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Phylogenetic Species Recognition and Species Concepts in Fungi

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Taylor, J. W., Jacobson, D. J., Kroken, S., Kasuga, T., Geiser, D. M., Hibett, D. S., and Fisher, M. C. 2000. *Fungal Genetics and Biology* 31, 21–32. Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics and Biology* 31, 000–000. The operational species concept, i.e., the one used to recognize species, is contrasted to the theoretical species concept. A phylogenetic approach to recognize fungal species based on concordance of multiple gene genealogies is compared to those based on morphology and reproductive behavior. Examples where Phylogenetic Species Recognition has been applied to fungi are reviewed and concerns regarding Phylogenetic Species Recognition are discussed.

Index Descriptors: phylogenetic species concept; species recognition; biological species concept; morphological species concept; evolutionary species concept; congruence of gene genealogies.

This article is concerned more with how to recognize a fungal species than with species concepts. However, some mention of species concepts at the outset is necessary and should be useful. Mayden (1997) surveyed species concepts and characterized them as either theoretical or operational. Unlike theoretical species concepts, operational species concepts can be used to diagnose or recognize species. Operational species concepts outnumber the theoretical type; of the concepts reviewed by Mayden, the only primarily theoretical one was the Evolutionary Species Concept (Simpson, 1951, 1961; Wiley, 1978). Although many theoretical species concepts are possible, ranging from those based on divine creation to those based on Darwinian evolution, it is hard for an evolutionary biologist to take issue with the ESC. That is because it defines a species as, "... a single lineage of ancestor-descendent populations which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate" (Wiley, 1978). The more commonly discussed species concepts, Morphological Species Concept, Biological Species Concept, and Phylogenetic Species Concept, are considered by Mayden to be secondary to the ESC. They also are compatible with the ESC in the sense that all three are trying to recognize evolutionary species (Avise and Wollenberg, 1997). Unfortunately, when it comes to identifying species, the ESC is not helpful because it has no recognition criteria. In contrast, the many secondary, operational species concepts, e.g., the MSC, BSC, and PSC, do specify criteria for recognizing species. To emphasize the distinction between theoretical and operational species concepts, we will reserve the term "species concept" for the theoretical variety and use the term "species recognition" for the operational ones, i.e., Morphological Species Recognition, Biological Species Recognition, and Phylogenetic Species Recognition.

As Mishler and Donoghue pointed out (1982), biologists adopt a method of species recognition that is based on their own experience with organisms and systematic char-

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acters. None of the methods of species recognition can recognize the moment that individuals in an ancestral species are split into progeny species, because time must pass before changes in morphology, mating behavior, or gene sequences occur and can be recognized in the progeny species. Under the ESC, species have been recognized by MSR, BSR, or PSR, but our experience with fungi is that the phylogenetic analysis of variable nucleic acid characters currently comes closer than the others to recognizing species consistent with the Evolutionary Species Concept. PSR performs best because, once progeny evolutionary species have formed from an ancestor, changes in gene sequences occur and can be recognized before changes have occurred in mating behavior or morphology.

Our aim is to examine PSR as it relates to fungi by (1) comparing it to morphological and biological species recognition, by (2) examining recent examples in which PSR has been applied to fungi, and by (3) considering concerns that have been raised about PSR. The topic of fungal species concepts and speciation has been reviewed ably (Brasier, 1987, 1997; Burnett, 1983; Natvig and May, 1996; Petersen and Hughes, 1999; Harrington and Rizzo, 1999). With the exception of that by Harrington and Rizzo, these reviews have focused on BSR and MSR and have not thoroughly examined PSR. Much has been written about the PSC and PSR in general (Avise and Ball, 1990; Avise and Wollenberg, 1997; Cracraft, 1983; Mayden, 1997; McKitrick and Zink, 1988). With fungi, Harrington and Rizzo (1999) advocate a type of PSR that diagnoses species as " . . . the smallest aggregation of populations with a common lineage that share unique, diagnosable phenotypic characters," which is nearly identical to that proposed by Nixon and Wheeler (1990). However, Harrington and Rizzo did not include several recent fungal studies that have used PSR based on the concordance of gene genealogies to diagnose species (Geiser *et al.*, 1998; Kasuga *et al.*, 1999; Koufopanou *et al.*, 1997, 1998; O'Donnell *et al.*, 1998). These studies demonstrate that PSR by genealogical concordance is well suited to fungi and likely to become very popular with mycologists.

