CHAPTER 3. DEFINING SPECIES IN THE FUNGI

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3.1 Introduction

The species concept is central to biology and has received considerable debate, yet a universal definition of a species has not found widespread agreement. Much of the discussion has centered on animals and plants, while mycologists and their organisms have generally been peripheral to the debate. A recent symposium dedicated to species concepts in clonal organisms (Mishler and Budd, 1990) did not include the fungi, though they probably have more apomictic taxa than any other major group of organisms (Blackwell, 1993). Inclusion of the fungal kingdom, with its broad array of reproductive strategies, may complicate the debate, but new insights could be gained by using fungal models. This chapter attempts to bring fungi into the debate and aims to arrive at a workable definition for fungal species.

We take an admittedly practical view in defining species and will not delve deeply into the extensive literature on the philosophical and theoretical underpinnings of systematics and the meaning of species (e.g., Baum and Donoghue, 1995; de Queiroz and Donoghue, 1990; Ereshefsky, 1992; Hull, 1997; Nixon and Wheeler, 1990; Davis, 1997). While recognizing that organisms are dynamic entities, a useful species concept should define an organism at a single period in time and serve as a means of communication among biologists. From our applied point of view, plant pathologists, medical practitioners, and others must be able to diagnose, name, and communicate about the organisms they encounter (Brasier, 1997; Miller and Rossman, 1995). Accurate delimitation of fungal species is critical in the establishment of quarantine regulations, tests for plant resistance to pathogens, specification of organisms for pharmaceutical production and patent applications, and preservation of biodiversity and ecosystem function (Brasier, 1997). Delineation of taxa may sometimes appear arbitrary, but we believe that species are real and that a meaningful species definition can be applied to the fungi.

In the search for a practical species definition, strict devotion to any particular species concept will be difficult. Much of the discussion has been theoretical (Hull, 1997), and few of the recently proposed phylogenetic species concepts have actually been put into practice. In our attempt to apply these concepts to the fungi, we have come to three main conclusions: 1) a species concept for the fungi should be based on characters and populations (or lineages); 2) the characters used to delineate species

should be phenotypic, including physiological; and 3) the biological species concept and phylogenetic analyses may be more appropriate for identifying lineages than as a means to delineate species.

3.2 General species concepts and speciation

Species definitions and concepts have been debated from both biological and philosophical positions (Claridge *et al.*, 1997; Ereshefsky, 1992). Many of these species concepts have undergone extensive revision, and they continue to evolve. Mayden (1997) discusses 22 different species concepts, and Table 1 lists a small subset. Species definitions have been based on phenotypic similarity, ecological parameters, reproductive isolation or cohesion, evolutionary principles, and various combinations of the above. Some see two broad philosophical approaches: mechanistic concepts vs. history-based concepts (Luckow, 1995).

Mechanistic species concepts integrate the processes of speciation into the species definition. Examples include the biological species concept (Mayr, 1963), the ecological species concept (Van Valen, 1976), and the cohesion species concept (Templeton, 1989). The classic mechanistic approach is the biological species concept, in which reproductive isolation is assumed to be fundamental to speciation and is incorporated into the species definition. Mechanistic concepts are complicated by the likelihood that many factors may lead to speciation. Some of these factors affect the origination of new variants and others are important in the maintenance and cohesion of this variation in a new lineage (Endler and McLellan, 1988; Templeton, 1989). Because of these numerous speciation mechanisms, some suggest that there can be no universal way of defining a species using mechanistic concepts (Ghiselin, 1987; Luckow, 1995). Others (e.g., Cracraft, 1983, 1997) have questioned the utility of the biological species concept, and, as will be discussed later, this concept has limitations in delimiting species of fungi.

History-based species concepts view species as historical entities that are the endpoint in evolution (Baum and Donoghue, 1995; Baum and Shaw, 1995; Luckow, 1995). These concepts are generally neutral in terms of speciation mechanisms and are, therefore, potentially universal in their applicability (Baum and Donoghue, 1995; Luckow, 1995; Davis, 1997). History-based concepts are generally based on phylogenetic principles, and several have been referred to as ‘the phylogenetic species concept,’ but the various authors begin with different assumptions in defining species (e.g., Baum and Donoghue, 1995; Baum and Shaw, 1995; Cracraft, 1983; Davis, 1997; Luckow, 1995; Nixon and Wheeler, 1990; Table 1). Mishler and Donoghue (1982) and Mishler and Brandon (1987) have proposed a species concept that incorporates aspects of both the history-based and mechanistic approaches. They advocate the use of monophyly to group organisms, while using other attributes (e.g., ecological and reproductive) to rank organisms as species, genera, etc. Such pluralistic approaches suggest that a number of mechanisms may be important in holding a species together and in distinguishing it from other species.

Table 1. Definitions and major features of various species concepts.

Concept	Definition	Major Features
Biological (Mayr, 1963)	Species are groups of interbreeding natural populations that are reproductively isolated from other such groups.	· Species defined in terms of isolating mechanisms. · Ecology and morphology assumed to be congruent with potential interbreeding. · Not applicable to asexual organisms.
Ecological (Van Valen, 1976)	A species is a lineage that occupies an adaptive zone minimally different from that of any other lineage in its range and that evolves separately from all lineages outside of its range.	· Control of evolution is largely by ecology and the constraints on individual development. · Applies to sexual and asexual organisms.
Cohesion (Templeton, 1989)	The most inclusive population of individuals having the potential for cohesion through the intrinsic cohesion mechanisms of genetic and/or demographic exchangeability.	· Genetic exchangeability includes factors that define the limits of spread of new genetic variants through gene flow. · Demographic exchangeability includes factors that define the fundamental niche and limits of spread of new genetic variants through genetic drift and natural selection. · Applies to sexual and asexual organisms.
Monophyletic (Mishler and Brandon, 1987)	A species is the least inclusive taxon in which organisms are grouped by evidence of monophyly and ranked as a species by processes that are dominant in producing and maintaining lineages	· Evidence of monophyly is based solely on the presence of derived characters (autapomorphies). · Ranking of taxa may be based on many criteria; i.e., it is pluralistic. · Applies to sexual and asexual organisms.
Genealogical (Baum and Shaw, 1995)	A species is the smallest exclusive monophyletic group.	· Species exist at the boundary of reticulating and hierarchical relationships. · Species are the smallest apomorphic unit. · Emphasis on the monophyly of genes rather than organisms. · Applies to sexual and asexual organisms.
Phylogenetic (Nixon and Wheeler, 1990)	A species is the smallest aggregation of populations (sexual) or lineages (asexual) diagnosable by a unique combination of character states in comparable individuals.	· Constant character states are seen as evidence of the distinction between reticulating and hierarchical relationships. · The terms monophyletic and paraphyletic do not apply to species; no phylogenetic structure exists within a species. · Applies to sexual and asexual organisms.

