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Review

# Fungal species and their boundaries matter – Definitions, mechanisms and practical implications

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ABSTRACT

Recent scientific and technological advances have improved and streamlined our ability to recognise and describe fungal species. Detailed comparative genomics studies have also expanded our understanding of species boundaries. Against this background, we explore the nature of fungal species and consider how this impacts our understanding of their genetics and evolution. The current body of evidence suggests that fungal species are unique evolutionary units that are separated from one another by boundaries that are "porous" under certain conditions ("semipermeable" in analogy to the differential permeability of membranes). Overall, the penetrability of these boundaries depends on the relatedness between donor and recipient species, the spatial proximity of related species to one another during their evolution, and the evolutionary potential associated with the breach of a boundary. Furthermore, the semipermeable nature of species boundaries fundamentally affects the population genetics of a species, with potentially profound effects on its overall evolution and biology. This also influences the methodologies used in taxonomy, because some species appear capable of maintaining their genetic isolation despite extensive penetrability of their boundaries. Most analytical procedures are also not able to distinguish the signals of species boundary permeability from those associated with incomplete lineage sorting or intraspecific diversity. Collectively, these issues greatly complicate how we study and name fungi. An awareness of the nature of species, their boundaries and the biological and genomic signatures of boundary breaches, will enhance our ability to identify them and, perhaps more importantly, to develop realistic strategies to manage and manipulate their growth and distribution.



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## 1. Introduction

The importance of fungi in agriculture, forestry, medicine, industry and ecosystem functioning is widely recognised, yet only a small fraction of the millions of species suggested to inhabit our planet have been formally described (Blackwell, 2011). This lag in documenting fungal diversity will likely speed up because of developments in DNA sequencing technologies and the streamlining of naming schemes, which simplifies species discovery and description (Crous *et al.*, 2015; Hibbett and Taylor, 2013). Despite these advances, however, the identification and delineation of new species will not necessarily become easier. This is primarily due to complexities regarding their distribution and behaviour – fungi occur everywhere on earth, they can interact with one another and other organisms across the Tree of Life, and given appropriate circumstances, benign or beneficial species can become harmful to their associates and *vice versa*. This heterogeneity and ability to adapt to new environments complicate taxonomy, especially in recently evolved taxa, because the type and magnitude of change needed for bringing about a new taxon vary on a case-by-case basis. This is clearly evident in emerging pathogens, the evolution and establishment of which, is typically unpredictable and associated with extreme agricultural, medical or environmental impacts.

All studies of fungi, including those dealing with ecology, genetics or any other branch of mycology, rely to some extent on the identification and analysis of one or more species. The philosophical and theoretical aspects of these taxonomic units has been the topic of intensive debate for the greater part of the last century (Wilkens, 2010; Ereshefsky, 2010). As a consequence, numerous concepts of what species are have been proposed (e.g., Mayden, 1997). Practical application of these existing ideas and concepts for delimiting fungal species, however, is not straightforward (e.g., Harrington and Rizzo, 1999; Taylor *et al.*, 2000). Although there is generally an intention to recognise units approximating those occurring in nature, taxonomic studies are almost always limited and delimited by the available biological samples and experimental/analytical resources. Given these realities, species delineation and description commonly involve some degree of qualitative judgement (e.g., weighting of certain delineation criteria over others) or subjective interpretation (e.g., of divergence estimates) (Leavitt *et al.*, 2015; Taylor *et al.*, 2000). In order to delineate fungal species, taxonomists also utilize a range of approaches that can differ markedly in how species are conceptualized (e.g., Cai *et al.*, 2011; Crous *et al.*, 2015). Collectively, these issues (i.e., the human element and the multitude of species concepts) represent significant hurdles when seeking a definition for fungal species that is biologically meaningful and that would allow unambiguous delineation of these units.

Here we explore the nature of fungal species and examine how this might impact upon our understanding of their genetics and evolution and our ability to accurately recognise and potentially control them. We first consider how species are conceptualized. The nature of the so-called "species boundary" and its apparent permeability is then systematically scrutinized and aligned with our expectations for the population biology of fungal species. We illustrate how this influences methodologies for species identification, especially those that are plant and animal pathogens. By dissecting the basic units of fungal diversity in this way, the potential to improve our knowledge of their genetic makeup and the forces driving their evolution is highlighted. Where possible, we refer to the wealth of literature on fungal systematics, population biology and comparative genomics that has accumulated in recent times.

## 2. What are fungal species?

Wiley (1978) defined a species as "a lineage of ancestral descendant populations which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate". De Queiroz (2007) later refined this ontological definition with the General Lineage concept, which describes species as segments of separately evolving metapopulation (interconnected subpopulations making up an inclusive population) lineages. By viewing species from a population genetic perspective, Mallet (1995, 2007a) introduced the Genotypic Cluster concept that defines species as multi-locus genotypic clusters, separated from one another by discontinuities in the characters and character states used to recognise them. The clusters in Mallet's concept are thus comparable to De Queiroz's lineage segments, while Mallet's character and character state discontinuities are outcomes of the specific evolutionary processes responsible for shaping the trajectory of that lineage through time.

Both the General Lineage and Genotypic Cluster concepts are applicable to all organisms on Earth because they do not stipulate criteria for the species category (Pigliucci 2003; Ereshefsky 2010). Other than being independent evolutionary units, specific processes (e.g. reproductive isolation and ecological adaptation) are not required to recognise the existence of a species. Therefore, both concepts are suitable to delimit fungal species using the commonly employed species recognition criteria (Table 1). In the case of the General Lineage concept, the criteria would be used to show that a species is a segment of an independently evolving lineage. For the Genotypic Cluster concept, the species in question would be shown to consist of a cluster or group of individuals that share commonalities at multiple loci across their genomes, while at the same time lacking certain shared characters with other such groups. Despite these differences in philosophical perspective, both concepts thus allow empirical discovery of species using multiple independent lines of evidence, which is a common practice in modern fungal taxonomy (see Table 2).

