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Fungal species: thoughts on their recognition, maintenance and selection

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When it comes to fungal species and speciation, it is hard to find anything to say that has not already been said in several excellent recent reviews. The most comprehensive source of information is Burnett's recent book (Burnett, 2003), which expands upon the themes from his British Mycological Society Presidential Address (Burnett, 1983). In addition to reviewing mycological species concepts and speciation, he describes enough about basic mycology and the methodology of evolutionary studies to make chapters on defining fungal individuals and populations, or on the processes of evolution in fungi, useful for mycologists interested in evolution and for evolutionary biologists interested in fungi. Burnett's review of the early literature in fungal speciation is particularly helpful in the present age, when it seems as if literature that is not online is forgotten. A second source of information is Brasier (1997), who explored three of what he considered to be the four main elements contributing to fungal speciation: original interbreeding populations, natural selection on populations and reproductive isolation between populations. He left a discussion of mating systems to others. Brasier's discussion of natural selection is particularly good, and his figure comparing the narrow range of growth rates of dikaryotic hyphae taken from *Schizophyllum commune* fruiting bodies to the much broader range of growth rates for dikaryons synthesized from their haploid progeny is as clear a demonstration of the effects of selection as one could want. In the same era, Natvig & May (1996) considered biological species in terms of the physical scales and life histories

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appropriate to fungi. Their discussion of the great variety of fungal mating systems (including pseudohomothallism) and the ability of fungi to make both clonal and recombined offspring is particularly useful when trying to fit fungal data into the Procrustean bed of evolutionary thought emerging from studies of other eukaryotes. Petersen & Hughes (1999) then focused on Agaricales and the biological species concept with an emphasis on practical aspects of fungal species recognition. When comparing species recognized by phylogenetic and biological approaches, they noted that many genetically and geographically distinct species are not reproductively isolated, a point that we will explore below. An additional comprehensive source of information is Worrall's book (Worrall, 1999), and especially the chapter on defining fungal species contributed by Harrington & Rizzo (1999). These authors also discuss the problem of assigning a taxonomic rank to populations that are genetically isolated but kept from mating only by geography. Harrington & Rizzo (1999) decided to recognize a taxon as a species only if it exhibits a unique, diagnosable, phenotypic character; this is another point that we will consider below. Finally, although it is based almost entirely on examples from the animal and plant kingdoms, we would be remiss to omit mention of Coyne & Orr's recent and thorough volume on speciation (Coyne & Orr, 2004). In light of the many recent contributions to knowledge of fungal species and speciation, commentary here will emphasize research that has been published subsequently to these works.

A quarter of a century ago, Burnett rendered a perhaps overly dismal picture of research on fungal speciation (Burnett, 1983), famously stating, 'Mycology and mycologists, on the whole, have contributed very little to the mainstream of ideas concerning the modes of origins of species.' Fourteen years later, Brasier echoed Burnett's sentiments before sounding a more hopeful note (Brasier, 1997), that '. . . fungi provide superb material with which to study evolution in action . . .' Just three years ago, Burnett again synthesized results from many studies (Burnett, 2003), but noted that most of them involved fungi having, '. . . populations whose structure is largely determined by agricultural activities'. Among the fungi that will be featured here, species of *Coccidioides, Histoplasma, Aspergillus* and *Neurospora* appear not to be so influenced. Species of the first three genera are important pathogens of animals and humans, but the evidence to date indicates that agriculture is more likely to influence fungal biogeography (and therefore speciation) than human disease (which is not to say that human activities have had no effect on the evolution of medically important fungi, as will be noted below).

Burnett lamented (Burnett, 1983) that, 'A real constraint is imposed by the incomplete understanding of the taxonomy, ecology and biogeography of the fungi compared with that available to those concerned with ... plants [and] animals ...' The situation is changing and, for some fungi, the phylogeny and biogeography are relatively well understood, although our understanding of fungal ecology is still lagging and has become a factor that limits our understanding of adaptation and speciation. In this chapter, we will begin by discussing recent work on species recognition, and then proceed to a discussion of speciation processes and processes that maintain species by reducing or ablating gene flow among populations. Finally we will consider the interplay of these processes and natural selection.

