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Fungal species concepts in the genomics era

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Abstract: The 140 000 or so fungal species reported so far are heterogeneously defined based on varying criteria such as morphological, physiological, mating, and (or) molecular features. Incongruences are common among traits used to separating closely related species and it is often difficult to compare fungal taxonomic groups defined based on different species recognition criteria. Though DNA sequence-based classification and identification have been made, a consensus has not been reached, primarily due to intrinsic limitations in the proposed one or a few genes. Here, I argue that the fundamental reason for the observed inconsistencies is that speciation is a stochastic process with the emergence and fixation of different traits influenced differently by many non-deterministic factors such as population size, random mutation, mode(s) of reproduction, selection imposed by interacting biotic and abiotic factors, and chance events. Each species concept attempts to capture one or a few traits emerged in the continuous process of speciation. I propose that a genome sequence-based classification and identification system could unify and stabilize fungal taxonomy and help integrate taxonomy with other fields of fungal biology. The genomic species concept could be similarly argued for other groups of eukaryotic microbes as well as for plants and animals.

Key words: species concepts, yeasts, molds, mushrooms, trait evolution, genome sequence, genome species concept.

Résumé : Il existe environ 140 000 espèces fongiques rapportées à ce jour, mais elles ont été définies sur des bases hétérogènes selon divers critères, que ce soit morphologiques, physiologiques, de reproduction ou de propriétés moléculaires. Les incohérences sont nombreuses parmi les caractères employés pour séparer des espèces proches et il est souvent difficile de comparer les groupes taxonomiques de champignons définis sur des bases différentes en matière de reconnaissance des espèces. Bien que la classification et l'identification fondées sur des séquences d'ADN ait été faite, aucun consensus n'a encore été trouvé principalement en raison des limitations intrinsèques liées au(x) gène(s) proposé(s). Dans cet article, l'auteur avance que la raison principale pour les incohérences observées est que la spéciation est un processus stochastique où l'émergence et la fixation de différents caractères est influencée par plusieurs facteurs non-déterministes distincts tels que la taille de la population, une mutation aléatoire, le mode de reproduction, la sélection imposée par des facteurs biotiques et abiotiques, ainsi que le hasard. Chaque concept de l'espèce tente de capturer un ou plusieurs caractères apparus au cours du processus continu de spéciation. L'auteur propose qu'un système d'identification et de classification fondé sur la séquence du génome pourrait unifier et stabiliser la taxonomie des champignons et aider à intégrer la taxonomie avec d'autres champs d'étude en biologie des champignons. Le concept d'espèce fondée sur le génome pourrait également s'appliquer à d'autres groupes de microorganismes eucaryotes, de même qu'aux plantes et aux animaux. [Traduit par la Rédaction]

Mots-clés : concepts d'espèce, levures, moisissures, champignons, évolution des caractères, séquence du génome, concept d'espèce fondée sur le génome.

Introduction

Species is a fundamental term in biology. However, species can mean different things for scientists in different fields and (or) working on different groups of organisms. While most biologists know approximately what species means to them, no one definition has satisfied all biologists. In macroorganisms such as plants and animals, features that can be seen by the human naked eye have been the dominant criteria used to define species. Indeed, most species of plants and animals were primarily recognized based on macromorphological features by the end of the 19th century. However, due to their intrinsic diversity, different sets of morphological features have been used to define species for different groups of plants and animals. In contrast, taxonomy of microscopic organisms such as those in the Bacteria and Ar-

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chaea Domains did not start until the end of the 19th century when methods for identifying their micromorphological features, and later their structural, biochemical, and genetic features, became available. Fungal taxonomy contains elements and criteria used for both macroorganisms and microorganisms. For macroscopic fungi such as those that produce fruiting bodies (e.g., mushrooms) that are visible to the naked human eye, the morphological species concept dominated this group of fungi in the early literature and their taxonomic history largely paralleled those of plants and animals, dating back to the 18th century (Linnaeus 1753). In contrast, for microscopic fungi such as yeasts and filamentous molds, their taxonomic history parallels those of prokaryotes, with major advancements starting around the early 20th century (Kurtzman et al. 2011; Kendrick 2014).

