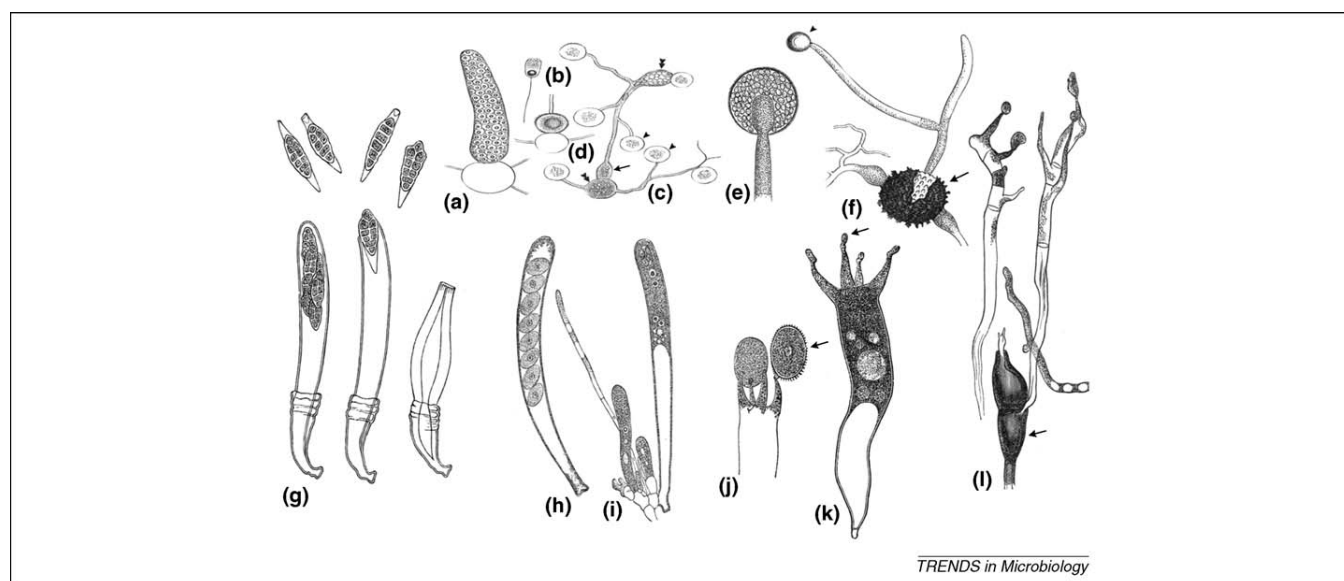
Figure 1. The defining features of the major groups of *Fungi*. These illustrations from the 1880s by de Bary and his students [72] are fully informative for characterizing taxa today. (a–d) Chytridiomycota, *Polyphagus euglenae*: (a) zoosporangium with discharge vesicle, (b) uniflagellate zoospore, (c) conjugating thalli (double arrowheads) initiating a resting spore (arrow) and attached to parasitized *Euglena* cysts (arrowheads), (d) maturing resting sporangium. (e,f) Zygomycetous fungi, *Mucor mucedo*: (e) sporangium and (f) germinating zygosporangium (arrow) between suspensors with germ sporangium (arrowhead). (g–i) Ascomycota: (g) *Macrospora scirpi* and (h,i) *Pyronema confluens* with (g) bitunicate asci before, during and after ascospore discharge and (i) unitunicate asci forming and (h) mature. (j–l) Basidiomycota: (j,k) *Aleurodiscus amorphus* and (l) *Puccinia graminis* with basidia with (k) asymmetrically forming and (j) mature basidiospores (arrows) or (l) arising from the overwintered teliospore (arrow).

Box 1. Classification of the fungi and slime molds by Anton de Bary

Groups that diverge from a class or are of doubtful position are indicated by an asterisk (*). Except for the uncertain placement of Protomyces and Ustilagineae, this classification from the 1880s [72] remained accepted for much of the 20th century.

Fungi

- Phycomycetes
  - Peronosporeae
  - Saprolegnieae
  - Mucorinii or Zygomycetes
  - Entomophthoreae
  - *Chytrideae
  - *Protomyces and Ustilagineae
- Ascomycetes
- Uredineae
  - *Basidiomycetes

Mycetozoa

- Myxomycetes
- Acrasieae

The class Phycomycetes – fungi with algal characteristics – was introduced by de Bary. This included aquatic and nonaquatic taxa, chytrids, water molds and their relatives, and zygomycetes. The class persisted for about 100 years. Subdivision of the aquatic fungi by Sparrow [20] based on motile cell structure began the unraveling of aquatic members of the *Fungi* from those species more closely related to algal groups, such as the Oomycota. However, the zygomycetous fungi, although also a para-

phyletic group, could not be sorted out until much later when molecular data became available [21].