PREVAILING SPECIES CONCEPTS AND RECOGNITION

The dominant fungal operational species concept is the MSC and the dominant method of diagnosing species is MSR. With few exceptions, the ca. 70,000 described fungi are diagnosed by morphological characters (Hawksworth *et al.*, 1996) or other phenotypic characters, e.g., growth at different temperatures or water activities (Pitt, 1979), the production of secondary metabolites (Frisvad and Filtenborg, 1990), or the presence of pigments (Besl and Bresinsky, 1997). The great strength of MSR is that it has been applied widely so that comparisons can be made among existing taxa and between new and existing taxa. The weakness of the MSR is that species diagnosed by MSR often comprise more than one species when diagnosed by BSR or PSR. The classic example is the morphological species *Armillaria mellea sensu lato*, which contained over a dozen species as determined by mating tests (BSR) (Anderson and Stasovski, 1992; Anderson and Ullrich, 1979; Korhonen, 1978). A more recent study of another basidiomycete, the shiitake mushroom (*Lentinula edodes*), also found more species by PSR than were found by MSR or BSR (Hibbett *et al.*, 1995), and provided a discussion of species concepts and recognition from the perspective of conservation biology (Hibbett and Donoghue, 1996). Most recently, medically important morphological species have been found to contain between two and three species as diagnosed by PSR (Taylor *et al.*, 1999a). In the human pathogenic fungus *Histoplasma capsulatum* (meiosporic state = *Ajellomyces capsulatus*), six genetically isolated groups diagnosed by congruence of gene genealogies (PSR) were found in this one morphological species and they were correlated with biologically relevant features such as geographic distribution and pathogenicity (Kasuga *et al.*, 1999). Therefore, as widespread as is MSR, and as straightforward as it is to apply, it cannot be counted on to diagnose evolutionarily meaningful species in fungi. In fact, if the recent examples are representative, MSR can be counted on to lump two or more species as recognized by BSR or PSR in each morphological species.

The BSC as articulated by Mayr (1940) diagnosed species as " . . . groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups." The BSC and BSR hold a prominent place in mycology. More than a decade before Mayr's article, the criteria of the as-yet-to-be-named BSC were used by Shear and Dodge (1927) to show that the morphological species *Monilia sitophila* harbored three species that could be diagnosed by mating tests: *Neurospora crassa, N. sitophila,* and *N. tetrasperma.* In 1965, Pore *et al.* invoked the BSC to show that *Arthroderma quadrifidum* harbored another species, *A. lenticularum,* and by 1987 Brasier could list almost 40 cases where morphological species harbored more than one genetically isolated

biological species (Brasier, 1987). In 1999, Petersen and Hughes discussed more examples of the BSC from mushrooms, highlighting studies on *Armillaria, Pleurotus, Xeromphalina, Omphalotus,* and the aforementioned *Lentinua.*

In fungi, BSR has been used to identify groups of mating compatible individuals, which have been equated with species. However, mating tests are impossible to apply to fungi that lack meiospores. This problem is a serious one because approximately 20% of fungi are morphologically asexual and do not produce meiospores (Reynolds, 1993). Other fungi are homothallic and will produce meiospores without a partner. With these fungi, the presence of meiospores is not sufficient to infer mating, instead genetic markers must be used to ensure that the progeny have two parents. In addition, some heterothallic fungi cannot be coaxed into mating in cultivation, and many fungi cannot be cultivated. Here, too, it is impossible to apply BSR.

There is deeper problem with BSR. Even when BSR can be applied to fungi, there is evidence that the criterion of reproduction lumps together groups of fungi that are genetically isolated in nature, but that retain the ancestral character of interbreeding. Even though the BSC emphasizes actual gene flow, BSR by mating tests measures potential gene flow. The aforementioned study of *Lentinula* (Hibbett *et al.,* 1995; Hibbett and Donoghue, 1996) offered one example of the conflict between actual and potential gene flow. Another example is found in a study of the *Pleurotus ostreatus* complex of oyster mushroom species. With *Pleurotus,* Vilgalys and Sun (1994) identified eight intersterility groups or biological species in the *P. ostreatus* complex and used variation within the ribosomal repeat unit to make a phylogenetic analysis of individuals from each biological species. Three of the biological species (I, II, and VI) contained more than one phylogenetic species and in each case the phylogenetic species were found on different continents in the Northern Hemisphere. What were considered to be three biological species using BSR would be considered to be nine phylogenetic species using the PSR. The difference is not simply academic because each of the phylogenetic species showed genetic and geographic isolation. As Petersen and Hughes (1999) note, " . . . these species do not exchange genes in nature despite the potential to do so."