Species recognition

Species recognition is a human endeavour that is essential to subsequent study of the evolutionary processes leading to speciation. Placing individuals in genetically isolated species is a necessary prerequisite for studies of reproductive mode, hybridization, gene flow and selection. Traditionally, all fungal species were recognized by morphology. Where morphological species were broad, and where the need to distinguish species was great, mycologists employed other phenotypes for species recognition; for example, substrate utilization and growth rate on different media at different temperatures are important to systematics of yeasts and penicillia (Pitt, 1979; Kurtzman & Fell, 1998). Now that we know that multiple fungal species may share easily observed phenotypes and still be genetically isolated, other methods of species recognition are needed. An obvious choice would be species recognition by mating compatibility tests, or Biological Species Recognition (BSR). Unfortunately, given that only *c*. 11% of fungal species have been cultivated and that *c*. 20% do not reproduce sexually in cultivation (Ainsworth *et al*., 2001), BSR by mating tests is not broadly applicable. In its place, measurement of genetic isolation by molecular Phylogenetic Species Recognition (PSR), using the concordance of gene genealogies, has provided a popular solution to the problem of fungal species recognition (Avise & Wollenberg, 1997; Taylor *et al*., 2000).

To carefully implement PSR, it is necessary not only to find several genomic regions with sufficient polymorphism to build well-supported gene genealogies, but also to assemble a collection of individuals from throughout the range of the fungus. When the sequence data have been obtained, the resulting gene genealogies can be compared to discover the

transition from concordance among gene genealogies (due to lineage specific loss of ancestral variation following genetic isolation) to conflict among gene genealogies (due to recombination within populations) and thereby recognize species. Species recognition by concordance of gene genealogies has had a dramatic impact on fungal taxonomy, particularly for socially important fungi. Its first application in mycology was with *Coccidioides immitis*, where comparison of five genes sequenced from 17 individuals showed two phylogenetic species in what had been considered to be one morphological species (Koufopanou *et al.*, 1997). Similar studies of medically and agriculturally important fungi have proven the wide applicability of PSR.

Recently, web-based Multilocus Sequence Typing (MLST) schemes (Maiden *et al.*, 1998; Taylor & Fisher, 2003) have been established to bring together PSR research conducted throughout the globe for a single fungal species. MLST works because a gene sequence is 'portable' in the sense that sequences determined in different laboratories can be combined, unlike RAPD or fingerprinting approaches. In addition, sequencing a region of DNA avoids ascertainment bias, that is, the problem that polymorphic loci discovered for one set of individuals may prove to be fixed (and, therefore, uninformative) in individuals used in subsequent studies. With DNA sequence, of course nucleotide positions polymorphic in the first population may be fixed in the second, but other nucleotide positions may be polymorphic in the second population, and so on. It seems very likely that each socially important fungus will have its own MLST scheme in the near future, which will enable truly global studies of fungal species and greatly enhance our knowledge of fungal biodiversity. A note of caution might be sounded concerning the generality of knowledge about species recognition, population structure, speciation, and species maintenance gained from socially important fungi, because many of them appear to rely heavily on clonal reproduction. For this reason it seems advisable also to experiment with fungi that are not human pathogens and that are not overly clonal, for example, outbreeding *Neurospora* species.

Intraspecific variation

Variation in DNA sequences can also be used to search for genetic variation within a species, but more variable markers are often needed. In this regard microsatellite loci have proven valuable. Fungal microsatellites were discovered in yeast (Field *et al.*, 1996; Field & Wills, 1996), but their first thorough application to elucidating fungal populations again was with *Coccidioides*. Here, with 9 microsatellite loci and nearly 170

individuals from throughout the New World range of the fungus, the two species proposed by MLST were confirmed, and populations were discovered in each species; at least two populations were found in *C. immitis* and at least three in the newly described species *C. posadasii* (Fisher *et al.*, 2002a). Microsatellites also are portable and can be used for Multilocus Microsatellite Typing (MLMT) schemes (see Chapter 16, this volume). Microsatellite loci can be used to discover populations by phylogenetic analysis, or by Bayesian assignment methods (Pritchard & Feldman, 1996; Falush *et al.*, 2003). In either case, once a MLMT scheme is in place, new individuals can be assigned to populations by using assignment methods (Rannala & Mountain, 1997), as has been demonstrated for isolates of *Coccidioides* species that were obtained from patients who lived far from the endemic areas (but whose travel histories showed that they had visited the endemic areas) (Fisher *et al.*, 2002b). Both MLST and MLMT schemes gain power as more researchers use them and they should prove very popular with both plant pathologists and medical mycologists. Prior to their broad application in *Coccidioides* species, the utility of microsatellies was evaluated by comparing them to sequence variation for *c.* 20 individuals from *C. posadasii* and *C. immitis* (Fisher *et al.*, 2000). An important result of that study was the finding that single microsatellites can be misleading owing to hypervariability and attendant homoplasy. The same conclusion was reached in a comparison of ten microsatellites in *Neurospora* with sequence variation for nearly 150 individuals (Dettman & Taylor, 2004). Therefore, microsatellies are best employed to extend multilocus sequence studies from species into populations; if the aim is to accomplish species recognition and population characterization simultaneously, many microsatellites should be used (Fisher and colleagues (Fisher *et al.*, 2004) used 20 in their study of *Penicillium marneffei*).