Over the past century, a variety of species concepts have been proposed and applied to define species in different groups of organisms. These include the quasispecies concept for viruses (Domingo and Perales 2019); the micromorphological, physiological, ecological, and DNA–DNA hybridization-based species concepts for Bacteria and Archaea (Murray and Holt 2001); and the morphological, phylogenetic, evolutionary, and reproductive species concepts in eukaryotes, including fungi (Cai et al. 2011; Zachos 2016). However, different groups of fungi (and other organisms) are often defined based on different species concepts using different criteria, making comparative studies among species often difficult to perform. In addition, the same group of organisms may show different patterns of grouping using different sets of criteria or species concepts (e.g., Singer 1986; Dettman et al. 2001; Kwon-Chung et al. 2017). As a result, taxonomic revision is a common feature in the current fungal taxonomic literature, often involving moving organisms into different genera and even families (e.g., Cai et al. 2011; Wu et al. 2016; indexfungorum.org). In this paper, I explore the potential reasons for the inconsistency among the species concepts and propose a potential solution for moving forward. I first briefly describe the commonly used fungal species concepts. I then outline the major changes that could happen during speciation and how those changes may be related to the different fungal species concepts. This is followed by highlighting a few features of fungi that distinguish them from plants, animals, and prokaryotes and how these features may have contributed to complications in fungal taxonomy and fungal species recognition. I finish by discussing how genomics can help bring the diverse fungal species concepts together and integrate fungal taxonomy into the broad field of fungal biology.

Commonly used species concepts in fungi

Both macro- and micromorphological features have dominated the fungal taxonomy literature, especially in its early history (Singer 1986). Some fungal species are defined completely based on macromorphological and (or) microscopic features, while others include morphological features for at least part of their species descriptions (e.g., Linnaeus 1753; Singer 1986; Kurtzman et al. 2011; Wu et al. 2016). Indeed, dichotomous keys for species recognition based on morphological features permeate the fungal taxonomy literature even today (e.g., Zheng et al. 2019). Though not intended nor substantiated, such dichotomous keys often give the impression for the order on the emergence of those specific morphological traits.

Since the beginning of the 20th century, with advances in technology and biomedical sciences, other features have been progressively introduced to help define species, including fungal species. Those features include ecological factors (both abiotic and biotic interacting factors), physiology, reproduction, and DNA sequences. Parallel to these developments were expanded discussions and conceptual changes in how species should be defined and recognized. There are over 30 species concepts in the biological literature (Zachos 2016), including common ones such as the morphological, ecological, phenetic, biological, recognition, evolutionary, genotypic cluster, and phylogenetic species concepts that have been applied to delineate fungal species (Table 1). These concepts emphasize different but often overlapping features of organisms. Each of the criteria has been and continues to be featured in defining fungal species. Example literatures showing fungi defined based on different criteria and using different species concepts are presented in Table 1. These literatures reported some of the environmentally, economically, and medically very important fungi, including the cultivated button mushroom *Agaricus bisporus* (biological species concept, Kerrigan et al. 1994; recognition species concept, Callac et al. 2003); the common indoor mold *Penicillium chrysogenum* (genotypic cluster species concept, Banke et al. 1997); the wild gourmet mushrooms *Tricholoma nauseosum* from Scandinavia (ecological species concept, Kytövuori 1988) and *Thelephora ganbajun* from Southwestern China (morphological species concept, Zang 1987); and the human fungal pathogens in the genus *Cryptococcus* such as *Cryptococcus deneoformans* and *Cryptococcus decagattii* and other sister species (phylogenetic species concept, Hagen et al. 2015).

Since the late 1990s, phylogenetic analyses based on DNA sequences at one or multiple loci have been increasingly used to define fungal species (Taylor et al. 2000; Schoch et al. 2012). In an increasing number of cases, DNA sequence or genotype information has become the main or only information used to separate strains into distinct species (e.g., Weir et al. 2012; Hagen et al. 2015). This approach has led to the identification of a large number of new and cryptic species within previously recognized fungal species. For example, a recent review of 51 studies involving fungi from 16 genera of diverse taxonomic groups showed that each of the originally

Table 1. Commonly used species concepts in fungi.

Species concept	Key criterion	Early proponent	Fungal examples
Biological	Interbreeding producing viable and fertile offspring	Mayr 1942	Kerrigan et al. 1994
Ecological	Sharing a niche containing the same biotic and abiotic interacting factors	Van Valen 1976	Kytövuori 1988; Trudell et al. 2017
Evolutionary	Shared evolutionary history, tendency, and fate	Simpson 1951	Kwon-Chung et al. 2017
Genotypic cluster	Distinct genotypic clusters that maintain cohesion within individual cluster but with no or few intermediates between clusters	Mallet 1995	Banke et al. 1997
Morphological	Distinct morphological feature(s)	Regan 1926	Linnaeus 1753; Zang 1987
Phenetic	High overall phenotypic similarity (quantitative)	Michener 1970	Singer 1986; Kurtzman et al. 2011
Phylogenetic	Monophyly: irreducible group of organisms descended from a common ancestor, with all members of a species possessing certain derived traits	Rosen 1979	Hagen et al. 2015; Wu et al. 2016
Recognition	Shared mate recognition	Paterson 1985	Callac et al. 2003

described species contained an average of three cryptic species (Matute and Sepulveda 2019).