In this commentary, we mainly use a paradigm of species that closely resembles Mallet's Genotypic Cluster concept. In part, this is because it resonates strongly with a widely used definition for fungal species: "group[s] of individuals separated by heritable character discontinuities" (Hawksworth, 1996). It also aligns with the innate human capability to perceive biodiversity as being discontinuous and consisting of clusters or groups of like individuals, where individual clusters are separated from one another by gaps in the continuum

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**Table 1 – Criteria commonly used to recognise fungal species.**

| Criterion[a] | Basis for species recognition and current application |
|---|---|
| Phenotypic/genetic cohesion | Individuals of species form distinguishable groups based on their shared observable attributes (e.g. morphology and other cultural traits, as well as DNA-based characters such as electrophoretic profiles and marker alleles). Although this is widely used in fungal systematics, most of the experimental procedures employed to evaluate this criterion lack resolving power. The criterion is useful to obtain a preliminary perspective of potential species limits, but is normally not used as principal tool for delineation. |
| Reproductive biology | Species are interbreeding populations that are reproductively isolated from other such populations. Despite its historical use in fungal taxonomy, this criterion is not applicable to all fungi (e.g., many fungi are apparently asexual and some do not require mates to complete the sexual cycle). Many species also retain the ability to mate despite being distinct taxonomic units. Not surprisingly, few contemporary studies employ reproductive biology as a main criterion to delineate fungal species. |
| Ecological cohesion | Species consist of individuals adapted to a specific niche. Although this criterion is useful for pathogenic and symbiotic fungi, the ability of most fungi to occupy diverse environments limits its taxonomic value. |
| Phylogenetic diagnosability | Species are irreducible clusters of individuals that are characterized by unique combinations of character states. In fungal ecology, this criterion is widely applied to develop species hypotheses from DNA barcoding data. Because species limits are subjectively interpreted from divergence estimates, this criterion only provides a preliminary appraisal of the potential species units under consideration. |
| Genealogical exclusivity (also known as genealogical concordance) | A species consists of a cluster of individuals whose gene sequences coalesce exclusively, and more recently, with one another than with those of other species. Because of its relatively straightforward implementation and objectivity in determining species limits, genealogical exclusivity is widely used as a principal criterion for delineating fungal species. |

[a] These were derived from the secondary or operational species concepts reviewed by Mayden (1997) and later emphasized for fungi by Taylor *et al.* (2000). For fungus-specific perspectives on ecological speciation and phylogenetic diagnosability in barcoding data, see the reviews of Giraud *et al.* (2010) and Rydberg (2015), respectively.

of traits observed (see Ereshefsky, 2010; Pigliucci, 2003). Furthermore, the notion that the members of a Genotypic Cluster share unique commonalities at multiple loci across their genomes, directly correlates with the practice of utilizing multiple and diverse lines of evidence to delineate fungal species (i.e., traits encoded from multiple genetic loci; see Table 2). Finally, "clusters" are less abstract constructs than "lineage segments", and the concept of species as multi-locus genotypic clusters would potentially simplify discussions regarding the dynamic nature of species boundaries.

## 3. Fungal species boundaries are semipermeable

When species are viewed as multi-locus genotypic clusters embedded within biodiversity continua, the character and character state discontinuities separating them denote species boundaries. In other words, the species boundary is that abstract line demarcating a species and separating it from other species (Fig. 1). In fungi, this boundary is thought to be the result of so-called "species isolation mechanisms" (Table 3) that limit or prevent the exchange of genetic material among non-conspecifics. However, it is not a completely impregnable barrier. Experimental evidence from plants and animals clearly show that species boundaries are naturally penetrable and that species can evolve independently, despite often maintaining significant levels of interspecies genetic connectivity (Harrison and Larson, 2014; Wu and Ting, 2004).

In fungi, the penetrability of species boundaries is reflected by the common occurrence of hybrid species. Well-studied examples include those that emerged from natural genetic crosses between non-conspecifics in genera, such as *Saccharomyces* (Leducq *et al.*, 2016), *Epichloë* (Shoji *et al.*, 2015), *Verticillium* (Inderbitzin *et al.*, 2011) and *Zymoseptoria* (Stukenbrock *et al.*, 2012). Various studies have also reported leakage of foreign DNAs into the gene pools of well-delimited fungal species through the process of introgressive hybridisation (Table 3). Notable fungal examples include introgressions from *Microbotryum silenes-dioicae* into *M. lychnidis-dioicae* (Gladieux *et al.*, 2011), from *Heterobasidion annosum* into *H. irregular* (Gonthier and Garbelotto, 2011) and into *Neurospora tetrasperma* from closely related *Neurospora* species (Sun *et al.*, 2012). In addition, a growing body of evidence suggests that foreign genetic elements readily cross the fungal species boundary and become established in populations via processes typically implicated in horizontal gene transfer (HGT) (Table 3) and as reviewed by Fitzpatrick (2012) and Soanes and Richards (2014).

Broad scale empirical evidence remains lacking for fungi, but their species boundaries, like those of other eukaryotes (reviewed by Harrison and Larson, 2014), are probably permeable only in certain situations. To account for the apparent

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**Table 2 – Some of the names for taxonomic approaches that use multiple independent lines of evidence to recognize and describe fungal species.**

| Name | Description of the approach | Selected references |
|---|---|---|
| Polyphasic taxonomy | The term was initially introduced for bacteria to take "into account all available phenotypic and genotypic data and integrates them in a consensus type of classification". The term is similarly used in studies aiming to delineate fungal species. | Vandamme *et al.* (1996), Wicht *et al.* (2012), Stadler *et al.* (2014) |
| Consolidated and consilient species concepts | Two formalizations of the polyphasic approach, both of which are based on "the convergence of multiple, independent data sets, as a means of delimiting species". In fungal studies, both terms refer to the use of phylogenetic, morphological, physiological and ecological data to delineate species. | Jančič *et al.* (2015), Quaedvlieg *et al.* (2014) |
| Integrative taxonomy | The term was initially introduced for animals and is defined as "the science that aims to delimit the units of life's diversity from multiple and complementary perspectives". Although not commonly used in fungal systematics, the term has been applied in situations where information on evolutionary process (e.g, phylogeography and coevolution) was used in addition to molecular, morphological, and ecological data to delineate fungal species. | Dayrat *et al.* (2005), Millanes *et al.* (2014), Zamora *et al.* (2015) |