We are primarily interested in a practical definition of a fungal species, but we recognize that a species concept should serve as a hypothesis for testing modes of speciation and evolution. Many agree that the process of speciation is a population-level phenomenon, and that the initiation of speciation may occur by a number of mechanisms (Levin, 1993; Templeton, 1989). For example, the process may begin with a unique set of alleles that allow for adaptation to a new ecological niche. Genetic drift, gene flow, natural selection, and other processes alter the frequency of new variants or adaptations within and between populations (Endler and McLellan, 1988). In the fungi, a variety of genetic systems, life-history patterns, and intraspecific interactions allow for ample generation of new variants and maintenance of unique adaptations (Andrews, 1995; Brasier, 1995; Ramsdale, this volume; Rayner *et al.*, 1995). Fungi may speciate at a very rapid rate as compared to other organisms, which may explain why traditional characters (i.e., morphology) have frequently proven inadequate for delineating fungal species. Analyses of extant populations of fungi, using either population genetics or phylogenetic analyses, will allow us to gain an understanding of the mechanisms of evolution at the species level (Bruns *et al.*, 1991) and should suggest where to look for diagnostic characters in delimiting species.

3.3 Species concepts in the fungi

Fungal species concepts, in practice, have evolved from strictly morphological descriptions, through the embrace of the biological species concept, to a call for phylogenetically based species concepts (e.g., Blackwell, 1993; Hibbett *et al.*, 1995; Vilgalys, 1991; Vilgalys and Sun, 1994). Brasier (1997) has used a population-based approach to define 'operational species units' or "... the principal population units sharing a common gene pool and exhibiting a common set of physiological, ecological and morphological attributes...." We agree that a population-based approach is key to any successful species concept in the fungi, as is evolutionary history, and we emphasize phenotypic characters in delineating species. The most important diagnosable characters would be those associated with ecological niche, though minor morphological differences, often quantitative, are most commonly used for delimiting species of fungi.

Though there has been increasing use of intersterility and molecular markers in diagnosing fungal species, we will emphasize the use of phenotypic characters. Reproductive isolation from other sympatric populations is important to maintain cohesion in a species. It is also essential that the individuals that share salient phenotypic characters are of a single genealogical lineage, or polyphyletic species would result. Thus, identification of lineages using molecular markers may prove important in identifying potential species, but, as will be pointed out, the use of reproductive and molecular criteria in delimiting species is far from straightforward. In this section we will present a population-based approach to delimiting species, first addressing reproductive isolation, the monophyletic and genealogical species concepts, and the problems with asexual species.

3.3.1 Reproductive isolation

Sympatric, outcrossing species must have some barriers to gene flow in order to maintain species cohesion, and reproductive isolation is explicitly or implicitly a part of most species concepts. The biological species concept and the phylogenetic species concept (Table 1) are similar in that they emphasize the lack of gene flow between populations as an important determinant of species status (Davis, 1997; Luckow, 1995). The biological species concept, however, concentrates on intrinsic isolating mechanisms to delimit species; i.e., reproductive isolation is the sole defining criterion of a species. In contrast, the phylogenetic species concept makes no assumptions about intrinsic isolating mechanisms because species delimitations require only diagnostic characters, which cannot be fixed in a species if there is substantial gene flow with other populations. Genealogical species concepts use gene trees to identify phylogenetic lineages; theoretically, such lineages would not be seen at the species level if they were not reproductively isolated.

3.3.1.1. Biological species in the fungi

The biological species concept is most applicable with sympatric, sexually outcrossing populations. Representatives of two populations may be crossed and any viable progeny studied for fitness, or population genetic analyses can be used to look for evidence of gene flow between the two populations. If there is no gene flow between such populations, then it is logical to assume that either hybrids between the two are unfit or there is some intersterility barrier preventing sexual reproduction. A reproductive barrier, i.e., intersterility, may arise through natural selection, presumably because of the poor fitness of hybrids. Thus, reproductive isolation strongly suggests that the two populations have differing ecological adaptations.

The biological species concept has generally been accepted more by animal biologists than plant biologists. The frequent hybridization among related plant species suggests that development of intrinsic isolating mechanisms are not a major factor in plant speciation; tests for sexual compatibility and gene flow between populations may not prove informative in delimiting plant species. The evidence to date suggests, however, that fungi are more like their animal cousins when it comes to hybridization; hybrids are rare in the fungi. There is morphological evidence that introduced rust species in the genus *Melampsora* have formed hybrids on *Populus* species in New Zealand (Spiers and Hopcroft, 1994). A hybrid between the fir and pine forms of *Heterobasidion annosum* has been reported (Garbelotto *et al.*, 1995), and rare hybrids between *Ophiostoma ulmi* and *O. novo-ulmi* have been documented (Brasier *et al*., 1998). Aside from studies of possible hybrids between grass endophytes in the genus *Epicloe* (Tsai *et al.*, 1994), there is little indication that hybridization has been important in the evolution of fungal species. In general, intersterility barriers between closely related species of fungi are strong.

Although we are not advocates of the biological species concept, we do feel that mating tests play an important role in our understanding of the biology of sexually

reproducing fungi and in identifying barriers to gene flow. But the biological species concept has some major limitations. Obviously, it does not apply in defining asexual species. Difficulties also arise when interpreting mating in distinctly allopatric populations. If no gene flow occurs naturally, how is one to interpret a successful laboratory mating of two geographically separated organisms? Would the individuals freely interbreed if in sympatry? A further problem with many of our best fungal models (namely, plant pathogens in *Phytophthora*, *Fusarium*, the Dutch elm disease organisms, the rusts, and the smuts) is that the studied populations are not endemic but, rather, occupy their present geographical ranges because of introductions by humans. Tests for gene flow between individuals from unnatural populations may tell us little of speciation and gene flow in endemic populations. Also, partial interfertility between fungal species is common in the laboratory and leads to ambiguities. Nonetheless, with the proper caution in interpretation, tests for gene flow and intersterility are important tools in fungal taxonomy.