By the 1960s cell wall chemistry and biochemical pathways began to clarify relationships among fungi (Figure 2) [22]. *Fungi* were defined by amino acid biosynthesis via the diaminopimelic acid pathway and cell walls of chitin and often β-glucan, while fungus-like organisms used the aminoadipic acid pathway in amino acid synthesis and had different cell wall compositions. With these advances the modern outlines of the *Fungi* as a monophyletic group began to emerge.

Fungi and the kingdoms of the eukaryotes: mid-19th century to present

Early classifications divided all organisms into two major groups, the plant and animal kingdoms. Fungi were included in the plant kingdom by de Bary because of their morphological similarities, although this point of view was not universally accepted [19]. Whitaker [23] was first to recognize *Fungi* as a distinct kingdom. He based his classification on cell structure, levels of tissue organization and nutritional mode. Although Whitaker's classification was heavily influenced by ecological considerations it had a major impact on thinking about fungi. A monophyletic kingdom of *Fungi* and its alignment with *Animalia* emerged in the 1990s with molecular sequence data [1,24]. The inclusion of animals and *Fungi* in the Opisthokonta is supported by all large datasets with broad species coverage and by a limited number of cellular synapomorphies; these include flattened mitochondrial cristae, a single posterior flagellum on motile cells, and similarities in the flagellar apparatus [24]. Although formerly treated
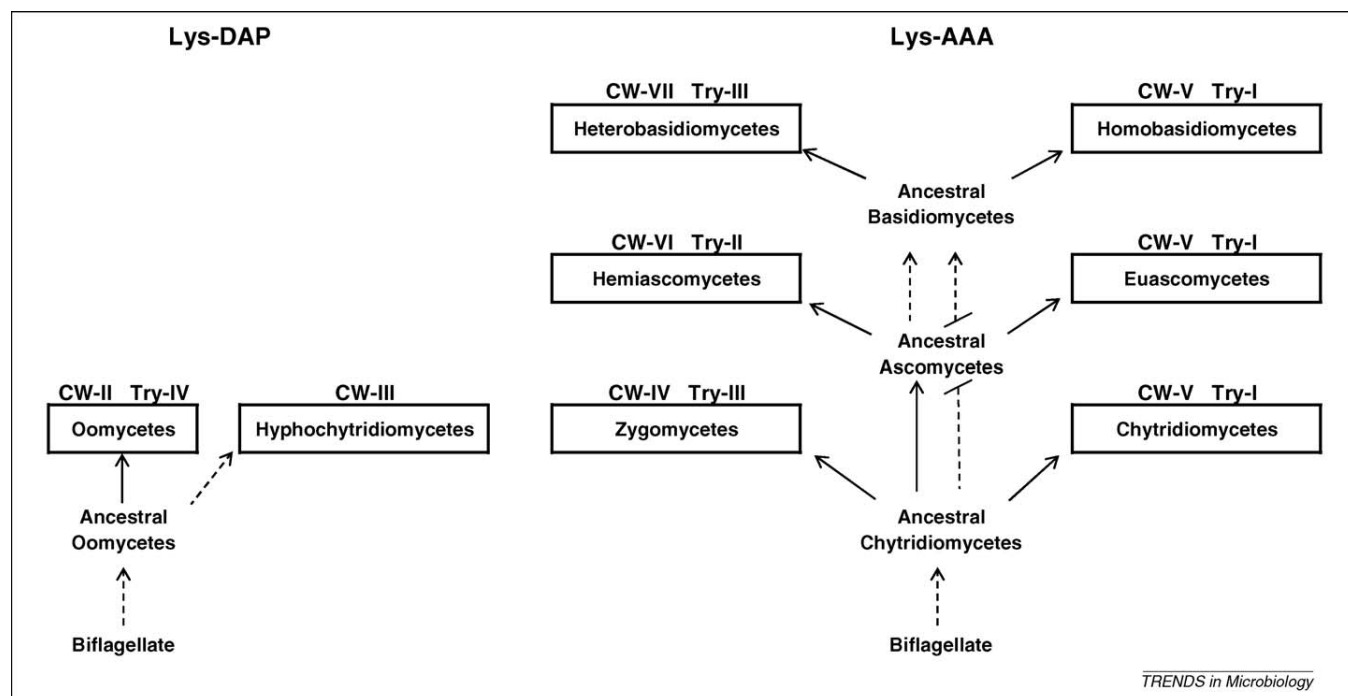
Figure 2. Hypotheses of fungal evolutionary relationships from 1969. Relationships are based on cell wall composition and biosynthetic pathways from Bartnicki-Garcia [22]. Lysine synthesis (Lys) can occur via the diaminopimelic acid (DAP) or aminoadipic acid (AAA) pathways. Cell wall (CW) types range from type II to VII: II, cellulose–β-glucan; III, cellulose–chitin; IV, chitin–chitosan; V, chitin–β-glucan; VI, mannan–β-glucan; VII, chitin–mannan. There are also four types of sedimentation patterns of tryptophan biosynthesis enzymes (Tryl–IV). Reproduced with permission of Academic Press [22].