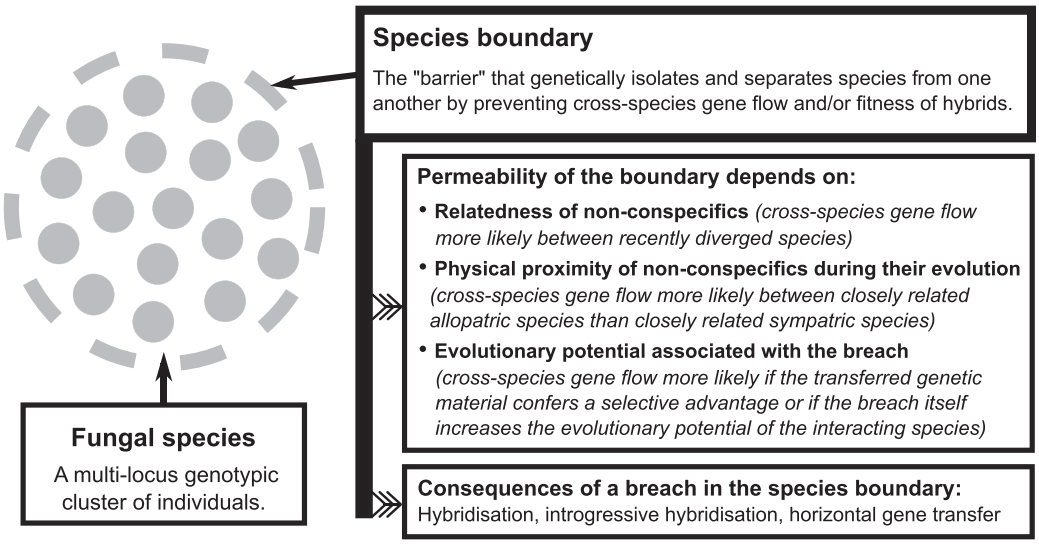


**Fig. 1 – An overview of the fungal species category using the paradigm of species as multi-locus genotypic clusters with semipermeable boundaries. The collection of grey dots denotes such a species or genotypic cluster and the dashed line encircling them depicts the selectively penetrable boundary.**

selectivity of the process, the adjective "semipermeable" (used in cell biology in reference to the differential permeability of membranes) is favoured over "porous" or "permeable" to describe the boundary (Harrison and Larson, 2014). Nonetheless, based on what we know, the penetrability of fungal species boundaries seems to depend on at least three factors: (i) phylogenetic relatedness between the non-conspecifics whose genetic material crosses the species boundary; (ii) the degree to which selection has reinforced reproductive isolation of the interacting non-conspecifics; and (iii) the evolutionary potential that accompanies a species boundary breach. Each of these factors is briefly outlined below (also see Fig. 1).

### *Phylogenetic relatedness*

Penetrability of the species boundary firstly depends on the relatedness between the interacting species during hybridisation or between genetic donors and recipients during HGT. Because the penetrability of the boundary is contingent on the evolution of suitable species isolation mechanisms (Table 3), its overall stringency generally increases with time since the species diverged. In other words, cross-species transfers of genetic material are more likely between closely related fungal species than between more distantly related individuals. This is reflected by the numerous examples of breaches in the species boundary involving

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hybridisation and introgressive hybridisation between closely related non-conspecifics (reviewed by Stukenbrock, 2016). The extent of HGT among fungi seems to follow a similar pattern (see Qiu *et al.*, 2016 and the references therein). Therefore, the frequency and likelihood of HGT-based breaches across fungal species boundaries also depend to some extent on the relatedness between the donor and recipient species (Qiu *et al.*, 2016), as have been shown for prokaryotes (Popa and Dagan, 2011).

### *Reinforcement of reproductive isolation*

The physical proximity of evolving species to one another can affect the permeability of their boundaries. This is particularly noticeable when allopatric species that evolved in separate and non-overlapping geographic locations are compared with sympatric species that evolved in close proximity to one another (Coyne and Orr, 2004; Wu and Ting, 2004). The boundaries between sympatric species are typically reinforced and generally less penetrable than those between allopatric species (Turner *et al.*, 2011). In contrast to allopatric species, the evolution of sympatric species involves direct selection for genetic isolation, which enhances the mechanisms responsible for their isolation. Most previous studies on the role of reinforcement in fungi have focused on how cross-species gene flow is limited during sexual encounters between sympatric species (Coyne and Orr, 2004; Turner *et al.*, 2011), but it has been suggested to also buttress the boundary between sympatric species that interact vegetatively (Hu *et al.*, 2014). It is thus not surprising that most known fungal examples of hybridisation and introgressive hybridisation have involved species that diverged in allopatry (Fisher *et al.*, 2012; Giraud *et al.*, 2010; Gladieux *et al.*, 2011; Leducq *et al.*, 2016; Leroy *et al.*, 2016).

### *Evolutionary potential*

Previous work on fungi suggests that the genetic elements (i.e., genes/chromosomes/genome regions) involved in species boundary breaches are sources of genetic innovation and adaptive evolution (Arnold, 2004; Gladieux *et al.*, 2014; Soanes and Richards, 2014). The populations, introgressed lines or hybrids harbouring such elements are often more fit than the parental species. This is evident in fungi such as *Cryptococcus neoformans* (Kavanaugh *et al.*, 2006), and species of *Fusarium* (Ma *et al.*, 2013) and *Coccidioides* (Neafsey *et al.*, 2010), where the genetic elements transferred across species boundaries encode for genes allowing improved niche exploitation. Acquisition of these elements may further allow for the regeneration of loci in which deleterious mutations have accumulated (e.g., the mating-type determining chromosome of *N. tetrasperma*) (Corcoran *et al.*, 2016). They can also confer adaptive architectural changes to the genome. For example, hybridisation among *Saccharomyces* species causes chromosomal rearrangements that result in postzygotic reproductive isolation and subsequent ecological divergence (Leducq *et al.*, 2016).