3.3.1.2 Tests for intersterility

There are two basic approaches for testing if two sympatric populations are reproductively isolated. The first uses various phenotypic or genotypic markers in tests for gene flow or in grouping related individuals. Alternatively, the sexual compatibility of individuals from two populations may be tested directly through experimentation. Both approaches have been used extensively, with the latter particularly popular with mycologists. Many fungi are readily amenable to laboratory tests to indicate sexual compatibility, and mycologists have long used such tests to diagnose species (e.g., Anderson and Ullrich, 1979; Hallenberg, 1984; Korhonen, 1978a, b; Petersen, 1995; Shear and Dodge, 1927; Vilgalys and Miller, 1983;). Reproductive isolation has often, but not always, been congruent with other diagnostic characters (Brasier, 1997; Harrington and McNew, 1998; Petersen, 1995; Vilgalys, 1991; Vilgalys and Sun, 1994).

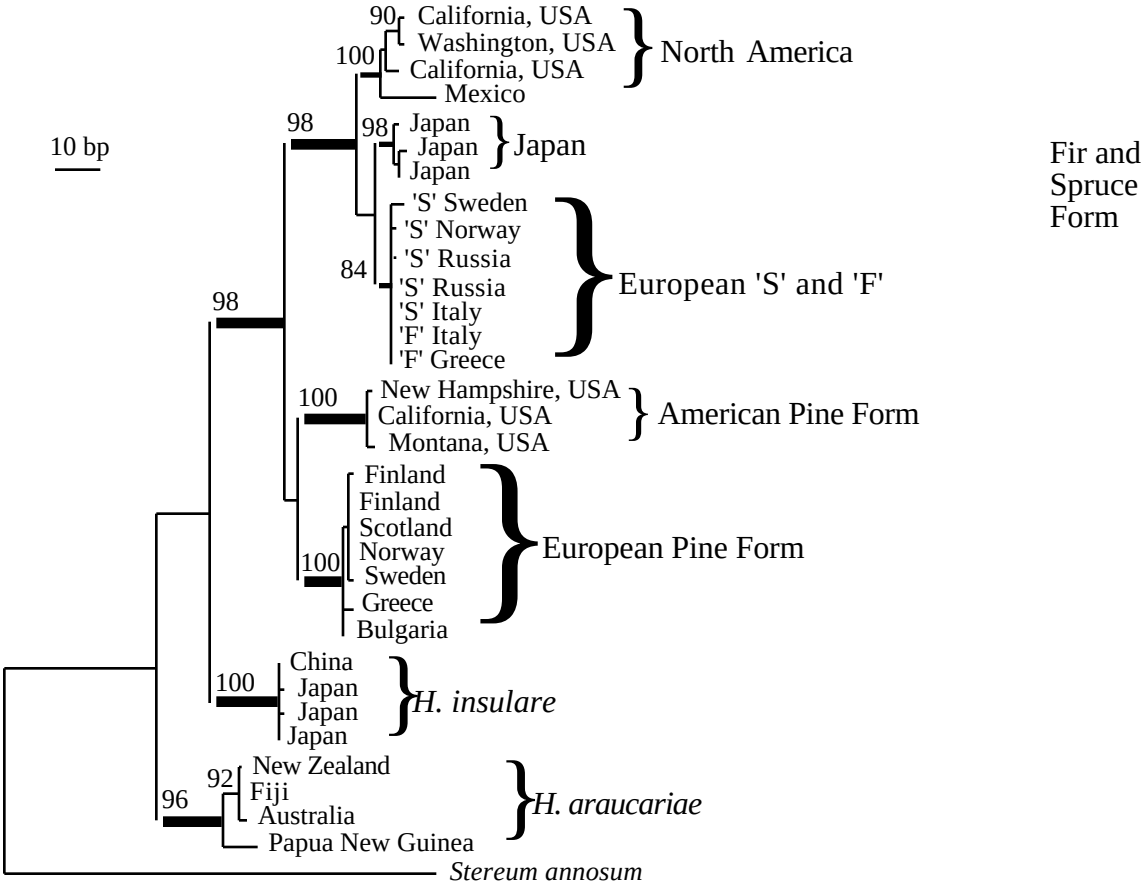
Tests for intersterility have proven highly valuable in suggesting where to look for phenotypic differences between difficult to separate species. Delimitation of species in *Pleurotus* (Petersen, 1995; Vilgalys and Sun, 1994), *Heterobasidion* (Korhonen, 1978a), and *Armillaria* (Korhonen, 1978b), for instance, was greatly facilitated by tests for sexual compatibility. Many species of *Armillaria* occur sympatrically; occasionally several species may even be found on the same host substrate (Rizzo and Harrington, 1993). Until recently, these morphologically similar species were considered by plant pathologists to be part of a single, variable species, *Armillaria mellea*. Intraspecific variation was noted in virulence, basidiome morphology, and rhizomorph production, but it was not until barriers to gene flow were identified (Anderson and Ullrich, 1979; Korhonen, 1978b) that many of these phenotypic traits were considered to be characters of distinct species. Since tests for intersterility have been applied, eight additional species in the *A. mellea* complex have been described and others newly recognized by classic morphological characters (Berube and Dessureault, 1989; Cha *et al.*, 1994; Volk et al, 1996; Watling *et al.*, 1991).

Although reproductive isolation is often considered to be absolute in the fungi, regardless of whether it is pre- or postzygotic in nature (Brasier, 1987; Burnett, 1983), partial interfertility is commonly observed in laboratory pairings (Harrington and McNew, 1998; Perkins, 1994; Petersen, 1995). Interpretation of partial interfertility and its application to identifying species has been rather straightforward with ascomycetes, where tests are usually followed through to analysis of viable ascospore progeny. Early work with *Neurospora* species (Shear and Dodge, 1927), through work with the Dutch elm disease pathogens *Ophiostoma ulmi* and *O. novo-ulmi* (Brasier and Mehrota, 1995), and sibling species in *Ceratocystis* (Harrington and McNew, 1998) have shown that closely related species with minor morphological differences or distinct ecological niches form few or no viable progeny in laboratory pairings.

With dikaryon-forming basidiomycetes, however, interfertility often has been determined by observation of only the first step in mating, the formation of clamp connections, and experiments have generally not been carried through to observe the production of viable offspring from crosses. It is not surprising that closely related species, or populations of the same species that have been geographically isolated for extended periods, would show partial interfertility. Clamp connection tests have been incongruent with clear ecological, morphological, and/or molecular differentiation in delineating species in a number of genera. Problems in interpretation of such tests in *Pleurotus* have been well documented (Petersen, 1995). In the agaric genus *Lentinula,* a number of species have been described from Asia and the South Pacific based on morphological characters, and these morpho-species represent different evolutionary lineages (Hibbett *et al.*, 1995), but mating studies have indicated that many of these species are fully intercompatible based on dikaryon formation (Petersen, 1995).

