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Toward a Fully Resolved Fungal Tree of Life

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Abstract

In this review, we discuss the current status and future challenges for fully elucidating the fungal tree of life. In the last 15 years, advances in genomic technologies have revolutionized fungal systematics, ushering the field into the phylogenomic era. This has made the unthinkable possible, namely access to the entire genetic record of all known extant taxa. We first review the current status of the fungal tree and highlight areas where additional effort will be required. We then review the analytical challenges imposed by the volume of data and discuss methods to recover the most accurate species tree given the sea of gene trees. Highly resolved and deeply sampled trees are being leveraged in novel ways to study fungal radiations, species delimitation, and metabolic evolution. Finally, we discuss the critical issue of incorporating the unnamed and uncultured dark matter taxa that represent the vast majority of fungal diversity.

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INTRODUCTION

Fungi are a distinct, diverse, and ecologically important branch of the tree of life. These hardworking organisms play a vital role in ecosystems as diverse as soil, leaves, rocks, and pelagic zones of the ocean, yet their roles are primarily enacted behind the scenes, literally as hidden layers within their substrate. Distinguished from plants by their heterotrophic nature, and also distinct from animals by their external rather than internal digestion, fungi diverged from their sister kingdom the animals ~1.3 billion years ago (11). They have mostly marched via stepwise codiversification with the plants, their intertwined partners in numerous symbiotic interactions (71). Although there is no trait that is uniquely shared by all fungi and defines the fungal kingdom (88), they are generally characterized by a chitinous cell wall and a form of nutrient uptake called osmotrophy in which secreted enzymes break larger substrates and molecules into smaller ones that can be passed through the cell wall in an active manner (90). This mode of nutrition (sloppy eating) poses numerous and increasing threats to ecosystems when wielded by pathogenic species that attack crops, wildlife, and even humans (10, 14, 32). In order to outcompete each other and other microbes, fungi have evolved numerous strategies to degrade hard-to-digest substrates, such as lignin, cellulose, and pollen, while combating competitors using an arsenal of bioactive metabolites, such as the familiar antibiotics, ethanol, and organic acids (94, 96).

Fungi also have served a crucial role as model organisms for biological inquiry, such as brewer's yeast, *Saccharomyces cerevisiae* (43); and pink bread mold, *Neurospora crassa* (23). Major insights, such as the nature of the gene, autophagy, control of cell cycle, and how telomeres function, have been made by leveraging these morphologically simple organisms with a complex cellular machinery similar to that of our own cells. Due in part to their typically small genome sizes and life cycle stages with free-living haploid states (112), fungal genomes are easy to obtain, and fungi have served as models for genome evolution and reconstruction of phylogenetic relationships using genome-scale data. Groundbreaking comparative genomic studies that take advantage of these features have already been published (33, 58, 95, 108). These pioneering studies are just the prelude to the period that is upon us now. The arrival of next-generation sequencing technology allowing fungal genomes to be sequenced for as little as a few dollars now means that most phylogenetic studies, including those at the species level, can be conducted using genome-scale data.

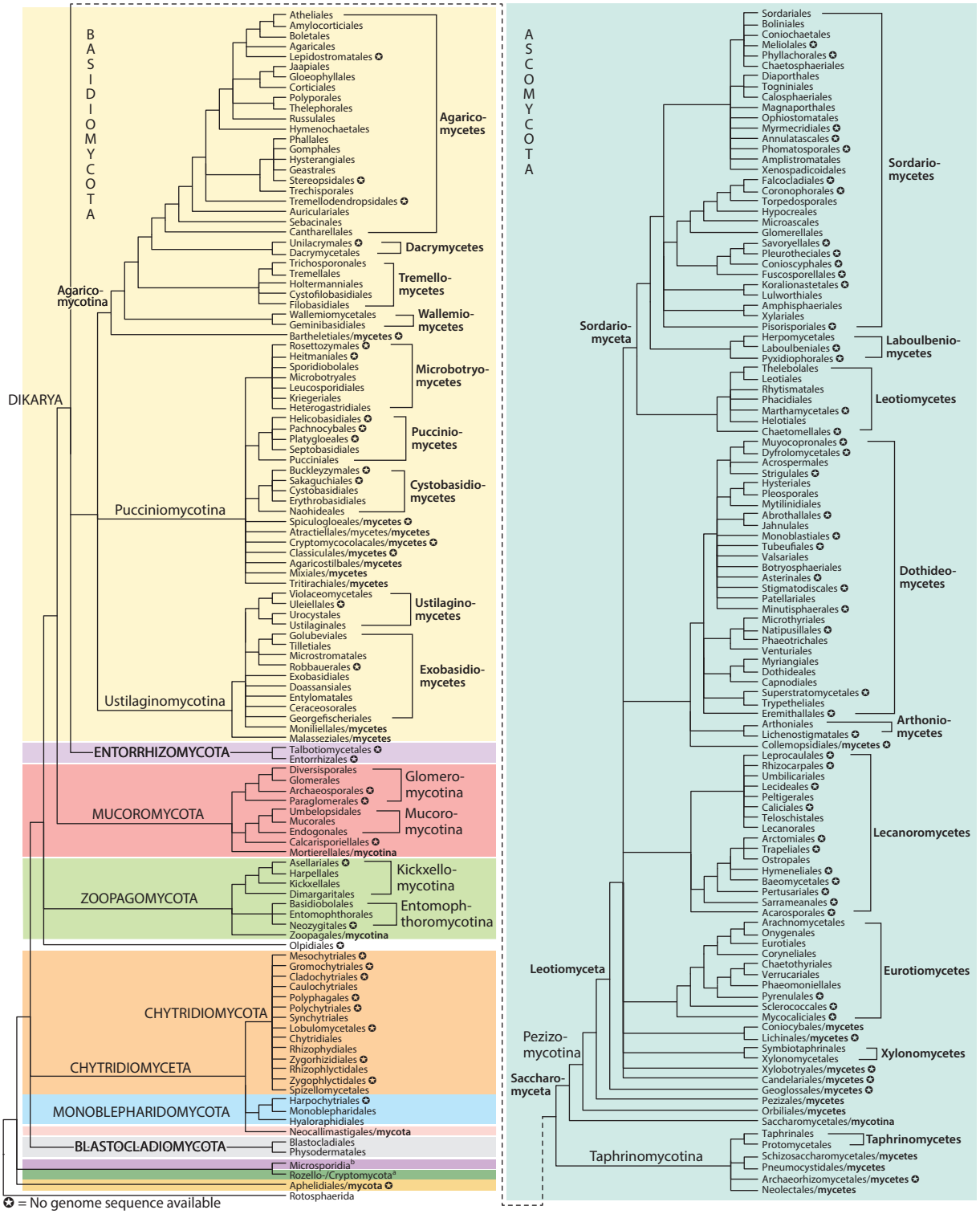
Despite the early embrace of molecular systematics by mycologists, both the discovery and classification of fungi are still in great flux, particularly among the more basal branches of the tree, whose true diversity is only now coming to light from genomic analyses and environmental DNA surveys. Dramatic changes in higher-level taxonomy have occurred in the last 20 years, as