**Box 2. What do we mean when we use 'fungi' and '*Fungi*'?**

What do we mean by 'fungi'? When the term 'fungi' is used it conveys a historical meaning of all groups that have fungal or fungal-like characteristics. Thus, besides the species in the monophyletic kingdom *Fungi*, it includes the water molds and white rusts (i.e. Oomycota), some orders previously included in the Trichomycetes (i.e. the zygomycetous fungi that form symbiotic relationships with aquatic invertebrates) and slime molds. The organisms that fall outside kingdom *Fungi* are now classified in other kingdoms. Pseudofungi has been proposed as a subphylum for Oomycetes and Hyphochytriomycetes [73] but the term 'pseudofungi' can be applied to any of these fungal-like organisms; this term is not needed. These fungi are no more 'pseudofungi' than the non-monophyletic organisms that comprise the algae or bacteria are pseudo-members of each of these groups. In these cases the name has an ecological meaning, not a systematic one. A possible solution to the confusion caused by 'fungi' is to qualify the term as is done with the algae and use 'true fungi', 'chromistan fungi', etc. To avoid confusion *Eumycota* has been introduced for *Fungi*, but the preference of most mycologists is to retain the better-known term *Fungi* for this kingdom [40].

as fungi by many authors, the cellular and acellular slime molds (Mycetozoa), together with lobose amoebae (Lobosa), form the sister clade to the Opisthokonta [24]. Taken together these clades have been referred to as the Unikonts (Keeling et al., 2005). The relationship among the *Eukarya* continues to be refined; however, placement in the Unikonts is a reasonably supported hypothesis of the relationships of the *Fungi* [24,25]. Clarification of which taxa belong in the kingdom *Fungi* has led to a nomenclatural problem that continues to cause confusion (Box 2).

Ultrastructural and molecular data and phylogenies: 1950s to present

The advent of ultrastructural data in the late 1950s and of molecular data in the 1990s has clarified the distinctions between fungal groups and revealed numerous cases of parallel or convergent evolution (homoplasy). But neither type of data has fully resolved the FToL. Structural data are incomplete with only a limited number of species studied in any phylum; new subgroups of *Fungi* revealed by molecular phylogenetic studies [26] are only now being examined structurally. Molecular data are similarly limited and have yet to resolve fully the deeper nodes of the FToL.