However, significant issues have also emerged in these approaches. At present, there is no standard as to which gene(s) should be analyzed, how much sequence divergence is needed, and what statistical support is required at both the individual locus and the combined concatenated sequence levels to call different strains as belonging to different species. In the review by Matute and Sepulveda (2019) on identifying fungal species boundaries based on DNA sequences, the mean number of loci used to define species was ~4 (range 1–15), with over half of the studies relying on 1–2 loci. Similarly, the amount of sequence divergence and statistical support separating closely related species based on individual genes varied significantly. In addition, most taxonomic studies and new species descriptions rely on relatively few samples. Indeed, further analyses of some of the closely related DNA sequence-based "phylogenetic or genotypic cluster species" with larger sample sizes revealed abundant signatures of recombination among them, indicating that some of these species in fact belong to the same reproductive group in nature and their separate species designations represent sampling artifacts (e.g., Xu et al. 2016). Furthermore, comparative analyses of genome sequences from both closely and distantly related fungi often show evidence of recombination, hybridization, and introgression (Steenkamp et al. 2018). At present, there is significant heterogeneity among fungal taxonomists in the criteria by which different strains are called different species. Often, the convenience and availability of molecular markers and the tradition(s) of the specialist taxonomists working on the specific group of fungi play a large role in determining how species are defined for most fungal groups (Matute and Sepulveda 2019).

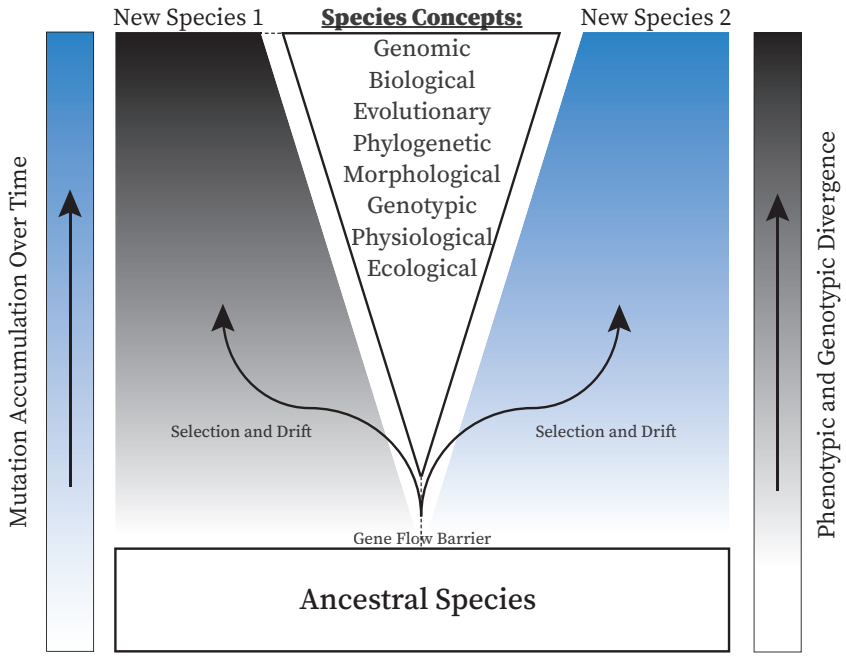
Fungal speciation and relationship to species concepts

A species is the product of speciation, a dynamic and ongoing process. At present, the mechanisms for fungal speciation are little investigated. However, similar to the speciation mechanisms observed in prokaryotes, other eukaryotic microbes, plants, and animals, both geographic separation and ecological specialization have likely played significant roles in generating fungal diversity, contributing to allopatric and sympatric speciation. In all groups of cellular organisms, including fungi, a fundamental feature of both sympatric and allopatric speciation is the establishment of a barrier for gene flow between sub-populations due to the separation of the ancestral population into sub-populations, either ecologically or geographically. Over time, the sub-populations diverge, through mutation accumulation, selection, and (or) drift, and form distinct geographic, ecological, morphological, and (or) reproductive groups (Fig. 1).