Mutational processes associated with hybridisation and HGT can also introduce variation in the genomes of fungi. For example, a breach in the species boundary can activate transposable elements, thus mediating DNA insertions and excisions, as well as genomic rearrangements (Daboussi and Capy, 2003). Even the ploidy variation typically associated with hybridisation or HGT-based acquisition of whole chromosomes can be mutational (Depotter *et al.*, 2016; Stukenbrock and Croll, 2014). In such cases, the sudden multiplication of genome copies gives rise to a type of "genome shock" that causes rapid genome-wide mutation and transcriptome reorganization, which in turn drives adaptive evolution (Cox *et al.*, 2014).

## 4. Semipermeable species boundaries affect the population biology of fungi

The semipermeable nature of fungal species boundaries manifests most clearly in the population biology of these organisms. The term "population biology" refers to the diversity and distribution of genetic variation within and across the populations of a species (Halliburton, 2004), all of which are dependent on the various forces that drive the evolution of a species (McDonald and Linde, 2002). Accordingly, a breach in the boundary of a species (i.e., cross-species gene flow involving the movement of genes or genomic regions from one genome to another [Mallet, 2001]) could influence the structure of its populations. It could also influence how selection and recombination would change the amount and distribution of variation across the populations of such as species. A boundary breach may further influence the effective population size ($N_e$) of a species and its overall vulnerability to genetic drift. The effects of species boundary semipermeability on each of these factors are briefly outlined below.

### *Cross-species gene flow and genetic variation*

Compared to mutation, cross-species gene flow is anticipated to influence the population biology of a species more dramatically. Apart from increasing genotype diversity (Burnett 2003; Halliburton 2004), cross-species gene flow has the added effect that it can facilitate *de novo* mutation. For example, mutations can arise due to the activity of transposons encoded on the genetic elements acquired via HGT (Soanes and Richards, 2014), or mutation can be a consequence of genome shock following polyploidization or the acquisition of foreign chromosomes (Calo *et al.*, 2013; Morrow and Fraser, 2013). Mutational processes accompanying species boundary breaches can, therefore, be expected to provide some (if not most) of the genetic variation on which selection and drift (see below) can act, to ultimately facilitate the establishment of more fit populations, hybrids or introgressed lines.

### *Natural selection*

Natural selection largely determines the fate of a population after a breach in the species boundary. Selection changes the frequency at which alleles/phenotypes occur in populations and the magnitude of these changes is partly dependent on the adaptive nature of the respective alleles/phenotypes (Halliburton, 2004). Following a species boundary breach,

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**Table 3 – Concepts relevant to the discussion of fungal species and the semipermeable nature of their boundaries.**

**Horizontal gene transfer (HGT)**

HGT refers to the transfer of genetic material between species (Soanes and Richards, 2014) via processes other than hybridisation (Mallet, 2005). In fungi, any cell type can participate in HGT, because, unlike in certain other multicellular eukaryotes (Richards *et al.*, 2011), they do not sequester their heritable genetic material to particular cell lines. Therefore, any cell of a fungal individual can acquire foreign elements that, together with the cell's own genetic material, can be passed on to neighbouring cells or individuals and subsequent generations to ultimately facilitate invasion of the foreign material into the species' gene pool.

Foreign genetic elements (e.g., stretches of DNA, genes, gene clusters and whole chromosomes or parts of chromosomes) can enter a fungal individual's genome during unstable anastomosis (fusion of vegetative cells or hyphae) and heterokaryosis (occurrence of genetically different nuclei in a mycelium) involving non-conspecifics (Burnett, 2003; Fitzpatrick, 2012; Richards *et al.*, 2011). Under these conditions, the interacting non-conspecifics exchange cytoplasmic/organellar DNAs/RNAs, thus facilitating the potential integration of these sequences into a new genome (Soanes and Richards, 2014).

Recent evidence also suggests that foreign genetic elements can enter the genome of a fungal individual through processes typically associated with prokaryotic HGT (Richards *et al.*, 2011; Thomas and Nielsen, 2005). These include transformation or uptake of naked exogenous DNA from the environment (e.g., Nevoigt *et al.*, 2000; Mentel *et al.*, 2006), as well as conjugative bacterium-to-fungus (e.g. Heinemann and Sprague, 1989; Sawasaki *et al.*, 1996) and transductive virus-to-fungus (e.g., Liu *et al.*, 2010; Taylor and Bruenn, 2009) transfer of genetic material (Fitzpatrick, 2012; Richards *et al.*, 2011; Soanes and Richards, 2014).

**Hybridisation and hybrids**

Hybridisation refers to the mating of non-conspecific individuals and the production of viable recombinant offspring (Mallet, 2007b; Kirk *et al.*, 2008). In fungi, such matings can occur during sexual and asexual/vegetative encounters (see table entry below on sex and parasex) between non-conspecific individuals (Schardl and Craven, 2003; Stukenbrock, 2016). This is followed by the "genomic merger" of the interacting individuals, from which offspring with unique chromosomal compositions and architectures is produced (Albertin and Marullo, 2012; Mallet, 2007b; Stukenbrock, 2016). Homoploid hybrids are those with chromosome numbers similar to that of a parent, allopolyploid hybrids have chromosome numbers approaching the sum of the chromosome numbers of the parents, while the chromosome number of aneuploid hybrids differ by one or a few chromosomes from those of their parents (Albertin and Marullo, 2012; Giraud *et al.*, 2008).

**Introgression and introgressive hybridisation**

Introgession refers to the invasion of new genetic material into a species' gene pool by means of hybridisation and backcrossing (Harrison and Larson, 2014; Mallet, 2005). Backcrossing and recombination of a transient or short-lived hybrid with individuals of one of the parental species allows for the incorporation of new genetic material (originating from the other parental species) into the genome of that parental species (Baack and Rieseberg, 2007; Harrison and Larson, 2014; Stukenbrock, 2016). The process, by which hybridisation-derived genetic material is introgressed into the gene pool of one of the parental species, is referred to as "introgressive hybridisation" *sensu* Anderson and Hubricht (1938).