Challenging morphological species recognition with phylogenetic species recognition

A recent study that highlighted the potential for conflict between phenotypic and genotypic methods of species recognition used the fungus *Histoplasma capsulatum* (the mitosporic name for *Ajellomyces capsulatus*) (Kasuga *et al.*, 2003). This species was known to be phenotypically complex and was divided into three varieties based on host, geographic range and disease symptoms (Kwon-Chung & Bennett, 1992). The first variety, *Histoplasma capsulatum* var. *duboisii*, was confined to Africa and, in humans, caused bone and skin infections in addition to primary lung infections; *H. c.* var. *farciminosum* was found in Eurasia and caused skin

lesions in horses and donkeys; *H. c.* var. *capsulatum* was found in the New World and caused pulmonary infections in humans. When the sequences for four loci were obtained from more than 130 individuals assigned to the three varieties, they were found to form at least seven genetically isolated clades. These seven as yet undescribed species showed a strong correlation with geography but not with host or symptoms (Fig. 15.1): North America 1 and North America 2, Latin America A (which includes a Eurasian subclade) and Latin America B, Africa, Australia, and Indonesia (Kasuga *et al.*, 2003). The African species was shown to harbour members formerly assigned by symptoms to varieties *H. c.* var. *duboisii* and *H. c.* var. *capsulatum*; therefore, *H. c.* var. *dubiosii* is not a monophyletic taxon. Individuals assigned by phenotype and host to *H. c.* var. *farciminosum* were found in three different clades; in the Eurasian clade, all individuals had the same genotype. *H. c.* var. *farciminosum* is a polyphyletic collection of clonally propagating lineages that have independently made the jump to horses or donkeys as hosts. Finally, individuals formerly assigned to *H. c.* var. *capsulatum* are found in all clades; again the historical variety *H. c.* var. *capsulatum* is not a monophyletic taxon. Although the phenotypes used to describe the old varieties are not useful for identifying the new clades, it is likely that other phenotypes may be found that are diagnostic of the new groups, as will be noted below for *Aspergillus flavus*.

Species divergence and geologic time

Sequence data can be used to estimate the dates of divergences among species and populations and to compare these dates to recent geologic, archaeological or historical events to make hypotheses about the evolutionary history of fungi. Estimates of geologic time have been applied to three studies of *Coccidioides* species. The first example concerned the divergence of the two *Coccidioides* species, which was estimated to have occurred between 10 and 12 million years ago (MYA) (Koufopanou *et al.*, 1997). A second study estimated divergence times between *Coccidioides* and the closely related genus *Uncinocarpus*, and among phylogenetic species recognized in both genera, and showed that the divergence between *Coccidioides* and *Uncinocarpus* was at least an order of magnitude older than divergences among phylogenetic species in either genus (Koufopanou *et al.*, 2001) (Fig. 15.2). Morphological species typically appear to harbour two or more cryptic species with divergences of the order of 3–10 MYA (Burt *et al.*, 2000) while, in these cases, the nearest morphologically distinct species seem to have diverged on the order of 30–100 MYA. If divergences recognizable by genetic