The *Heterobasidion annosum* complex, a group of root-rotting basidiomycetes, is of particular interest, as this is the only fungal model for which specific genes for intersterility have been identified (Chase and Ullrich, 1990). The pine and fir forms of *H. annosum* in western North America are clearly distinct species based on their ecology, morphology, and phylogeny (Harrington *et al.*, 1998; Korhonen, 1978a; Figure 1), yet up to 10% of the laboratory pairings of monokaryons of these respective forms will form clamp connections *in vitro* (Harrington *et al.*, 1989). Occasionally, such hybrids will form basidiomes and viable basidiospores (Chase and Ullrich, 1990), and one putative hybrid was found in nature (Garbelotto *et al.*, 1995). The ‘S’ and ‘F’ types of the fir form of *H. annosum* in Europe could not be distinguished by ribosomal DNA (rDNA) spacer sequences (Harrington *et al.*, 1998; Figure 1), but they are distinguished by morphology (Mugnai and Capretti, 1989), RAPD markers (La Porta *et al*., 1994), and isozyme markers (Karlsson and Stenlid, 1991; Otrosina *et al*., 1993). Allopatric ‘S’ and ‘F’ type isolates are largely interfertile based on dikaryon formation; ‘S’ strains from Europe formed clamp connections with fir form isolates from America in 97% of the pairings (Harrington *et al*., 1989). With sympatric populations of ‘S’ and ‘F’ type from central Europe, about 24% of the pairings were interfertile, while pairings between northern European ‘S’ strains and southern European ‘F’ strains were about

Figure 1. One of 24 most parsimonious trees of the rDNA spacer regions and 5.8s gene of the genus *Heterobasidion*. The formally recognized *H. annosum* is comprised of at least three well-delineated clades: the American pine form, the European pine form, and the fir and spruce form. Three geographical lineages are seen within the fir and spruce form, but the 'S' and 'F' types in Europe are not distinguished by these data. The sequences were taken from Harrington *et al.* (1998), with the 5.8s, ITS and IGS-1 data combined. Ambiguously aligned regions were eliminated, leaving 584 bp for parsimony analysis. Shortest tree = 298, CI = 0.893, RI = 0.952. Bootstrap values greater than 80% are shown above the appropriate branches.



72% interfertile (Korhonen *et al*., 1992). Thus, the further apart the sampled populations of the 'S' and 'F' types, the greater their compatibility.

The reduced morphology of fungi and the hesitation to use physiological characters to define fungi has led many mycologists to use tests for intersterility and tests for gene flow between populations to identify potential species. The value of these criteria in teasing apart morphologically similar species in the fungi is obvious, but limitations preclude their use in actually delimiting species. Perhaps the biggest limitation is that most fungi cannot be tested experimentally for compatibility, either because the species do not form meiotic spores readily in the lab or because they are asexual species.

3.3.1.3 Asexual species

Asexual reproduction is extremely common in the fungi, especially in the ascomycetes, and phylogenetic analysis has shown that strictly asexual species can be derived from sexual species (Lobuglio *et al.*, 1993; Witthuhn *et al.*, 1998). Strictly asexual organisms always have presented difficulties for species concepts, and some biologists (including some mycologists, see Perkins, 1991) do not believe asexual organisms can form species. Asexual organisms often are considered to be evolutionary dead ends. "Muller's ratchet" suggests that accumulation of deleterious mutations will cause clonal lineages to go rapidly extinct (Muller, 1964). Most lineages, perhaps all, will go extinct, whether they are sexual or asexual, so the difference in extinction between sexual and asexual lineages may be just a matter of time, and it is inappropriate to deny species status to a group of organisms based solely on hypothesized extinction rates. Asexual species are a real phenomenon and often show cohesive groupings as strong as those of sexual organisms, although often with less variation. No workable species concept for the fungi can exclude asexual species.

Although thought to be clonal and, therefore, identical, putative asexual fungi have shown through population studies a surprising amount of variation (Correll and Gordon, this volume; Kohn, 1995). Polymorphic traits have been found in vegetative compatibility (VC) groups, allozymes, DNA fingerprints, mitochondrial haplotypes, and other markers (Gordon, 1993; Kohn, 1995). These markers may be viewed as defining 'individuals' much as in sexual organisms, but these clonally reproducing 'individuals' would have a much greater geographic distribution than sexual individuals (e.g., see Koenig *et al.*, 1997).

Phylogenetic analyses using genotypic markers could potentially identify each individual of an asexual species as a terminal, so taxonomically ranking lineages, particularly at the species level, is problematic without ecological or morphological criteria (Mishler and Brandon, 1987; Mishler and Donoghue, 1982). However, genetic markers have proven highly valuable in grouping asexual individuals and suggesting genealogical lineages that may be diagnosed as species. In the three varieties of *Leptographium wageneri*, which attack the living xylem of different members of the

Pinaceae in western North America, genetically identical, or nearly identical individuals are found across many hundreds of kilometers; and there is a strong correlation of phenotypic and genotypic divergence among these three varieties (DeScenzo and Harrington, 1994; Witthuhn *et al.*, 1997; Zambino and Harrington, 1989, 1990). In contrast, in *Fusarium oxysporum,* a pathogen of agricultural crops that likely has been moved by humans throughout the world, VC groups and fingerprints often are not congruent in delimiting lineages, and additional evidence has suggested that some physiologically specialized forms (*formae speciales*) based on host range may be polyphyletic (Jacobson and Gordon, 1990; Koenig *et al.* 1997). Here, incongruence between identified lineages and diagnosable phenotypic characters is due to the difficulty in delimiting lineages without knowledge of endemic populations. With proper sampling and the use of highly variable molecular markers, lineages of asexual fungi should be easy to identify. Genealogical populations or lineages in asexual fungi may be considered equivalent to sexual populations in a search for fixed diagnosable (phenotypic) characters for species delimitation.

3.3.2 Monophyly and genealogy

Phylogenetic analysis has been explicitly invoked to designate species in several species concepts (e.g., de Queiroz and Donoghue, 1990; Mishler and Brandon, 1987), most recently in the genealogical species concept of Baum and Shaw (1995). Although the utility and power of DNA sequences and phylogenetic analyses are not questioned, Nixon and Wheeler (1990), Luckow (1995), Davis (1997), and others have argued strongly against monophyletic or 'autapomorphic species concepts.' Many of the criticisms of monophyletic and genealogical concepts have been theoretical, but there are practical limitations as well.