Other examples of this problem with BSR include *Heterobasidion annosum* (Chase and Ullrich, 1990) and *A. mellea* (Anderson *et al.,* 1980), and neither is this difficulty with BSR limited to Basidiomycota. In the ascomycete genus *Neurospora,* BSR has been more broadly applied than in any other fungus, with over 4500 isolates in the Fungal Genetics Stock Center (http://www.fgsc.net/) collection that have been identified by mating to tester strains (Perkins and Turner, 1988; Perkins *et al.,* 1976; Turner *et al.,* 2001). However, there are some signs that BSR in *Neurospora* also lumps genetically isolated groups into biological species. For example, for both *N. crassa* and *N. intermedia,* more than one pair of testers may be needed to identify new isolates because not all members of each species will mate with one pair of testers. And, in the case of *N. intermedia,* there is a population of isolates that is associated with food or food by-products and that has large, yellow macroconidia (Perkins and Turner, 1988). Although these isolates mate with *N. intermedia* testers in the lab, it is possible that they are genetically isolated in nature. Because BSR by mating tests does not distinguish actual gene flow in nature from the potential for gene flow, it should not be surprising that it may define species that encompass more than one genetically isolated group. Among allopatric sibling species, many traits might diverge before the ancestral ability to mate would be lost, particularly since there could be no selection against it. This concept and others related to sympatric speciation have been explored for fungi by Natvig and May (1996).

The PSC and PSR extends phylogenetics through the boundary of genetically isolated groups into interbreeding individuals. The boundary was recognized by Hennig, who contrasted the diverging clades of phylogenetics with the anastomosing networks of tokogenetics (Hennig, 1966). Mayden (1997) points out that there are several versions of the PSC. Cracraft's original version (Cracraft, 1983) equated a phylogenetic species with " . . . the smallest diagnosable cluster of individual organisms within which there is a pattern of ancestry and descent." The criterion of monophyly arose with the requirement that a derived character be shared by the members of the species, i.e., that there be a synapomorphy (Rosen, 1978), and a combination of the two ideas of a diagnosable cluster and a shared apomorphy was advocated by McKitrick and Zink (1988). The drawback of this type of PSC is that individuals are grouped very well, but the decision about where to place the limit of the species is subjective. For example, if a gene is polymorphic and has two alleles (A, B) among individuals in a species, and the gene is used for PSR, individuals sharing allele A might be called a species to the exclusion of those sharing allele B (Fig. 1). Of course, including more information, such as the ability to interbreed would rectify the confusion (Baum and Donoghue, 1995). However, as has been mentioned, many fungi cannot be tested for the ability to interbreed. And, without the

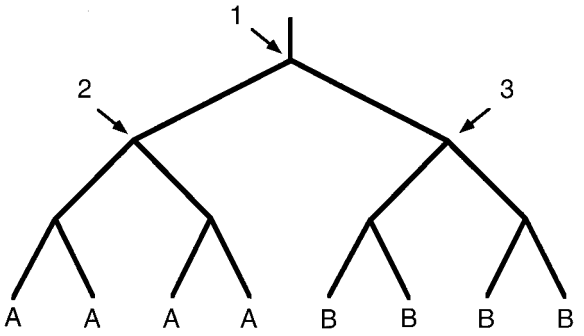

FIG. 1. Uncertainty about the limits of species in a single gene genealogy. Phylogenetic analysis of single genes can be used to group individuals into monophyletic groups, but not to rank them into species. For example, should the limit of a species be at arrow 1, implying that alleles A and B represent intraspecific variation? Or, should the species limits be placed at arrows 2 and 3, implying that alleles A and B are fixed in two genetically isolated species?

additional information one would not know if the gene was polymorphic within a species, or fixed for alternate alleles in two species.

PSR can avoid the subjectivity of determining the limits of a species by relying on the concordance of more than one gene genealogy. Mayden labels this type of PSC the Genealogical Concordance Concept. It was proposed by Avise and Ball (1990) and carefully discussed by Baum and Shaw (1995) based on ideas of coalescence and lineage sorting developed earlier by many authors (e.g., Avise *et al.*, 1987; Hudson, 1990; Slatkin and Maddison, 1990; Templeton, 1989). Remembering the distinction between species concepts and species recognition, we will use the term Genealogical Concordance Phylogenetic Species Recognition. The strength of GCPSR lies in its comparison of more than one gene genealogy. A requirement of each gene genealogy is that recombination does not occur within the gene, and in practice, parts of genes are often used to construct the genealogies. Where the different gene trees are concordant they have the same tree topology due to fixation of formerly polymorphic loci following genetic isolation; these concordant branches connect species. Conflict among the gene trees is likely to be due to recombination among individuals within a species, and the transition from concordance to conflict determines the limits of species (Fig. 2).