evidenced by a tripling of fungal phyla from 4 to 12. The hidden and microscopic nature of many fungi also means that their diversity is undersampled, and perhaps less than 5% of the estimated two to four million species have been formally described (13, 45). A fully resolved, comprehensive fungal tree of life (FTOL) will be an essential component of the future of fungal research. The FTOL has a number of utilities, such as facilitating comparative biology, allowing the functional and morphological prediction of newly discovered or dark matter taxa, facilitating study of evolutionary processes, and providing a robust evolutionary framework for their accurate (and natural) classification. To their credit, mycologists have embraced the concept of a natural classification system grounded in phylogeny and have made great progress toward creating a broad sketch of the fungal tree. However, most of the task of producing the comprehensive phylogeny lies ahead, and there are a number of challenges that need to be addressed by the community. For example, the great volume of data in the genomic revolution is both a blessing and a curse. Without careful consideration of analytical methods, it becomes easy to arrive at robust support for the wrong phylogeny (21, 82). Additional analytical challenges involve adjusting for horizontal gene transfer, gene duplications, and population-level processes to determine the true species phylogeny. Finally, how do we reasonably incorporate taxa that cannot be cultured and may never have been observed into the growing tree? This question is paramount, as ecological studies indicate that such taxa may dominate some fungal communities. In this review, we discuss the current state of the fungal tree, novel research being done using fungal phylogenies, and technical challenges that must be overcome to derive a fully resolved tree.

THE CURRENT STATUS OF THE FUNGAL TREE OF LIFE

Our synthesis of the current state of the FTOL and classification down to the ordinal level is summarized in **Figure 1**. We recognize 224 orders organized into 12 phyla. Orders, in mycological systematics, are typically monophyletic, are well circumscribed, and likely reflect a stable clade with identifiable characteristics (47). Our assembly of orders into a higher-level phylogeny derives primarily from 46 key papers produced in the last 10 years that either helped resolve the backbone of the fungal tree through multigene or phylogenomic approaches or included sampling of key taxa (**Supplemental References 1**). We recognize six major groups of Fungi.

1. Most of the diversity in terms of described species (>97%) is concentrated in the subkingdom Dikarya, composed of Ascomycota, Basidiomycota, and Entorrhizomycota (**Figure 2*a***). The first two phyla each contain three monophyletic subphyla. These fungi have the synapomorphy of heterokaryotic cells with unfused nuclei dividing conjugately after mating for a short (Ascomycota) or long (Basidiomycota) period.
2. Mucoromycota, a clade of mostly plant-associated taxa with coenocytic hyphae and zygospores, is sister to the Dikarya. Despite earlier evidence that the Dikarya and Glomeromycota might form a clade (51), the Glomeromycotina instead are related to Mucoromycotina and Mortierellomycotina (110).
3. Zoopagomycota, another clade of fungi primarily having coenocytic hyphae and zygospores, is composed of three subphyla of fungi in parasitic or predatory (sometimes saprobic) association with animals, protists, or other fungi.
4. Blastocladiomycota forms a distinct clade of fungi with zoospore ultrastructure with a nuclear cap of ribosomes, and it is the only clade in Fungi with sporic meiosis or alternation of haploid/diploid generations (53).
5. Chytridiomyceta is recognized as a subkingdom comprising most zoosporic (flagellated) fungi in the phyla Chytridiomycota, Monoblepharidomycota, and Neocallimastigomycota.



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Consensus phylogeny among orders of Fungi. Relationships conflicting among studies or with minimal support are shown as unresolved. In some cases, notably the lone order Saccharomycetales in the subphylum Saccharomycotina, taxonomists have conservatively avoided describing additional orders or higher-rank taxa, even though recent phylogenomic results suggest they may be justified (108). [a]Rozellomycota (or Cryptomycota) is a diverse group where intraphylum taxonomy has not been established, because most species are known only from environmental DNA. [b]Microsporidia nomenclature is not covered by the International Code of Nomenclature for algae, fungi, and plants. Taxa indicated with a star are those in which no nuclear genome sequence is available through either GenBank or the Joint Genome Institute MycoCosm portal. A list of references used to assemble the phylogeny can be found in **Supplemental References 1**.