3.3.2.1 Genealogy-based species concepts

Inherent assumptions in phylogenetic analyses lead to questions of their application in delimiting species (Nixon and Wheeler, 1990). Phylogenetic analyses require homology of characters and knowledge of levels of homoplasy (e.g., convergence, reversals, and parallelisms), which rarely are known with species-delimiting characters. Monophyletic species concepts (e.g., Mishler and Donoghue, 1982) emphasize the detection and analysis of apomorphies (derived characters), which, if limited to phenotypic characters, are difficult to identify in the fungi. However, most phylogenetic analyses today utilize genotypic markers, namely DNA sequences. In contrast to the monophyletic species concept, the genealogical species concept (Baum and Shaw, 1995) relies exclusively on gene trees to delineate species. Recognition of the difference between species trees and gene trees has been pointed out as a serious limitation with strictly genealogical species concepts (Doyle, 1997; Luckow, 1995; Davis, 1997). Ancestral polymorphisms in gene sequences that predate speciation events can lead to incongruence between gene trees and species trees through lineage sorting (Brower *et al.*, 1996; Doyle, 1997; Maddison, 1997). Other sources of incongruence among gene trees include introgression/hybridization and horizontal transfer of genes (Brower *et al.*, 1996).

The use of multiple gene trees to examine for coalescence has been suggested as a way to overcome these difficulties with genealogical approaches for defining species (Avise, 1994; Baum and Shaw, 1995). The genealogical species concept, as originally defined (Baum and Shaw, 1995), does not dictate the choice of genes for analysis and phylogeny reconstruction. Therefore, depending on the regions of the genome selected for analysis, a coalescence approach may be too conservative in many cases to distinguish species (Doyle, 1997; Davis, 1997). Another problem with basing species definitions on phylogenetic analysis is how to deal with unresolved relationships among taxa. For example, a species recently derived from a peripheral population of a widespread species (i.e., peripatric speciation) leaves a parental species that is paraphyletic (another species included in its lineage) rather than monophyletic (Mishler and Donoghue, 1982). Special terminology has been introduced to account for such unresolved analyses (Donoghue, 1985; Olmstead, 1995), but this would leave many organisms not belonging to any well-defined species.

3.3.2.2 Phylogenetic analyses and fungal species

Sequences of conserved DNA regions have successfully resolved broad evolutionary relationships in the fungi at the family, order, and class levels, but species-level relationships require highly variable DNA regions. The transcribed spacer regions in the rDNA operon (the internal transcribed spacer regions, ITS1 and ITS2) and the non-transcribed spacer regions between the tandem repeats of the rDNA operon (the intergenic spacer regions, IGS) have been successfully used to identify distinct lineages of fungal species and resolve relationships between closely related species (e.g., Hibbett *et al.*, 1995; Nakasone, 1996; Nakasone and Sytsma, 1993; Witthuhn *et al.*, 1998; Vilgalys and Sun, 1994; Yan *et al.*, 1995).

There may be an over reliance on rDNA and their spacer regions for phylogenetic analyses of the fungi, especially for species-level comparisons. One of the attractions of nuclear rDNA genes and spacers is that they occur in high copy number, in tandem, and the uniformity of these copies is generally maintained through concerted evolution. However, the complexity of this concerted evolution, the potential for pseudogenes, and other implications for phylogenetic analyses are just now being realized (Buckler et al., 1997; O'Donnell and Cigelnik, 1997). Other genes (e.g., beta-tubulin, histones) have been identified that may provide information at the species level due to variation in their introns or the third base of the codons (Glass and Donaldson, 1995; O'Donnell and Cigelnik, 1997). Relatively few species or species complexes of fungi have been analyzed with an adequate number of individuals to identify population level variation at the DNA sequence level. Still fewer studies have contrasted the sequences of rDNA spacer regions with other genes.

Even with highly variable regions of DNA, sibling species that have diverged relatively recently may not show differences in their rDNA spacer sequences. For instance, Harrington and Potter (1997) and other studies cited by Seifert *et al.* (1995) report

identical ITS sequences for morphologically distinguishable species. In the mushroom genus *Armillaria*, *A. gallica* is a circumboreal, largely saprophytic species occurring in a variety of hardwood forest types, but particularly common in oak forests. In the birch-beech-maple forest type of northeastern North America, a sister species of limited distribution, *A. calvescens,* replaces *A. gallica*. Although these two species differ in ecology (Blodgett and Worrall, 1992; Harrington and Rizzo, 1993; Rizzo and Harrington, 1993) and morphology (Berube and Dessureault, 1989), IGS sequences do not adequately separate these two (Harrington and Wingfield, 1995). Likewise, the 'F' and 'S' types of *Heterobasidion annosum* in Europe discussed above cannot be distinguished by ITS or IGS sequences (Harrington *et al.*, 1998; Figure 1). For an ascomycete example, two species of *Ceratocystis* associated with bark beetles in the genus *Ips*, *C. polonica* and *C. laricicola,* have identical ITS sequences yet are intersterile and differ in an isozyme electromorph and in their hosts (spruce and larch, respectively) (Harrington and McNew, 1998; Harrington *et al.*, 1996; Witthuhn *et al.*, 1998). It is interesting that isozymes are generally conservative markers, yet the 'S' and 'F' types of *H. annosum* (Karlsson and Stenlid, 1991; Otrosina *et al*., 1993), and *C. polonica* and *C. laricicola* (Harrington *et al.*, 1996), can be distinguished by isozymes but not ITS sequences.

In contrast to the above examples where phylogenetic analysis of DNA sequences failed to separate phenotypic species, not all identified lineages deserve species rank. Interfertile, allopatric populations may exhibit the same phenotypic characters and occupy virtually the same ecological niche, but molecular markers indicate essentially no gene flow. If the populations have been geographically isolated for sufficient time, phylogenetic analysis may suggest that they are separate lineages (Avise, 1994; Maddison, 1997; Slatkin, 1994). In *Heterobasidion annosum*, for instance, isolates occurring mostly on spruce and fir from North America, Japan, and Europe appear as three distinct geographic lineages based on phylogenetic analysis of rDNA spacer regions, presumably due to geographic isolation (Harrington *et al.*, 1998; Figure 1). When tested in the laboratory, however, isolates representing these geographically separated lineages are sexually compatible; they also have a similar morphology and, with the exception of the 'F' and 'S' types of *H. annosum* from northern Europe, they are ecologically similar (Harrington *et al.*, 1989; Hood, 1985; Korhonen, 1978a). Similar geographic lineages are seen in the widespread *Armillaria mellea sensu stricto* (Coetzee, Harrington, and others, unpublished) and in the *Lentinula* example (Hibbett *et al.*, 1995) discussed above.