The types of cellular structures that have proven phylogenetically informative among fungal phyla include septal pore organization, nuclear division and spindle pole body (SPB) form, and the organization of motile cells (Figure 3). These characters have been used in phylogenetic analyses [27] but are often incompletely known within phyla [28]. Until the basal branches of the FToL are fully resolved it may be difficult to interpret the evolution of some structural characters, such as SPBs. The multiple losses of centrioles in basal fungi [26] could imply multiple independent origins of SPB structure in basal groups, but not necessarily in the Ascomycota and Basidiomycota that are sister clades. Bioinformatics is an essential tool for utilizing both structural and molecular data in phylogenetic reconstruction. Comparison of structural characters is best achieved with scientific community input into a common database, for which the Structural
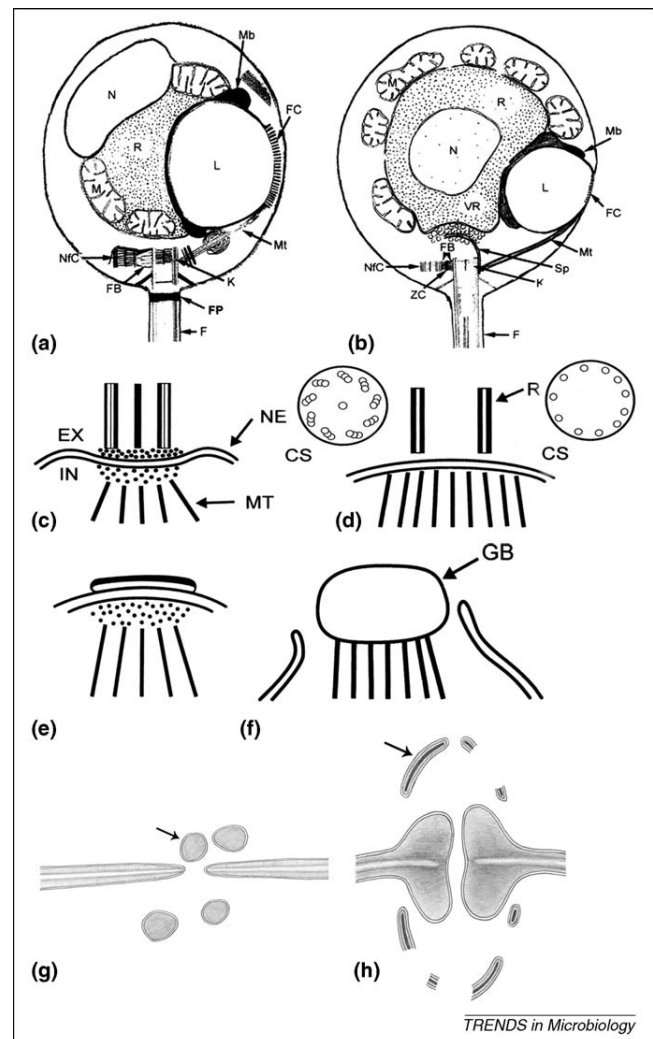
Figure 3. Examples of phylogenetically informative subcellular structures. These structures were used for elucidating fungal clades at the ordinal to subphylum or phylum level [17,28]. **(a,b)** Zoospore types in Chytridiomycota: **(a)** Chytridiales and **(b)** Rhizophydiales. Abbreviations: F, flagellum; FB, fibrillar bridge; FC, fenestrated cisterna; FP, flagellar plug; K, kinetosome; L, lipid globule; M, mitochondrion; Mb, microbody; Mt, microtubular root; N, nucleus; NfC, nonflagellated centriole; R, ribosomes; Sp, spur; VR, vesiculated region; ZC, zone of convergence. **(c–f)** Spindle pole body forms at metaphase–anaphase and their relationship to the nuclear envelope in **(c)** Blastocladiomycota, **(d)** zygomycetous fungi, **(e)** Ascomycota, and **(f)** Basidiomycota. Abbreviations: CS, cross section of the kinetosome; EX, extranuclear area; GB, globoid spindle pole body; IN, intranuclear area; MT, spindle microtubules; NE, nuclear envelope; R, ring with microtubules but lacking nine-fold symmetry. **(g,h)** Septa and septal pore organization in hyphae of **(g)** Ascomycota with Woronin bodies (arrow) and **(h)** Basidiomycota with septal pore cap (arrow) and pore swelling. Reproduced from Refs. [17] **(a,b)** and [28] **(c–h)** with permission.

and Biochemical Database (see http://aftol.umn.edu) has been developed to provide character and character state data in an exportable format for use in phylogenetic analysis programs [28]. This database reveals the limitations of the available data and will guide future data acquisition.

Molecular phylogenies of the *Fungi* initially were based on single locus trees of nuclear ribosomal DNA (rDNA). Two-locus trees of *Fungi* began to appear soon after (in 1992), but it took until 1997 for these phylogenetic studies to be based on three loci and an additional three years before more than four loci were used [29]. Indeed, more

than 75% of all fungal trees published each year until 2003 were still based on a single locus. Recently, the availability of whole genomes has permitted the application of phylogenomics to fungal phylogeny. The complete genomes of *Saccharomyces* species were used to determine the number of genes needed to develop a robust phylogeny [30,31]. Phylogenomics is now being extended to a broader sampling of taxa [32,33] for phylogenetic reconstruction across the *Fungi*. The large number of genes now available for phylogenetic studies of the *Fungi* has provided several new bioinformatic challenges, including the need for interactive databases with increasing levels of sophistication (e.g. Provenance, Ref. [34]), large scale data set assembly and visualization (such as WASABI, Ref. [35]; and Mesquite, http://mesquiteproject.org), phylogenetic search methods that can be implemented on supermatricies of thousands of taxa (e.g. RaxML, Ref. [36]), and efficient bioinformatic tools to visualize large-scale phylogenetic trees (such as PhyloWidget, Ref. [37]) and the information they contain (e.g. the database *mor*, Ref. [38]).

The FToL in the 21st century

Fungal systematics received a boost early in the 21st century from two National Science Foundation-sponsored projects, the Deep Hypha Research Coordination Network (RCN) and the AFTOL1 (Assembling the Fungal Tree of Life) project [39]. Deep Hypha supported a series of meetings of fungal systematists from 2001 to 2006 that enabled the community to share information and plan research. However, Deep Hypha did not directly support data-gathering activities. Plans for AFTOL1 were developed in the context of Deep Hypha, and benefited greatly from the community network that was formed through the RCN. The AFTOL1 proposal included a very large number of supporting letters, most from Deep Hypha participants, and the project adopted a policy that all donors of material would be invited to be coauthors on publications that reported new data derived from those materials. This policy recognizes the significant mycological expertise required to find and identify organisms and to archive voucher specimens and cultures. As a consequence, many of the AFTOL1 publications have numerous coauthors, examples being Lutzoni *et al.* [29], James *et al.* [26], and Hibbett *et al.* [40] respectively with 44, 70 and 67 coauthors.