A drawback to the GCPSR is that the transition from concordance to conflict cannot occur for a truly clonal species, and as much as one-fifth of fungi are mitosporic and have been thought to be clonal. This concern may be misplaced, however, because recent examination of several mitosporic fungi has found they are recombining in nature in addition to reproducing clonally (Anderson and Kohn, 1998; Taylor *et al.*, 1999a, b), and even a low level of recombination leads to conflict among gene genealogies. In fact, a very extensive study of a primarily clonal fungus, *Sclerotinia sclerotiorum*, successfully compared gene genealogies to identify the limits of the species, to find patterns of clonal descent, and to identify some instances of recombination (Carbone *et al.*, 1999). At present, it seems that exclusively clonal fungi, should they be found, will not be common as compared to recombining fungi. In that case, they could still be accommodated via the GCPSR by defining them in relation to their recombining relatives. In phylogenetic studies, putatively clonal fungi have been shown to have close sexual relatives (LoBuglio *et al.*, 1993), which indicates that this means of accommodating clonal species in the GCPSR would be an expedient one.

FUNGAL EXAMPLES OF PHYLOGENETIC SPECIES

There are now several fungal examples of species diagnosis by GCPSR. The *Gibberella fujikuroi* (mitosporic states = *Fusarium*) complex provides a good example with many taxa (O'Donnell *et al.*, 1998). O'Donnell *et al.* assembled a large collection of isolates and used nucleotide sequence of the beta tubulin gene to sort the isolates into clusters. They then added nucleotide sequence for two other genes for one representative of each cluster. Phylo-

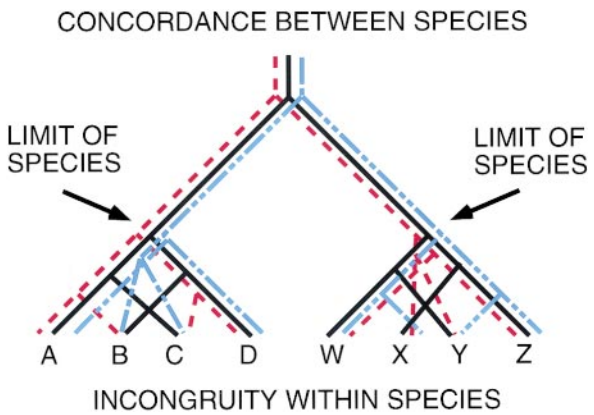

FIG. 2. Simultaneous analysis of three gene genealogies shows how the transition from concordance among branches to incongruity among branches can be used to diagnose species.

genetic analysis of all three genes revealed 45 species in the complex, 23 of them new to science. Among the 45 species were more than half-a-dozen species that previously had been identified as biological species by mating tests (Hsieh *et al.,* 1977; Leslie, 1995). In all cases, the biological species and the phylogenetic species were identical, which indicates that PSR can provide the same information as BSR. None of the other species had been diagnosed by BSR because they would not mate in cultivation, and 23 of the species had not been diagnosed by MSR, although morphological characters were found after the fact (Nirenberg and O'Donnell, 1998). The *G. fujikuroi* species complex is not a complete example of GCPSR because only one of the three genes was sequenced for all isolates. It is possible that these taxa, which were recognized by alleles of the beta tubulin gene, would not be supported by other genes. However, this was not the case for any of the *G. fujikuroi* biological species and a number of the strictly mitosporic species. *Candida albicans* (Sullivan *et al.,* 1995), *Botryotinia fuckeliana* (mitosporic state = *Botrytis cinerea*) (Giraud *et al.,* 1997), *Lentinula lateritia* (Hibbett *et al.,* 1995), and *Filobasidiella neoformans* (mitosporic state = *Cryptococcus neoformans*) (Franzot *et al.,* 1999) represent other recent cases where nucleic acid variation and phylogenetics have helped to discover cryptic phylogenetic species in one morphological species, albeit without using concordance of genealogies.