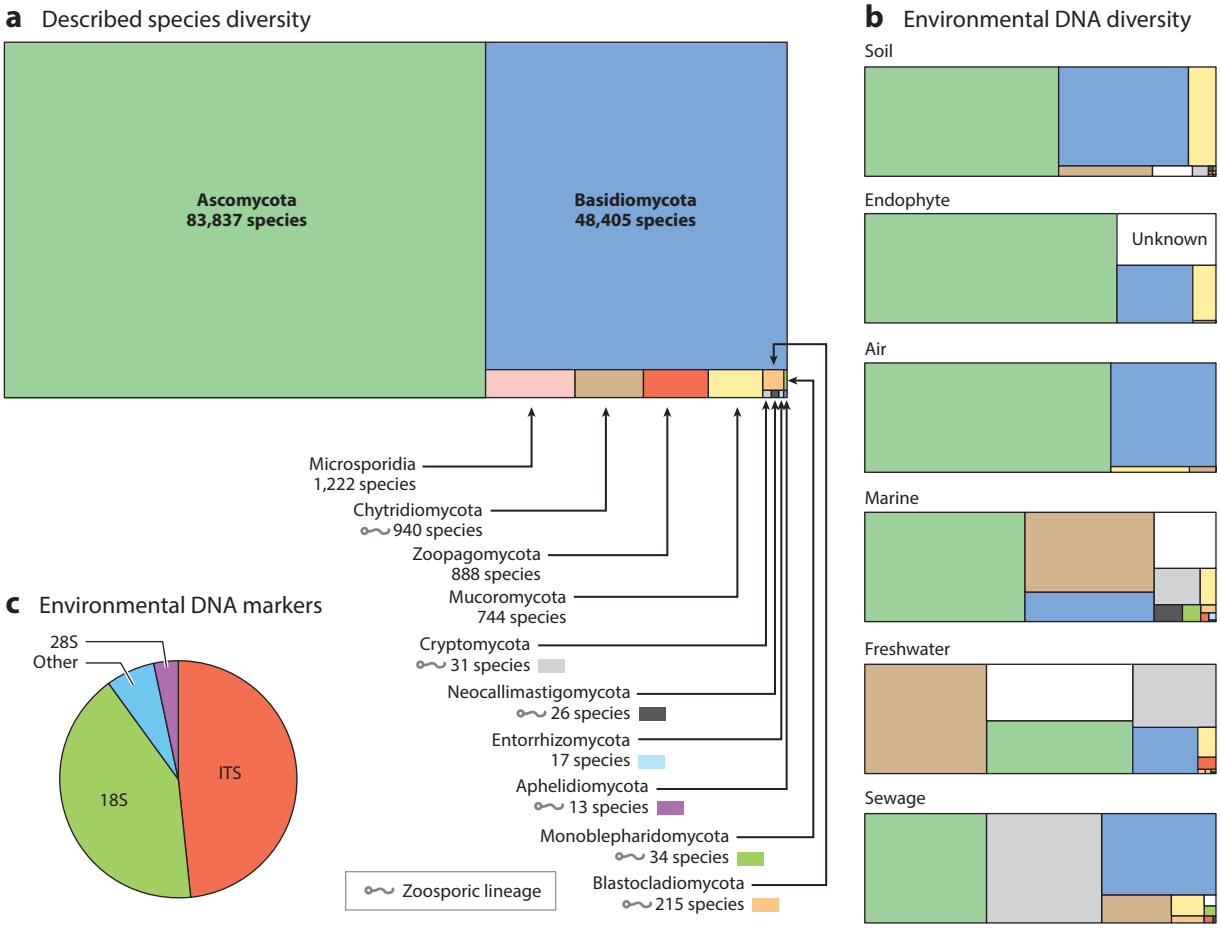


Figure 2

Comparison of described fungal diversity at the phylum level with estimated fungal diversity using environmental DNA studies. (*a*) Species diversity of fungi was obtained from the Catalog of Life 2019 (**http://www.catalogueoflife.org/annual-checklist/2019**). (*b*) Treemaps represent proportion of environmental DNA sequences identified at the phylum level for each habitat. Ten studies per habitat were summarized, with the list of studies in **Supplemental References 2**. (*c*) The molecular markers of the 60 environmental studies are summarized, with most studies using ribosomal RNA regions and other including either protein-coding genes or metagenomic sequencing. Abbreviation: ITS, internal transcribed spacer.

6. Opisthosporidia is possibly a paraphyletic group composed of Aphelidiomycota, Cryptomycota/Rozellomycota, and Microsporidia. These endoparasites are the basal branch(es) of Fungi.

Genomics has had a major impact on resolving the tree shown in **Figure 1**. Phylogenomic approaches have been essential for resolving nodes deeper in geological time (15, 20, 28), yet they are also particularly useful for resolving relationships involving short divergence times. That is not to say that phylogenomic approaches using nuclear genome sequencing are the only game in town. A great deal of progress has been made in elucidating the fungal tree using mitochondrial genomes (68, 140), transcriptomics (9, 121, 129), a combination of a few common protein-coding genes (e.g., *RPB1*, *TEF*) and ribosomal RNA (rRNA) genes (75, 128, 135, 141), or even just rRNA genes themselves (106). Although much progress has been made sampling subsets of genes to fill out the leaves of the major branches, it is clear that they often lack the power to resolve the higher-level relationships, even the interordinal ones (**Figure 1**).

In recent years, two major separate efforts have aimed to dramatically increase the sampling of genomes in the FTOL. The first effort is the 1000 Fungal Genomes Project (**http://1000.fungalgenomes.org/home**), which aims to sequence 1,000 fungal genomes across the FTOL by specifically targeting taxa in lineages that were underrepresented or absent in genomic databases. So far, this project has contributed more than 500 reference genomes toward the more than 1,500 now available for the kingdom. Additional low-coverage sequencing has generated more draft genomes focused on primarily chytrid and zygomycete lineages. The second effort, known as the Y1000+ Project (**https://y1000plus.wei.wisc.edu**), is aimed to sequence the genomes of all ∼1,000 known species of budding yeasts (phylum Ascomycota, subphylum Saccharomycotina). The most recent output of this project has been an analysis of genomic data from 332 budding yeast species, including 220 new genomes (108).

What are the major unanswered questions across the FTOL? Here we emphasize the deeper branches, as these are the ones that are likely to be most difficult to resolve and will likely require not only large amounts of sequence data but also the use of state-of-the-art analytical computational workflows and models of sequence evolution. From the base of the FTOL upward, the following are major unresolved branches that have withstood multiple attempts at resolution:

1. Aphelidiomycota (aphelids). Aphelids have a number of similarities with the group known variously as Cryptomycota/Rozellomycota/Rozellida/Rozellosporidia (88), such as being endoparasites that use phagocytosis for nutrition (55). While the relationship between Cryptomycota/Rozellomycota and Microsporidia appears certain (7, 85), it is unclear whether Opisthosporidia is a clade or whether the Aphelidiomycota branches before or after the divergence of Cryptomycota/Rozellomycota/Microsporidia from the fungal stem (56, 121). Transcriptome data show that Aphelidiomycota have a genome composition more similar to that of free-living fungi (121), but this information does not inform on the order of branching and only makes the resolution more useful for interpreting genome evolution.
2. Blastocladiomycota. Genome-scale trees place Blastocladiomycota as diverging either just before Chytridiomyceta or just after (20, 28, 52, 120). Blastoclads have characteristics more similar to those of the terrestrial fungi, such as well-developed hyphae, closed mitosis, cell walls with β-1–3-glucan, and a secretory vesicle complex known as the Spitzenkörper (25, 98). Understanding the true branching order has large implications for life cycles, mitosis, and cell wall biology in the fungi.
3. Neocallimastigomycota [or anaerobic gut fungi (AGF)] and Monoblepharidomycota. AGF comprise a phylum of zoosporic fungi that is distinct in several ways. They are the most potent cellulose degraders, and the fungal kingdom's only obligate anaerobes that live in

the guts of ruminants, although they are increasingly recognized as also present in non-ruminant organisms (66). Unlike any of the other zoosporic true fungi, members of this phylum can have multiple flagella on a single spore. Phylogenetic data have placed AGF squarely near Chytridiomycota but as a distinct lineage. Importantly, the Monoblepharidomycota also appear distinct from Chytridiomycota, and the relationship between the Monoblepharidomycota, Chytridiomycota, and AGF is uncertain (20, 68), despite the similarity of zoosporic ultrastructure of the former two (34).