In an idealized situation, during speciation, allele frequencies between diverging populations will change in response to selection and (or) genetic drift, leading to significant population genetic differentiation and generating different genotypic clusters in different populations and with no genotype intermediates, creating different genotypic cluster species (Mallet 1995). Genotypic clusters and genetically differentiated fungal populations are commonly found in nature in many fungal species where no (few) alleles are shared between populations (e.g., Banke et al. 1997; Xu et al. 1997; Ashu et al. 2018; B. Wu et al. 2019). However, in some of these studies, the identified genotypic clusters were called different species (e.g., the common mold *Penicillium*, Banke et al. 1997), while in others, due to other shared features such as being sexually compatible, they are not elevated to species level but are referred to as differentiated subpopulations or different varieties of the same species (e.g., the commercial button mushroom *Agaricus bisporus* var. *bisporus*, var. *burnettii*, and var. *eurotetraspora*; Kerrigan et al. 1994; Xu et al. 1997, 1998; Callac et al. 2003).

During evolution and population divergence, random mutations continuously emerge, and separate populations accumulate different mutations which may be

Fig. 1. Simplified and idealized diagram of speciation and its relationship to fungal species concepts. As populations separate by a geographic and (or) ecological barrier to gene flow, independently acting forces such as mutation, selection, and drift would lead to sub-populations with distinct evolutionary trajectories. Over time, these genetically differentiating sub-populations may acquire different features in their morphology, ecology, physiology, genotypes, and (or) mating system, which have traditionally served as evidence for species delimitation for different species concepts. In different fungal groups or different sub-populations, these features do not necessarily arise at the same time or in the same order. Consequently, species defined based on different species concepts may be inconsistent with each other, especially during early stages of speciation. A genomic species concept would eliminate the inconsistencies in fungal taxonomy and help integrate diverse fields of fungal biology.



fixed through either selection and (or) genetic drift. If the new mutations impact morphological, ecological (e.g., substrate utilization for saprophytes or host specialization for pathogens and symbionts), or sexual reproductive traits and these traits become fixed in different populations, then morphological, ecological, and biological species as inferred based on their respective species concepts and criteria could be recognized (Regan 1926; Mayr 1942; Van Valen 1976). Because new mutations influencing morphological, ecological, and reproductive traits can happen at any time, and with no particular order, during population divergence, novel species could be recognized at different times based on the different species concepts (Table 1). Consequently, inconsistencies among the different species concepts could result in contradictory species delineations (e.g., Dettman et al. 2001). Even for the morphological species concept, the use of different morphological features can provide different classifications for the same organisms. The inconsistency and confusion in fungal taxonomy was especially prevalent for fungi where no asexual reproductive structure was observed during initial species description. Previously grouped to the large phylum "Fungi Imperfecti" for organisms where only asexual reproductive structure was observed, each organism was given an anamorphic species name. If the sexual form was later discovered, an additional teleomorphic species name and classification would be provided for the same organisms, often in different genera, family, or even higher taxonomic rank (e.g., Cai et al. 2011; Qiao et al. 2011; Kwon-Chung et al. 2017).

Given sufficient time, the diverging populations may accumulate mutations that impact most or all types of traits, causing each individual sub-population to have its own distinct morphological, ecological, and reproductive features. With the accumulation of a large number of mutations, samples from the diverging populations based on sequences at one or multiple loci could be separated into different, statistically well-supported monophyletic groups, or phylogenetic species (Rosen 1979). However, it is also possible that despite extensive sequence divergence, there might be no or little change in morphological, ecological, or reproductive traits between the populations such as in the human pathogenic *Cryptococcus gattii* species complex (e.g., Hagen et al. 2015; Kwon-Chung et al. 2017; You and Xu 2018). Indeed, different species concepts use different phenotypic and (or) genotypic features, with some of the features may have emerged in one group of fungi but not in others. Even if multiple classifiable features are present among members of a target fungal group, their evolutionary relationships inferred based on one feature may not be the same as those infered based on other features due to the potentially independent evolutionary trajectories of differ-

ent traits. Consequently, consistencies among species concepts should not be an expected outcome.