H. capsulatum (meiosporic state = *A. capsulatus*), an ascomycetous human pathogen, provides an example with fewer species than *Gibberella,* but with more nucleotide sequence data. In this example, there were four complete gene genealogies with sequence from 46 isolates for every gene (Kasuga *et al.,* 1999). Prior to the work of Kasuga *et al., H. capsulatum* was thought to comprise three varieties, *duboisii, capsulatum,* and *farciminosum.* Mating reactions among individuals of the three varieties had been cited as one reason for keeping them in a single species (Kwon-Chung *et al.,* 1974). There also were arguments for raising *H. c.* var. *duboisii* to species based on yeast cell morphology, site of infection, and the fact that *H. c.* var. *duboisii* is limited to Africa, while *H. c.* var. *capsulatum* was considered to be found primarily in the New World (Kwon-Chung, 1975). *H. c.* var. *farciminosum* was also originally described as a species based on the fact that it infects horses, mules, and donkeys and is acquired via the skin while the other varieties affected humans and were acquired via the lungs (Kwon-Chung and Bennett, 1992). Previous work on *H. c.* var. *capsulatum* from Kobayashi's group had discovered classes of isolates with different pathogenicities (Medoff *et al.,* 1986; Spitzer *et al.,* 1990) and nucleic acid variation (Spitzer *et al.,* 1989; Vincent *et al.,* 1986), but had not addressed the question of multiple species. Using four gene genealogies, Kasuga *et al.* (1999) could identify six genetically isolated taxa among the 46 isolates representing all varieties and classes. One of the phylogenetic species was identical to the African *H. c.* var. *duboisii.* Five of the phylogenetic species were found in *H. c.* var. *capsulatum,* and some of them were identical to the aforementioned classes. *H. c.* var. *farciminosum* isolates did not form a phylogenetic species. Instead all isolates had the same genotype, and that genotype fell within one of the two South American species of *H. c.* var. *capsulatum.* Apparently this genotype has the ability to cause skin disease and is passed clonally from animal to animal. With *H. capsulatum,* it certainly seems that GCPSR has performed better than MSR or BSR.

Coccidioides immitis, another ascomycete human pathogen, provides an example of phylogenetic species found through GCPSR in a mitosporic fungus. Koufopanou *et al.* (1997, 1998) used five gene genealogies to examine 17 individuals from throughout the New World distribution of this fungus. One branch that was common to all five genealogies separated the isolates into two species, one in California, and the other outside the state. Within each species, the gene genealogies were in conflict, but not significantly so because most of the variation was found between the two species and not within them (Koufopanou *et al.,* 1997, 1998). Using a collection of arbitrary single nucleotide polymorphisms, Burt *et al.* (1996) showed that the non-California species was recombining in nature, and Fisher *et al.* (2000a) have used the same approach to the same end in the California species. Using the same polymorphic loci developed to test for reproductive mode, Burt *et al.* (1997) showed that gene flow between the two species was nonexistent, which supported the two species discovered by Koufopanou *et al.* Subsequent sequence analysis of a proline-rich antigenic protein also supported the genetic isolation of the two species (Peng *et al.,* 1999). Unlike *H. capsulatum,* there were no previously known morphological or physiological differences between the two *Coccidioides* phylogenetic species. However, knowledge that there are two species may now lead to the discovery of differences, as will be mentioned later.

Aspergillus flavus provides a third example where concordance of gene genealogies revealed cryptic, phylogenetic species, also in a mitosporic fungus. Geiser *et al.* (1998) used five gene genealogies to examine 16 unique genotypes found among 33 isolates taken from Australian peanut fields. When all five genealogies were combined,

one strongly supported branch emerged that divided the isolates into two species. In one of the species, there were enough isolates and there was enough variation to demonstrate intraspecific incongruity; again a mitosporic fungus was found to be recombining in nature. *A posteriori,* it has been seen that variation in the presence and size of sclerotia and variation in mycotoxin production correlates with the phylogenetic species (Geiser *et al.,* 2001; D. Carter, personal communication).

These examples show that GCPSR can be applied to fungi that are both meiosporic (*Gibberella, Ajellomyces, Botryotinia, Filobasidiella*) and mitosporic (*C. immitis, A. flavus, Candida, Fusarium*). In all cases, PSR revealed species not detected by MSR, and in some cases PSR revealed species not seen by BSR. In *Fusarium* (meiosporic state *Gibberella*), a system with well-characterized biological species, PSR and BSR were in agreement, and PSR detected the many other mitosporic species that could not be diagnosed by BSR. Despite these successes, GCPSR is far from universally accepted, and some of the arguments against it are discussed below.

UNCERTAINTY ABOUT PSR AND GCPSR

1. When using PSR, as the genetic markers become more polymorphic, won't the number of species rise? This concern is raised by Mayden in his comparison of species concepts (Mayden, 1997). If just one character, or gene, were used to recognize species, this concern would be valid. For example, if a microsatellite locus with many alleles was used as the locus, there could be clusters of individuals with each allele and one could consider each to be a phylogenetic species. The solution to this problem is to use more than one polymorphic locus. Here, different individuals will have different combinations of alleles, and different genes will have different genealogies within a species, but between species the genealogies should be concordant due to the effects of genetic isolation and drift and associated lineage sorting and coalescence. In fact, genealogies of nine highly polymorphic microsatellite loci diagnose the same phylogenetic species that are found using gene genealogies in *C. immitis* (Fisher *et al.,* 2000b).