4. *Olpidium*. This genus comprises the most morphologically simple, endoparasitic, zoosporic fungi. Earlier zoospore ultrastructural studies suggested a relationship with Spizellomycetales and *Rozella* (6). However, *Olpidium* fundamentally differs from *Rozella*, because it grows as an endoparasite but with a (presumably chitinous) cell wall during all stages of parasitic growth. *Olpidium* has been placed outside of the core chytrids, apparently closer to the terrestrial fungi, with the precise relationships as of yet uncertain (51, 104).
5. Subphyla of Basidiomycota. Most phylogenies with multiple genes place Ustilaginomycotina sister to Agaricomycotina, but this is still debated (21, 46, 81, 84). Similarities in septal pore ultrastructure support this relationship.
6. Entorrhizomycota. One of the most controversial placements in the FTOL is the placement of *Entorrhiza*, a gall-forming root parasite of Poales. The fungus has strong similarities to Ustilaginomycotina in septal pore ultrastructure and teliospore germination (8). Entorrhizomycota was erected after a five-gene phylogeny placed *Entorrhiza* as sister to the other Dikarya, though alternative placements within or sister to the Basidiomycota could not be rejected (8). A more recent analysis of six genes recovered *Entorrhiza* with high support as the sister branch to all Basidiomycota (141). Because of the morphological similarities to smuts, the low number of genes employed, and the low statistical support, this placement is controversial. Convincing resolution of this placement will require a genome-scale phylogeny, which is much needed to understand the evolution of cytology and the events that led to the emergence of the most successful lineages of fungi with dikaryotic hyphae.
7. Classes in Taphrinomycotina. Taphrinomycotina form the earliest diverging clade of Ascomycota. Many of the genera (including the model fission yeast genus *Schizosaccharomyces*) are highly distinct morphologically, and the clade is not speciose, such that classes are mostly monotypic (i.e., only have a single genus). Phylogenetic analyses provide very little resolution among the classes (78, 97).

FUNGAL CLASSIFICATION AS A DYNAMIC OBJECT

Phylogeny and classification are two separate but interacting frameworks. While we assume there exists a true species tree of the fungi, how that tree is used to determine a system of taxonomic classification is subject to debate. A functioning community of researchers uses consensus to solve this issue, understanding that competing classifications should exist. Classification systems are usually outlined in landmark, influential papers or books (4, 47, 48, 63). Many users of classification merely wish to adopt the community consensus, and reference sources for fungal classification exist, such as the *Dictionary of the Fungi* (last published in 2008; 57) and the related online resource Index Fungorum (**www.indexfungorum.org**). Many researchers also look to GenBank [part of the International Nucleotide Sequence Database Collaboration (INSDC)] as an authoritative source of higher classification, because most taxonomic studies are associated with DNA sequence data. The sequence data in GenBank and the underlying phylogenies they support were leveraged by the Open Tree of Life project (50) to assemble and visualize data for 2.3 million tips of the entire tree of life, including 297,000 fungal sequences. The approach uses graph theory to connect and

assemble highly curated phylogenies to resolve the underlying GenBank taxonomy, but it is not intended to provide a formal classification scheme. The latest *Dictionary of the Fungi* classification (57) was largely based on the publication of Hibbett et al. (47), which produced a classification of all fungi down to order, but the classification has been in great flux since this time. More recent higher-level classifications have been reviewed by Tedersoo et al. (118), Spatafora et al. (110), and Naranjo-Ortiz and Gabaldón (77). Regardless of the uncertainty of the structure of the FTOL, there are a couple burning issues in fungal classification worth mentioning.

First, circumscription of fungi became quite contentious with the discovery of multiple lineages that branch near the base of the tree that do not have the normal set of fungal traits (54). These include the taxa grouped into Opisthosporidia (55), as well as a novel chytrid-like clade of marine diatom parasites (19). Critically, all of these groups appear to lack cell walls during the primary part of the trophic phase, and some are known to have a phagocytosis-like mechanism for consuming host cytoplasm (55, 83). Because of this latter characteristic, many taxonomists have sought to exclude these groups from true fungi (17, 55). This debate has been much discussed, but it appears that most mycologists accept these endoparasitic lineages as fungi. Among the logical reasons for their inclusion are, firstly, there is no synapomorphy for the Fungi (11, 88) and, secondly, mycologists had already accepted the highly reduced, nonfungus-like Microsporidia as a part of the kingdom (47), and inclusion of aphelids and rozellids that possess flagella and chitinous cell walls is a logical extension of a growing concept of Fungi. It is interesting to contrast the acceptance of basal forms in the taxonomy of the Fungi to that of Metazoa: The unicellular choanoflagellates are to animals as Opisthosporidia are to Fungi, yet choanoflagellates are not included in Metazoa, because they lack the homoplastic character of multicellularity.

The second classification controversy relates to a recently published revision of higher classification of the Fungi by Tedersoo et al. (118). The goal of their study was to produce a "fungal classification as a user-friendly tool for both taxonomists and ecologists" (118, p. 136). The primary justification for the revised classification was dissimilarity in ages of higher taxa, with basal clades of the tree underrepresented at higher ranks relative to their divergence times from the stem. To generate a time-calibrated phylogeny for which divergence time could be used to translate into taxonomic ranks, the authors used a sample of small and large nuclear rRNA subunit genes for the fungi and calibrated a derived phylogeny with fossils at five nodes. Ultimately, the divergence times suggested a classification with 8 new subkingdoms and 18 phyla, 10 of which were newly created or resurrected. While it is hard to argue against the principle that higher taxonomic ranks should be equivalent in age, the Tedersoo study relies on a data set (rRNA) that is well known to be subject to lineage-specific rates and to support relationships at odds with the consensus tree (such as placing Glomeromycotina as sister to Dikarya). Moreover, once the true diversity of basal lineages is revealed by sequencing of uncultured lineages, the approach would likely result with numerous phyla of early-diverging fungi and only 3 of the Dikarya. On the other hand, the Tedersoo approach provides what is likely a rightful recognition of the true phylogenetic (and possibly physiological/ecological, etc.) diversity of the basal fungal lineages. Time will tell whether the fungal community will adopt this new classification or a more conservative one; currently, the INSDC considers the basal lineages to be *incertae sedis* in likely anticipation of a community consensus emerging.

OVERVIEW OF PHYLOGENOMIC INFERENCE APPROACHES

As the number of fungal genomes continues to rapidly increase in genomic databases, reconstruction of the FTOL is becoming increasingly reliant on phylogenomic inference approaches, which aim to reconstruct the fungal tree (or its parts) using genome-scale sets of gene markers (e.g., 24, 84, 92, 108, 110, 114, 139). For example, a recent phylogenomic study of the budding yeast

subphylum by the Y1000+ Project team analyzed a data matrix comprising more than 2,400 gene sequence alignments from 332 budding yeast species (108). The state-of-the-art practice in phylogenomics involves application of concatenation and coalescence, two approaches that, in recent years, have become standard for phylogenomic inference (**Figure 3**).