In nature, speciation unlikely fits the idealized situation described above (Fig. 1). Given the high dispersal abilities of fungal propagules, gene flow in fungi can obscure the geographic and ecological barriers, resulting in frequent geographic and (or) ecological overlaps among fungal populations. This is especially true for fungi closely associated with humans such as opportunistic human fungal pathogens *Aspergillus fumigatus* (Ashu et al. 2017) and *Candida tropicalis* (J.Y. Wu et al. 2019), and the globally cultured mushroom *A. bisporus* (Xu et al. 1997, 1998) where anthropogenic factors such as human travel and commercial trade have likely contributed the spread of fungal genotypes throughout the globe. Such gene flows create admixture populations where hybridization among diverging populations could occur. Indeed, hybridization has been frequently reported in fungi, even between phylogenetically well-separated species (Mixao and Gabaldon 2018; Samarasinghe and Xu 2018; Steenkamp et al. 2018).

Another complicating factor for classification in fungi is their developmental and phenotypic plasticity in many traits. Indeed, variations among individuals of the same species and overlaps in trait values between species are common for virtually all fungal traits, including morphological, ecological, reproductive, and developmental traits (e.g., Weir et al. 2012; Hagen et al. 2015; Wu et al. 2016; Zheng et al. 2019). At the genetic level, incomplete lineage sorting could also result in false signals in population relationships (Steenkamp et al. 2018). With increasing commercial trade and human travel, as well as our increasing influence on the environment, both the genotypic and phenotypic features of fungal populations and species could be severely impacted (e.g., Xu et al. 1997; Chen et al. 2016; Samarasinghe et al. 2020). In the section below, I highlight some of the features of fungi and their implications for fungal species concepts.

Features of fungi

The term fungi (singular: fungus) has been in our lexicon for centuries; however, there is no universally accepted definition of fungi (Richards et al. 2017). Traditionally, fungi were defined as a kingdom of heterotrophic eukaryotic organisms with cell walls containing chitin but not cellulose (Kendrick 2014). However, the features of heterotrophy and chitinous cell wall without cellulose are not exclusive to fungi but are shared by several other groups of non-fungal eukaryotic microorganisms. Investigations so far have identified no unambiguous morphological, subcellular, or biochemical synapomorphies of Fungi (Richards et al. 2017). Instead, the emerging consensus among biologists for what constitute the "Fungi" is a phylogenetic definition which defines Fungi as "the smallest crown clade containing *Rozella allomycis*, *Batrachochytrium dendrobatidis*, *Allomyces arbusculus*, *Entomophthora muscae*, *Coemansia reversa*, *Rhizophagus intraradices*, *Rhizopus oryzae*, *Saccharomyces cerevisiae*, and *Coprinopsis cinerea*" (Hibbett et al. 2018). Indeed, the lack of unambiguous synapomorphies to define Fungi also trickles down to lower taxonomic ranks, including at the species level for many groups of fungi (Richards et al. 2017). Not surprisingly, partly due to this reason, the number of estimated fungal species on Earth varies widely, from around 700 000 to over 12 million (Mueller 2011; Blackwell 2011; B. Wu et al. 2019). Several features of fungi have contributed to the lack of a consensus fungal species concept and the broad variations in the estimated number of fungal species.

The first feature is the broad diversity of morphological forms among fungi. Fungal morphological forms range from unicellular yeasts to multi-cellular molds and macroscopic mushrooms. Unlike plants and animals, of which the adult individuals are often visible to the human naked eye during most of the individual's life span, microscopic fungi such as yeasts and molds are usually not visible. As a result, new species recognition often requires advanced tools that may not be readily available to the broad community. For macroscopic fungi, though their fruiting bodies can be visible to the naked eyes, for several reasons, their fruiting bodies can be difficult to find: (*i*) fruiting body development is highly sensitive to a variety of environmental conditions, (*ii*) different stages of fruiting body development can be morphologically very different, and (*iii*) the fungal fruiting body represents only a brief (and in a sense, non-essential) stage of their life cycle.

The second feature is the modes of reproduction and reproductive barriers in fungi. Like other microorganisms, all fungi are capable of asexual reproduction to produce genetically identical spores or vegetative hyphal cells. This feature distinguishes fungi from most plants and animals. On the other hand, unlike prokaryotes, many fungi are also capable of sexual reproduction through mating and meiosis. However, interfertility among natural strains can be often difficult to assess in the laboratory or nature due to our limited knowledge of the conditions conducive for their mating and (or) meiosis, and the potential rapid loss of sex for strains in both natural and laboratory environments (Xu 2002). On the other hand, divergent populations with significant genome sequence and chromosome structure differences can mate and generate viable sexual progeny (e.g., Samarasinghe and Xu 2018; Steenkamp et al. 2018; You and Xu 2018). Furthermore, sterile fungal hybrids can reproduce asexually by mitosis and generate abundant genetic diversity through mitotic recombination, contributing to their rapid adaptations to novel environmental conditions (Mixao and Gabaldon 2018; Dong et al. 2020; Samarasinghe et al. 2020). Indeed, the uniqueness of fungal reproduction has made the application of bio-

logical species concepts to define fungal species often not practical (Singer 1986; Kurtzman et al. 2011).