2. If different phylogenetic species share the same neutral alleles at any polymorphic locus, how can they be considered to be distinct species? This concern has been raised by Doyle (1995, 1997). Although it seems logical that shared alleles at even one locus would provide evidence of continued genetic exchange among species, that is not the case because genetic isolation precedes the loss of shared polymorphisms. Following genetic isolation, alleles sort randomly due to genetic drift and the proportion of loci for which the polymorphisms are shared changes in inverse proportion to the time since genetic isolation began. This scenario is true as long as other factors, such as population size, remain constant. Therefore, in genetically isolated species one would expect to find some variable nucleotide positions where polymorphism is still shared by both sibling species, other positions where only one species is polymorphic and the other is fixed for one allele, and others where both species have fixed loci (Fig. 3). This is just what is seen in the data sets of the examples reviewed above. Interestingly the rarest class seems to be the one where both species still share the polymorphism, at least in the cases cited before. Therefore, this concern can be reversed to make the argument that discovering a few loci, or even one, that shows fixation in one or the other of the phylogenetic species is evidence of genetic isolation.

3. Neutral loci seem well suited to PSR, but wouldn't loci subject to balancing selection confound PSR? Doyle (1995) raised this concern for loci in the mammalian major histocompatibility complex and the answer could be "yes" if loci subject to balancing selection unknowingly were used in GCPSR. Fortunately, recognizing loci under balancing selection is straightforward if enough individuals are sampled from the taxa in question. For example, studies of fungal mating loci (May *et al.,* 1999) or self/nonself-recognition loci (Wu *et al.,* 1998) have produced phylogenies showing unusually long coalescence times or trans-species polymorphisms or both, the hallmarks of balancing selection. In these genealogies, major clades are monophyletic for functionally different alleles and individuals from each species in question may be found in each clade. Within a species, each functional allele may show neutral variation, as has been seen in *Coprinus* mating loci (May *et al.,* 1999). It should be possible to use the neutral variation among functionally equivalent alleles to build gene genealogies for GCPSR. However, the situation can be complicated when alleles that are phylogenetically distant display the same function, as has been seen in the *Neurospora* heterokaryon incompatibility locus *het-c* (Wu *et al.,* 1998). Although it should be possible to use neutral variation of functional alleles for GCPSR, it is safer to employ loci that show no evidence for balancing selection.

4. Shouldn't species have a unique phenotype, preferably one that is related to the role of the species in nature? This concern is a major feature of Harrington and Rizzo's

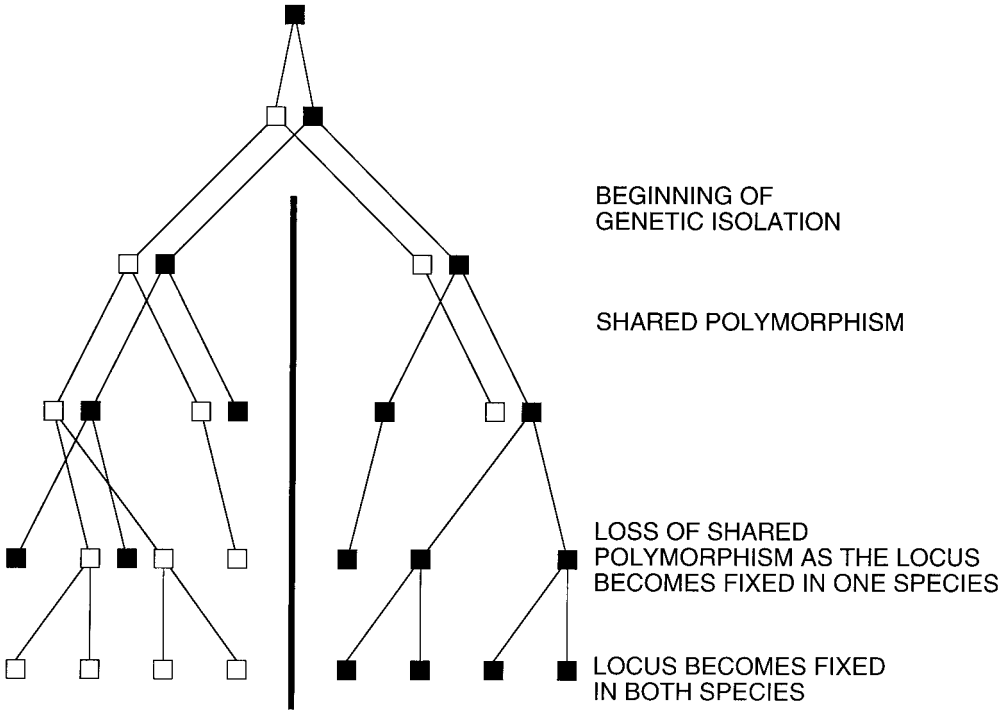

FIG. 3. Lineage sorting at one polymorphic locus following genetic isolation proceeds through several stages leading to loss of polymorphism in both species. Polymorphism is shared between the two newly isolated species; then it is lost in one species and finally in the other.