The concatenation approach relies on the analysis of the entire phylogenomic data matrix using site-homogeneous models of sequence evolution (i.e., models that assume that all nucleotide or amino acid sites in a gene sequence alignment have evolved under the same substitution process), or site-heterogeneous ones (i.e., models where each site in the alignment is allowed to have its own substitution process), under the assumption that all individual genes share the same evolutionary history. However, it is well established that the histories of genes can differ from each other and from the species tree (e.g., 42) due to the action of biological processes, such as horizontal gene transfer (e.g., 137), hybridization (e.g., 115), positive selection (e.g., 1), gene and whole-genome duplication and/or loss (e.g., 74), and incomplete lineage sorting (e.g., 113).

In contrast, the coalescence approach utilizes a model that explicitly accounts for the presence of incomplete lineage sorting among individual gene trees to infer the species phylogeny (e.g., 67). Under the coalescence approach, the tree for each gene in the phylogenomic data matrix is reconstructed, and the resulting individual gene trees are then used as input to estimate the species phylogeny. The major shortcoming of this approach is that estimation of individual gene trees, especially for deeper branches, can be error prone due to the small number of sites in individual gene alignments and the often large amounts of missing data (e.g., 111). Moreover, for the resolution of deeper branches, coalescence approaches tend to function like consensus methods across gene trees.

Which of the two approaches is more accurate is a matter of active debate (e.g., 29, 111). As a result, several recent fungal phylogenomic studies employ both approaches and explicitly discuss the disagreements between the phylogenies inferred using the two approaches (e.g., 24, 108, 114). Given that the inference of gene trees tends to be more accurate for shallow branches than for deeper ones, a priori, it would seem that the coalescence approach might be most useful for inferring shallow branches of the fungal tree (e.g., at or below the genus level), whereas concatenation might be most useful for deeper ones.

It is important to emphasize that the availability of genome-scale amounts of data for inferring the fungal phylogeny is not a panacea and that several examples already exist where different phylogenomic analyses have generated conflicting results. For example, concatenation analysis of a 1,233-gene, 96-taxon phylogenomic data matrix provided absolute clade support for the family Ascoideaceae as the closest relatives of the families Phaffomycetaceae + Saccharomycodaceae + Saccharomycetaceae (all from Ascomycota, subphylum Saccharomycotina) (109). In contrast, concatenation analysis of a 1,559-gene, 38-taxon phylogenomic data matrix robustly placed the family Ascoideaceae as sister to a broader clade composed of the family Pichiaceae, the CUG-Ser1 clade, the family Phaffomycetaceae, the family Saccharomycodaceae, and the family Saccharomycetaceae (92). In such cases, where different data matrices contradict one another in their support for specific relationships, examination of the phylogenetic signal stemming from each gene or site in the phylogenomic data matrix can shed light onto the causes of the observed incongruence (107). For example, a recent examination of phylogenetic signal across genes and individual sites of a fungal phylogenomic data matrix (as well as of plant and animal data matrices) revealed that support for contentious branches often rests on the phylogenetic signal emanating from one or a handful of genes or one or a few sites in each gene (107).

Several measures are now routinely used to assess and quantify conflict in phylogenomic data, including bootstrap support (31), internode certainty (142), and concordance factor (4) approaches. Bootstrap support is by far the most popular, but also the least appropriate and most

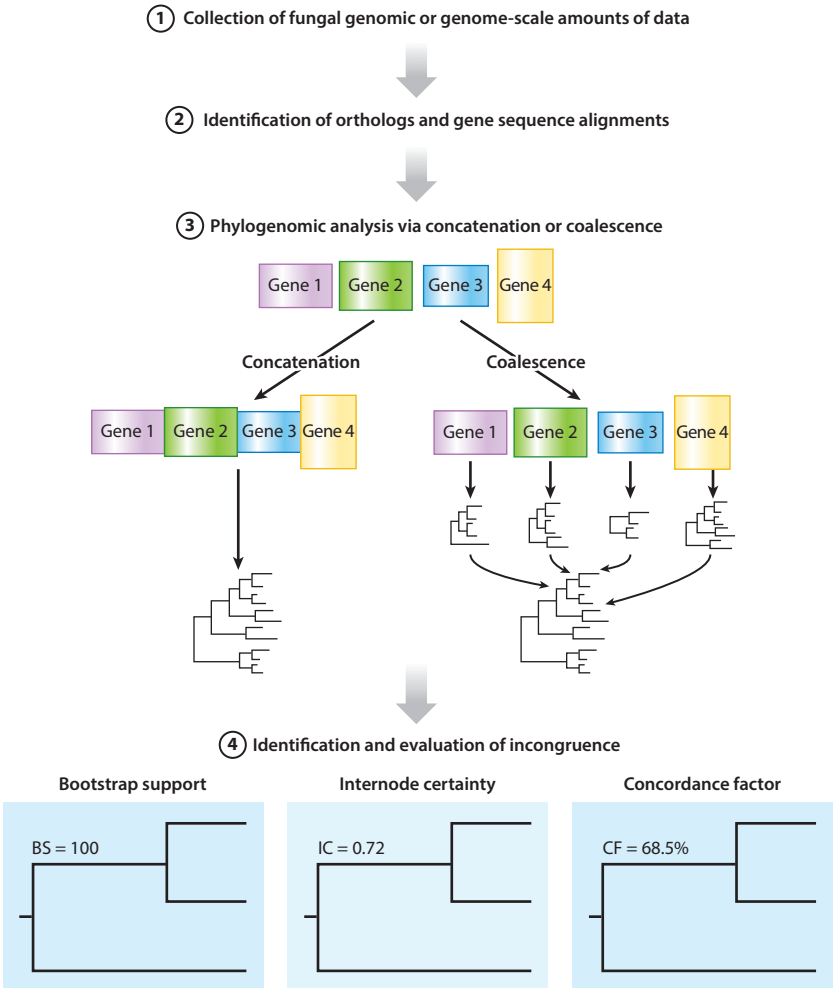


Figure 3

Key steps of a fungal phylogenomic analysis. (①) Once fungal genomic (or other genome-scale amounts of) data have been collected, (②) groups of single-copy orthologous genes are identified, each of which is aligned. (③) Phylogenomic analysis can then be performed either on (*left*) the concatenated data matrix that consists of all the genes analyzed or on (*right*) each gene individually. Genes have different amino acid or nucleotide lengths (box widths) or different numbers of taxa with missing data (box heights). Concatenation produces a single species phylogeny (under the assumption that all genes have the same history as their species), whereas coalescence uses the individual gene trees (whose histories can vary) as input to generate the species phylogeny. Different phylogenomic analyses produce conflicting results for a small fraction of their internal branches, necessitating that (④) each branch of the species phylogeny is examined for the presence of incongruence. This can be done using a wide variety of methods, such as bootstrap support (BS) (which is typically not able to identify incongruence in large data matrices), internode certainty (IC) (142), and concordance factor (CF) (4) approaches. The last two have been developed specifically for phylogenomic studies and are better able to identify major conflicts between different sites or genes in a phylogenomic data matrix. Adapted with permission from Reference 26.