The third feature is related to the history and traditions of the mycology community. Aside from the few early mycologists who worked on the taxonomy of many groups of fungi, most mycologists, especially those working over the last 30 years, have tended to focus on a relatively small group of fungi through their research career. In addition, they often follow traditions established previously for the same or closely related groups of fungi in identifying and describing new fungal species. This is frequently necessary to determine whether the new specimens belong to previously described species or represent potentially new taxa. Consequently, over time, increasing knowledge and experience are required for individual taxonomists, with most such knowledge applicable only to certain taxonomic groups. As such, over the last 200 years, taxonomists working with different groups of fungi have diverged in the characters that they have used to identify and describe different groups of fungi, contributing to making broad comparisons among different fungal groups difficult. For example, as mentioned briefly above, most fungal taxonomists working on microscopic fungi such as yeasts tend to use traits similar to those in prokaryotes, including micromorphological traits, substrate utilization patterns, DNA–DNA hybridization, DNA sequences at the ribosomal RNA gene cluster, and mating and meiotic progeny production and viability (e.g., Kurtzman et al. 2011; Qiao et al. 2011; Hagen et al. 2015; Zheng et al. 2019). In contrast, those working on macrofungi such as mushrooms often rely on a specific set of morphological traits such as the shape, size, and colour of various parts of mushroom fruiting bodies, the size and shape of spores and spore-production tissues, and more recently DNA sequences at one or multiple gene loci (Singer 1986; Wu et al. 2016). Such a trend is not unique to fungal taxonomy but can also be found in the plant and insect taxonomy literatures (https://www.itis.gov/). Though not a mandatory requirement (Seifert and Rossman 2010), an emerging theme among all fungal taxonomists is the increasing use of DNA sequences, based on primarily variations in the ribosomal RNA gene cluster, in supporting the taxonomic uniqueness of individual taxa.

On the other hand, significant progress has been made in understanding the underlying mechanisms for a number of phenotypic traits in fungi, such as mating system (for model species), virulence factors (for economically or medically important fungi that are pathogenic to plants, animals, or humans), mycotoxins (for toxic fungi), and key secondary metabolites (e.g., for antibiotic producers). However, little is known about the genetic and environmental bases for variations in many of the traditional traits used to discriminate closely related fungal species such as the size and shape of the mushroom caps, the length and diameter of mushroom stipes, the surface texture and coloration of mushrooms, the conidiophore structure of molds, or the size, shape, and surface texture patterns of both sexual and asexual spores in yeasts, molds, or mushrooms. Coupled with the high phenotypic plasticity for many traits within individual fungal species and their sensitivity to environmental influences, our lack of understanding in these areas severely undermines the stability and usefulness of some of these characters in fungal taxonomy, contributing to frequent revisions in fungal taxonomy at the species, genera, family, and even higher levels (indexfungorum.org).

Fungal DNA barcoding and sequence-based classification and identification

Since the late 1980s, DNA sequence information has become a common element in fungal taxonomy (Hibbett et al. 2016; Xu 2016). DNA fragments located in the nuclear ribosomal RNA gene cluster, especially the internal transcribe spacer (ITS) regions, have been the most frequently used, which eventually led to the recommendation in 2012 of ITS as the consensus fungal DNA barcode (Schoch et al. 2012). Several features contributed to this recommendation, including its multi-copy nature in fungal genomes (which allows analysis of small quantities of original materials), the highly conserved sequences flanking parts or the entire ITS regions allowing PCR amplification of diverse fungi (White et al. 1990), and an obvious barcode gap between many closely related species. With the development of metagenome sequencing, a large number of ITS sequences showing significant divergence from the ITS sequences of known fungal taxa have also been identified (B. Wu et al. 2019). Coupled with the issues described above for the specimen/culture-based species concepts, the new developments in DNA sequencing technologies and in metagenomics have led to the call for a sequence-based classification and identification (SBCI) system for fungi (Kõljalg et al. 2013; Hibbett et al. 2016).