(1999) thorough discussion of the PSC, and the reason that they restrict their definition of the PSC to phenotypic characters. Again, genetic isolation precedes the divergence of character states, whether due to drift or selection. So, it is not expected that recently genetically isolated species will show immediate phenotypic differences, although over time they should. In the cases of *Gibberella, Histoplasma, Candida,* and *Aspergillus,* the phylogenetic species discovered through gene genealogies displayed phenotypic differences, including pathogenicity. However, for *Coccidioides* no morphological or physiological differences were known when the two phylogenetic species were discovered. There were, however, differences in the amino acid composition of proteins that were discovered during the phylogenetic studies (Koufopanou *et al.*, 1997, 1998) or in subsequent studies (Peng *et al.*, 1999). These differences in the primary structure of proteins constitute a phenotypic difference and may affect the activity of the protein or its antigenicity. To ensure that differences in antigenicity do not confound vaccine protection, proteins from both species are being used to develop vaccines (Peng *et al.*, 1999). It is also possible that there may be undiscovered morphological or physiological differences between individuals in the different species of *Coccidioides.* So far, one such example has been found; growth rate in high salt media is significantly different between the two species (M. C. Fisher and G. Koenig, unpublished data). Now that physicians can recognize the two phylogenetic species, it will be interesting to see if there are biologically meaningful differences in pathogenicity between them.

5. Isn't determining the limits of a species inherently subjective because there is a continuum of genetic differentiation following genetic isolation that is proportional to time and inversely proportional to population size? Yes. As seen in Fig. 3, each gene that is polymorphic in the ancestral population will go through several stages leading to fixation. Certainly, there will be cases where genetic isolation cannot be recognized because only a few ancestrally polymorphic loci have become fixed in one or the other progeny species. In this case, species that conform to the ESC could not be diagnosed by PSR, but neither should they be diagnosed by MSR or BSR. The fact that PSR cannot diagnose all evolutionary species is a failing, but in this regard PSR appears to be superior to MSR or BSR. A fungal example of this problem involves the Texas isolates of *C. immitis.* Using 12 single-nucleotide polymorphisms discovered in the Arizona isolates, Burt *et al.*

(1997) found evidence for a small but significant barrier to gene flow between *C. immitis* in Arizona and Texas. This differentiation, however, did not support a Texas clade when GCPSR was applied to *C. immitis* (Koufopanou *et al.*, 1997, 1998). It is possible that the Texas genotypes are evolutionary species, but they cannot be recognized by PSR. Considering the three stages of differentiation shown in Fig. 3, genetically isolated groups might be defined as species when a statistically significant fraction of bialleleic loci that were polymorphic among the species were fixed for alternate alleles. Where clades were found with weak statistical support, they would diagnose populations within the species. This criterion is arbitrary, and the outcome could change as more loci were examined, but it can be applied in an objective manner to a given data set. There is, of course, another explanation for shared polymorphisms between two species, gene flow in the form of hybridization or introgression. Gene genealogy concordance is well suited to identifying hybrid individuals because hybrids should group with different species in different single-gene genealogies, and lie at the base of the tree in combined gene genealogies (O'Donnell *et al.*, 2000).

6. Couldn't the GCPSR describe reproductively isolated disjunct species that may at some time in the future come together? Yes. If genetically isolated species as recognized by GCPSR retain the ability to interbreed and come together, they could reticulate into one evolutionary species. The concept of an evolutionary species accommodates divergence to form new species as well as hybridization. Using GCPSR, we can only describe species in the present; we cannot predict the future.