**Phylogenetic incongruence**

Incongruence among phylogenies inferred from different loci is often used as evidence for a historical breach in the boundary of a species, but may also reflect instances of incomplete lineage sorting or analytical shortcomings (Wendel and Doyle, 1998). In contrast to these shortcomings that can be overcome with appropriate experimental design, incomplete lineage sorting is an evolutionary phenomenon. Also known as deep coalescence, it refers to situations where the "common ancestry of gene [sequences] at a single locus extends deeper than speciation events" (Maddison, 1997). Rather than having evolutionary trajectories that coalesce with ancestral sequences in their own lineages, the incompletely sorted loci of non-conspecifics coalesce with that of the homolog in their ancestor. Accordingly, incompletely sorted loci typically group closely related non-conspecifics together instead of separating them. Various analytical procedures, particularly those based on coalescence (i.e., the stochastic process of merging ancestral alleles backward in time) (Rosenberg and Nordborg, 2002), have been developed for distinguishing the signatures of species boundary breaches from those associated with incomplete lineage sorting (reviewed by Yu *et al.*, 2011).

**Sex and parasex**

Sex and parasex are processes that involve the fusion of parental cells, karyogamy or fusion of nuclei, chromosomal crossover and ploidy changes to produce recombinant progeny (Kirk *et al.*, 2008; Ni *et al.*, 2011; Schardl and Craven, 2003). The primary differences between sex and parasex pertain to the parental cell types involved, and how chromosomal crossover and ploidy changes are achieved. Sex involves the fusion of reproductive cells from sexually compatible partners (Ni *et al.*, 2011), while parasex involves the fusion of cells of vegetatively compatible individuals (Aanen *et al.*, 2010). In sex, meiosis facilitates chromosome crossover and chromosome reduction to the homoploid condition (i.e., the same ploidy as the parents) (Schardl and Craven, 2003). This is true for intraspecies and some interspecies crosses, although irregular meiosis during the latter could also generate hybrid offspring with chromosome numbers different from those of their parents. In parasex, which is meiosis-independent, chromosome crossover of the fused nuclei occurs during mitosis and ploidy reduction happens apparently stochastically via random chromosome loss (Pontecorvo, 1956; Schardl and Craven, 2003).

**Species isolation mechanisms**

These mechanisms restrict or prevent the sharing of genetic material among non-conspecific individuals. They include systems that limit the transfer/acquisition of foreign genetic material or that subsequently prevent its incorporation and persistence in the new genome.

In fungi, limitation of transfer/acquisition of foreign genetic material is facilitated by sexual and vegetative incompatibilities among non-conspecific individuals (Aanen *et al.*, 2010; Giraud *et al.*, 2008; Glass and Kaneko, 2003), and restrictions in the potential for non-conspecifics to encounter one another (e.g., through differential ecological specialization or preferential self-fertilization) (Giraud *et al.*, 2008). Genetic isolation can also be facilitated via modulations of reproductive mode (e.g., preferential selfing with limited outcrossing), and mechanisms that cause the inviability of hybrids (Giraud *et al.*, 2008). Although little is known regarding the mechanics and regulation of horizontal gene transfer (HGT; see table entry above) in fungi, it is likely that these organisms also employ processes to limit the acquisition of foreign genetic material from the environment, viruses and other microorganisms (e.g., limits on the availability of suitable receptors and specificities of nucleic acid uptake and transfer mechanisms) as have been demonstrated for prokaryotes (Thomas and Nielsen, 2005).

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**Table 3 (*continued*)**

Should any of the initial isolation mechanisms fail, the incorporation and persistence of the acquired genetic material in the new genome is dependent on the activity of genome defence systems and genomic epistasis. Defence systems that protect genomes against or purge them of foreign genetic elements (Richards *et al.*, 2011), include repeat induced point mutation (RIP), methylation induced premeiotically (MIP) and RNA interference-based systems like meiotic silencing of unpaired DNA (MSUD) and quelling (Dang *et al.*, 2011; Romano *et al.*, 1992; Selker, 2002).

Genomic epistasis refers to the functional compatibility among interacting alleles at unlinked loci (Phillips, 2008). These interactions are typically broken down during the intragenomic conflict that occurs in individuals whose species boundary was breached (Richards *et al.*, 2011; Stukenbrock, 2013). The incompatibility among allele or gene combinations (i.e., negative epistatic interactions) in such an individual's genetic background might reduce its fitness or cause it harm, thus allowing its loss from the population and simultaneously eliminating the foreign genetic material from the species' gene pool (Brown and O'Neill, 2010; Giraud *et al.*, 2008; Gladieux *et al.*, 2014; Stukenbrock, 2016).

selection would thus determine whether or not the acquired phenotypes/genes/genome regions would be retained in the population. And, depending on the adaptive value of these elements, directional selection might increase their frequencies until they become fixed. Furthermore, because gene flow between non-conspecifics can introduce variation much faster than mutation alone, natural selection would probably favour the inherent permeability of a species boundary. Substantial evidence for this is lacking, but various well-documented fungal cases illustrate how cross-species acquisitions of genetic variation have facilitated "abrupt evolutionary changes" (Stukenbrock and McDonald, 2008; Stukenbrock, 2016). These have led to the emergence of populations, hybrids or introgressed lines that have niches broader than those of their parents (e.g., Inderbitzin *et al.*, 2011), that can outcompete their parents (e.g., Farrer *et al.*, 2011), that occupy new niches (e.g., Leducq *et al.*, 2016) or that infect new crops (Menardo *et al.*, 2016). In all these examples, a breach in the species boundary represented a convenient mechanism for establishing genetic variation from which selection could drive the emergence of new (or "improved") multi-locus genotypic clusters (or metapopulation lineage segments) over exceptionally short timescales (Mallet, 2007b; Gladieux *et al.*, 2015; Stukenbrock and McDonald, 2008).

