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REVIEW

What are fungal species and how to delineate them?

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Abstract
This is the opening paper in the special issue of *Fungal Diversity,* which collates the data on defining species. Defining and recognizing species has long been a controversial issue. Since Darwin's proposed origin of species, over 30 species criteria have been brought forth and used to define species boundaries. In recent times, phylogenetic analyses based on multiple loci have been extensively used as a method to define species boundaries. However, only a few mycologists are aware that phylogenetic species criteria can mask discordances among fungal groups, leading to inaccurately defined species boundaries. In the current review, we discuss species recognition criteria, how and where these criteria can be applied along with their limitations and derived alternatives. In order to delimit fungal species, authors need to take into account not only the phylogenetic and phenotypic coherence, but also the timing of events that lead to fungal speciation and subsequent diversifications. Variations in the rate of phenotypic diversifications and convergent fungal evolution make it difficult to establish a universal species recognition criterion. The best practice can only be defined in the context of each fungal group. In this review, we provide a set of guidelines, encouraging an integrative taxonomic approach for species delimitation that can be used to define fungal species boundaries in the future. The other papers in this special issue deal with fungal speciation in *Ascomycota, Dothideomycetes*, *Basidiomycota*, basal fungi, lichen-forming fungi, plant pathogenic fungi, and yeasts.

Keywords Allopatry · Speciation · Species concepts · Species criteria · Species recognition · Sympatry

Introduction

This paper is the introductory paper of this special issue entitled "What is a Species?", which is aimed at discussing mechanisms of fungal speciation, and conceptual frameworks and associated metrics for fungal delimitation. The current review discusses fungal speciation, the applicability of existing species criteria in defining species boundaries and possible adaptations to the fungal nomenclature with omics data. Considering different species recognition criteria, we recommend a set of approaches for defining species under the existing criteria. In other contributions to this special issue, Maharachchikumbura and co-authors review the current species recognition criteria used for *Ascomycota* with their advantages and disadvantages. Furthermore, they outline the drawbacks in the traditional phylogenetic methods that lead to ambiguous conclusions and propose an integrative and pragmatic approach for identifying species boundaries in *Ascomycota*. The ecology, taxonomy and diversity of the largest and most diverse class in *Ascomycota*, *Dothideomycetes*, is discussed by Pem and co-authors. They discuss the current species criteria used to define species boundaries in several highly confused fungal taxa and provide basic guidelines for standard *Dothideomycetes* taxonomy based on morphology and phylogeny. Cao and co-authors discuss the species recognition criteria used in *Basidiomycota* together with the difficulties faced during species recognition and present recommendations for species delimitations. Species recognition criteria applicable

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to yeasts are reviewed by Boekhout and co-authors. They discuss the genetic and evolutionary processes that challenge the definition of species boundaries, application of two-barcode systems, the impact of comparative genomics for introducing taxonomic novelties, practicalities involved when describing yeast species and propose a scheme for recognizing species boundaries in yeasts. Both Manawasinghe and co-authors and Jayawardena and co-authors deal with the species boundaries in pathogenic fungi. While Manawasinghe and co-authors focus on species identification approaches below the species level such as forms and races, Jayawardena and co-authors reviewed the species recognition criteria used to define species boundaries in plant pathogenic fungal genera, emphasizing the examples from the pathogenic genera *Bipolaris*, *Colletotrichum Curvularia*, *Diaporthe*, *Diplodia*, *Meliola*, *Plasmopara* and *Trichoderma*, and provide guidelines for species delimitation in pathogenic genera. Lücking and co-authors provide a comprehensive overview of different aspects of alpha and gamma taxonomy in lichen-forming fungi. They present a more practical framework together with a set of recommendations to assess taxonomic novelties and species boundaries by integrating phenotypic characters with phylogenetic and phylogenomic approaches. Filling the gap between the higher fungi and early-branching fungi, Voigt and co-authors focus on the taxonomy, ecology and distribution of basal fungal lineages. The study discusses the species concepts in several basal fungal classes and provides novel insight into their phylogeny and evolution.

One of the most fundamental problems in biology is to understand speciation and define species boundaries. The term 'species' has different meanings in different disciplines in biology and these definitions vary among taxonomists, phylogeneticists and evolutionary biologists (De Queiroz 2005, 2007; Jeewon and Hyde 2016; Aldhebiani 2018; Xu 2020). The fungal kingdom is estimated to comprise between 2.2 to 3.8 million species, yet less than 10% of them are named and classified (Hawksworth and Lücking 2017; Chethana et al. 2020; Hyde et al. 2020a). Understanding speciation and thus the process of how these species emerged is of fundamental importance. Even before Darwin published the 'On the origin of species', speciation was highly debated among biologists (De Queiroz 2005). Many introduced definitions for species and recognition criteria later (Taylor et al. 2000; De Queiroz 2005, 2007; Jeewon and Hyde 2016; Aldhebiani 2018; Xu 2020). Although much progress has been achieved in fungal taxonomy, the application of molecular techniques, combined with morphological and ecological approaches have identified profound deficiencies of proposed species delimitation criteria (Jeewon and Hyde 2016; Xu 2020).

With their diverse morphologies, ecological roles and nutritional modes, fungi exhibit enormous species diversity. They play vital roles in ecological stability and have numerous industrial applications (Hyde et al. 2019). Thus, almost all of the mycological subdisciplines rely on accurate identification of species (Chethana et al. 2020). During the last two decades, the rate of new species introduction has increased to more than 2,000 fungal species per year (Willis 2018). Their identification has been based either on morphology or phylogeny or a combination of both. Use of morphology alone in species identification can be misleading due to hybridization, cryptic speciation, convergent evolution and phenotypic plasticity (Matute and Sepúlveda 2019; Chethana et al. 2020). For instance, use of morphology alone is inadequate when linking asexual and sexual morphs of the same fungus. The confusion can be resolved by using molecular data, resulting in correspondence between these two stages. Using only molecular phylogeny for fungal identification is also problematic. One of the main problems is that most of the fungal species described before the 1990s (which include most of the type species) are based only on morphology (Yang 2011; Papagianni 2014; Dayarathne et al. 2016). Another issue relating to the sole dependence on molecular phylogeny is the lack of sequence data or use of unverified sequences in public databases, which can result in misleading phylogenies and erroneous interpretations (Nilsson et al. 2012). In addition to morphological and molecular approaches, many other techniques such as chemotaxonomy and omics approaches have been also used to define species (Kuhnert et al. 2015; Jeewon and Hyde 2016). Selecting the best approach for a particular fungal group is challenging.

Species and species recognition criteria

Species is the most fundamental and most widely applied unit in almost all disciplines in biology. It has been the most debated term for centuries. The earliest definition for species was provided by the English scholar John Ray, who stated that "No matter what variations occur in the individuals or the species, if they spring from the seed of the same plant, they are accidental variations and not such as distinguish a species permanently; one species never springs from the seed of another nor vice versa" (Ray 1686). Following this, several other species definitions surfaced (Linnaeus 1753; De Candole 1813; Darwin 1859). The most contemporary one was introduced by the zoologist Mayr (1942). In his book '*Systematics and the Origin of Species',* he defined species as "groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups''. The adoption of this definition for fungal taxa stimulated critiques and brought forth proposals for many alternatives (Sokal and Crovello 1970; De Queiroz 2005). The above definition requires sexual reproduction, thus individuals of a species need to interbreed. According to this

definition purely asexual groups of organisms cannot be considered species, which is far from the reality (Ereshefsky 2007). Further, Mayr's species definition is impractical to apply in some instances to allopatric populations (Cronquist 1978; Stace 1989). The advent of molecular technologies, DNA sequences, genomic and transcriptomic data provided additional support for species definitions. Mallet (1995) proposed a new definition for species, in which he incorporated molecular data that defines species as "groups of individuals with few or no intermediates" (Michener 1970). This formulation resulted in the genotypic cluster definition, which considers a species as a "genotypic cluster that can overlap without fusing with its sibling" (Mallet 1995). Similar to Mayr's definition, genotypic cluster definition also has some undesirable repercussions, such as the identification of genetically different clones of a species as separate species (Coyne and Orr 2004). Despite this, the above definition is considered as one of the most useful, as the majority of species criteria result in species that form genotypic clusters at one stage of their lifespan. Even though there are many definitions for fungal species, the use of these definitions depends on the fungal group, the availability of information, as well as the individual preferences of the authoring fungal taxonomists or mycologists (Seifert and Rossman 2010; Jeewon and Hyde 2016; Boekhout et al. 2020; Xu 2020).