time consuming for phylogenomic analyses. Bootstrapping is a very useful measure of sampling error when the amount of data analyzed is small (e.g., in phylogenies of single or a few genes) (100). However, use of bootstrap in the analysis of phylogenomic data sets can be highly misleading, not only because sampling error is minimal but also because its application will, even in the presence of notable conflict or systematic error, almost always result in 100% values (e.g., 100). In contrast, methods like internode certainty, which aims to quantify topological disagreement or incongruence among a set of trees (e.g., a set of gene trees), and concordance factors, which aim to estimate the proportion of the genome that is consistent with each of the branches of the species phylogeny, are much faster to implement and much more appropriate for comparing and evaluating the phylogenetic agreement and disagreement stemming from the use of hundreds to thousands of markers.

Alternative approaches to sequence-based phylogenetics, such as the use of rare genomic changes (e.g., genetic code alterations, transposon integrations, whole-genome or segmental duplications, insertions/deletions: 93), are useful to consider because they avoid some of the issues, such as model misspecification and long branch attraction. These approaches have yet to become popular, partly because of the lack of computational methods that streamline their identification and partly because of the lack of availability of models for their evolution. The use of presence or absence of gene families seems attractive at phenetic characterization of taxa and may be predictive of ecology, though from a theoretical perspective, it should be highly subject to homoplasy (60, 84). Others have used genome sequences but applied alignment-free approaches to derive the FTOL, such as frequency of protein motifs (21, 72, 127). Despite the fact that phylogenomic analyses fail to use most of the sequence data because paralogs or orphans are discarded, it is actually the sequence evolution that provides the signal. Therefore, the alignment-free approaches seem to generate multiple spurious results, such as Neocallimastigomycota and Microsporidia not grouping with Fungi, among other inconsistencies with the accepted FTOL (21). Both horizontal gene transfer and extreme genome reduction (3, 22, 76) are aspects that likely drive erroneous placements based on gene or protein motif content.

Finally, it will be important to keep in mind that, despite the advent of phylogenomics, it is highly likely that certain branches of the FTOL (particularly those that are short and are deep in time) may be challenging to resolve. Indeed, some short branches may involve so much incomplete lineage sorting, introgression, and hybridization that the search for a single tree may not even be biologically appropriate. It will be important to emphasize in our phylogenomic studies those parts of the fungal tree that remain unresolved or uncertain. A lot of the (often unnecessary) debate about the placement of branches in the fungal tree of life concerns branches that are poorly supported. Clearly identifying challenging-to-resolve branches of the FTOL has the potential to focus the efforts of fungal researchers to test improved and more sophisticated algorithms as they are developed and to analyze data matrices with more comprehensive sampling of taxa.

THE EXPANDING UTILITY OF TREES IN FUNGAL BIOLOGY RESEARCH

Trees have always served not only to guide systematics and classification but also to provide the framework for studying biological mechanisms. They have been widely used by fungal biologists to study the evolution of morphology and ecology (5), gene family evolution (33, 108), and the diversification of fungi through time through incorporation of fossils into time-calibrated phylogenies (12, 70, 114). Now that we have the ability both to generate phylogenetic trees for fungi and to endow each leaf with massive amounts of data (i.e., omics), what have been some examples of the new developments in utilization of trees and insights that have come from them?

The ever denser sampling of genomes from an ever more diverse array of fungi has increased our appreciation for the occurrence and impact of rare recombination events in evolution, such as horizontal gene transfer and hybridization. Both processes were once thought to be rare or absent from fungi, but as the amount of genomic data has grown, the evidence has mounted to support their occurrence across the fungal tree and hint at their ecological importance (35, 89, 101). Examples of the ecological relevance of horizontal gene transfer in fungi abound, and they include the acquisition genes contributing to novel functions [e.g., evolution of fungal gravitropism (79)], genes aiding in ecological arms races [e.g., evolution of detoxification mechanisms (50, 131)], and genes providing new metabolic capacities [e.g., biosynthesis of new secondary metabolites (59, 132]. Phylogenies demonstrated that deep hybridization followed by gene loss have shaped the diversification of the saccharomycete yeasts (74), and multiple hybridization events leading to diploidization on more recent timescales are prevalent in the extremotolerant halophile *Hortaea werneckii* (36).

Low-cost genome sequencing is democratizing and accelerating progress toward discovering, characterizing, and describing the tips of the FTOL. Decades ago, fungal researchers were early adopters of rRNA sequencing to support taxonomic descriptions (62, 130), and they are now leading the way to applying genome sequencing to taxonomy (D. Libkind, N. Čadež, D.A. Opulente, Q.K. Langdon, CA Rosa, et al., unpublished manuscript). Taxogenomic approaches that unify the description of new species with the simultaneous publication of their genome sequences have several advantages over traditional approaches, at least when there are sufficient genome sequence data available for the backbone of the relevant clade. Although it is mainly possible in the subphylum Saccharomycotina, the necessary genomic sampling across taxa to enable taxogenomics will likely soon be available for many other fungal genera (e.g., *Aspergillus*, *Mortierella*) and clades [e.g., subphyla Taphrinomycotina, Agaricomycotina (122)].

How could taxogenomics improve fungal systematics? First, as discussed above, genome-scale phylogenies afford the most reliable placement of a species. Thus, newly discovered taxa are more likely to be stably assigned to a genus, improving nomenclatural stability. Second, genome sequence data put to rest concerns about whether the new species being described is really distinct from known species or whether it might be an interspecies hybrid. For example, the discovery and description of *Saccharomyces eubayanus*, the wild ancestor of hybrid lager-brewing yeasts (*S. cerevisiae* × *S. eubayanus*), were coupled with genome sequencing to lay to rest a persistent and seemingly intractable taxonomic debate about the interpretation of multilocus data from several complex hybrids (65). More than a dozen recent yeast species descriptions have followed suit (D. Libkind, N. Čadež, D.A. Opulente, Q.K. Langdon, C.A. Rosa, et al., unpublished manuscript). In cases where lineage sorting is not complete and hybridization is ongoing, the power of genome-scale data can help in species delimitation.

