Hurdles in fungal taxonomy: Effectiveness of recent methods in discriminating taxa

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Abstract

The fungal kingdom is estimated to comprise between 2.2 to 3.8 million species with only about 7% named and classified. Novel biochemical, physiological and molecular techniques have been utilized to improve the systematics of fungal taxa and estimates of their diversity. Multidisciplinary approaches should be used for resolving species and higher taxa of the fungi. However, even with all the benefits of the new techniques, they are also providing unclear results and taxonomic instability. Taxonomists should be aware of these issues and should follow pragmatic approaches. In order to overcome these taxonomic challenges, cooperation and communication among mycologists worldwide are crucial for the study of fungal diversity.

Introduction

The kingdom fungi has an enormous species diversity with varied morphologies, ecologies and nutritional modes, many of which have biotechnological, medicinal and industrial importance (Hyde et al. 2019). With this extraordinary species richness, fungal systematics and classification face huge challenges. Taxonomy is a part of the scientific practice that deals with naming, describing and classifying fungal species (Katoch & Kapoor 2014). The current classification system, which is based on Linnæan ranks, has been criticized by many taxonomists and a new dialogue has been opened to discuss higher ranks in the classification system. However, even with all the benefits of the new techniques, they are also providing unclear results and taxonomic instability. Taxonomists should be aware of these issues and should follow pragmatic approaches. In order to overcome these taxonomic challenges, cooperation and communication among mycologists worldwide are crucial for the study of fungal diversity.

First, we discuss differences between phylogenetics and phylogenomics and when to use which approach. Next, we focus on issues of how to define higher ranks in the classification system. Then we discuss future directions in mycology, specifically towards exploring undiscovered fungal taxa. Finally, we conclude with a positive note and look at ways we can integrate the study of fungal taxonomy with applied aspects.

01. Single and multi-gene phylogenetics vs. phylogenomics: What & when to use?

Traditional approaches in taxonomy and systematics were mainly based on morphology. There are several aspects that affect the results of a morphology-based classification such as pleomorphism, homoplasy, phenotypic stasis and cryptic speciation within fungal taxa. With the discovery of these aspects, the taxonomy of the organisms has become less certain. As a result, in the early 1990s, DNA sequence data (especially 18S ribosomal DNA), emerged as a new approach for delineating species. In addition to DNA sequence data, other molecular techniques such as amplified fragment length polymorphism, restriction fragment length polymorphism and randomly amplified polymorphic DNA, have been employed in the classification and taxonomy of various organisms in different kingdoms (Williams et al. 1990; Roeckel-Drevet et al. 1997). DNA sequence data from fungi has accumulated exponentially and resulted in the establishment of many accepted relationships between fungi (Wingfield et al. 2012; Persoh 2015; Balint et al. 2016; Samarakoon et al. 2016; Spatafora et al. 2016; Tedersoo et al. 2018). Another benefit of the molecular approach is that it has revealed fungal taxa that would not have been detected using traditional culturing techniques.
Phylogenetics is a dynamic discipline that reconstructs and analyses the evolutionary relationships of species or taxa (Wiley & Lieberman 2011; Young & Gillung 2020). Initially, most phylogenetic papers were based on nuclear ribosomal RNA genes (rDNA) (Bruns et al. 1991; Hibbett 1992; Hwang & Kim 1999; Schoch et al. 2009, 2012) as these multicopy genes are highly conserved within a species and can vary among species (Bruns et al. 1991; Hibbett 1992; Xu 2006). As different regions of these rDNA genes evolve at different rates, they are useful to resolve lineages at different taxonomic levels, for example, SSU and LSU rDNA are generally applied at higher taxonomic level delineation (Schoch et al. 2012; Zhang et al. 2017). However, protein-coding genes and non-functional DNA fragments are better suited when performing comparisons within a species as they are more informative than the highly conserved rDNA genes. This less informativeness in rDNA genes has resulted from the strong concerted evolution pressure, while the non-coding sequences and third base substitutions have been subjected to fewer constraints (Lutzoni et al. 2004; Xu et al. 2006). However, these single copy protein-coding genes together with the rDNA genes provide relatively higher resolution compared to single gene phylogenies (Liu et al. 1999; Hong et al. 2005; Zhang et al. 2012). Multi-locus phylogenetic analysis was developed by combining all available phylogenetic data and has been considered as a better alternative to single gene phylogeny as it increases the informativeness, helps resolve nodes, nodal branching and improves phylogenetic accuracy (Zhang et al. 2006; Chen et al. 2015; Maharachchikumbura et al. 2016; Hyde et al. 2017a, 2018). With the advent of new sequencing strategies and bioinformatic approaches for analyzing large amounts of data (Karp et al. 2002; McDermott et al. 2005), together with the continuing decrease in sequencing costs (Margulies et al. 2005), there has been an exponential growth in the availability of fungal genomic data. These fungal whole genome or transcriptome data intertwined together with the developments of phylogenetics have resulted in the emergence of the new field of phylogenomics, which is the study of evolutionary relationships based on comparative analyses of genome scale data (Tatusov et al. 2003; Chan & Ragan 2013).