In the absence of phenotypic divergence, there seems to be little justification to separate geographic lineages as species. On the other hand, if the lineages have been sympatric for an extended period, phenotypic differences would suggest that there has been some intersterility barrier to gene flow or that hybrid progeny between the populations are not fit. In either case, natural selection would seem to be playing a role in the maintenance of the separate lineages, and a closer examination of morphological or physiological characters would be warranted.

3.3.3 A population-based approach, the phylogenetic species concept

One view of a species is that it exists at the boundary of reticulate evolution (population genetics, relationships within a species) and hierarchical descent (phylogenetics, relationships among species) (Baum and Shaw, 1995; Davis, 1997; Hennig, 1966; Luckow, 1995). While several species concepts make this distinction, we will use the 'phylogenetic species concepts' of Cracraft (1983, 1997) and Nixon and Wheeler (1990) as points of discussion. Nixon and Wheeler define a species as ". . . the smallest aggregation of populations (sexual) or lineages (asexual) within which there is a pattern of ancestry and descent, and which is diagnosable by a unique combination of character states in comparable individuals (semaphoronts)." Phylogenetic history is important, but species are diagnosed by comparing attributes among populations; character states or combinations of character states that are diagnostic for a species are expected to be the point of difference between reticulating and hierarchical systems (Davis and Nixon, 1992). Two key factors of such phylogenetic species concepts are 1) the focus on populations (or lineages) and 2) the emphasis on the results of speciation processes, not the processes themselves (Cracraft, 1983, 1997; Davis, 1997; Luckow, 1995; Nixon and Wheeler, 1990). Cracraft's (1983) discussion of the phylogenetic species concept refers to phenotypic characters for species diagnosis, and for the fungi, an emphasis on phenotypic characters, rather than genotypic characters, will prove key.

Because of the emphasis on distinguishing characters and character states, this species concept has been called the 'diagnostic species concept' (e.g., Baum and Donoghue, 1995; Hull, 1997). Characters are the attributes of organisms that diagnose species; they are the way common ancestry is inferred and hypotheses proposed about evolutionary history (Davis, 1997; Luckow, 1995). Different forms of a character are known as character states. Nixon and Wheeler (1990) consider all inherited attributes of organisms to be either characters (found in all comparable individuals within a terminal lineage) or traits (not universally distributed among comparable individuals within a terminal lineage). Diagnostic characters are generally qualitative with one or more discrete states (Luckow, 1995). Unlike the characters in the monophyletic species concept, characters under the phylogenetic species concept of Cracraft (1983, 1997) and Nixon and Wheeler (1990) need not be autapomorphies (derived characters); any unique combination of plesiomorphic (ancestral) and derived character states is acceptable. It should be emphasized that recognition of phylogenetic species *sensu* Cracraft (1983) and Nixon and Wheeler (1990) is not dependent on the execution of a phylogenetic analysis; phylogenetic species serve as the starting point for cladistic analyses, not the endpoint (Davis, 1997; Davis and Nixon, 1992).

Davis and Nixon (1992) proposed 'population aggregation analysis' of population polymorphism to search for fixed characters that aggregate populations into phylogenetic species. They recognize a number of potential sources of error in delimiting species, including failure to detect homology in characters, undersampling of attributes, undersampling of individuals, and undersampling of populations.

Undersampling makes the distinction between characters and traits difficult. For example, undersampling of individuals in a population may result in failure to recognize character polymorphisms, which may lead to the recognition of too many different species. In contrast, two different phylogenetic species that are initially considered as part of the same population may lead to the conclusion that certain characters are polymorphic traits. These opportunities for error point towards the necessity of increased rigor in the study of populations and characters.

Cracraft (1983) clearly had phenotypic characters in mind in searching for phylogenetic species, but that was before the onslaught of RFLPs, PCR, and automated DNA sequencing. This newfound wealth of characters and their power to identify down to the level of genotype has shifted the focus from fixed phenotypic characters in populations (e.g., Cracraft, 1983) to more genealogical approaches (e.g., Baum and Donoghue, 1995; Baum and Shaw, 1995). We have previously discussed the limitations of a genealogical approach to species delimitation in the fungi. The use of molecular characters in the same way as phenotypic characters in the phylogenetic species concept also can lead to difficulties.

3.4 Characters in delimiting fungal species

In our species concept, it is important that the individuals that comprise the species are derived from a common ancestor and that these individuals are reproductively isolated from sympatric populations of related species, but unique phenotypic characters delimit the species. The phenotypic characters most valuable as delimiting characters would be those associated with the ecological adaptations that circumscribe the niche of the species. Molecular markers frequently correlate well with the species-delimiting phenotypic characters, thus providing excellent identification tools, but they are not the final word in species delimitation. In the following sections, we outline major points concerning diagnostic characters, including their role in defining the ecological niche of the species. Morphological, physiological, and other phenotypic characters will be discussed and contrasted with molecular characters.

3.4.1 Phenotypic characters

Under the current, formal species concept, the holotype concept, when a fungal taxon is formally designated, a representative specimen that shows diagnostic features is deposited in an herbarium (Greuter *et al.*, 1994). It is this dried specimen, rather than the author's description, that represents the species. Many have pointed out limitations to this typological system, including lack of recognition of variability in fungal species and restriction of the observable specimen characters to morphology. A population-based approach to the study of characters is needed to include the inherent variation in the species, and the acceptance of non-morphological (physiological) characters in defining species also will be necessary if we are to improve our current concepts of fungal species.

3.4.1.1 Ecological niche

In asexual taxa, species cohesion is maintained primarily by factors that define the ecological niche, and the spread of new genetic variants is controlled primarily by genetic drift and natural selection (Templeton, 1989). The role of niche in maintaining species cohesion may be just as important in sexual species (Templeton, 1989), where gene flow within species and intersterility between species facilitate species cohesion, but ecological constraints play a large role in the fate of the species. Natural selection will determine whether and when the suite of characters used to define the species become untenable and the species goes extinct, or if a new suite of characters results in a better-adapted population, and a new species can be recognized.

If niche plays a major role in determining the development and maintenance of fungal species, then any phenotypic character associated with the niche should be useful in defining species. Morphological attributes used to define fungal taxa may be related to ecological niche. Among the most prominent of these characters are adaptations for effective spore dispersal. However, such adaptations are generally useful at the genus, family, and higher taxonomic levels. Adaptations to particular substrates, temperature conditions, or moisture conditions, and competition with other microbes probably play larger roles than morphology in speciation and cohesion. Physiological characteristics may more accurately define a fungal species than the morphological features evident in a holotype specimen.