Third and most importantly, genome sequencing allows the rich physiological and metabolic data collected as part of taxonomic descriptions to be leveraged to understand the genetic mechanisms that underlie these traits. In the premolecular era, these traits were the main characters available for phylogenies and classification. Although these characters are highly homoplastic and have little phylogenetic signal (80), they now have new potential to provide insight into the genes and traits that differentiate close relatives, as well as the evolutionary mechanisms that led to this diversity. For example, the well-known budding yeast *Yamadazyma* (*Candida*) *tenuis* can consume the disaccharide lactose, while its newly described relative *Yamadazyma laniorum* cannot due to the loss of genes encoding a lactose permease and a β-galactosidase (39). As the FTOL becomes increasingly populated by genomes and genotype-phenotype relationships become clearer, we expect taxogenomics not only to facilitate the formal description of fungal biodiversity but also to provide increasingly concrete insight into its making.

Whole-genome sequencing is also providing the data required to finely dissect closely related species into genetically isolated units. The human pathogenic *Cryptococcus* species complex has been extensively studied for its disease importance, with many hundreds of isolates collected globally from environmental, animal, and human infections. The lineages have been subjected to study with a variety of techniques from serotypes based on not only whole-genome sequencing but also antigen tests, multi-locus sequence typing, and proteome spectroscopy. Initial classification had a single species (*C. neoformans*) with four serotypes (A–D) that were later given species or variety names (e.g., *C. neoformans* var. *grubii*, *C. neoformans* var. *neoformans*, and *C. gattii*) (41). Further work examining global isolates with multilocus sequence typing and whole-genome sequencing provided clear evidence for further subdivision into discrete genetic clusters, and one group of authors proposed additional naming of species based on estimates of divergence times and some measurable molecular characteristics (40). Currently, there are seven named species of *Cryptococcus*, but recent sampling and the discovery of new lineages provide evidence within the *C. gattii* complex for a further splitting into an eighth and ninth species of the *Cryptococcus* species complex (30). The renaming of each lineage into species remains contentious because clonality leads to the formation of linkage disequilibrium (119), and the current system lacks a standard for how hybrids, which are common among the A and D lineages, are to be called (61). The molecular and sequence-based methods are enabling the discovery of additional lineages from cultured isolates, and there could be additional lineages discovered should appropriate markers or metagenomic methods be applied to additional environmental or zoonotic reservoirs. In contrast, another study recognized at least four species among what was the *Histoplasma capsulatum* complex, where there are strong phylogeographic patterns among the isolates, which simplified the arguments for naming (105). How names are chosen and applied to recognize species in a complex requires a balance acknowledging the utility of stable names in communicating about disease, but also the need to clarify differences when there are genetically distinct microbes.

Phylogenies give insight into the process of diversification, such as estimates of rates of speciation and extinction (91). Particularly when coupled with character or ecological traits, phylogenies can test relationships between traits and diversification. However, proper use of phylogenies for estimation of these parameters requires densely or unbiasedly sampled trees. A recent example of a densely sampled tree is the megaphylogeny of the mushroom fungi based on 5,284 species (125). A phylogenomic tree of 104 mushroom taxa was used as a backbone constraint for the time-calibrated phylogeny, with the leaves mostly composed of species with data from three loci. The results suggested an increase in diversification rate beginning in the Jurassic and continuing today that coincided with the origin of the typical mushroom fruiting form with a stalk and cap. These results and those of Hinchliff et al. (49) provide a powerful demonstration that massive phylogenies are within reach and can be valuable for studying the relationship of traits to diversification. Ideally the fungal research community should expand upon the structural and biochemical databases (18) to develop a kingdom-wide trait database that could be used to study nutritional modes, ecological niches, and biogeography on a massive scale.

Finally, genome-scale phylogenies have enabled reconstruction of the evolution of both primary and secondary metabolic pathways (133), especially in lineages where the sampling of genomes is dense and the metabolic traits in question have been well characterized. For example, one study (108) recently examined the evolution of 45 distinct metabolic traits (and, in some cases, the associated genes and pathways) in budding yeasts of the subphylum Saccharomycotina. This study revealed widespread loss of traits and pathways in organisms from the budding yeast common ancestor to present-day taxa, suggesting that reductive evolution (i.e., evolution through loss of traits) has significantly contributed to the evolutionary diversification of budding yeasts. The development of a robust phylogenetic hypothesis allowed for reconstruction of some of the

metabolic traits present in the common ancestor of the budding yeasts and estimation of rates of loss and gain of carbon metabolism across the tree.

THE UNCHARTED TERRITORY: DARK MATTER TAXA AND THE UNCULTURED MAJORITY

A complete FTOL should represent the true diversity of the fungal kingdom, sampled for phylogenetic markers in a way that allows a robust phylogenetic hypothesis to be generated. Two important questions to answer are, How well do we actually know fungal diversity, and how do we go about filling out the leaves? First, how completely have we sampled the FTOL for genetic data? As of the end of the year 2019, there were 2,061 fungal species with some form of nuclear genome assembly in GenBank, many of which were collated by the Joint Genome Institute's MycoCosm website (**http://jgi.doe.gov/fungi**; 37). As impressive as this might be, there are 125,000 described fungal species and an estimated two to five million more are undescribed (13, 45), meaning there is a long way to go before the far reaches of the tree are elucidated. On the other hand, there is some form of sequence data for 45,404 completely identified fungal species. If one adds in environmental or incompletely determined samples, there are 104,307 fungal TaxNodes in GenBank that are not assigned to species and represent mostly rRNA genes from environmental studies or from cultures that are incompletely identified or novel species. The amount of diversity with respect to actual species is hard to determine from these environmental TaxNodes, but the alpha diversity of the environmental data in GenBank is clearly greater than that of the named species. Summarizing GenBank data provides opportunities for understanding gaps between named and environmental sequences, and analysis of the existing data provides an opportunity for megaphylogenies. For example, analysis of the 9,329 fungal 18S rRNA sequences available from the SILVA SSU Ref database revealed 33% of higher taxa (orders and above in GenBank classification) were not monophyletic (136). This is not too poor of a result for a single gene; however, more promising are reference trees such as those provided by the Open Tree of Life project, which have the potential to use supertree methods to combine highly supported reference trees based on phylogenomic approaches with single gene trees from monographic studies or environmental metabarcoding studies (49).