AFTOL1 sought to generate molecular data of seven loci [nuclear large and small subunit and 5.8S ribosomal RNA genes, subunits 1 and 2 of RNA polymerase II (*rpb1*, *rpb*2), elongation factor 1-α, and mitochondrial ATP synthetase (*atp6*)] from about 1500 species representing all groups of *Fungi*, as well as ultrastructural characters from selected taxa. Molecular data from AFTOL1, including primer sequences and reference alignments, are available through a web-accessible database (http://aftol.org/data.php). Most of the AFTOL1 molecular data have been published and are in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html) that includes 4478 nucleotide sequences from 1106 species that can be retrieved with the keyword AFTOL.

Much of the output of AFTOL1 is summarized in four key references, including two kingdom-wide multilocus

analyses [26,29], a collection of phylogenetic studies on diverse groups of *Fungi* in the Deep Hypha issue of *Mycologia* [2,21,28,39,41–60], and a novel higher-level phylogenetic classification of the *Fungi* [40] that has been adopted by the mycological community and beyond, thus facilitating scientific communication.

The analysis of James and colleagues [26] included six of the seven AFTOL1 target loci (excluding only *atp6*) that were sampled in 199 species. The major conclusions of this study concerned the phylogenetic disposition of the 'basal fungal lineages', a paraphyletic assemblage containing multiple clades of chytrids and zygomycetes. The analysis also suggested that the Glomeromycota (traditional zygomycetes, including arbuscular mycorrhizal fungi) is the sister group of the Dikarya (a clade containing Basidiomycota and Ascomycota that is named from the synapomorphy of dikaryotic hyphae), although support for the Glomeromycota–Dikarya clade was weak.

One of the most contentious issues addressed by James *et al.* [26] concerns the number of losses of the flagellum among the *Fungi*. Several clades of chytrids form a paraphyletic assemblage at the base of the *Fungi* that is consistent with the view that the presence of flagella is an ancestral character state in the *Fungi*. Two groups of non-flagellated taxa appear to be nested among the chytrids and probably represent independent losses of the flagellum. One is *Hyaloraphidium curvatum*, an enigmatic planktonic organism that was first shown to be a member of the *Fungi* by Ustinova and coworkers [61]. The analysis of James *et al.* [26] suggests that *H. curvatum* is nested in a clade that includes free-living chytrids (Chytridiomycota *sensu stricto*) and anaerobic rumen symbionts (Neocallimastigomycota). The other group of non-flagellated taxa that appears to be nested among the basal chytrids is the Microsporidia, which are obligate intracellular parasites notable for their highly reduced genomes, degenerate mitochondria, and accelerated rates of molecular evolution [62]. The analysis of James *et al.* [26] suggests that a clade containing Microsporidia and the chytrid *Rozella allomycis* (an endoparasite of other chytrids) is the sister group of all other *Fungi*. Several other studies have suggested that the Microsporidia are nested within the *Fungi* or could be the sister group of the *Fungi* [24,63,64]. The apparent number of losses of the flagellum is also influenced by the position of *Olpidium brassicae*, a soil-dwelling chytrid that is a pathogen of plant roots. Surprisingly, *O. brassicae* was placed as a close relative of the zygomycete *Basidiobolus ranarum*, a filamentous species that functions as an animal pathogen or saprotroph.

Considering its complexity it is unlikely that the eukaryotic flagellum could be regained after having been lost. Applying this principle, the optimal trees produced by James *et al.* [26] imply five independent losses of the flagellum, two on the lineages leading to *H. curvatum* and Microsporidia, and three among the zygomycetes (owing to the position of *O. brassicae*). However, alternative placements of Microsporidia and *O. brassicae* resulted in trees that imply only two or three losses, and these could not be rejected. An analysis of data on *rpb1* and *rpb*2 published at about the same time as the James *et al.* study suggested that the Microsporidia are the sister group of the

Fungi and that the traditional zygomycetes are monophyletic, and therefore concluded that there was only a single loss of the flagellum in fungal evolution [63]. However, this analysis did not include *R. allomycis, H. raphidium,* or *O. brassicae*.