### *$N_e$ and genetic drift*

$N_e$ refers to the number of individuals in a population whose genetic information may be inherited by progeny. It thus reflects the overall genetic diversity that is transferable to the next generation (Burnett, 2003; Stukenbrock, 2016; Wright, 1931). Therefore, the $N_e$ of a fungal species determines the degree to which cross-species gene flow and selection would ultimately affect its population biology (McDonald and Linde, 2002). Generally, cross-species gene flow can be expected to affect the population biology of a species with a small $N_e$ more than one with a large $N_e$ (Burnett, 2003). Also, when $N_e$ is larger, natural selection can be expected to more efficiently drive the acquired phenotypes/genes/genomic regions to fixation, especially when they convey fitness advantages (Lanfear *et al.*, 2014). In turn, the loss of such elements, via the stochastic processes of genetic drift, is expected to be much more pronounced when $N_e$ is smaller (Burnett, 2003; Lanfear *et al.*, 2014).

### *Recombination and new allelic combinations*

An immediate consequence of recombination following cross-species gene flow is increased population diversity, especially under conditions of restricted sexual reproduction. For example, when dispersal extrinsically restricts meiotic recombination (Taylor *et al.*, 2015), the generation of new allelic combinations from the resident and foreign genetic material would be an important source of novel variation. Here, the new combinations may be brought about by mitotic recombination that presumably occurs during DNA processing and repair (Aguilera *et al.*, 2000; Andersen and Sekelsky, 2010) or during parasex (see Table 3) (Calo *et al.*, 2013; Hickman *et al.*, 2015; Schardl and Craven, 2003). A similar scenario would be true when sexual reproduction is intrinsically restricted to mating strategies that involve selfing (homothalism) or high levels of inbreeding (Taylor *et al.*, 2015). Under such conditions, and following a breach in the species boundary, new allelic combinations may arise from the resident and foreign genetic material via either meiotic or mitotic recombination. Overall, however, such increases in genetic variation would differ from case to case and their impact on the population would depend on the combined effects of $N_e$ and selection (e.g., Anderson, 2005; Mandegar and Otto, 2007).

### *Recombination and reproduction*

Recombination following cross-species gene flow can indirectly affect the population biology of a species by changing its reproductive mode. For example, rare sexual recombination between an inbreeding or homothallic species and other closely related species would afford the inbreeder/homothallic all the benefits of outcrossing (increases in $N_e$ included) (Billiard *et al.*, 2012; Taylor *et al.*, 2015). Such rare cross-species recombination events can conceivably also allow for the formation of new combinations of fungal mating type genes. This could potentially generate a homothallic species from heterothallic or obligate outcrossing species and *vice versa*, and might explain some of the frequent thallism switches observed among closely related fungal species (e.g., Gioti *et al.*, 2012; Strandberg *et al.*, 2010). Additionally, cross-species recombination can give rise to hybrids with small $N_e$ values and that are reproductively isolated from their parental species due to large-scale differences in chromosome content and organization (Yakimowski and Rieseberg, 2014). Although the latter situation was initially thought to be true only for allopolyploid hybrid fungi (see Hybridisation and hybrids in Table 3) (Burnett, 2003; Giraud *et al.*, 2008; Stukenbrock *et al.*, 2012; Stukenbrock, 2016), it was recently also shown to occur in a homoploid hybrid fungus (see Hybridisation and hybrids Table 3) (Leducq *et al.*, 2016).

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## 5. Molecular basis of fungal species boundaries

Examination of the character and character state discontinuities that separate individual species may reveal the precise determinants of the initial emergence and subsequent maintenance of species boundaries. This implies that the molecular basis of fungal speciation and, in effect, the intrinsic nature of the species boundary itself can be determined empirically. Based on evidence from various eukaryotes, including fungi, the process of speciation (i.e., the formation of new species boundaries) may be driven by two primary groups of molecular mechanisms: those involving specific genes and those involving chromosome-level changes (reviewed by Nei and Nozawa, 2011). The effects of both groups on the species boundary are likely to be dramatically pronounced under conditions of cross-species interaction and/or genetic exchange (see below).

Chromosome-level changes are thought to allow almost instantaneous establishment of new species boundaries in fungi (Albertin and Marullo, 2012; Stukenbrock, 2016). For example, in *Botrytis*, *Cryptococcus* and *Epichloë* (reviewed by Giraud *et al.*, 2008), allopolyploid hybrids are genetically isolated from their homoploid parental species (i.e., ploidy differences prevent backcrossing of hybrids with parents). Chromosomal rearrangements accompanying fungal hybridisation can also facilitate the development of species boundaries. This was elegantly illustrated in a recent study of wild *Saccharomyces* species (Leducq *et al.*, 2016), where the rearranged chromosomal sequences in homoploid hybrids resulted in their genetic isolation (i.e., differences in chromosome architecture prevent backcrossing with parents). Chromosome-level genomic co-linearity thus represents an important component of the fungal species boundary, the semipermeability of which could lead to the emergence of a new species with its own boundary.

The genic determinants involved in the initial formation of a species and its boundary have been termed "speciation genes". This is because their divergence and/or products cause emergence of the incipient species (Harrison, 2012; Nosil and Schluter, 2011; Wu and Ting, 2004). Although the identification of speciation genes has generally been elusive, studies from model plants, animals and *Saccharomyces cerevisiae* suggest that they may bring about speciation because their products affect the survival of hybrid progeny (Blackman, 2016; Louis, 2011; Nosil and Schluter, 2011). This is particularly true for those genes whose fitness effects depend on the genetic background in which they occur (see "genomic epistasis" under Species isolation mechanisms in Table 3). For example, by making use of experimentally evolved incipient species derived from *S. cerevisiae*, Anderson and Sekelsky (2010) showed that the co-occurrence of certain gene variants severely impeded hybrid fitness due to negative epistatic interactions. The same outcome was observed when certain mitochondrial proteins, respectively transcribed from the nuclear and mitochondrial genomes, co-occurred in *S. bayanus* × *S. cerevisiae* hybrid backgrounds (Lee *et al.*, 2008). Apart from reducing the fitness of hybrids, genomic epistasis would likely also determine the retention of HGT-derived foreign genetic elements in the recipient genome. All extant hybrids, HGT-derived or introgressed lines represent instances where negative genomic epistasis was overcome, with the genes or gene variants facilitating this, representing integral components of the species boundary.