What do we know that can explain the huge gap between the named and the estimated fungal species? One possibility is that we know all of the major groups of fungi, but the community is only moving slowly through the discovery and description phase. Alternatively, there may be a huge disconnect between the lineages of fungi that have been described and those that exist in the environment. Studies of environmental DNA shed light on the taxa that are missing from the classification and gaps in sampling (**Figure 2*b***). Since the turn of the century the number of studies investigating fungal communities using environmental DNA has skyrocketed. In the early days of environmental DNA studies, a number of lineages were identified that suggested undescribed class- or phylum-level diversity (102, 123, 124). Uncultured and undescribed fungal species and lineages have been called dark matter fungi in parallel with usage by bacteriologists (38, 99). The analogy to bacterial diversity may be quite appropriate. In a global soil metabarcoding study using ribosomal internal transcribed spacer (ITS) sequences, only 9.8% of the 45,000 operational taxonomic units (OTUs) matched vouchered and named material (117). Whether this value is globally applicable depends on what type of habitat is sampled, such as the human mycobiome, where most species are known (73), or marine ecosystems, which are a highly unstudied environment from the mycological perspective (87).

Moreover, the values obtained from metabarcoding studies might underestimate fungal diversity. Because of well-known issues with primer biases, several lineages are highly biased against

depending on primers or loci employed (63, 86, 97, 116). Further, criteria for defining species from DNA surveys are ad hoc, making it unclear whether they are estimates of species diversity. A better estimate of true fungal diversity would come from approaches that are less biased, such as metagenomics. However, these studies have yet to be conducted for fungi (**Figure 2*c***), in part because their genome assembly and identification are more challenging (but see, e.g., 27). One interesting approach to understanding biases in the vast metabarcoding literature based on rRNA was to estimate fungal diversity in soil using PCR amplification of the *RPB2* gene (126). Using a mock community researchers found that *RPB2* was less biased than an ITS rRNA-estimated community, and they detected potentially novel lineages of fungi from soil using the *RPB2* marker. These biases notwithstanding, one takeaway of the environmental DNA studies of fungi is that most of the deep branches in the kingdom have been discovered, and the new diversity will be described closer to the tips (**Figure 2*b***).

Marker-based and metagenomic studies, even in ideal situations, suffer from the fact that the linkage among markers is unknown. Therefore, while gene function and taxonomies can be profiled, it is difficult to estimate total species richness and phylogenetic affinities from these data. A promising recent development is the use of single-cell genomic methods, which, when applied to complex samples, allow individual genomes to be partially sequenced (134). Access to genome sequences from individual cells makes it then possible to accurately place unculturable fungi in the FTOL (2, 9, 24). The method could be appealing to grow the FTOL, but as currently applied, it requires the ability either to sort the cells into individual droplets or wells using flow cytometry or to microdissect the cells. The former method is scalable to high throughput, but the latter method allows a visualization of the cells. Being able to both use fluorescence-activated cell sorting to sort as well as visualize cells is a much-needed technological advance. Moreover, efforts are needed to develop methods to work directly with mycelium (the most common fungal biomass in most ecosystems).

Ideally, genomes would be tied to images, ecosystem metadata, and environmental DNA sequences to provide as much data as possible on taxa that cannot be brought into pure or dual culture with a host. For well-known taxa, such as the enigmatic *Entorrhiza*, genome sequencing from individual teliospores would provide much-needed data to resolve their phylogenetic placement. Single-cell genomics, however, just like metagenomic approaches, presents a major conundrum for taxonomy because mycology exists in a morphology-based classification system. Species are named and validated under a rather thorough process that requires a type to exist (48). As the number of taxa in GenBank known only from DNA sequence continues to grow and dwarf the fully named taxa, it has become attractive for mycologists to consider a taxonomy that includes the use of sequence-only evidence. A number of proposals have been made, including using DNA sequences as types (44) or using candidate taxa in an informal system, as done by bacteriologists (138). This topic is one of considerable rancor and difference of opinion (69, 99, 103). The utility of a formalized naming system for environmental DNA sequences is self-apparent; meaningful biological entities could be tracked across sites and studies. Moreover, there would be a means of quantifying and identifying missing taxa and gaps in taxonomic knowledge. From our perspective, it seems that provisional names are sufficient to circumscribe environmental DNA sequences, such as the LKM11 clade, Soil Clone Group 1, or UCL8_022698. In most cases, there seems to be little reason to inject ghost taxa into the formal classification, just as placeholder Wikipedia pages lack utility. However, there may be specific instances when one would like to formally describe a new *Aspergillus* species when overwhelming evidence points to its existence, though it has never been directly observed, and the mechanism to support formal naming of unobserved taxa should be discussed. For example, it may be acceptable to allow naming of species when the genomes or metagenomes of closely related taxa can be used as evidence. Even so, any system that

merely catalogs novel DNA sequence types without clear scientific reasoning for naming new species seems of low taxonomic utility. One possibility is to allow use of provisional names clearly indicated as such, perhaps with quotation marks, and a formal registry of those names that can be tied to sequences, preferably without the complicating rules of priority that can lead to taxonomic arms races as scientists compete to be the first to name species. Care should also be taken so that new taxonomic proposals do not discourage the deposition of preserved or living type tissue whenever possible; this long-standing practice ensures replicability and will ultimately enable connections between genotype and phenotype in a way that DNA sequences cannot do alone.

CONCLUSIONS

From the tips to the root, there is much to be revealed about the structure and shape of the FTOL. Yet, more than ever, the structure of the tree and how to assemble it are being clarified. Genomic methods have revealed that with enough data, most relationships can be resolved. These include species delimitation and identification of recent or ancient hybridization events as well as deeper branches. However, it is important to identify systematic biases in the data, because traditional methods, such as bootstrapping, can provide strong support for incorrect relationships (100). More appropriate are measures of internode certainty and concordance factors, which address support for branches of the species phylogeny across genes. Massive phylogenies are being assembled thanks to the use of supertree methods fueled by both phylogenomic analyses at deep levels and easily acquired marker-gene data, which often are sufficient to resolve relationships among related species. As phylogenies increase in size, we need to develop the tools to visualize them, improve and annotate them by community consensus, add trait data to them, and position environmental sequences in the mix, such as the Tree-Based Alignment Selector toolkit (16). Repositories of reference user trees will make major contributions to reconciling the huge difference between known and dark taxa and leverage phylogenies for understanding character evolution and as a guide for bioprospecting. Although single-cell genomics may allow us to fully place dark taxa into a robust phylogenetic framework with potential metabolic data, effort must be directed into visualization of uncultured cells as well as going from sequence to ecological function, a so-called reverse ecology (64). While we identify multiple controversial aspects to fungal classification, we encourage these healthy debates about how to input trees into a usable classification. Despite these advances and challenges, a synopsis of the current higher-level fungal phylogeny shows that much work needs to be done to resolve the branching order at the base of the FTOL, and we identify a number of difficult-to-resolve relationships, such as placement of Blastocladiomycota, that require more sophisticated models or branches that require more taxon sampling or genomic data, such as placement of Aphelidiomycota and Entorrhizomycota. It is an exciting time to be a fungal biologist to witness the dramatic changes that are occurring in how phylogenies are produced and the stories that they tell.

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