One of the major goals of AFTOL1 was to formalize our understanding of fungal phylogeny by the introduction of new classifications. At the time that AFTOL1 and Deep Hypha were initiated there were substantial differences among the major classifications for *Fungi*, with different names often being applied to the same clades and some taxa lacking monophyly. Examples of the competing classifications included the *Dictionary of the Fungi* series [5] and the classification employed by GenBank. Under the auspices of Deep Hypha and AFTOL1 a consensus classification containing only strongly supported monophyletic groups was developed, with reference to 102 phylogenetic studies published between 1998 and 2007. Again, this was a community-based endeavor, including experts on diverse groups and the authors and administrators of major taxonomic resources [40]. The 'AFTOL classification', that includes 129 orders as its terminal taxa, is now embodied in the current *Dictionary of the Fungi* [5], the GenBank classification, the Tree of Life Web Project (http://tolweb.org/tree/), the Myconet classification of Ascomycota (http://www.fieldmuseum.org/myconet/), and the Catalogue of Life annual checklist (http://www.catalogueoflife.org/annual-checklist/search.php). Reflecting uncertainty about the earliest branching events in the *Fungi*, the classification has a large polytomy at its base, including Dikarya, Glomeromycota, and eight other groups containing chytrids, zygomycetes, and Microsporidia (Figure 4).

Future prospects for fungal phylogeny
The immediate future of phylogenetics of the kingdom *Fungi* involves the analyses of genomic and subcellular data to address hypotheses pertaining to long-standing, enigmatic questions regarding the FToL. Major hypotheses to be addressed include (i) the placement of Microsporidia among the *Fungi*, (ii) resolution of the early diverging lineages of *Fungi* traditionally classified as chytrids and zygomycetes, (iii) more definitive ancestral character reconstruction associated with multiple losses of the flagellum, (iv) the placement of the Glomeromycota relative to other major clades of terrestrial, plant-associated *Fungi*, and (v) resolution of several problematic internal nodes of the Ascomycota and Basidiomycota that are crucial to the understanding of the diversification of fungal structure and ecology. All of these hypotheses represent questions in fungal evolutionary biology that have eluded traditional approaches using standard molecular systematics and observational studies of subcellular traits; novel approaches will be necessary to develop robust and testable explanations successfully.

Based on results from AFTOL1 (Figure 4), a second phase of AFTOL (AFTOL2) recently proposed a targeted set of taxa for sampling that will explicitly address problematic nodes and the hypotheses summarized above. Importantly, sampling of subcellular and genomic characters will overlap for a core set of taxa so as to maximize the explanatory power of the combined data. Subcellular characters to be sampled include septa of vegetative hyphae and meiosporangia, the nuclear division apparatus, SPB cycle, and the Spitzenkörper. In addition to the collection of subcellular data for target taxa, AFTOL2 is developing ontologies for these characters so that homologies can be communicated more accurately across disparate groups of taxa.

Advancements in genome sequencing technologies have resulted in a rapid increase in the availability of genomic data for *Fungi* [65] (see http://fungalgenomes.org/genome), setting the stage for the convergence of the fields of phylogenetics and genomics [66,67]. These studies include evolutionary analyses of genome organization that have recently provided additional support for placement of Microsporidia among the *Fungi* [64], and the phylogenetic analyses of a large amount of primary nucleotide or amino acid data [33,68]. The accurate determination of orthologous sequence data is central to the phylogenetic analyses of genomic data. The problem of paralogy and misinterpretation of homology is significantly higher with genomic data as compared to PCR-directed gene sequencing. Numerous analytical approaches have recently been developed for determination of orthologous sequences, and Kuzniar *et al.* [69] provided a comprehensive review of the strengths and weaknesses of currently available programs and databases. In addition to ortholog determination, early phylogenomic studies also observed potential conflicts among gene trees [30,68], systematic biases associated with taxon and character sampling [31], and difficulty in the assessment of nodal support [33,67,68]. Guided by these preliminary studies, AFTOL2 initiated a study to identify a kingdom-wide set of orthologous markers and facilitate acquisition and analyses of these data.