3.4.1.2 Morphological characters

Although morphological characters are central to species descriptions and identifications, diagnostic, morphological characters are often elusive. Clearly, the simple morphology of fungi limits the number of potential characters available. If fungal speciation is driven primarily by physiological adaptation, then there may be little or no selection pressure for qualitative or quantitative changes in morphology. Morphological differences between sister species may arise slowly, only through genetic drift, and recently diverged species need not differ morphologically. We are typically confronted with ecologically distinct species that show only minor quantitative differences in morphology, with considerable overlap in the size ranges of spores and fruiting structures.

Quantitative morphological characters always have been an important part of species descriptions and can be used to define phylogenetic species (Luckow, 1995). Of the quantitative characters associated with fungi, spore size is probably the most often used, though it is rarely critically analyzed, and many closely related species show considerable overlap (Parmasto and Parmasto, 1992). Too much overlap in quantitative characters between species may place an individual into two or more species. With rigorous sampling, multivariate statistical analyses of several overlapping quantitative characters (e.g., spores, hyphae, sterile elements) may distinguish closely related species by unique associations of character sizes (Luckow, 1995; Parmasto and Parmasto, 1992).

While morphological variation within individual taxa is well known, the source of this variation often is not. The relative effects of genetics and environment on small morphological changes are unknown for many fungal taxa. It is known that conidiophore and conidial morphology of many asexual taxa is strongly influenced by culture media, which has led to differing species limits depending on how a fungus is maintained in culture (Booth, 1971). Standardized procedures have helped considerably in some well-known groups (e.g., *Fusarium*, Nelson *et al.*, 1983).

It appears that relatively few genetic changes are necessary to significantly alter the gross morphology of fungal fruiting structures. Interpretation of such phenotypes can be difficult without direct knowledge of genetic and/or population-level phenomena. For example, a change from an agaric phenotype (exposed hymenia) to a secotioid phenotype (enclosed hymenia) may be due to a single allele at a single locus. In the cases of the agarics *Lentinus tigrinus* and *Suillus grevillei*, molecular, developmental, and geographical data suggest that secotioid phenotypes are not fixed in any particular population and are, therefore, traits rather than characters; the secotioid forms are conspecific with the respective agaric phenotypes (Hibbett *et al.*, 1994; Kretzer and Bruns, 1997).

Pleomorphy and dimorphism also have tended to complicate the delineation of fungal species. Many fungal groups, perhaps most commonly the ascomycetous fungi and the rust fungi, produce distinct sexual and asexual stages, and these different stages can be recognized by distinct species names. Individual collections or isolates of a species may not contain all of the characters that diagnose the species. Blackwell (1993) has pointed out the utility of Hennig's (1966) concept of the semaphoront (character bearer) for systematic studies in the fungi. The semaphoront is basically defined as, "the individual at a certain brief period of time" (Hennig, 1966). This allows all stages of the life cycle of an organism to be utilized in diagnosing a species. In the fungi, anamorphic and teleomorphic stages are thus easily integrated into the species diagnosis, though current rules of nomenclature allow separate names for these stages.

As discussed earlier, there are a number of examples of intersterility groups or 'biological species' that have been discovered within morphological species. Careful examination of those biological species have, however, generally shown the biological species to be morphologically distinct. Genealogical lineages within putative morpho-species also may point to better morphological characters for species delimitation. One of the most dramatic examples of 'biological species' is the *Armillaria mellea* story, where a wide range of morphological and ecological variability was lumped under one species until intersterility revealed the cryptic species. There are, however, apparent cases where morphology cannot distinguish ecologically distinct species. *Ceratocystis laricicola* and *C. polonica* are ecologically but not morphologically distinct throughout their range across Eurasia (Harrington and Wingfield, 1998).

Some of the most problematic genera are agriculturally important ascomycetes, many of which are known morphologically only by their reduced anamorphic states (Leslie, 1991; Sherriff *et al.*, 1994; Van Etten and Kistler, 1988). Reduced morphology is part of the problem in these cases, but it also is possible that these fungi speciate rapidly in the artificial ecosystems created by humans (Brasier, 1995), and morphological differentiation lags behind physiological differentiation. As discussed earlier under asexual fungi, the individuals selected for study are typically from introduced populations, and speciation and species limits may be clearer if these fungi were studied in their endemic ranges. Nonetheless, we should not limit species-delineating characters to morphology.

3.4.1.3 Physiological and other characters

Many developmental and physiological characters are available for use in defining species and have been used in an informal sense. Potential developmental characters include spore ontogeny (Minter *et al.*, 1983), though such characters are more useful at higher taxonomic levels. Secondary metabolites have been used at the species level in the Xylariaceae (Whalley and Edwards, 1995), and a number of biochemical features have been used in classification of the lichenized fungi (Purvis, 1997). Unique temperature optima, minima, or maxima for growth or survival also may help define taxa and have been given as diagnostic characters (e.g., *Penicillium*; Pitt, 1995).

Isozyme or allozyme markers are phenotypic characters, and as such they can reflect fixed differences for delimiting fungal species. Differences in electrophoretic mobility *in vitro* may differentiate morphologically similar populations and often are congruent with ecological adaptations to specific climatic conditions or pH of substrate. As previously mentioned, *Ceratocystis polonica* and *C. laricicola* have similar geographic distributions, are vectored by related bark beetle species, are indistinguishable morphologically, and have the same sequence in the ITS regions of the rDNA (Witthuhn *et al.*, 1998). However, the two species differ in their host relationships and differ at a single isozyme locus (Harrington *et al.*, 1996). Isozymes also have proven useful in separating asexual taxa that differ little in morphology, such as in the varieties of *Leptographium wageneri* (Zambino and Harrington, 1989).

Because of their heterotrophic lifestyle, fungi are excellent examples of Dawkins' (1982) concept of the extended phenotype; i.e., gene expression in the fungus can lead to characters that are observed at a distance from the fungus. Host specificity commonly has been used to recognize parasitic fungal species. Plant pathologists and others have used a *forma specialis* concept for informal designation of host-specific fungi that lack sufficient morphological distinction to warrant species designation. Mutualistic associations, such as mycorrhizae, plant endophytes, and various insect-associated taxa, also may demonstrate various degrees of host specificity. Other examples of fungal phenotypes at a distance may be seen where the fungus modifies the substrate in some distinctive way. In wood-rotting basidiomycetes, different types of white rot have been observed: a uniform white rot, in which all host

cell wall components are removed, and a delignifying decay, in which lignin is preferentially removed (Blanchette, 1995). This is one of the primary distinguishing characters between the very closely related *Phellinus gilvus* (uniform white rot) and *P. senex* (delignifying decay) (Rizzo and Gieser, unpublished). Modifications of host phenotype also can be seen with rust fungi that can cause distinctive changes in the morphology of stems and flowers (e.g., Roy, 1993).