Speciation genes can also facilitate divergence by preventing gene flow between incipient species (Blackman, 2016; Nosil and Schluter, 2011). This can occur when alternative speciation gene alleles, or the presence of a particular speciation gene in one of the species, allow for survival in contrasting environments (where contact between the diverging species is limited). In fungal pathogens, for example, effector genes (i.e., genes encoding secreted compounds that modulate host–pathogen interactions) (Plissonneau *et al.*, 2017) have been suggested to represent speciation genes because their divergence or acquisition via HGT, hybridisation or introgressive hybridisation would accompany ecological differentiation and speciation (Giraud *et al.*, 2010; Stukenbrock, 2013). However, exceptionally few candidate speciation genes have been conclusively shown to cause speciation in fungi and, based on current data, they may be represented by many diverse classes of genes (Nosil and Schluter, 2011; Presgraves, 2010).

The genic determinants of the intrinsic systems and mechanisms that maintain the segregation of species have been termed "barrier genes" (Harrison, 2012; Noor and Feder, 2006). These genes ensure integrity and maintenance of species boundaries. They determine the phenotypic variation underlying the genetic isolation of non-conspecifics. Genes involved in many of the species isolation mechanisms known in fungi (see Table 3) potentially belong to this class. As a result, barrier gene products may be involved in diverse biological processes, including niche differentiation and host/vector specificity, as well as reproductive strategy and mode. This class of genes may even encode components of DNA metabolism; e.g., deactivation of the DNA mismatch repair system erodes the boundary between *S. cerevisiae* × *S. paradoxus* (reviewed by Louis, 2011). Work on animal and plant models has also identified several housekeeping and regulatory loci that contribute to or maintain species boundaries (Noor and Feder, 2006; Rieseberg and Blackman, 2010).

Designation of barrier or speciation genes is probably not absolute. The barrier genes in one species (e.g., the mating type genes in a homothallic species that maintain isolation through the promotion of selfing/inbreeding) may represent speciation genes in another (e.g., the mating type genes acquired via hybridisation can cause divergence by genetically isolating the hybrid from one or both parental species) and *vice versa*. Research aimed at identifying and untangling the molecular processes and pathways responsible for the development of species boundaries (i.e., those encoded by speciation genes) from the processes responsible for boundary maintenance (i.e., those encoded by barrier genes) would thus allow elucidation of the genetic basis of a fungal species' initial emergence and its subsequent continued separation from non-conspecifics.

Molecular dissection of the speciation process and the nature of the species boundary in non-model fungi is increasingly feasible using genome-based approaches. Numerous

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recent studies have reported the mosaic nature of fungal genomes (e.g., Gladieux *et al.*, 2011; Leducq *et al.*, 2016; Ma *et al.*, 2013; Stukenbrock *et al.*, 2012). In almost all these cases, semipermeable species boundaries led to the development of genomes consisting of "patchworks" of sequences with unique evolutionary histories. Within such a genomic "quilt", the distribution of the foreign "patches" is determined by the format of the species boundary breach. Genomes with numerous and widely distributed foreign "patches" typically characterize those of hybrids, while the hallmark of introgressive hybridisation and HGT are genomes with fewer and more localized foreign "patches". Detailed examination of the distribution and composition of these "patches" may reveal specific mechanisms underlying the speciation process. Candidate speciation and barrier genes may be identified using forward genetics approaches based on comparative genome analyses of recently evolved species and their relatives (Blackman, 2016). Once identified, however, their precise roles in the speciation process require confirmation via functional and evolutionary characterization.

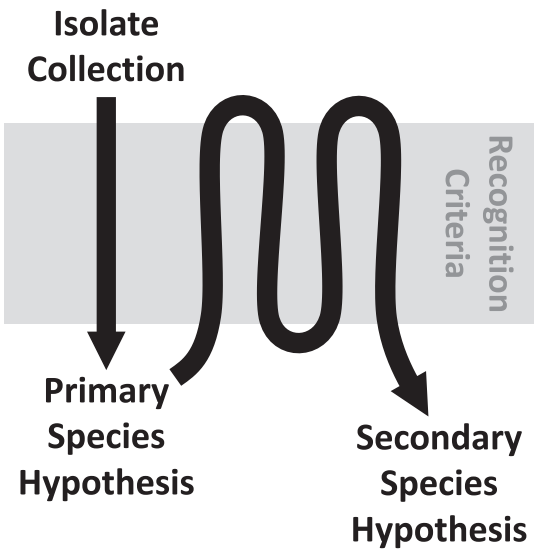


**Fig. 2 – Synopsis of the process used to delineate fungal species. Robustness of the species hypothesis ultimately proposed is ensured by utilizing different types of data and analytical procedures to evaluate a range of species recognition criteria (see Table 1). The first phase of the process involves development of one or more primary species hypotheses from the collection of isolates examined. These primary hypotheses reflect the preliminary species limits that are mostly identified using recognition criteria such as phenotypic/genetic cohesion or phylogenetic diagnosibility. During the second phase of the delineation process, robust secondary species hypotheses are developed from the primary species hypotheses by subjecting the latter to additional rounds of scrutiny using added recognition criteria (particularly genealogical exclusivity). In this way, multiple independent lines of evidence support the secondary species hypotheses that subsequently lead to the formal description and naming of species.**

## 6. Implications for fungal taxonomy

Described fungal species represent formal hypotheses about how the diversity of these organisms are structured. In other words, given the available biological resources and analytical capacities, a species hypothesis is the best explanation for the genetic patterns observed in the empirical data examined. To ensure robustness of these hypotheses, contemporary mycologists employ a wide range of criteria and procedures (Table 1), the results of which they then integrate to establish hypotheses that optimally match the experimental observations (see Table 2 for the integrative taxonomic approaches used in fungi). However, the overall process is usually separated into two phases (Fig. 2). In the first phase, putative species boundaries are proposed and primary species hypotheses formulated (e.g., putative species boundaries are inferred based on phenotypic data or phylogenetic relatedness; see Table 1). In the second phase, these primary hypotheses are subjected to more extensive rounds of experimentation using an array of species delineation criteria. This leads to secondary species hypotheses that are supported by multiple characters associated with diverse biological attributes (e.g., morphology, pathology, physiology and ecology). It is these robust secondary species hypotheses, supported by multiple independent lines of evidence, that are formally named.