The use of either phylogenetic or phylogenomic approaches depends on the user perspectives, the amount of the data available for the study and the objectives of the study. If the study objective is to perform species delimitation for several fungal taxa or even for many fungal taxa using multiple gene regions (<10), then a multi-gene phylogenetic approach is more suitable. However, if the study requires to infer phylogenetic relationships between taxa to understand the mechanisms of molecular evolution or to use multispecies phylogenetic comparisons to infer putative functions for DNA or protein sequences, then phylogenomic approaches are justifiable. These phylogenomic approaches work on genomic data, which are 10–1000× larger than the typical multi-gene phylogenetic dataset. Therefore, phylogenomics has the potential to improve resolution by reducing the stochastic error resulting from the finite length of sequences used in the inference (Som 2015; Nafy & Szollosi 2017). Due to the shallow relationships existing among higher ranks, phylogenomic approaches can be used for better resolution in the tree of life (Spatafora et al. 2017; Ewers-Saucedo et al. 2019). Therefore, if the resources and the time is available, phylogenomics can be used to infer the relationships or the classification of higher ranks. In recent studies, divergence times based on a molecular clock has been used as an effective and robust approach for taxonomic distinction (Zhao et al. 2016, 2017). Phylogenomic studies have been used to resolve fungi with controversial evolutionary relationships. For example, Fu et al. (2020) suggested the use of genome coverage and genome mapping ratios as reliable, universal taxonomic criteria at the genus level for fungi.

Despite holding considerable promise, phylogenomics also has some pitfalls due to the limitations of the existing phylogenetic reconstruction methods, prevalence of non-phylogenetic signals and inability to form fundamental assumptions in genome scale data sets (Jeffroy et al. 2006). Even with these pitfalls, there is evidence supporting the importance and the usefulness of phylogenomic analyses to resolve evolutionary patterns and relationships between the higher ranks in the fungal taxonomy. For example, evidence for the relationship between Leotiomycetes and Sordariomycetes (Fitzpatrick et al. 2016), positioning of Xylonomycetes among the fungi (Gazis et al. 2016), the paraphyletic nature of Zygomycota (Liu et al. 2009), and the construction of kingdom-wide fungal phylogeny including the early lineages, Ascomycota and Basidiomycota (Ebersberger et al. 2012), can be listed as examples resolved by phylogenomic analyses. Therefore, it is highly recommended to utilize more genomic scale data to improve phylogenetic resolution and form a foundation for fungal taxonomic and systematic research. The drive towards next-generation phylogenomics has been initiated and entered a period of improvement. More than 1000 genome projects using phylogenomic techniques have been initiated and are being used to provide answers to long-standing phylogenetic problems at all levels of the tree of life. Despite its pitfalls, phylogenomic data have helped resolve many long-standing questions. Genome-scale datasets yield a dramatic increase in our statistical confidence of inferred relationships, resulting in maximally supported species trees. Therefore, if the data and resources are available, phylogenomic approaches should be employed to resolve fungal relationships.
02. Defining higher ranks in the classification system: can we justify monospecific families and can all taxonomists have a common ground?

Higher taxonomic ranks, such as classes, orders and families are not natural entities, but human constructs that are defined for the benefit of taxonomists (Laurin 2010; Liu et al. 2016; Hyde et al. 2017). However, based on evolutionary aspects, there is an argument that these entities do naturally exist and the inability to recognize these evolutionary relationships has led to the conclusion of higher ranks being artificial human constructs (Ereshefsky 2007; Lücking 2019). When we discuss the delineation of higher taxa, their consistency is based on evolutionary theories. According to evolutionary theory, phenotypic stasis or stability is the result of natural selection and genetic drift or caused by mutation and recombination events among populations (Lücking 2019; Mallard et al. 2019). However, this evolutionary consistency can clearly be observed at the genus level and below. Therefore, when defining families, orders and classes, taxonomists should follow a combined approach that best reflect their individual evolutionary history.