Species concepts do not define what a species is, but clarify what drives speciation (Aldhebiani 2018). Historical examples have shown how species concepts and definitions can yield different conclusions (Agapow et al. 2004; Isaac et al. 2004), resulting in frustration and misconceptions (Isaac et al. 2004; Hausdorf 2011). Species definitions describe entities, whereas 'species concepts' correspond to criteria used for delimiting them (Taylor et al., 2000; De Queiroz 2007; Hey 2006; Giraud et al. 2008). Almost all 'species concepts' established to date outline practical criteria for distinguishing species (Aldhebiani 2018; Taylor et al. 2000; Xu 2020). Therefore, using the term 'species concept' to represent species criteria is misleading. We agree with Taylor et al. (2000) that the term 'species concept' should be abandoned for operational species criteria. Mayden (1997) characterized the existing 'species concepts' as either theoretical or operational. Evolutionary species concept is the only primarily theoretical species concept that does not provide any recognition criterion (Simpson 1951; Kwon-Chung et al. 2017). Most of the proposed 'species concepts' are compatible with the evolutionary species concept. The evolutionary species concept is the only 'species concept' that actually conceptualizes what a species is, but lacks operational criteria to delimit species across space and time. In contrast, operational concepts specify criteria for species identification. Commonly used 'concepts' that are classified under this category include morphological, biological, and phylogenetic species concepts. To avoid confusion between theoretical and operational concepts, Taylor et al. (2000) proposed to reserve the term 'species concept' for the theoretical ones and 'species recognition' for the operational 'concepts', i.e., morphological species recognition, biological species recognition, phylogenetic species recognition, and genealogical concordance phylogenetic species recognition. Therefore, hereafter in this review, we will use the term 'recognition criteria' to represent all the operational 'concepts'.

There are around 30 established species recognition criteria, some of which overlap to some degree (Zachos 2016; Xu 2020). The most common species recognition criteria used in fungal taxonomy include biological (Mayr 1942; Kerrigan et al. 1994), morphological or phenetic (Regan 1926; Linnaeus 1753; Michener 1970; Kurtzman et al. 2011), ecological (van Valen 1976; Trudell et al. 2017), genotypic cluster (Mallet 1995; Banke et al. 1997) and phylogenetic (Rosen 1979; Wu et al. 2016) characters.

Driving forces in speciation

Speciation is a dynamic and ongoing process, whereby populations diverge and become distinct species (Kohn 2005; Giraud et al. 2008). Speciation constitutes one of the most debatable topics since Darwin's time to the current molecular era. Almost all discussions about evolutionary biology begin with Darwin's theory of evolution based on natural selection. Species arise from the divergence in a population at a particular ancestral point (Darwin 1859). A number of studies (both genetics and population genetics) have investigated the mechanisms underlying speciation based on interfertility and hybridization (Anderson et al. 1980; Coyne and Orr 2004; Kohn 2005), and phylogeography (Avise 2000; Hare 2001; Kohn 2005).

Species divergence is the result of the interplay between several evolutionary forces such as natural selection, accumulation of genetic mutations, migration and genetic drift (Kohn 2005; De Queiroz 2005). Natural selection is one of the core mechanisms responsible for the evolutionary changes of adaptive features that increases individuals' survival and reproductive output or fitness, resulting in phenotypic and genetic diversity within a population (Milgroom 2017). This genetic variation first originates from random processes, such as point mutations or genetic recombination (Gregory 2009). When evolutionary processes act on these mutations, allele frequencies within the population change over generations via genetic drift or natural selection (Milgroom 2017). Genetic drift is a random, long term fundamental process that creates divergence in the genetic composition of a population. Founder effect (limited number of colonizers) and bottleneck effect (dramatic reduction in population size) are two of the speciation hypotheses that result from

genetic drift and may lead to speciation by accelerating the formation of new allelic combinations (Yeung et al. 2011). Later, with the developments to the theory of population genetics (Fisher 1930; Mayr and Provine 1998), new information came into light on the process of speciation. Several factors that drive speciation were found including biogeography, gene flow, genetic drift, natural selection and reproductive isolation (Kohn 2005). Even though we discuss these factors separately most of them are linked to each other, hence their influence on speciation is synergistic rather than independent. The relative importance of different driving forces of speciation may vary among different groups of fungi. For instance, the host is an important driving force in the evolution in plant pathogens, whereas the geographic isolation is for aquatic fungi. Because of this, and because divergence accumulates in populations, eventually giving rise to different species, it is often challenging to decide between inter- or intra- specific divergence from the available phylogenetic, morphological, or ecological evidence.

Geographical context or biogeography is a major factor that affects speciation (Kohn 2005; Xu 2020). Mayr (1963) proposed that geographically isolated populations would accumulate sufficient genetic differences that ultimately could lead to speciation. In order for geographical isolation to occur a barrier to gene flow between subpopulations must be present, thus allowing them to diverge independently (Kohn 2005; Xu 2020). Speciation of geographically isolated populations is known as allopatric speciation and it is the most widely occurring process (Kohn 2005; Price 2007; Xu 2020). In allopatric populations, species divergence may occur either by natural selection or genetic drift, and both may lead to reproductive isolation. Taylor et al. (2006) discussed the evolution of cryptic species in geographically non-overlapping areas as a result of allopatric speciation. For example, cryptic species in the *Ophiocordyceps unilateralis* complex that diverged on different continents have been identified based on molecular markers (Araújo et al. 2018). Genomics and population genetic studies suggest that new varieties of *Cryptococcus neoformans* diverged allopatrically. Specifically, *C. neoformans* var. *neoformans* originated in temperate regions in the northern hemisphere, while *C. neoformans* var. *grubii* originated in Africa (Litvintseva et al. 2011; Xu 2014). Given enough time, these geographically isolated populations or varieties can undergo sufficient divergence to form new species. Gostinčar et al. (2014) confirmed speciation from varieties using genomic data. According to their study, the genomes of *Aureobasidium pullulans* varieties described in 1918 are substantially different to justify their redefinition as separate *Aureobasidium* species.

Speciation may also occur in geographically overlapping populations, which is known as sympatric speciation (Via 2001). Even though the occurrence of sympatric speciation in nature is rare and thought to be extremely difficult (Coyne and Orr 2004), a few compelling examples do exist. A specific population living in sympatry with another may become subject to natural selection and diverge into different species. In these cases gene flow between populations is disrupted. For example, wheat-adapted *Mycosphaerella graminicola* diverged sympatrically from wild grasses populations to newly domesticated cereal crops (Stukenbrock et al. 2007). This study suggested that *M. graminicola* sub-populations diverged sympatrically via reinforcement. Reinforcement is an active mechanism that increases prezygotic reproductive isolation, leading to selection against interspecific mating due to reduced hybrid fitness (Dobzhansky 1951). Sympatric divergence has been shown in some studies, such as in *Rhizoplaca melanophthalma* species complex (Leavitt et al. 2011), grass endophytes *Epichloë typhina* and *E. clarkii* (Treindl and Leuchtmann 2019), ecologically and genetically diverse *Didymella rabiei* subpopulations (Frenkel et al. 2010), and genetically diverse cryptic sub-populations of *Botrytis cinerea* (Fournier and Giraud 2008). Parapatric speciation is another speciation scenario, which refers to non-overlapping populations in contiguous, yet geographically separated areas with a zone of contact for gene flow between diverging populations (Nosil et al. 2002; Niemiller et al. 2008). Even though this phenomenon has been observed in several organisms (Niemiller et al. 2008), this kind of speciation has not yet been documented in fungi.

As previously discussed, natural selection is a central factor affecting speciation (Milgroom 2017). It is the driving force of the evolution of adaptive features and plays an important role in producing phenotypic and genetic diversity within a population. Natural selection is the force that acts on existing features of two populations with reference to ecological differences of their habitats and leads to adaptations. When the genes are under positive selection pressure, they are more likely to diverge rapidly and create variants upon which natural selection can act (Stukenbrock and McDonald 2009) and these changes may prevent populations from mating and exchanging genes (Nosil 2012). Thus, with time they diverge into separate species.

Development of a reproduction barrier known as 'reproductive isolation' or evolution of a different type of reproduction is one of the main drivers for speciation. According to the biological species concept, species arise when individuals from different populations are no longer capable of mating. Mayr (1942) further explained that this new species formation involves the evolution of reproductive barriers. These barriers are of utmost importance because they determine the level of isolation of reproductively-isolated populations and whether the population completed the speciation or not. According to Nosil (2012), reproductive isolation is a weak force during the early stages of speciation, whereas it becomes stronger and complete during the later stages.

These reproductive barriers can either be prezygotic, where reproduction between populations is prevented by geographical isolation or incompatible interactions; or postzygotic, whereby incompatible genetic variation among populations produces offspring with reduced fitness (Boekhout et al. 2020). This scenario has been described in *Cryptococcus* species. Genomic studies have shown that sub-complexes of *Cryptococcus* species complex accumulated significant genetic variation, limiting inter-specific gene flow among their species. This is consistent with the reproductive isolation idea between the species (Desjardins et al. 2017; Rhodes et al. 2017). However, several studies found that gene flow still occurs in the form of inter-species hybridization among *Cryptococcus* species, though they resulted in meiotic progenies with low germination rates or spores with compromised viability (Sun and Xu 2007). Smut pathogenic *Microbotryum* species complex also exhibit a similar scenario. Gladieux et al. (2011) showed that co-existing, yet divergent populations of *Microbotryum lychnidis-dioicae* and *M. silenes-dioicae* initially diverged in allopatry, but later introgression occurred when brought in secondary contact by human activities.