AFTOL2 identified a core set of 71 genes that are ubiquitously distributed across the *Fungi* and are good candidates (e.g. length of predicted proteins, sequence variability, single or low copy-number gene family) for large-scale phylogenomic analyses (see http://www.aftol.org). Twenty-five of these genes have been included in other phylogenomic studies [30,70] or tree of life projects (http://atol.sdsc.edu/projects), and provide cross-reference data points for global studies of the Tree of Life. The remaining 46 genes were identified by AFTOL2 using a Markov clustering approach [33] and target the FToL. To facilitate working with such large datasets AFTOL2 developed a semi-automated PERL wrapper to integrate and articulate existing algorithms for ortholog identification, multiple protein alignments, model of evolution assessment, and phylogenetic analyses of individual and concatenated super alignments (*Hal*: see http://aftol.org/pages/Halweb3.htm; beta versions of *Hal* are available from J.S. upon request). This approach not only uses data from completely sequenced genomes but it is also able to incorporate identified orthologs from heterogeneous genome resources such as expressed sequence tag (EST) libraries. The result will be a supermatrix whereby some genes are missing for some taxa, but will permit a broader and more inclusive approach to taxon sampling. In addition, to facilitate the rapid expansion of additional phylogenetic markers for use in fungal phylogenetics, AFTOL2 is also
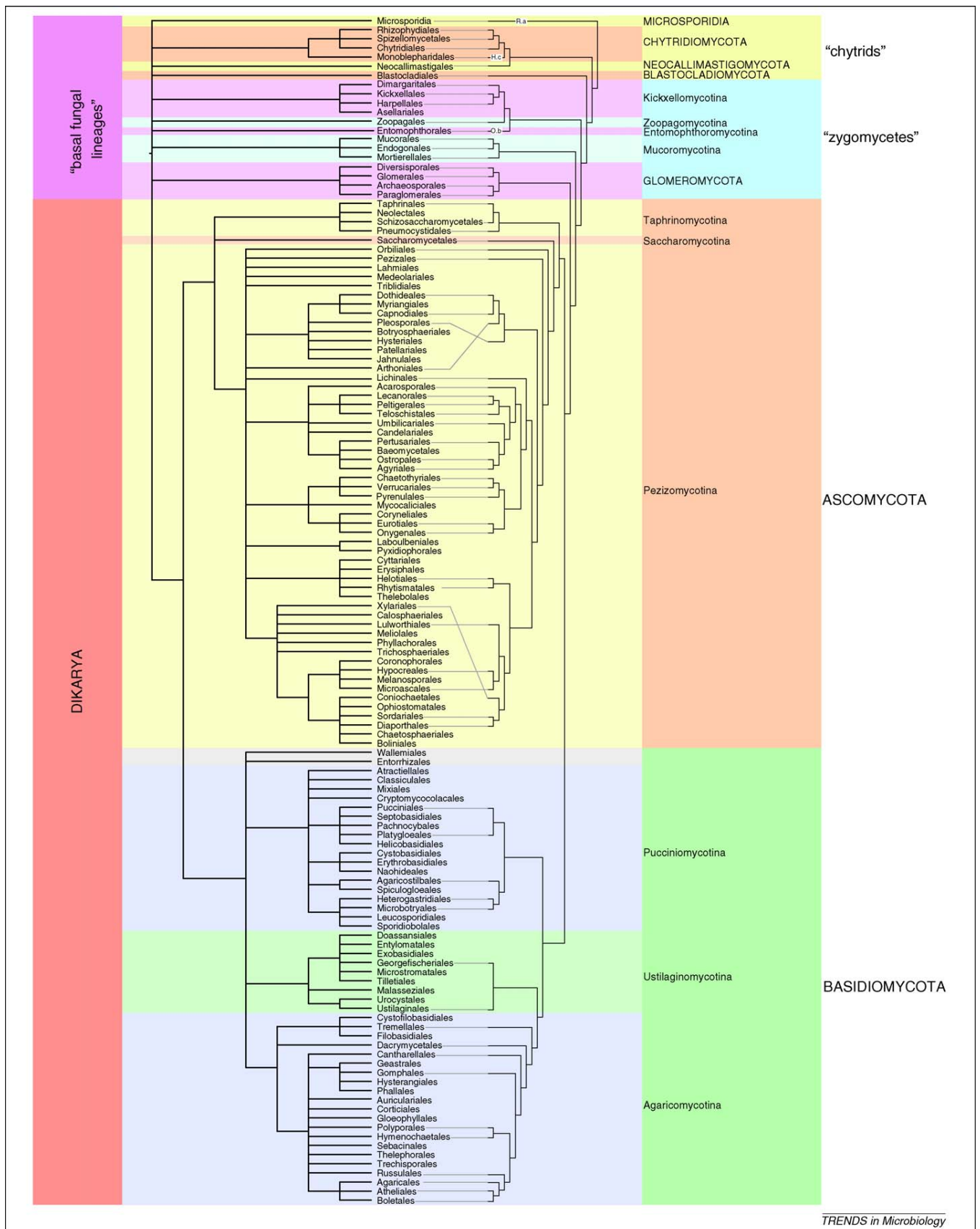
Figure 4. Phylogeny and classification of *Fungi*. The tree on the left represents the AFTOL classification. Only nodes corresponding to formally named taxa are resolved. Phyla (suffix -mycota), subphyla (-mycotina) and subkingdom-level taxa (Dikarya) are labeled. Names in quotation marks are informal, non-monophyletic groups. The tree on the right reflects taxon sampling and tree topology from James *et al.* [26] (the AFTOL classification was developed with reference to many additional studies). Positions of *Rozella allomycis*, *Hyaloraphidium curvatum*, and *Olpidium brassicae* estimated by James and coworkers are indicated by R.a, H.c, and O.b, respectively.