In higher-level taxonomy, another major challenge faced by taxonomists is the justification of monospecific families. The accepted number of fungal families has risen from 536 to 886 during the past decade, mainly due to the rapid increase in the use of DNA sequence data in defining families (Wijayawardene et al. 2017, 2018a, b). There are families with over 1000 species and also some with only a single known species. There are 57 such monospecific fungal families (Kew Report 2018). A monospecific family is described based on a single genus with a single species. From a conservation point of view, these families are important as they may represent species-rich relic taxa from early lineages, that hold a unique gene pool. For example, Bartheletiaceae is a family with a single species, Bartheletia paradoxa. These families deserve considerable attention as if we ignore them, there is a possibility that they could be lost forever (Samarakoon et al. 2016). Limitations associate with locating and loaning the herbaria of these monospecific families include, lack of reference data due to loss or damage, or not being available in the public domain and ruined or damaged fungal material (Dayarathne et al. 1998). For example, the herbarium of the monospecific Hypocreodendron sanguineum Henne. (1897) deposited by Hennings was destroyed by fire. Another issue with studying monospecific families is that their re-collection is difficult due to the destruction of their original habitats by human interference (Hyde et al. 1998).

In recent years, the introduction of monospecific families has increased due to the sole dependency on DNA sequence data. Ascocylindraceae (Ariyawansa et al. 2015b) is an example. In some instances, these monospecific families have resulted because of inadequate sampling. Therefore, mycologists and taxonomists should consider following a polyphyletic approach to define higher ranks, such as families, orders and classes. However, to date, there is no universal taxonomic criterion for defining higher ranks in fungal taxonomy (Samarakoon et al. 2016). Even though mycologists have attempted to establish more reliable methods, it has always been contentious, subjective and resulted in unnecessary conflicts (Liu et al. 2016; Divakar et al. 2017; Hyde et al. 2017b). For example, the three families namely Endemelanconiopsisaceae, Pseudofusisococcumaceae and Septorioidaceae were treated as genera pending further sampling and phylogenetic support by Liu et al. (2016) due to their few morphological or ecological characters. However, Crous et al. (2017) and Wijayawardene et al. (2018a) accepted nine families including the above in Botryosphaeriales. Phillips et al. (2019) then used a polyphasic approach (morphological studies, phylogenetic analyses and evolutionary study) and synonymized Endemelanconiopsisaceae, Pseudofusisococcumaceae and Septorioidaceae under Botryosphaeriales, Phyllostictaceae and Saccharataaceae, respectively.

In order to avoid the confusions related to monospecific families some taxonomists have placed monospecific genera such as Pseudoxylomyces under Dothideomycetes genera incertae sedis rather than introducing new families (Ariyawansa et al. 2015a, b). In another approach to addressing this problem, Phillips et al. (2019) employed the morphology of sexual morph, phylogenetic relationships and evolutionary divergence times of lineages in relation to major events in the evolution of their hosts on a geological timescale for the re-assessment of families in Botryosphaeriales. Following the same integrative approach, Lücking (2019) proposed to use comparative phenotype divergence combined with branch length pattern analysis, in order to reflect the individual evolutionary history of higher-level taxa. This means that nomenclature of higher taxa has to find a balance between scientific evidence of evolutionary relationships and the arbitrary decision of what rank a higher taxon should receive (Hyde et al. 2017b).

03. Exploring the undiscovered based on environmental sequences: valid or invalid?

Fungal taxonomy pursues discovering, describing and classifying all species of fungi and also provides tools for their identification. It is necessary to accelerate the pace of species description, to develop a complete catalogue of fungal diversity within a reasonable time frame, before the species become extinct. However, it is unlikely that
this goal will be achieved in the near future due to the disadvantages of traditional morphology-based taxonomy and the massive number of active taxonomists required (Hibbett et al. 2011).

The most common limitation of traditional morphology-based taxonomy analysis is limited or overlapping morphological characters. The development of molecular phylogeny has revealed an unexpectedly high fungal diversity. For example, studies with molecular phylogenetics have demonstrated that many morphologically similar taxa might represent distinct lineages, and many well-known species are in fact species complexes, such as in the genera *Colletotrichum* and *Diaporthe* (Dai et al. 2015). However, traditional molecular tools are most applicable to cultivable and fast-growing species isolated from the environment, whose DNA can be extracted from single spore isolates or from fresh specimens. This approach, however, cannot be applied to unculturable fungi (Hongsanan et al. 2018). This limitation has now been overcome via culture-independent techniques, specifically metagenomics (Blackwell 2011).

Next-generation sequencing approaches have resulted in a huge number of unidentified fungal taxa or operational taxonomic units (OTUs) from various habitats (Davison et al. 2018). These OTUs generally consist of ITS and more recently SSU and LSU sequences obtained from environmental genomic DNA (Womack et al. 2015; Hongsanan et al. 2018). Even though the assignment of sequences to OTUs are obtained by different algorithms and threshold levels, the resulting OTUs can be the same across different habitats (Schmidt et al. 2014). Due to lack of taxonomic information in fungal databases, most OTUs are not often classified at different taxonomic ranks and are considered as “unclassified” in the analyses. Wu et al. (2019) estimated that the total fungal diversity is about 12 million species based on both culture dependent and independent methods.