The discovery of pure hybrid species in nature suggests that incompatibilities resulting from gene flow among populations can be overcome in some circumstances (Greig et al. 2002; Stukenbrock 2016). Stukenbrock (2016) discussed hybridization as a major force in the evolution of fungal phytopathogens. Genomic studies on hybrid genetics have validated this concept and provided evidence for the importance of introgressive hybridization, which transfers genes responsible for adaptive traits (Arnold 2004). Menardo et al. (2016) demonstrated the importance of hybridization for the evolution and adaptive diversity of *Blumeria graminis* f. sp. *triticale*. Introgressive hybridization has been observed and documented in grass endophytic *Epichole* (Schardl 2001), grass pathogen *Zymoseptoria pseudotritici* (Stukenbrock et al. 2012a), the Dutch Elm pathogens *Ophiostoma ulmi* and *O. novo-ulmi* (Brasier and Kirk 2010), and smut pathogens *Microbotryum silene-dioicae* and *M. lychnidisdioicae* (Gladieux et al. 2011).

In addition to clonal divergence and hybridization, crop domestication is another avenue associated with speciation of fungal pathogens (Stukenbrock 2013). Domestication of host plants not only influences their genetic structure, but also the physical, biotic and genetic composition of the associated fungal taxa (Dettman et al. 2003; Kohn 2005; Stukenbrock et al. 2007). As mentioned above, Stukenbrock et al. (2007) provided evidence that divergence of the wheat pathogen *M. graminicola* from an ancestral population occurred on wild grasses. They also demonstrated, using population genetic approaches, that divergence and domestication of the fungal pathogen occurred simultaneously with the domestication of the agricultural crop host. Stukenbrock (2013) stated that the patterns of speciation via host domestication differ significantly among fungal pathogens. For instance, speciation in *Pyricularia oryzae* is associated with host shifting, loss of sexual reproduction, and clonal speciation (Couch et al. 2005), whereas the factors associated with *Zymoseptoria tritici* are strong host specialization, and frequent sexual recombination (Stukenbrock et al. 2012b).

Sexual selection or the type of reproduction also plays a role in speciation (Nosil 2012). Asexual reproduction is predominant in most fungal taxa. Extensive asexual reproduction facilitates clonal speciation under selection (Anderson and Kohn 1995; Carbone and Kohn 2001). Parasexuality in fungi has been explored as another mechanism for interspecies hybridization, which accelerates the genetic divergence leading towards speciation in many asexual reproduction dominant fungi (Schardl and Craven 2003; Kohn 2005). Heterokaryotic multinucleate cells result from anastomosis of vegetative hyphae during the parasexual cycle, which may later undergo nuclei fusion and mitotic chromosomal crossovers, resulting in chromosomal losses on the way, can generate novel genotypes from the original heterokaryon (Caten 1981; Schardl and Craven 2003). Brasier (1995) suggested that this parasexual genetic exchange between species facilitate introgression or genomic rearrangements, giving the new hybrids recombined characteristics from their parents or an entirely new set of characters. Parasexual hybridization has been observed, particularly in grass endophytes in *Hypocreales* and mutualistic arbuscular mycorrhizal fungi (Schardl et al. 1994; Tsai et al. 1994). Parasexual hybridization between *Neotyphodium* and *Epichloë* species has given rise to several asexual endophytes of grasses (Moon et al. 2002; Schardl et al. 1994; Tsai et al. 1994). In fungi, polyploidization is another process that may lead to speciation. Ploidy level, specifically polyploidy and aneuploidy, may have resulted in the evolution of new species as in *Allomyces* (Kohn 2005).

Even though we discuss each of the factors separately, they do not act in isolation. Interaction among these mechanisms drives speciation in natural fungal populations. Up to now, we discussed the mechanisms and some of the driving forces of speciation in fungi. Next, we will be looking at how taxonomists identify species boundaries in fungi. There are many species recognition tools and in the following sections, we will focus our attention on the most commonly used species recognition tools in fungal taxonomy.

Commonly used species recognition criteria for fungi

Species recognition criteria define the standard and the practical approach for delineating species (Taylor et al. 2000). Around 30 species recognition criteria have been proposed

each based on a specific character (Zachos 2016). For example, interbreeding ability is emphasized in biological species recognition, whereas distinct morphological characters dominate morphological species recognition. Often, the criteria used for different species recognition overlap as in morphological, phylogenetic and more modern consolidated species recognition. The distinguished criteria for the first two recognitions overlap with the latter one. Due to the high diversity and variabilities of fungal taxa, adopting a universal species recognition for fungi is difficult. Some studies suggest that this is because speciation is an extended process that differs among fungal groups with regards to time and most importantly the mechanism leading to speciation. Some argue that because speciation events occur without any chronological order in different populations, we cannot use the same recognition criteria to judge them all. Also fungal characteristics, such as asexual reproduction, limit the application of some species recognition criteria. Therefore, it is fundamentally impossible to apply a single species recognition criterion to all groups of fungi. Selection of the most suitable single or multiple species recognition criteria depends on the fungal group, its history of speciation and the degree of achieved divergence (Giraud et al. 2008). Having more species recognition to determine species status is not a negative notion, but a result of increased knowledge on fungi in the scientific community and the increased availability of different types of data available for taxonomists to use.

Morphological species recognition

Cronquist (1978) defined species as the smallest group distinguished by morphological characters (Aldhebiani 2018). These characters have occupied a central position in taxonomic studies, and the use of macro-and micro- morphological characters dominated fungal identification. Thus, morphological species recognition defined "species as a community or related communities with distinct morphological characters which are sufficient to entitle it, or them, to a specific name" (Regan 1925). This recognition applies to a broad range of fungi, including asexual, sexual and even fossil records (Cronquist 1978). To date, characters such as spore pigmentation (*Pestalotiopsis* and *Xylariaceae* species), asci shape, the presence or absence of pseudoparaphyses, and appendages used to segregate ascomycete fungi, while the data on the stipe, cape, and lamellae are used for basidiomycetes (Noble et al. 1995; Tang et al. 2007; Maharachchikumbura et al. 2011; Zeng et al. 2012; Desjardin and Perry 2015; Jeewon and Hyde 2016; Li et al. 2016). Before the introduction of DNA sequence data, this was the most used recognition criterion to define fungi. In fungal taxonomic studies, some used morphological species recognition as the only recognition criterion such as *Thelephora ganbajun* from Southwestern China (Zang 1987). Prior to the molecular era, many of the books, monographs and protologues described fungi based only on morphological criterion (Saccardo 1883; Fuckel 1872; Chupp 1954; Boerema et al. 1996).

Morphological characters are however, often elusive, subjective and their interpretation depends on the observer, and hence they have often proven to be inadequate (Petersen and Hughes 1999). Further, these characters can get influenced by the environmental conditions as in *Cladosporium* (Basilico et al. 2007), and also by the culture conditions as for lichen-associated fungi (Muggia et al. 2017). Hence, using only morphological species recognition for demarcating species limits may lead to falsely identified species. Problems arise when morphological characters are used for the identification of cryptic species or morphologically indistinguishable species and fungi whose identification is based only on one part of the fungal life cycle. Johnston et al. (2017) demonstrated that 23% of *Phoma* species previously identified based on morphology, now place in phylogenetically distinct genera in closely related families such as *Didymellaceae*, *Leptosphaeriaceae*, *Phaeosphaeriaceae* and *Cucurbitariaceae*. For example, morphological species recognition identified the species in *Gibberella fujikuroi* species complex as one morphospecies, whereas multi-gene phylogenetic analyses identified numerous cryptic phylogenetic species (Nirenberg and O'Donnell 1998; Hsuan et al. 2011; Crous et al. 2021). Even though the genera *Lophiostoma*, *Lophiotrema* and *Massarina* belong to different lineages, they are morphologically similar and in some cases, indistinguishable (Hirayama and Tanaka 2011; Zhang et al. 2009). Further, the divergence of species in species complexes such as *Cryptococcus neoformans/C. gattii* complex (Hagen et al. 2015), *Candida albicans/C. dubliniensis* complex (Sullivan et al. 1995), *Diaporthe* complexes of Udayanga et al. (2012) and the *Malassezia* complex (Guého et al. 1996), cannot be explained by morphological species recognition. Similarly, there are many basal fungal phyla such *Zoopagomycotina* where morphology alone, might be insufficient for species or even genus delimitation (Naranjo-Ortiz and Gabaldón 2019). Due to the observed similarities, descriptions of these numerous taxa that reside in this complex can hardly be distinguished from sister species based only on morphology. The simple morphology observed and documented for some fungi also challenged the ability to use the morphological species recognition, such as for *Kiskunsagia* and *Constatinomyces* which are characterized only by yellow to white aerial mycelia and hyphal morphology, respectively (Harrington and Rizzo 1999; Egidi et al. 2014; Bezerra et al. 2017). Therefore, in many cases, morphological species recognition have overestimated or underestimated the speciation events (Jeewon et al. 2004) such as for genus *Diplodia*, for which 1010 species are accepted in MycoBank (MycoBank 2021) from which only 25 species were confirmed with molecular data (Zhang et al. 2021).