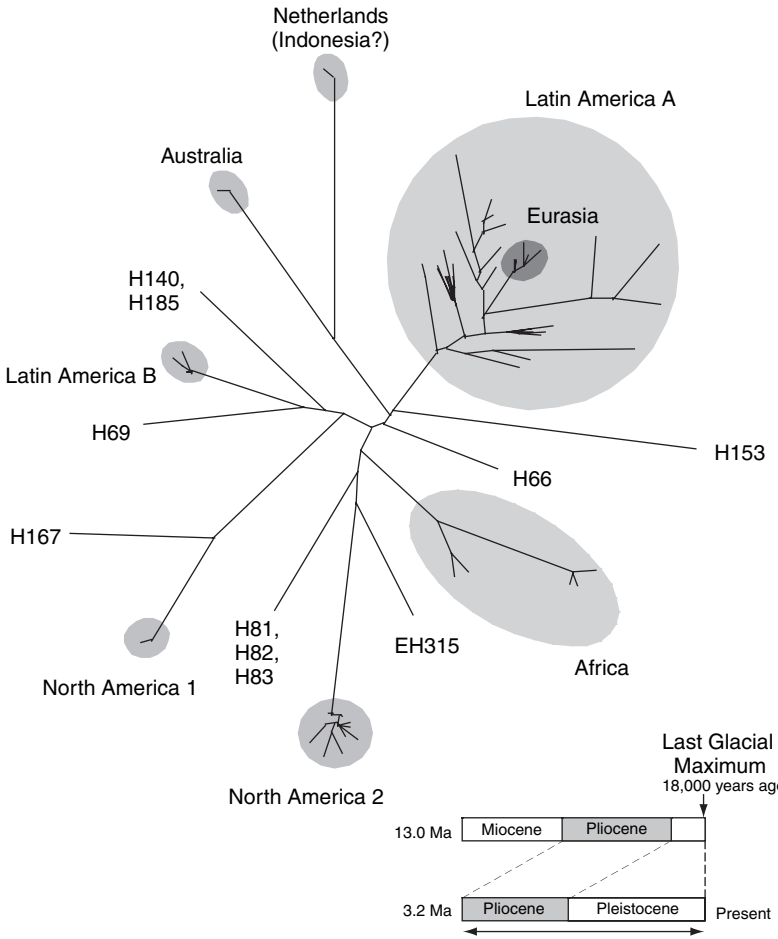

Fig. 15.1. Phylogenetic analysis of *Histoplasma capsulatum*, showing clades equivalent to cryptic species. The size of the clade is proportional to its genetic variation; the tropical clades, Latin America A and Africa, are the most diverse. This unrooted tree was made by neighbour-joining and shows a star phylogeny. The average genetic distance among distinct lineages (NAm 1, NAm 2, LAm A, LAm B, Australia + Indonesia, Africa, H81, and H153) was 2.8%. Half of this value corresponds to the time point of the radiation, which is 3.2 and 13.0 million years ago when DNA substitution rates of 4.3×10^{-9} and 1.1×10^{-9}, respectively, are used. Figure and legend from Kasuga *et al.* (2003) with the permission of the authors and publisher.

isolation occur every 3–10 MY, but divergences recognizable by morphology are found every 30–100 MY, very few genetically isolated clades persist to the point where they are morphologically distinct. The logical conclusion is that it must be far easier to form a new species than it is to maintain one. The third and most recent example concerns *C. posadasii*. A comparison of genotypes and genotypic diversity in populations from Arizona, Texas, Mexico and Latin America indicated that those from North American showed a significant positive correlation between genetic and geographic distances, as is typical of geographically stable species. However, when individuals from Latin America were included in the analysis, the correlation disappeared. This observation, and comparison of genotypic diversity in populations from North and South America, suggested that fungal individuals from Texas may have moved to Latin American as recently as 0.13 MYA, possibly in association with migrating humans (Fisher *et al.*, 2001).

Geologic time has also been useful in making hypotheses about the evolutionary history of *Histoplasma* species. The two tropical phylogenetic species of *Histoplasma*, the African clade and the Latin America clade A,

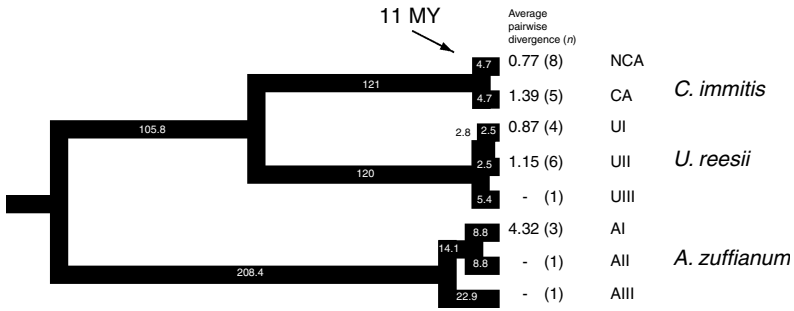

Fig. 15.2. Comparison of genetic distances among cryptic species and morphological species for *Coccidioides immitis, Auxarthron zuffianum* and *Uncinocarpus reesei*. If every cryptic species persisted, the tree ought to have far more branches and species. Estimates of the divergence among cryptic species (NCA and CA) in *Coccidioides immitis* is no greater than 11 MYA. Divergence between *Coccidioides* and *Uncinocarpus* is at least 20-fold greater than among their cryptic species. This maximum-parsimony tree is based on three genes with a 'molecular clock', enforced. Uncorrected average pairwise divergences within cryptic species are also shown for comparison. Both branch lengths and divergences within taxa are nucleotide substitions per 1000 sites; *n* is the number of isolates. Figure and legend adapted from Koufopanou *et al.* (2001) with the permission of the authors and publisher.