The pros and cons of a SBCI system have been debated among mycologists. One of the contentious issues is the choice of gene(s) for SBCI. While ITS seems like an obvious candidate in such a system, its multi-copy nature and the potential variations among copies within strains can make obtaining clear ITS sequences difficult (Schoch et al. 2012; Stielow et al. 2015; Xu 2016; Zhou et al. 2019). In addition, the overall relationship between ITS sequence variation, other species-delimiting criteria, and whole-genome sequence diversity across fungi have not been critically evaluated. In certain fungal groups such as the human pathogenic *Cryptococcus* species complex and the ubiquitous molds of the genus *Fusarium*, despite up to 10% of average nucleotide difference at the whole-genome level, there is little or no sequence variation at the ITS regions (Xu et al. 2000; O'Donnell et al. 2015; Hagen et al. 2017; Kwon-Chung et al. 2017). To overcome

these problems, several other genes (e.g., *tef1*, *rbp1*, and *rbp2*) have also been proposed as potential secondary fungal barcodes for different groups of fungi (e.g., Stielow et al. 2015). At present, the proposed SBCI system in fungi has not been accepted due to insufficient evidence for any DNA fragment(s) being broadly applicable to delineate closely related species across the fungal kingdom (Xu 2016; Matute and Sepulveda 2019).

Genome sequence-based classification and identification (GSCI)

The major issue of the choice of marker(s) in preventing a SBCI system from being adopted for fungi could be overcome in a whole-genome sequence-based classification and identification system. This system could apply not only at the species level, but also at below species levels (e.g., variety) and above species levels (e.g., genus, family, etc.). Indeed, such a system offers many advantages over all other species concepts and classification and identification systems. The increasing integration of genome sequence information into prokaryote taxonomy provides a glimpse of how such a system could work for fungi.

In prokaryotes, the universal barcode locus is the small subunit ribosomal RNA (SSU rRNA or 16S rRNA) gene. Sequence similarity at the 16S rRNA gene has been shown to be significantly correlated to DNA–DNA hybridization and to whole-genome nucleotide identity of homologous genes across phylogenetic groups. Specifically, though there are some exceptions, the species identification threshold of 97% sequence identity at the 16S rRNA gene represents a noticeable gap that corresponds well to 70% DNA–DNA hybridization and 97.5% whole-genome average nucleotide identity (Konstantinidis et al. 2006). In addition, there is typically only one or very few copies of the 16S rRNA gene within individual prokaryotic cells. Thus, unlike ITS in fungi, there is typically no sequence heterogeneity within individual prokaryotic strains for the 16S rRNA gene. Furthermore, unlike ITS in fungi, the 16S rRNA gene has a conserved secondary structure and function, making it possible to align 16S rRNA sequences from divergent prokaryotic taxa. Though there were disagreements among bacteriologists, especially during the early phase of its implementation, the consensus SBCI system in prokaryotes based on the 16S rRNA gene has contributed to the uniformity and stability of prokaryote taxonomy. So far, the genome sequences of almost 250 000 prokaryotes have been deposited in GenBank, representing most described prokaryotic species (https://www.ncbi.nlm.nih.gov/genome/browse/#!/prokaryotes/). Analyses of these genomes have provided a framework and a huge resource that have led to rapid advances in prokaryotic biology across diverse fields, from basic investigations in physiology, genetics, genomics, molecular biology, ecology, population biology, and evolution to applied fields in agriculture, forestry, and animal and human health.

A genome sequence-based classification system for fungi could similarly contribute to the stability of fungal taxonomy, enhance communication among fungal taxonomists, and accelerate discovery in diverse fields of fungal biology. This system should build on the existing fungal taxonomy foundation and progressively add whole-genome sequence information to new and existing taxa. At present, over 5600 fungal genome sequences are available in NCBI, representing over 1000 fungal species, including multiple strains in each of several closely related species that are of economic and (or) medical significance (https://www.ncbi.nlm.nih.gov/genome/browse#!/eukaryotes/). The relatively broad taxonomic coverage, as well as the presence of multiple strains within some species in the current fungal genome database, should allow for exploratory investigations to establish a potential cutoff for species boundaries. At present, a commonly used cutoff for sequences belonging to the same operational taxonomic unit (OTU) is >97% sequence identity at the ITS region(s) (B. Wu et al. 2019), similar to the boundary threshold of 97% sequence identity for the 16S rRNA gene in prokaryote species. Thus, a convenient cutoff may be 97% average nucleotide identity for all house-keeping genes (such as those involved in DNA replication, transcription and translation, energy production, and amino-acid and nucleotide biosynthesis). However, it should be noted that there is currently a great bias in the fungal genomes sequenced so far towards certain groups of species of economic and medical interests. In addition, for most sequenced fungi, there is usually only one representative strain in the database and thus their intra-specific genome sequence variations remain unknown. Moreover, even for species with multiple representative strains in the database, the species delimitations may still be subject to debates (e.g., the human pathogenic *Cryptococcus* species complex; Rhodes et al. 2017; Kwon-Chung et al. 2017; Hagen et al. 2017; Wang and Xu 2020). A comparative genome sequence analysis of the *Cryptococcus* species complex could serve as a model to define fungal genome species boundaries, and to identify consensus molecular markers for the classification and identification of novel species across the fungal kingdom.