Biological species recognition

Mayr (1942) originally coined the term biological species recognition based on the idea of reproductive isolation proposed by Dobzhansky (1937). Both argued that species are reproductively isolated units and replaced phenetic recognitions such as morphological species recognition with biological species recognition (Coyne 1994). According to Mayr (1942), 'Species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups'. Later on, revisions emphasized that the biological species recognition was applied best for sympatric taxa (Mayr 1963, 1982). Biological species recognition accentuates the lack of gene flow between populations resulting from intersterility barriers preventing sexual reproduction or producing unfit hybrids (Stenlid and Karlsson 1991; Aldhebiani 2018; Taylor et al. 2000). Biological species recognition has been used alone to delimit species in *Pleurotus* (Petersen 1995; Vilgalys and Sun 1994), *Heterobasidion* (Korhonen 1978a), and *Armillaria* (Korhonen 1978b) genera. Further, fungal species such as *Arthroderma uncinatum*, *A. quadrifidum* (Dawson and Gentles 1961), *Nannizzia incurvata* (Stockdale 1961), *Agaricus bisporus* (Kerrigan et al. 1994), *Armillaria mellea* (Anderson and Ullrich 1979), *Collybia dryophila* (Vilgalys 1991), *Pleurotus pulmonarius*, *P. ostreatus* (Petersen and Hughes 1993), and *Omphalotus* species (Petersen and Hughes 1998) have been introduced based only on the biological species recognition. Even before the establishment of biological species recognition, mating tests were used to separate fungal species and enhance the understandings of their biology and their barriers to gene flow, such as in *Monilia sitophila* (Dwidjoseputro 1961) and *Neurospora* species (Harrington and Rizzo 1999).

However, there are many restrictions on the application of biological species recognition widely for all fungi. As its definition states, biological species recognition deals with sexual taxa, hence, it is inapplicable to asexual and homothallic fungal taxa and impractical on allopatric fungal populations (Aldhebiani 2018). This difficulty is mainly because some fungal taxa lack meiospores and cannot be crossed even in experimental settings (Matute and Sepúlveda 2019). For some fungi, sexual reproduction occurs in nature, but it is infrequent or triggered by rare conditions (Wallen and Perlin 2018). Species complexes such as *Colletotrichum siamense* sensu lato do not have any known sexual stages, making it impossible to use the biological species recognition (Matute and Sepúlveda 2019).

Ecological species recognition

Ecological species recognition was established based on several assumptions. These are, (1) Genes play minor roles in evolution and are considered to the same degree as other molecules; (2) Evolution is controlled by ecology and constraints of individual development; (3) Selection primarily affects phenotypes which are the building blocks of communities (Van Valen 1976). Ecological species recognition defines species as 'a lineage or closely related set of lineages that occupies an adaptive zone minimally different from that of any other lineage in its range and evolves separately from all lineages outside its range' (Van Valen 1976). In this definition, a lineage represents a clone or an ancestral-descendent sequence of populations. Andersson (1990) stated that ecological species recognition explains speciation on a basis that is common to all organisms. Hence, this recognition focuses on adaptation to a particular ecological niche (Cai et al. 2011). Ecological species recognition has been applied to identify fungal taxa in *Tricholoma caligatum* group (Kytövuori 1988) and was shown to be the most useful criterion for separating *Hymenochaetaceae* species (Parmasto 1985).

The sole use of ecological species recognition or ecological information is deemed insufficient for species identifications. Most of the studies that use ecological data for species delimitations incorporate molecular and morphological data. The use of this combination is evident in most of the recent taxonomic studies as in *Ophiocordyceps unilateralis* species complex (Haelewaters et al. 2018), *Teratosphaeriaceae* species (Quaedvlieg et al. 2014), and *Hesperomyces virescens* species complex (Haelewaters et al. 2018). Morphologically similar *Ceratocystis laricicola* and *C. polonica* were identified based on their ecological distinctiveness and the support by molecular data (Harrington and Wingfield 1998). Kodsueb et al. (2016) described the fungal communities between a stream and the same host on the banks of the stream, and demonstrated that there was almost no overlap. Therefore, ecological evidence can be used further to support a new species from a habitat that was different from the former habitat where the fungus was previously found. Figure 1 illustrates a Neighbour Joining analysis for *Diaporthe eres* using multigene phylogeny. *Diaporthe eres* isolates with various ecologies have been selected for the analysis. If these selected isolates were identified based only on ecological species recognition, this may result in different species due to their significantly different ecologies. However, molecular data has shown that they all belong to the same species. Therefore, use of the ecological criterion alone is deemed insufficient to identify species, though they can be used to further support the establishment of a new species.

Phylogenetic species recognition

Many studies emphasize the need for defining species and species recognition criteria based on phylogeny instead of the reproductive relationship (Avise and Wollenberg 1997). With the proposal of Hennig (1965) phylogenetic theory, several researchers attempted to forge a recognition criterion

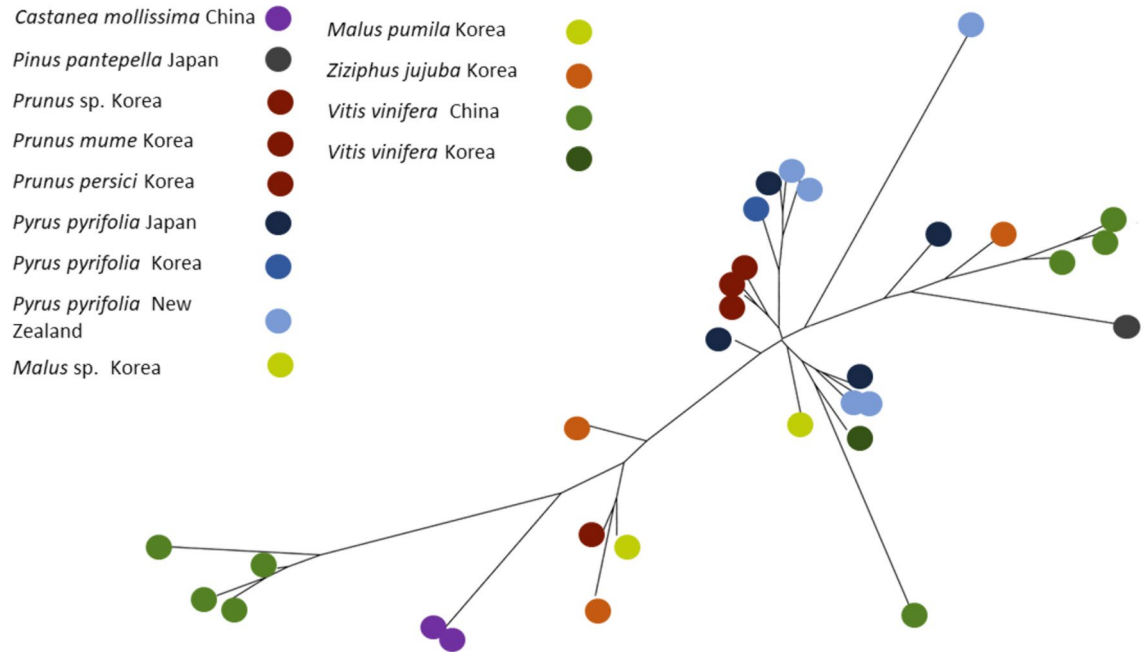

Fig. 1 Multi-gene NJ cluster analysis for *Diaporthe eres* isolates collected from different hosts and countries based on combined analysis of ITS, CAL, EF1-α, HIS, and TUB gene regions. The data set consisted with 32 *Diaporthe eres* strains. This figure shows intraspecies variation based on hosts and geography

compatible with the phylogenetic systematics or cladistics. This has led to several alternative recognition criteria, falsely identified as the phylogenetic species recognition (Nixon and Wheeler 1990), such as Hennigian species recognition, which incorporated biological species recognition with a historical component (Meier and Willmann 2000) and autapomorphic species recognition which describes a geographically constrained group of individuals with one or more unique apomorphies (Mishler and Theriot 2000). Many efforts were taken to improve or propose different species recognition criteria with regards to unique character patterns that are consistent with the ancestor–descendant relationships. As a result, Nixon and Wheeler (1990) defined the phylogenetic species recognition as "the smallest aggregation of a population (sexual) or lineage (asexual) diagnosable by a unique combination of character states in comparable individuals". Pivotal in the phylogenetic recognition is the monophyletic status of the fungal taxa. This recognition explains that populations of each species should share a common ancestor. Adapting phylogenetic recognition to the context of fungi, Harrington and Rizzo (1999) diagnosed a phylogenetic species as the "the smallest aggregation of a fungal population with a common lineage that shares unique, diagnosable phenotypic characters' which is similar to the one that was proposed by Nixon and Wheeler (1990).