Box 3. Outstanding questions

- **How has subcellular structure evolved in the *Fungi*?**

  The range of variation in subcellular structures within fungal phyla is unknown. Generalizations are based on minimal data (i.e. from one or a few species) but in better-studied subphyla a range of subcellular features is observed, for instance in motile cell organization in Chytridiomycota or SPB form and septal pore organization in Basidiomycota. Several SPB forms are known in zygomycetous fungi but the clades are still largely unstudied. To determine how SPB form has evolved in these fungi and its relationship to flagella loss in basal fungi a detailed analysis of nuclear division is needed for four zygomycete subphyla and the Glomeromycota. To understand subcellular evolution and characterize the genes in the many fungal genomes that are becoming available, a renewed focus will be required on fungal cytology, employing well thought-out sampling strategies. Improvements in bioinformatic resources for image labeling and storage will aid in comparative structural analyses and integration with molecular data.

- **What will be the next limiting factors for assembling the fungal tree of life?**

  Mycologists are entering a period where it will be as easy to sequence fungal genomes (often <40 Mb) as it was for prokaryotes over the last decade. The rapid sequencing of small genomes will permit finding the optimal set of genes to provide sufficient resolution to generate a FToL for all described species. The main challenges will be to obtain samples of all known species, necessitating coordination of effort and worldwide mycological expertise, as well as new bioinformatic and analytical tools. Another limiting factor will be the description and naming of the unknown fungal species, representing the great majority of the extant fungal species richness.

- **What are the key evolutionary innovations that took place during the evolution of the *Fungi* and their biological consequences?**

  For example, when and how many times did the lichen symbiosis originate? The origination of the lichen symbiosis might be associated with a rapid adaptive radiation early in the evolution of the Pezizomycotina (a subphylum representing nearly all filamentous ascomycetes). The statistical power of all current methods to infer ancestral traits using phylogenies is unknown. These methods are likely to be biased against changes occurring during rapid adaptive radiations (i.e. on very short internodes) because they all assume a constant rate of evolution across the entire phylogeny. Therefore, if lichen symbiosis originated during a rapid radiation, current methods are more likely to infer erroneously a more recent origin and, consequently, more numerous independent origins. This explains in large part (e.g. in addition to taxon sampling issues and branch length estimations) the high uncertainty associated with current estimations of the exact number of origins and their precise localization on phylogenetic trees.

- **Are current taxonomic practices adequate for describing fungal diversity and translating emerging phylogenetic hypotheses into classifications?**

  Fungal taxonomy is increasingly based on molecular phylogenies. Similarly, our knowledge of the diversity, distribution, and ecological roles of *Fungi* is expanding rapidly through molecular environmental studies. At the same time, new species descriptions and taxonomic proposals follow rules that were developed in the absence of phylogenetic perspectives, strongly emphasize morphology, and are scattered in the literature. Should current practices be enhanced or replaced by systems that emphasize phylogeny as the primary criterion for taxonomy, use centralized databases to update a global classification, and allow species descriptions based solely on sequence data?

developing PCR and sequencing primers for these target genes for use by the broader fungal systematics community.

Initial phylogenetic analyses of genome-scale data have provided increased support for controversial taxa (e.g. Taphrinomycotina) [71] and have continued to identify problematic regions of the FToL (e.g. the backbone of the Pezizomycotina) [33,68]. One limiting factor in all of these analyses, however, is taxon sampling. Although the number of sequenced genomes is rapidly increasing, most currently available genomes have been selected because they are human and plant pathogens or are central to the carbon cycle and energy concerns (e.g. mycorrhizae and wood decay fungi). Although these are important organisms for genomic sequencing, the initial result has been a bias in taxon sampling of phylogenomic analyses and there is an urgent need for genome sequencing of unsampled fungal lineages that are crucial to the understanding of deep divergences in the FToL.

In summary, in Darwin's day only a skeletal outline of the FToL was known and the fungi included unrelated taxa with similar morphologies and ecological roles. Understanding the relationships of these taxa, especially the basal taxa, took more than a century. In the second half of the 20th century, and especially in the past 20 years, the availability of biochemical, ultrastructural and genomic data has led to a sea-change in our understanding of the FToL. Recent studies have provided a well-corroborated phylogenetic tree for the *Fungi* and have permitted the development of a consensus classification. Deep branches within the FToL, as well as many internal branches, remain unresolved and are the focus of current multigene analyses; these are expected to resolve many of the uncertainties and provide guidance in interpreting character evolution and assistance in environmental studies and in identifying the probable large numbers of unknown species (Box 3).

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Trends in Microbiology, *Trends in Parasitology* and *Cell Host & Microbe* are jointly having a series on evolution to commemorate the 200th anniversary of Charles Darwin's birthday (12th February, 1809). The series focuses on aspects of evolution and natural selection related to microbes, parasites and their hosts. These are some of the articles that have already been published:

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