It does not matter if a single gene or multiple interacting genes are the determinants of substrate specificity. The ultimate result of specificity is due to gene expression in the fungus and, therefore, should be considered a phenotypic character. In our view, physiologically and ecologically distinguishable entities could be formally recognized as species if their putative substrate or vector specificity correlates with other phenotypic or genotypic characters, and the phenotypic characters show fixation at the population level.

3.4.2 *Molecular markers*

Molecular markers (e.g., RFLPs, RAPDs, AFLPs, SSCPs, DNA fingerprinting) have been used to distinguish fungal taxa that are difficult to characterize by traditional morphological means (e.g., Anderson, *et al.* 1987; Bruns *et al.*, 1991; Fukuda *et al.*, 1994; Harrington and Wingfield, 1995; Kohn, 1992). Such markers are frequently found to be fixed within species that are delimited by other characters and are invaluable for identification purposes. However, each individual could be characterized by unique DNA sequences, and several authors (e.g., Avise, 1994) have asked the question, where do we draw the line and will each population or individual become a new species under the phylogenetic species concept? Most molecular markers currently being utilized are traits *sensu* Nixon and Wheeler (1990), rather than characters, because they do not occur in all comparable individuals in the terminal lineage. While DNA fingerprinting probes can identify down to the level of individuals in fungi (e.g., DeScenzo and Harrington, 1994), the phylogenetic species concept emphasizes populations, not individuals (Cracraft, 1997).

Incongruence between molecular markers and phenotypic markers has been noted in the fungi and other groups of organisms, often with the assumption that the molecular characters are 'correct.' Molecular markers are generally more accurate than phenotypic markers in identifying lineages and in tests for gene flow between populations. For instance, isozymes may give misleading indications of high gene flow between populations while molecular markers such as RFLPs suggest genetic partitioning (Avise, 1994). Many of the commonly utilized molecular markers at the population level are selectively neutral, while phenotypic markers (such as isozymes) may be under varying amounts of diversifying or stabilizing selection pressure (Avise, 1994). Such selection pressures may result in continued heterogeneity in phenotypic markers that give the impression of gene flow between populations when in reality such gene flow has been greatly reduced.

It is clear that the genotype of the species is only an indirect indication of phenotype and ecological adaptation, and genotypic differences can be problematic in delimiting species, unless they are put into the context of phenotype. Fixation of genotypic characters as the sole criterion for species delimitation could potentially be taken to the ultimate reductionist level, fixation of a single nucleotide in a population. Any partially isolated population of a species would likely show at least a few unique nucleotide changes, or even some unique, trivial phenotypic character. For instance, the southernmost Sierra Nevada population of *Leptographium wageneri* var. *wageneri* has a unique electromorph for phosphoglucomutase, but this population is morphologically and ecologically identical to the other populations of this variety, and all individuals of the variety belong to a single vegetative compatibility group (Zambino and Harrington, 1989; 1990). At some point, common sense and practicality must prevail in the selection of diagnostic characters.

3.5 Conclusions

The fungi are an interesting challenge in biology. In many animal groups (particularly vertebrates) and plant groups, much of the species diversity in the world already has been discovered, and arguments about species concepts mostly concern how to reorganize already described taxa. For instance, should a single species be divided into two species or remain as subspecies? On the other hand, in groups such as fungi, other microorganisms, and insects, much of the world's biodiversity remains to be discovered (Hawksworth and Rossman, 1997). As mycologists attempt to describe new taxa, it becomes apparent that any species concept must have a practical focus as well as being intellectually satisfying. The wide array of fungal life cycles make it challenging to form an encompassing species concept, but we have adopted a simple approach. Slightly modifying Nixon and Wheeler's (1990) definition, we define species simply as ". . . **the smallest aggregation of populations with a common lineage that share unique, diagnosable phenotypic characters**." This is a phylogenetic species concept, incorporating the important features of population, lineage, and phenotype into a workable species definition.

We believe that the current holotype system is inadequate for characterizing the diversity apparent at the population level. Intraspecific variation has no application in formal systematics. In a population-based approach, however, variation is real and the holotype serves as an abstraction of the organism (Mayr, 1975). As mycologists incorporate many different types of data in defining species, the current system of nomenclature continues to hold back communication.

The ability to directly sample the genotype, via direct sequencing of nucleic acids, and the power of phylogenetic analyses have led some to think that species concepts should rely heavily on molecular markers. We view these markers differently than many. Genotypic characters that correlate with fixed phenotypic characters are valuable identification tools, and the use of gene trees and molecular data will become increasingly important in identifying lineages and reproductively isolated populations. Each gene will have its own history, and gene trees do not necessarily identify the

organism's history, but such phylogenetic analyses can point to lineages where diagnosable phenotypic characters may be found. All members of a species should be of the same lineage, whether the lineage is monophyletic or paraphyletic. But not all terminal lineages will prove to be species.

In order to facilitate a population-based approach to defining species, a stronger emphasis on all phenotypic characters is needed, the current emphasis on morphology notwithstanding. Ecological adaptations are key to the process of speciation, and characters associated with ecology should be used to define a species. Limiting formal species descriptions to the morphology of fungal fruiting structures ignores most of the biology of the species. If all phenotypic characters were acceptable, a much more comprehensive approach could be taken to defining fungal species. The classic example of an important physiological character would be host specificity in parasitic or mutualistic fungal species. As with morphological characters, substrate specificity is determined by a number of processes that are ultimately the result of gene expression in the fungus. There is no need, therefore, to consider physiology differently than morphology when defining appropriate characters for formal taxonomy. The informal rank of *forma specialis* for physiologically specialized organisms could be eliminated, and populations with such fixed phenotypic characters as host specialization should be elevated to the formal rank of species.

Our proposal may be met with skepticism on several fronts. Although we embrace a phylogenetic species concept, we find the genealogical species approach to be unworkable with the fungi. Intersterility tests are seen here as a tool rather than a defining criterion for fungal species. Questioning the holotype species concept will undoubtedly meet opposition, and some will point out the difficulty of carrying out population-based studies on all taxa. In cataloging the diversity of fungi in the world, many new species will be based on only a single collection. While it is important to get information on potential new taxa to the scientific community, we would caution against formalizing a taxon unless at least some indication of variation within a species is known. Physiological characters also will be a component of good species descriptions, species that will be recognized by other scientists and will not soon be split into new taxa.

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