Since the introduction of DNA sequence data studies in the early 1990s, phylogenetic species recognition has been used in many species delimitation studies (Taylor et al. 2000; Wu et al. 2016; Xu 2020). Application of phylogenetic species recognition for fungal taxa usually has two broadly defined forms, the coalescent-based species delimitation, and the strict genealogic concordance (SGC). Although these approaches are not mutually exclusive in phylogenetic analysis, their scopes are different (Matute and Sepúlveda 2019). Using the SGC approach, Dettman et al. (2003) predicted speciation to occur when all gene genealogies from unlinked loci are congruent. Further, if a group of putative species form a reciprocally monophyletic clade with bootstrap support above 70% and Bayesian support of 90% or above, then they are most likely to be reproductively isolated. The application of these rules has significantly helped the mycological community (Matute and Sepúlveda 2019). With these two rules, over 200 previously unknown cryptic species were identified (Matute and Sepúlveda 2019). The application of SGC is believed to be devoid of limitations, but it is still unclear to what extent it can be generalized to all taxa. In the genus *Beauveria*, species identification can be difficult due to a lack of phenotypic variations and structural simplicity. In a recent study, several *Beauveria* species were identified, and cryptic diversity in five species was confirmed using molecular phylogenetics (Bustamante et al. 2019). For newly formed asexual fungal species that are morphologically indistinguishable or not yet achieved morphological distinctness, phylogenetic species recognition can separate them into phylogenetically distinct lineages (Kohn 1995). There are many *Dothideomycetes*

families such as *Camarosporidiellaceae*, *Dothidotthiaceae*, *Pleosporaceae*, *Mycosphaerellaceae* that comprised of only asexual taxa (Videira et al. 2017; Wanasinghe et al. 2017; Senwanna et al. 2019). Most of them exhibit limited morphological characters (Guarro et al. 1999; Levetin et al. 2015). Hence, phylogenetic species recognition plays an important role in demarcating these species (Stielow et al. 2015). The examples where the importance of phylogenetic species recognition is highlighted include species segregation of the endophytic genus *Muscodor* (Samarakoon et al. 2020), *Lentinula* (Hibbett et al., 1995), identification of *Archaeorhizomyces* fungal taxa (Menkis et al. 2014), *coniothyrium*-like taxa (Verkley et al. 2014) and *camarosporium*-like taxa (Wanasinghe et al. 2017). This concept often involves concatenated sequence alignments and other methods such as the genealogical concordance phylogenetic species recognition (GCPSR) (Stewart et al. 2014). Coalescent-based methods, which incorporate lineage sorting and the incongruent genomic regions into phylogenetic estimation procedures, are also used for species delimitation. These methods have been used increasingly to identify cryptic speciation in *Cladosporium herbarum* complex (Schubert et al. 2007), species complexes in *Colletotrichum* (Damm et al. 2009; Jayawardena et al. 2016), speciation in *Didymellaceae* (Aveskamp et al. 2010), *Fusarium* (Summerell et al. 2010; Crous et al. 2021), and *Phyllosticta* (Wulandari et al. 2009; Norphanphoun et al. 2020).

A major drawback of the phylogenetic species recognition is its subjectivity in determining the species limits (Taylor et al. 2000). This was overcome by introducing a new derivation of the phylogenetic species recognition, namely genealogical concordance phylogenetic species recognition (GCPSR), which is an empirical method relying on the concordance of more than one gene genealogy (Taylor et al. 2000). Under this criterion, we assume that recombination within a gene does not occur, and in practice segments of genes are usually used to build the genealogies. Therefore, genealogical concordance phylogenetic species recognition involves the comparison of lineages resolved in each of these individual trees. Species limits among the fungal species are defined based on the point of transition from concordance to incongruity among branches (Taylor et al. 2000). The use of genealogical concordance phylogenetic species recognition is practical for species delimitation in morphologically reduced fungal taxa or taxa that exclusively exhibit their asexual stages, such as *Wallemia* (Nguyen et al. 2015).

Genealogical concordance phylogenetic species recognition has been immensely useful for fungi because it is more discriminating than other criteria, and the difficulty to demonstrate in vitro crosses between fungal taxa (Cai et al. 2011). In recent years, many species delimitation studies applied genealogical concordance phylogenetic species recognition (Liu et al. 2016). Identification of two phylogenetic species in the *Fusarium oxysporum* species complex (Laurence et al. 2014), 12 independent lineages in *Melampsora* rust species, cryptic species in the *Wallemia sebi* species complex (Nguyen et al. 2015), speciation in *Blastomyces dermatitidis* species complex (Brown et al. 2018), *Colletotrichum* species complexes (Liu et al. 2016), *Neurospora* species complexes (Dettman et al. 2003), and lichenized *Xanthoparmelia* species complexes (Leavitt et al. 2011) are few of the examples for successful application of GCPSR for resolving various fungal taxa. However, one of the drawbacks of genealogical concordance phylogenetic species recognition is the difficulty to determine species limits when there are conflicts among gene trees, which occur due to recombination within a truly clonal population. However, recent studies on truly clonal mitosporic fungi (Anderson and Kohn 1998) and *Sclerotinia sclerotiorum* (Carbone et al. 1999) revealed the occurrence of recombination at some instances in addition to clonal reproduction. Further, it was previously believed that the genealogical concordance phylogenetic species recognition cannot be applied for species in *Glomeromycota* as no evidence was available for the occurrence of recombination in these species (Rosendahl 2008). Later, with the advances in genomics, genome mining revealed several genes involved in meiosis in arbuscular mycorrhizal fungi, suggesting a cryptic sexual stage (Corradi and Lildhar 2012; Riley et al. 2014; Kamel et al. 2017).

Coalescent-based species recognition

Even though there are diverse recognition criteria for species delimitation, they are continuously being challenged by cryptic and allopatric species. The variations encountered in species are not only genomic and morphological but also geographical and ecological. Thus, it is necessary to apply different methods to identify distinct evolutionary lineages (Xu and Yang 2016). A recent study recommended coalescent-based approaches as one of the best approaches to identify cryptic species (Bhunjun et al. 2021), which is a derivation of the phylogenetic species recognition. Coalescent methods deviate from the genealogical concordance phylogenetic species recognition by integrating lineage sorting and incongruent genomic regions into their phylogenetic estimation procedures (Carstens and Knowles 2007). In this approach, a framework is designed based on morphology, gene flow and gene genealogies. These coalescent-based approaches incorporate analytical methods derived from population genetics (Fujita et al. 2012) and facilitate speciation events (Fujita et al. 2012; Sukumaran and Knowles 2017; Lutsak et al. 2020).

Species boundaries detected by coalescent-based methods are considered as initial hypotheses, which need to be validated further by other species criteria such as morphological species recognition (Fujita et al. 2012). There are

several coalescent-based methods available as summarised in Table 1. Coalescent-based approaches, such as General Mixed Yule Coalescent (GMYC) (Fujisawa and Barraclough 2013) and Poisson Tree Processes (PTP), are intended to delimit species from single-locus data. These are used often in studies for animal and plant taxa (Myers et al. 2013; Satler et al. 2013; Demos et al. 2014; Domingos et al. 2014; Hedin et al. 2015; Villamil et al. 2019), but have rarely been used for fungal studies (Millanes et al. 2014; Bhunjun et al. 2021). Similar to the other approaches, the coalescent-based methods also have limitations. However, these approaches are not the only method to define species, but this will sharpen the edges and fill the gaps created by other methods. One of the main problems associated with this method is its inapplicability for taxa without any genetic resources such as fossil taxa. Another limitation of coalescent-based approaches such as GMYC is the computational constraint to obtain an ultrametric tree from tree calibration methods. The accuracy of GMYC of a population declines with the increase in mean or variance (Fujisawa and Barraclough 2013). The GMYC has shown a tendency of over-splitting in some cases, especially when using the multiple threshold method. The GMYC is applied based on the assumption that species are monophyletic, however, in reality, this is not true as the divergence times of real species prevent gaining monophyly (Hudson and Coyne 2002).