With all its benefits, the use of environmental genomic DNA to define species also has its problems. A consequence of using environmental DNA to define species has resulted in introducing numerous dark taxa into light (Grossart et al. 2016). Dark taxa are fungal taxa introduced only from sequence data and cannot be linked to any physical specimen or resolved taxonomic name (Hongsanan et al. 2018; Ryberg & Nilsson 2018). Introduction of dark taxa is against article 38 of nomenclature and typification (Art. 7–10) (McNeil et al. 2012). Another major issue with environmental DNA sequence data is the inability to reproduce and verify; the latter will result in numerous erroneous sequences. However, the connection between a fungal species introduced based on morphology and phylogeny and species introduced as OTUs based on the similarity threshold is challenging and needs to be answered by the mycological community (Dissanayake et al. 2018; Hongsanan et al. 2018; Jayawardena et al. 2018). Therefore, efforts must be taken to obtain physical cultures of specimens as proposed by Hongsanan et al. (2018) and Wu et al. (2019). Introducing species based on mgDNA data at this time cannot be accepted. There is also a risk of describing already known species or the dark taxa. Although Hawksworth et al. (2018) was in favour of accepting environmental DNA as types, this was downvoted by the International Mycological Congress (IMC) nomenclatural session in 2018 and a special purpose committee was proposed to investigate this issue further and to report back in IMC 2022 (May et al. 2018). However, dark taxa cannot be ignored in the future, with the development of new technology and increased use of environmental barcoding in species delimitation. Therefore, Lücking et al. (2018) proposed that repositories should allow sequence-based names to be registered and should allocate an identifier for future communication as in the UNITE database (Nilsson et al. 2018). Sequence-based fungal nomenclature can be introduced with careful and strict guidelines to ensure high-quality data and reproducibility.

**Conclusion**

With all of the advanced technologies and newly developed techniques, there are still disagreements in species delimitation and species conceptualization. These disagreements have led to debates as to species definitions and methods used to infer both species boundaries and their numbers. There are many species concepts in work today including both conventional concepts (biological, morphological, ecological and phylogenetics) and modern concepts (consolidated species concept) and techniques (Genealogical concordance phylogenetic species recognition, coalescent based techniques and phylogenomic approaches). Projects have been initiated by ICFT and others towards establishing guidelines for a polyphasic approach to species boundaries which can be generalized towards all fungi and can also be applied to resolve specific species complexes.

Hyde et al. (2011) discussed the diminishing number of mycologists, especially in Europe and the USA, against a backdrop of the huge numbers of fungi awaiting discovery. With fewer fungal taxonomists, how could such large numbers of fungi be described before they become extinct? The answer seemed very gloomy. However, every cloud has a silver lining and there has been an explosive burst in the study of fungi in Asia and to a lesser extent in South America. There are many reasons for this, one being that fungi are greatly appreciated in Asia and especially in China. Mushrooms are consumed and
used as traditional medicine in astonishing amounts, and mushroom farms and medicinal production facilities have grown up across the region. It has also meant that fungal taxonomy has once again become important in the quest to discover new edible or medicinal taxa. Mushrooms are also gathered throughout Asia and again particularly in China. Therefore, it is important that people know what they are collecting and eating, and that poisonous species are also documented.

Novel chemical discovery has been a huge area of research, particularly when applied to plants. However, fungi are also a treasure trove of novel medicinal compounds which have been understudied and therefore research into this aspect has grown and will grow further. De Silva et al. (2012a,b, 2013) reviewed the research on ways in which the fungi can be used in cancer, diabetes and other illness treatments. Hyde et al. (2019) covered 50 ways in which fungi can be used in industry and most with research in its infancy. This included biocontrol, biofertilizers, novel packaging, dyes and novel drugs. With such a huge undiscovered diversity and so many applications it is no wonder more research is being carried and the fungi await serious exploitation.

The future of fungal taxonomy is challenging. Traditional morphological studies are still very important, as species names are needed in plant pathology, quarantine and most applied industrial aspects. Yet morphology alone is inadequate and far too subjective for some fungal groups such as Diaporthe, Colletotrichum, Pestalotiopsis, Coniella and many others. Therefore taxonomists must incorporate modern techniques, such as phylogenetics, genomics, metabolomics and evolutionary approaches. However, this increases costs and creative methods are needed to apply for funding. However, if all cultivable fungi are isolated and placed in repositories (i.e. culture collections) for other research, then value is added to the fungus diversity and biogeography in the greater Mekong Subregion.

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