For the genome sequence-based species delimitation system to work effectively and efficiently, a dedicated repository with annotation, comparative, and search functions tailored to community consensus criteria should be set up. NCBI would be an obvious choice for establishing and hosting this repository. However, care must be taken to ensure the accuracy of the submitted whole-genome sequences, including the associated metadata. Indeed, similar to misidentification of ITS sequences, misidentification of the submitted fungal genome sequences have been found in NCBI (Stavrou et al. 2018). Thus, stringent

filtering and curation protocols should be established to prevent the propagation of misinformation about fungal genomes in the literature. Within the database, whole-genome sequences from representative specimens for all currently described fungal species should be obtained. The priorities for genome sequencing should be given to holotype, lectotype, or epi-type specimens and cultures. At present, an estimated US$500 is needed to obtain a high-quality assembly of a 50 Mb genome, the average size of the known fungal genomes. With the ~140 000 described fungal species, the total sequencing cost would amount to about US$70 million. While this is a lot of money, the cost can be shared among countries, funding agencies, and organizations where special fund(s) could be established. After sequences are obtained, they need to be assembled and analyzed to derive appropriate statistics in support of the taxonomy as well as describe notable genomic features for potential future investigations. Annotation and analyses pipelines for eukaryotic genomes are already available on NCBI (https://www.ncbi.nlm.nih.gov/genome/annotation_euk/) and they could be adopted and modified to accommodate the genome sequence-based classification and identification for fungi. Alternatively, the current 1000 Fungal Genomes Project could act as a framework and model database (https://mycocosm.jgi.doe.gov/programs/fungi/1000fungalgenomes.jsf) for the genome sequence-based classification and identification system of fungi.

The fungal taxonomy community should require investigators to submit a whole-genome sequence of the type strain/specimen to the repository for each proposed new species. In papers describing new species, if the genome sequences of closely related species are available, comparisons between them should be provided, in a community-agreed standard format describing the results of such comparisons in table and (or) figure form. Financially, sequencing an individual fungal genome should not be an insurmountable burden for individual investigators. Specifically, the cost for obtaining a whole fungal genome sequence (~US$500 at present) amounts to less than half the cost for publishing an open access article in most journals. Indeed, an increasing number of fungal genomes have been published as genome announcements in a variety of journals. However, for certain mycologists who might have difficulty accessing a sequencing facility or the cost to obtain the whole-genome sequence might be prohibitively high, an application for funding could be made to a special fund dedicated for fungal taxonomy to help cover the cost.

The fungal genome database and sequence comparisons will not only provide a robust phylogeny for all fungi but also will likely unify fungal species concepts and help integrate fungal taxonomists with the broader mycological community. Specifically, the whole-genome sequences will likely reveal a large number of candidate genes potentially related to morphological, ecological, physiological, and reproductive differences among closely related, as well as divergent, species. Such information will allow fungal molecular and cellular biologists, geneticists, ecologists, and population biologists to efficiently design targeted investigations into the underlying mechanisms for the observed differences within and among species in specific traits/trait values. Whole-genome sequence comparisons, even between two strains, could provide insights into the history of genetic exchange/reproductive isolation between them in nature (Xu 2006). With increasing genome sequences, such a database and approach could be extended from the species level to higher taxonomic levels, with unambiguous genome-wide nucleotide similarity cutoffs for the establishments of genera, family, order, and class. Furthermore, with high-coverage sequencing, fungal DNA sequences can be directly obtained from environmental samples, assembled, and compared with known genomes to infer novel fungal genome species, even for unculturable fungi. The potential benefits of having a whole-genome sequence for each fungal species and the establishment of a genome-based classification and identification system for fungi are enormous!

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