Bayesian inference method (Heled and Drummond 2010), maximum likelihood method (Kubatko et al. 2009), and parsimony method (Maddison 1997) belong to a class of methods that explicitly model the coalescent process. However, these methods suffer from incomplete lineage sorting as a cause of incongruence (Yu et al. 2011). Phylogenetic networks emerge as models that allow deep coalescence events by simultaneously capturing vertical and horizontal genetic material transfers (Linder and Rieseberg 2004). This approach applies to data sets comprising evolutionary events such as hybridisation, horizontal gene flow, recombination and gene conservation/duplication (Vijaykrishna et al. 2015; Bastide et al. 2018). Phylogenetic networks are used to visualise incompatible datasets and to represent putative evolutionary events. However, in species delineation, the second aspect is used. There are different types of phylogenetic networks such as phylogenetic trees, reticulate networks and split networks. Some studies have defined phylogenetic networks as a specific type of network analysis (Linder and Rieseberg 2004; Gusfield and Bansal 2005). Recent studies on cryptic species used split networks to illustrate incompatible signals in datasets, thereby capturing conflicting signals (Huson and Bryant 2006). The study by Chethana et al. (2017) on *Coniella*, employed several species criteria for species delimitation in the *C. diplodiella-C. diplodiopsis* species complex. The study used morphology, phylogeny and coalescent-based techniques to define species boundaries in the species complexes. All three species that belong to the *C. diplodiella-C. diplodiopsis* species complex exhibit almost identical morphological characters, hence morphology alone cannot delimit species (Chethana et al. 2017). However, the phylogenetic divergence demonstrated by the *Coniella vitis* isolates is extremely high (Fig. 2a). Split networks generated for all species in the complex demonstrated clear species boundaries (Fig. 2b; $\Phi w = 1.0$; $\Phi w > 0.05$), and the study also provided evidence for significant recombination (within the species $\Phi w = 0.0001207$; $\Phi w < 0.05$) among the isolates belonging to the phylogenetically and pathogenetically diverse *C. vitis* species. Similar to Chethana et al. (2017), many studies employed split networks in defining species boundaries for cryptic species or species complexes (Huson and Bryant 2006; Crouch 2014; Quaedvlieg et al. 2014; Jayawardena et al. 2017; Bhunjun et al. 2019). Unlike phylogenetic trees, the internal nodes of the split networks do not always represent hypothetical ancestors, but provide an implicit picture of evolutionary relationships, whereas reticulate networks provide an explicit picture of evolution (Huson and Bryant 2006). The nodes in reticulate networks represent hypothetical ancestors and the edges represent lineages of reticulate events. Phylogenetic network analysis also has limitations such as overestimation of reticulate events and computational inefficiency (Huelsenbeck 1995; Park et al. 2010). The applications of all these deviations of the phylogenetic species recognition have led to the discovery of over 200 novel cryptic species, unravelling the unknown depths of fungal diversity.

Even with its limitations in delimiting recently evolved species (Fujita et al. 2012), coalescent-based methods have shown impressive improvements in the discovery, resolution, consistency, and stability of the taxonomy of species compared to the distance-based methods (Yu et al. 2017).

Distance-based species recognition

One of the limitations of phylogenetic species recognition is its subjectivity to interpretation or formation of poorly supported clades. In these circumstances, distance-based approaches such as the Automatic Barcode Gap Discovery (ABGD) can be used as supporting evidence (Puillandre et al. 2012). The ABGD is the most common distance-based method that delineates species. It is an analytical tool that detects a barcode gap in the distribution of pairwise distances and determines the number of operational molecular units within the dataset (Puillandre et al. 2012; Kekkonen et al. 2015). ABGD can be used to delimit species when the intra- and interspecific genetic distances overlap, and the method sorts sequences into hypothetical species (Puillandre et al. 2012; Postaire et al. 2016). Similar to ABGD, objective clustering is another distance-based method used often to determine the number

Table 1 Commonly used coalescent-based approaches in resolving fungal taxa

Coalescent-based approach	Analytical framework	Examples
General Mixed Yule Coalescent (GMYC)	Best-fit tree branching models, Transition point from species to populations, and estimate of species number	Pons et al. (2006), Parnmen et al. (2012), Fujisawa and Barraclough (2013), White et al. (2014), Bustamante et al. (2019), Bhunjun et al. (2021), Hilário et al. (2021)
Poisson Tree Processes (PTP)	Uses a single-rooted tree as input and models the number of substitutions which occur before a speciation event	Zhang et al. (2013), Bustamante et al. (2019), Hilário et al. (2021)
Split networks	Provides a comprehensive and interactive framework for estimating phylogenetic trees and networks. Also important for statistical inference	Huson and Bryant (2006), Crouch (2014), Jayawardena et al. (2017), Bhunjun et al. (2019)
Bayesian Phylogenetics & Phylogeography (BP&P)	Uses reversible-jump Markov chain Monte Carlo algorithms to estimate the posterior distribution of different species	Leavitt et al. (2011, 2013), Singh et al. (2015), Chethana et al. (2017), Lutsak et al. (2020)
Species Tree and Classification Estimation, Yarely (STACEY)	Bayesian method for inferring both species delimitations and species trees under the multispecies coalescent model using sequences from multiple loci	Kanz et al. (2015), Mark et al. (2016), Lutsak et al. (2020)
tr2-delimitation	Rapid method for multilocus species delimitation using Bayesian model comparison, with reduced complexity of likelihood calculations because trees are decomposed into rooted triplets	Lutsak et al. (2020)
Brownie	Maximum likelihood or gene tree parsimony	O'Meara (2010)
SpedeSTEM	Maximum likelihood and/or information theory	Ence and Carstens (2011), Singh et al. (2015)
Multispecies coalescent (MSC)	Statistical framework that incorporates multi-locus data to evaluate alternative hypotheses of divergence among lineages while allowing for gene tree discordance under a neutral model of genetic drift	Stewart et al. (2014), Liu et al. (2016), Achari et al. (2020)

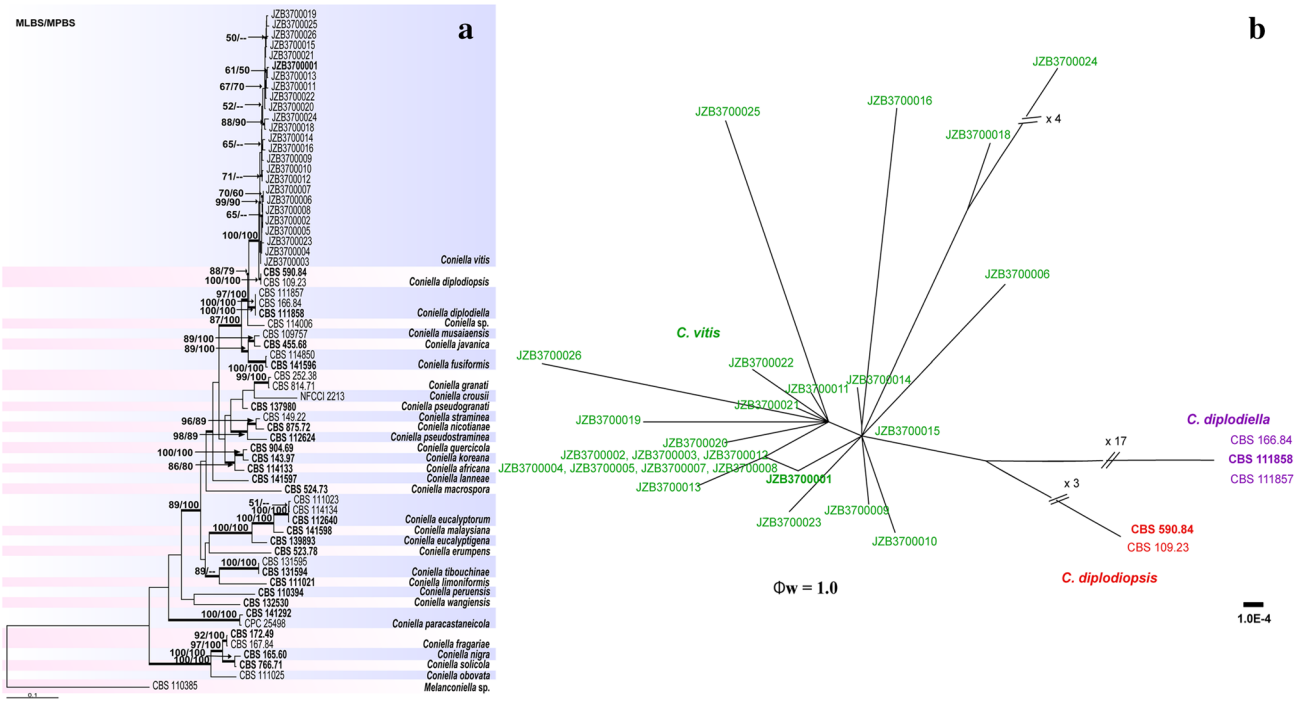

Fig. 2 High phylogenetic divergence among the members of the *Coniella diplodiella*, *C. diplodiopsis*, and *C. vitis* species complex. **a** Maximum likelihood analysis phylogram based on combined ITS, LSU, Histone and TEF 1-α gene regions supported with maximum parsimony and Baysian posterior probabilities. Maximum likelihood and maximum parsimony bootstrap values greater than 50% are indicated near the nodes, and branches with Bayesian posterior probabilities greater than 0.95 are given in bold. **b** Species boundaries confirmed by the split tree analysis conducted on the *C. diplodiella*, *C. diplodiopsis* and *C. Vitis* species. The Φ_w value < 0.05 indicates significant recombination. Reprinted with permission from Chethana et al. (2017)