Within this *status quo* taxonomic framework, the impact of semipermeable species boundaries becomes most apparent when attempts are made to convert primary species hypotheses into secondary species hypotheses. In situations where species boundaries have been breached, application of the standard set of species recognition criteria (Table 1) is fraught with complexity and may severely influence the accuracy of subsequent taxonomic decisions. This is primarily because the delineation process usually involves estimates of population differentiation or genealogical exclusivity, which are based on the assumption that populations of distinct species reproduce and evolve independently (Sites and Marshall, 2004). However, because of the semipermeability of species boundaries, expectations of extensive or absolute genetic isolation and evolutionary independence (especially for recently diverged or insipient species) are likely unattainable. Indeed, some previous reports of introgressive hybridisation (e.g., Brasier and Kirk, 2009; Gladieux *et al.*, 2011; Gonthier and Garbelotto, 2011; Sun *et al.*, 2012) and HGT (reviewed by Soanes and Richards [2014]) represent examples of where the species in question remained distinct, following gene flow from a non-conspecific or a breach in the species boundary. Practically, this means that all of the markers (e.g., phenotypes, genes, genome regions) used to delimit a species need not necessarily show patterns of genetic isolation or genealogical exclusivity.

Another complication is that the signatures of semipermeable species boundaries in empirical data are often not easily distinguishable from those associated with other evolutionary phenomena. For example, phylogenetic incongruence among multiple independent loci may provide evidence that the affected loci were acquired via HGT or hybridisation, but the pattern may also be a consequence of incomplete lineage sorting at these loci (see Phylogenetic incongruence in Table 3). A

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recent study of the causal agents of citrus brown spot (i.e., distinct lineages of *Alternaria alternata sensu lato*) provides and apt illustration (Stewart *et al.*, 2012). The authors showed that multi-locus phylogenetic incongruence was due to the combined effects of ancestral cross-species genetic exchange and incomplete lineage sorting, but that the causal fungi nevertheless represented at least two robust *Alternaria* species hypotheses. Rigorous interrogation of such difficult taxonomic cases allows detailed dissection of the various processes involved in the evolution of a species, and significantly enriches the value of fungal systematics studies.

Taxonomic studies that account for the semipermeable nature of fungal species boundaries, may lead to the delineation of taxa with exceptionally small $N_e$-values. Species falling into this category include those that emerged via the process of hybridisation, which also facilitated their genetic isolation from parental species (Stukenbrock, 2016). Another example includes apparently asexual fungi that maintain some level genetic connectedness, but that separate into clonal lineages (e.g., Ma *et al.*, 2013). In these instances, secondary species hypotheses might be represented by populations consisting of one or a few clones (e.g., Collado-Romero *et al.*, 2008; Jiménez-Gasco *et al.*, 2002). Formal recognition and description of such small-$N_e$ taxa might be construed as instances of "over-splitting" species (Coyne and Orr, 2004). Fortunately, their recognition is not prohibited in the nomenclatural code for fungi (McNeill *et al.*, 2012). Mycologists are, therefore, free to provide species names to the multi-locus genotypic clusters (or metapopulation lineage segments) they have identified. Formal recognition of these units would improve our understanding of fungal biodiversity and evolution, but it might also be essential, especially where pathogens and other economically important fungi are concerned.

## 7. Looking forward – risks in terms of pathogens

The semipermeability of fungal species boundaries is of paramount importance when considering pathogens, because it could affect the success of measures used to control their growth and distribution. This is partly because cross-species gene flow impacts the population biology of a pathogen, which in turn dictates its operational management and control (McDonald and Linde, 2002; Milgroom and Fry, 1997; Zhan *et al.*, 2015). More importantly, however, cross-species gene flow can enhance the evolutionary potential of a species, either indirectly by changing its population diversity and structure (McDonald and Linde, 2002), or directly through the mutagenic processes accompanying hybridisation and HGT. Therefore, because the evolutionary potential of fungal species is strongly linked to the emergence of new pathogens (Soanes and Richards, 2014; Stukenbrock, 2016), the semipermeable nature of species boundaries requires particular attention in the development of disease management strategies.

The dynamic nature of fungal species will likely remain a significant hurdle in biosecurity. Fungi are constantly being moved across the globe via human activity, and the biosecurity risk they pose is usually not immediately known (e.g., Fisher *et al.*, 2012; McTaggart *et al.*, 2016; Ploetz *et al.*, 2013; Wingfield *et al.*, 2015). This is mostly due to the initial lack of relevant life history information to make inferences of a species' evolutionary potential. But we know from experience that the probability of species boundary breaches escalates dramatically when closely related species that have evolved in allopatry are brought into the same environment where their niches overlap and where they might encounter one another. Numerous devastating fungal pathogens are known to have emerged under such conditions of secondary contact between allopatric species (Fisher *et al.*, 2012; Giraud *et al.*, 2010; Gladieux *et al.*, 2011; Leducq *et al.*, 2016; Leroy *et al.*, 2016). In an age when human-mediated ecological transformations are common and widespread, semipermeability of the species boundary is probably a key driver of speciation and pathogen emergence (Brasier, 2000; Fisher *et al.*, 2012; Thomas, 2013). Therefore, an awareness of the nature of the species boundary and the signatures of boundary breaches (e.g., unexpected sexual and vegetative compatibilities, unusual karyotypes and chromosomal architecture, genomic mosaicism) will lead to improvements in how biosecurity risks are assessed and fungal pathogens are ultimately controlled.

## Conflict of interest

The authors have no significant competing financial, professional or personal interests that might have influenced the performance, interpretation or presentation of the work described in this manuscript.

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