This article has focused on fungi, but GCPSR is not limited to fungi. Dykhuizen and Green (1991) first employed multiple-gene genealogies to investigate recombination in *Escherichia coli.* They also proposed GCPSR for bacteria, which was explored further and compared to Eukarya by Cohan (1994). Recently, GCPSR has been applied to socially important bacteria genera such as *Borrelia* (Dykhuizen *et al.*, 1993), *Neisseria* (Feil *et al.*, 1996; Holmes *et al.*, 1999; Smith *et al.*, 1999), and *Rhizobium* (Wernegreen and Riley, 1999) as well as ecologically important cyanobacteria (Rudi *et al.*, 1998; Urbach *et al.*, 1998). Among Eukarya, GCPSR has been more popular in fungi than in either plants or animals, probably because most fungi are haploid and because it is easier to survey multiple genes in fungi due to their having fewer paralogous genes and fewer complex multigene families. In plants, nuclear and chloroplast gene genealogies have been compared, but comparisons of more than one nuclear gene genealogy have not appeared. Unlike fungi, where genetic differentiation seems to precede morphological differentiation, studies of columbines (Hodges and Arnold, 1994; Fulton and Hodges, 1999), paintbrushes (Mathews and Lavin, 1998), and soybeans (Doyle *et al.*, 1999) show that morphological differences may precede easily detectable genetic differentiation. This situation could be due to strong directional selection for floral morphology, slow lineage sorting, or gene flow mediated by hybridization. In maize, it has been shown that selection for morphological traits important to domestication can accelerate molecular evolution in promoter regions of genes responsible for the trait while not affecting other genes, or even the coding region for the selected trait (Wang *et al.*, 1999). By inference, it seems possible that selection due to pollinator preference could effect similar dramatic changes only in genes controlling floral morphology (Fulton and Hodges, 1999).

In animals, comparison of nuclear and mitochondrial gene genealogies have also been common (Avise, 1994), and comparisons of several nuclear gene genealogies have begun to appear. For example, multiple nuclear gene sequences have been used to diagnose phylogenetic species in cichlid fish (Streelman *et al.*, 1998) and fruit flies (Caccone *et al.*, 1996). Conversely, when Hare and Avise (1998) used three nuclear gene genealogies to investigate oyster populations in the Atlantic Ocean and the Gulf of Mexico, they did not find genetic isolation, although previous work with mitochondrial DNA had done so. Once more single-copy genes are available, we expect that congruence of gene genealogies will be broadly applied in animals. The tremendous progress in animal developmental biology has begun to address mechanisms that maintain species. The molecular basis of prezygotic isolation has been studied in abalone with the finding that sperm lysin and egg receptors are compatible within species, but incompatible between them (Lee *et al.*, 1995; Nei and Zhang, 1998; Swanson and Vacquier, 1998), and the molecular basis of postzygotic isolation has been addressed in *Drosophila* with the finding that a homeobox gene thought to control spermatogenesis is compatible in intraspecific progeny, and incompatible in interspecific progeny (Ting *et al.*, 1998). Thus, the developmental processes that create and maintain species are due to genetic isolation, which allows incompatible alleles to arise between populations. This process, along with mutation, is fundamental to the process of evolution of species, and GCPSR is an excellent approach to studying it.

As a next to last word, the experience with the GCPSR in fungi, so far, has shown that it is straightforward in application and interpretation when compared to the MSR or the BSR. Of course, the studies to date are relatively small and the geographic sampling of taxa is sparse. As more thorough studies are reported, what now appears to be straightforward may become more complex. However, we are more optimistic than Petersen and Hughes (1999), who doubt that a "unified" fungal species concept is possible. We think that an Evolutionary Species Concept can be applied to all fungi and that GCPSR can be used to diagnose species for any fungi. We predict that additional data are likely to increase acceptance of GCPSR.

As a last word, the problem of the potential failure of the GCPSR to diagnose evolutionary species that have recently arisen due to the lack of sufficient time for lineages to sort, genealogies to coalesce, or loci to fix could lead one to question the very importance of species. If there is a continuum of genetic differentiation from the most similar populations through species and all the way to kingdoms and domains, why be so concerned with species? We take the view that species are important because they are the unit from which new species arise and, therefore, are the one taxon that can be ranked phylogenetically. However, we realize that there is a gradient of genetic differentiation between populations and species, which leads us to suggest that gradients are better described by numbers than by words. For example, pairs of populations could be described by the proportion of loci that show unilateral or reciprocal fixation, standard estimates of genetic distance, or statistics based on gene flow. Similarly, species names could be followed by the number of years estimated to have passed since their divergence from their closest sibling species (Koupopanou *et al.*, 1997, 1998). For that matter, the estimated number of years to the most recent common ancestor could be appended to any taxon name because time is the common currency of evolutionary biologists who work on different organisms and with different taxonomic characters. Methods for obtaining these dates for fungi are available (Simon *et al.*, 1993; Berbee and Taylor, 1993, 2000). Having names with divergence dates might make it easier for taxonomists to abandon ranks for taxa above the species level.

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