of hypothetical species in a dataset. Objective Clustering uses the pairwise distances of a dataset and calculates the *p*-distance or K2P distance of the taxa in the dataset (Meier et al. 2006; Lin et al. 2015), allowing users to compare the generated clusters with existing taxonomy (Hawlitschek et al. 2013; Lin et al. 2015; Bhunjun et al. 2020). As ABGD and objective clustering are based solely on pairwise differences, they do not rely on an underlying genealogical tree, which could be a limitation in some cases. Several issues have been discovered in distance-based species delimitation studies. In Bhunjun et al. (2020), ABGD was unable to detect a barcode gap in the distribution of pairwise distances of some of the datasets, hence could not reliably sort sequences into hypothetical species. Further, several studies criticized the lack of phylogenetic content, evolutionary justification of these methods, potential subjectivity in the selected distance thresholds that mark the species boundaries and its bias towards closely related, less divergent species (Moritz and Cicero 2004; Taylor and Harris 2012). Hence, results produced from distance-based methods require support from additional phylogeny-related approaches such as GCPSR or coalescent-based methods. Some commonly used distance-based methods are given in Table 2.

Regardless of the approach employed in species delimitation, their discoveries should be amenable to statistical analyses (O'Meara 2010), a feature that is not provided by distance-based methods. In contrast to the distance-based methods, coalescent-based methods exhibit their probabilistic approaches, providing clear and objective testing of alternative hypotheses of evolutionary independence (Fujita et al. 2012).

Phylogenomics in fungal species delimitation and genomic species recognition

Even though concatenated multi-gene phylogenies comprising a set of unlinked loci conducted under the phylogenetic species recognition and its derivatives provide objective, quantitative analysis on the consistency of traditional fungal groups (Ciccarelli et al. 2006), erroneous phylogenetic inferences can be produced (Fitzpatrick et al. 2006; Rokas et al. 2003; Phillips et al. 2004). Several studies have suggested the use of genomic data for species delimitations (Avise and Wollenberg 1997; Coyne and Orr 2004; Sobel et al. 2010; Steele and Pires

Table 2 Commonly used distance-based approaches in resolving fungal taxa

Distance-based approach	Analytical framework	Application
Statistical parsimony network analysis (SPN)	Estimates sequence polymorphism and shows the evolution history of sequences	Bustamante et al. (2019), Clement et al. (2000)
Automatic barcoding gap detection (ABGD)	Detects a barcode gap in the distribution of pairwise genetic distances and determine the number of molecular operational taxonomic units in the dataset	Puillandre et al. (2012), Kekkonen et al. (2015), Bustamante et al. (2019), Bhunjun et al. (2020)
Objective clustering	Uses pairwise distances to determine the intra- and interspecific genetic distances of cluster sequences	Meier et al. (2006), Lin et al. (2015), Bhunjun et al. 2020

2011; Bobay and Ochman 2017; Matute and Sepúlveda 2019) because genomic data limit the impact caused by individual genes on the topology of the phylogenetic tree and result in a phylogeny that truly represents the entire genome (Fitzpatrick et al. 2006). With the major advances in high-throughput sequencing technologies, the available fungal genomes exceed 7000, representing more than 2000 species (Gostinčar 2020). Many studies use whole-genome data for species delineations (Gostiňcar et al. 2014; Gladieux et al. 2015; Sepúlveda et al. 2017; Magain et al. 2017; Lorch et al. 2018; Kobmoo et al. 2019; Matute and Sepúlveda 2019; Nguyen et al. 2019; Morin et al. 2019; Haridas et al. 2020). Hence, Xu (2020) proposed genomic species recognition, a genome sequence-based classification for fungal taxa to eliminate the inconsistencies in fungal taxonomy and integrate diverse criteria. There are many approaches in phylogenomics that are used for fungal species delimitations (Delsuc et al. 2005; Wang et al. 2009; De Vienne et al. 2012; Leavitt et al. 2016; McCarthy and Fitzpatrick 2017).

Phylogenies derived from genomic data provide strong support for higher-level classifications rather than species-level discriminations (Fitzpatrick et al. 2006). In the super tree approach based on 42 fungal genomes, Fitzpatrick et al. (2006) provided a highly congruent phylogeny for *Ascomycota* and resolved its subphyla *Pezizomycotina* and *Saccharomycotina*. In their study, *Leotiomycetes* were placed closest to *Sordariomycetes* and further, identified two classes in *Saccharomycotina* that correlate to their sexual morphs. A similar study conducted using 81 genomes of medically important *Aspergillus* and *Penicillium* species provides a robust phylogenetic and evolutionary framework. The study provides strong support for most of the relationships and identified incongruences within the family (Steenwyk et al. 2019). Gryganskyi et al. (2018) identified *Rhizopus* species into three major clades using the phylogenomic approach. These are few among many studies that demonstrate the use of phylogenomics for delimiting species (Chang et al. 2015; Chibucos et al. 2016; Spatafora et al. 2016; Shen et al. 2016).

Studies have also used the comparative phylogenomics approach based on the principle that genetic information conserved in all organisms are responsible for functional and anatomical similarities and divergences among different species (Nobrega and Pennacchio 2004; Hardison 2003). For example, Hardison (2003) observed significant differences in the genomes of morphologically similar taxa *Caenorhabditis elegans* and *C. briggsae*. In addition to the delineation of fungal taxa, comparative phylogenomics suggests gene family expansions and contractions, providing clues for adaptive radiations, which results in producing taxonomic orders, and specific phenotypic changes that lead towards species or generic level adaptations (Whiston and Taylor 2016). Studies have utilized comparative phylogenomics for fungal delimitation studies such as for pathogenic and non-pathogenic fungal taxa in *Onygenales* (Whiston and Taylor 2016), *Rhizopus* taxa in Mucorales (Gryganskyi et al. 2018), and evolutionary studies (Delaux et al. 2014).

Guidelines for defining species boundaries for taxonomic novelties

There is still confusion and disagreement among taxonomists on introducing new species as can be seen in the papers of this special issue. Most fungal taxonomic studies employ one or several species criteria to define species. Based on these criteria, different 'morphospecies', 'phylospecies' and 'biospecies' have been introduced (Lücking 2020). As a consequence of the differences between the species recognition criteria, different species boundaries have been proposed depending on the recognition criterion that was adopted. Simply, species boundaries and species numbers of a fungal taxon are highly dependent on the recognition criteria that are used. For example, application of either version of the phylogenetic species recognition results in the identification of many more fungal species than adopting the biological species concept (Lücking et al. 2014; Matute and Sepúlveda 2019). As previously discussed, species criteria cannot be universal, specifically for fungal groups that

experience phenotypic plasticity and convergent evolution. As this is the introductory paper for this special issue, we provide some general guidelines that can be used to delimit fungal species and recommend an approach that uses empirical evidence and what is known on the biology the group under study to determine how species should be best delineated in that group of fungi. Furthermore, guidelines for fungal delimitation tailored to specific fungal groups such as *Ascomycota*, basal fungi, *Basidiomycota*, *Dothideomycetes*, lichen-forming fungi, plant pathogenic fungi and yeasts are addressed in other papers of this special issue.

Use a combination of species criteria based on the fungal taxa in question and include the guiding species recognition used into the species description

Fungal species live under different conditions and most of the time a given recognition criterion is favoured in a particular condition. Further, it is difficult to decide *a priori* which species criteria best applies to delimit species in a given fungal group, because their biological and phenotypic aspects can have practical and theoretical limitations. Hence, to recognize fungal species in any environment, broader speciation criteria should be applied. Combinations used for identification cannot be universal because the factors that influence species cohesion vary among species (Campbell and Reece 2002). Most commonly used species recognition are morphological, ecological, and phylogenetic species recognition, including both coalescent-based and genealogical concordance recognition. The most commonly used combination for fungi is morphological combined with phylogenetic species recognition (Rehner and Buckley 2005; Trejo et al. 2015; Ariyawansa et al. 2015; Hyde et al. 2020b). In addition to this, morphological and ecological species recognition (Kasuga et al. 2003; Das and Aminuzzaman 2017), phylogenetic and ecological recognition (Del-Prado et al. 2013), morphological, ecological and phylogenetic recognition (Sanders et al. 2006; Justo et al. 2011; Zhang et al. 2011) and more recently morphological together with coalescent-based phylogenetic recognition (Aldrovandi et al. 2015) have been used in fungal studies. Some studies have combined the latter combination with ecological criteria (Looney et al. 2020). Since the early 2000's using morphological, ecological and phylogenetic species recognition including either coalescent-based recognition or genealogical concordance recognition has been popular among the mycological community (Cai et al. 2009; Groenewald et al. 2013). Quaedvlieg et al. (2014) described this combination as a "consolidated species concept (CSC)". In this study, Quaedvlieg et al. (2014) successfully applied CSC to distinguish species in *Teratosphaeriaceae*. Following this, many studies used this combination to resolve fungal taxa especially in morphologically conserved genera such as *Bipolaris*, *Colletotrichum*, *Diaporthe* and *Hermatomycetaceae* (Bakhshi et al. 2015; Chethana et al. 2017; Karimi et al. 2017; Bhunjun et al. 2020; Phukhamsakda et al. 2020). Phylogenetic species concept used in these studies is either concordance-based (Taylor et al. 2000) or coalescent-based (Meier et al. 2006; Puillandre et al. 2012).

Henceforth, we recommend using a combination of species criteria for the identification of fungal taxa. Species criteria used in the study is decided based on the fungal taxa in question and the authors should state the guiding species concepts used in their respective species delimitation studies (Lücking et al. 2020).

Include multiple collections, whenever possible, to account for and describe phenotypic variation within a species

In most of the species introductions over the last few years, new species were introduced based on a single collection. This may be problematic and biased because our conclusions are based on phenotypic characters and molecular data of a single fungal collection. Variations in the informative regions of sequences affect our decisions on species boundaries. Both genetic variation and undetected errors caused during sequencing could account for sequence differences. Having multiple collections of the new species enables the easy detection of errors. Even though Jeewon and Hyde (2016) accepted a certain level of variation in sequence data for new species, they have not mentioned how many sequences need to be included in the analysis to overcome bias caused by errors. Therefore in this paper, we recommend using a minimum of three collections whenever possible to define a species. However, in a number of cases, the differences (either morphology or molecular data) can be compelling enough to describe a new species based only on one collection. In recent years, a new series was established to publish new collections of fungal species known as "*Asian Journal of Mycology*" to overcome this bias towards species introductions based on single collections (Hyde et al. 2020c; Chethana et al. 2021). Moreover, it is worth noting that this is not always attainable for all fungi and for some circumstances exceptions should be made, such as for rare taxa, taxa from remote regions or highly specialized niches, and for fossilized or non-culturable fungi. It may also be better to describe an obvious new species than risk it becoming extinct. In such situations, the number of collections can be flexible, however, authors need to provide evidence to support their decision on the newly described taxon.

When using the morphological species recognition criteria,

Examine the holotypes of the fungal taxa where applicable

Even though molecular data has dominated fungal taxonomy, morphology still holds an important role in fungal identification. When introducing new species or publishing records of an existing species, it is important to observe type specimens of fungal taxa, when possible. If the type specimens are not accessible, other authentic specimens or reference specimens are available to observe. Re-examination of the holotypes of generic specimens provides insight on their unique morphological characters.

For quantitative characters, study intraspecific variations of that character from multiple specimens

When describing fungal morphology, many studies have used quantitative morphological characters such as size of spores, conidia and asci to distinguish novel species. However, the environment influences some of these characters, bringing into question our conclusions on delineation of fungal taxa, which are based on these quantitative characters. Furthermore, it is important to be aware of the fact that characters, such as spores at different developmental stages exhibit different properties, such as colour and size. So, using these characters may hinder the accurate spore features, hence affect our identification, which is based on the morphological features of the spore, such as size, colour, and the layers of the cell wall (Brundrett et al. 1996). Therefore, if quantitative characters are used to delineate species, multiple specimens, or collections should be observed to determine the variability between species.

Species with incomplete descriptions, no molecular data or unambiguous molecular data, try to examine the specimen or consider epi typification or neo typification

When describing species, it is always important to compare the fresh collections with type specimens. However, due to paucity of taxonomic data, unavailability of the types, type specimens with incomplete descriptions, or unavailability of molecular data or unambiguous molecular data, reference specimens are being designated. If the type specimens are not available to loan or have been lost or specimens are depauperate, it is recommended to designate a neotype. In cases where the holotype, lectotype or neotype associated with the validly published name are not accessible or cannot be identified, epitypes should be designated. Dayarathne et al. (2016) discussed that when fresh or modern collections are designated as epitypes or neotypes, these newly designated specimens must incorporate DNA based phylogeny. Even though it is difficult and time-consuming to examine old specimens or to perform epi- or neo-typification, studying these specimens is necessary, rather than introducing them as new species, to avoid an undesirable increase in species names.

When using the DNA-based species recognition criteria,

Use broad taxon sampling to account for variation

The accuracy and reproducibility of phylogenetic inferences rely on appropriate taxon sampling. Incomplete, biased, or improper taxon sampling can lead to misleading and inaccurate phylogenetic inferences (Plazzi et al. 2010). Thus, taxon sampling is one of the critical steps in phylogenetic analysis. For example, *Diaporthe eres* is polyphyletic and exhibits high intraspecific genetic variation (Udayanga et al. 2014a). However, a few recent species introductions have ignored this high genetic diversity and have instead used only one or two representative strains of *Diaporthe eres* in their phylogenetic analyses. Underestimation of the phylogenetic diversity of *D. eres* has resulted in the introduction of several new phylogenetic species within the *D. eres* complex, which belong to *D. eres*. Yang et al. (2018) and Manawasinghe et al. (2019) showed that when the phylogenetic analysis includes more strains representing *D. eres*, species such as *D. ellipicola*, *D camptothecicola* and *D. mathothocarpus* are not resolved as new species, but were the result of incomplete taxon sampling. Therefore, we recommend broad taxon sampling when introducing novel taxa to cover intraspecific variation, and include a statement about the taxon sampling strategy in the methods section.

Use the multi-locus approach for phylogenetic analyses

Molecular data hold immense importance in species delimitation studies. Introduction of novel fungal taxa based on a single locus affects the reproducibility and accuracy of the phylogenetic inferences and species limits cannot be determined unambiguously, hence, is strongly discouraged. However, in unavoidable circumstances, such phylogenetic inferences must exhibit a considerable resolution, and authors should provide additional evidence to support the decisions. We recommend using multiple loci for the phylogenetic analyses, re-presenting both ribosomal and protein-coding genes. However, for some basal fungal lineages ribosomal genes provide sufficient resolution (Seto and Degawa 2018; Corsaro et al. 2020; Seto et al. 2020). The reliability of these phylogenetic reconstructions depends on the quality of sequence data. The use of sequences which are compromised or with low-resolution taxonomic annotations and substandard technical quality can result in incorrect fungal

delimitations. Therefore, when selecting sequence data for phylogenetic analyses, sequence data should be used from verified sources such as the UNITE database (Nilsson et al. 2014).

Use multiple data types or polyphasic approach for clear delineation of species

Before the molecular era, fungal taxa were introduced mainly based on morphology. These morphological characters are often limited (Frisvad 2015). Therefore, the use of a single data type is not enough to differentiate fungal taxa. There are many data types that describe different characters of fungal taxa such as morphological, physiological, ecological, and molecular. The polyphasic approach involves using multiple data types and methods when defining species boundaries for cryptic fungal species or species complexes (Hudler et al. 1998). Most of the studies suggest an integrative taxonomic approach combining evidence from genealogical, biological, phenotypic and phylogenetic approaches to include different aspects of fungal autecology, physiology, and biochemistry to resolve cryptic species or species complexes (Yang and Rannala 2010; Padial et al. 2010; Udayanga et al. 2014b; Haelewaters et al. 2018; Kruse et al. 2018; Bhunjun et al. 2020; Lücking et al. 2020). When defining species boundaries for cryptic species or for species complexes, it is recommended to apply genealogical concordance phylogenetic species recognition, coalescent-based or distance-based techniques together with other types of data such as morphological and ecological data. Further, additional approaches such as recombination analyses, phylogenetic incompatibility analyses and sequencing of restriction site-associated DNA markers (RADSeq) provide the evidence to support the decisions on the species delimitations. Employing different approaches for species delimitation does not promote competitiveness among the approaches, but rather provide several elements of a holistic strategy to define species boundaries.

When using the ecological species recognition criteria

Define the ecology as much as possible

When introducing new species, describe their ecology as much as possible. These ecological data, such as substrate, host, and locality, will facilitate future research.

Conclusions

In the current review, we discussed species definition, speciation, and species recognition criteria. We distinguished the difference between theoretical species concepts and operational species recognition criteria. Here, we followed the proposal of Taylor et al. (2000) by abandoning the term 'species concept' and replacing it with 'species recognition' for operational concepts. We discussed each of the species recognition criteria, their applications and the problems met during their applications. Herein, a set of guidelines are provided, which are to be followed when delineating fungal species. These guidelines do not resolve all problems faced during fungal identifications, nor do they offer a standard applicable to all fungi. They are, however, a general set of suggestions that should be considered when introducing a new species. More specific guidelines are necessary when dealing with different groups of fungi. We believe that guidelines for fungal identifications cannot be standardized and they should be tailored according to the fungal group in question.

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Declarations

Conflict of interest The authors declare that there is no conflict of interest.

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