have a much greater diversity of genotypes than do the more temperate clades. One hypothesis that could account for the different genotypic diversities involves the climate cycles of ice age glaciations. By this hypothesis, the temperate clades would endure population bottlenecks associated with population expansions from equatorial refugia into newly available temperate habitat and contractions back to the refugia when temperate habitat chilled; tropical clades would escape these bottlenecks. By using the rate of nucleotide substitution determined for Ajellomycetaceae by Kasuga *et al.* (2002), the radiation of the seven clades was estimated to have occurred between 3.2 and 13 MYA, well before the most recent series of ice ages (Kasuga *et al.*, 2003). Therefore it seems likely that individuals in extant temperate clades were forced to migrate in north–south corridors during the ice ages, and plausible that temperate clades endured genetic bottlenecks that tropical species escaped, thereby accounting for differences in their diversity.

Curiously, the Eurasian *Histoplasma* clade emerges from the large, lowland tropical, Latin American A clade and is more distantly related to the geographically closer African clade (Fig. 15.1). If the Eurasian clade originated from a single Latin American A individual, then it must have existed for at least 1.7 million years (Kasuga *et al.*, 2003). If, however, the clade originated by the transportation to Europe of several genetically different Latin American individuals, the date of this event could have been much more recent. Given that the largest assemblage of clonal individuals from horses and other equids is found in the Eurasian clade, it is not completely ludicrous to speculate that the fungus was brought from Latin America to Eurasia by animals owned by the first European explorers of the New World, some 500 years ago. On the other hand, if a variety of genotypes were brought across the Atlantic Ocean on horses or donkeys, there is no evidence that they persisted on these animals.

A posteriori *correlation of phenotype and genotype*

Traditional morphological concepts of species often are proven wrong when phylogenetic data become available. However, once phylogenetic species are found, it becomes possible to find new phenotypes that correlate with the genetically isolated groups. A good example is provided by *Aspergillus flavus*, in which PSR with five DNA regions associated with nuclear genes found at least four species-level clades in what had been thought to be one morphological species (Geiser *et al.*, 1998b). As with *Histoplasma*, there proved to be a correlation with phylogenetic clades and geographic range in that the deepest divergence in the combined gene

genealogies was consistent with a division between individuals collected in the Northern or Southern Hemispheres (Tran-Dinh *et al*., 1999). Once the phylogenetic groups were identified, subsequent study of two phenotypes (sclerotium size and aflatoxin production) found unique combinations of these phenotypes for each clade (Geiser *et al*., 2000). The ability to find taxonomically useful phenotypes once phylogenetic species are identified can allow species to be both evolutionarily sound and practical in the sense of Harrington & Rizzo (1999). In fact, a very recent example from Harrington's laboratory emphasizes this point (Engelbrecht & Harrington, 2005). The morphological species *Ceratocystis fimbriata* was shown to harbour several genetically and reproductively isolated groups, i.e. several phylogenetic and biological species. With this knowledge in hand, for each group phenotypic differences were found, i.e. the preferred host (sweet potato, sycamore or cacao) and the size and shape of meiosporic and mitosporic reproductive structures and spores. New species were described for the groups associated with sycamore (*C. platani*) and cacao (*C. cacaofunesta*). Interestingly, *C. cacaofunesta* encompasses two genetically and reproductively isolated groups that show some morphological differences but are kept together because they both infest the same agricultural host and the morphological differences were deemed trivial. When more is known about the natural hosts and additional phenotypes of *C. cacaofunesta*, it seems likely that another species will be recognized.

Correlation of phylogenetic species with geographic range

We have discussed examples of phylogenetic species that are strongly correlated with geography in the medically important species of *Coccidioides* and *Histoplasma*, and in the agriculturally important fungus *A. flavus*. There are many other such examples from species of the genera *Fusarium* (O'Donnell *et al*., 1998), *Botrytis* (Giraud *et al*., 1999), *Sclerotinia* (Carbone & Kohn, 2001a, b; Kohn & Carbone, 2001; Phillips *et al*., 2002) and *Magnaporthe* (Couch & Kohn, 2002), as well as many examples from Basidiomycota (Vilgalys & Sun, 1994; Hibbett *et al*., 1995; Petersen & Hughes, 1999; Johannesson & Stenlid, 2003). However, a strong geographic correlation, i.e. endemism, is not always found in fungal phylogenetic species. In *Aspergillus fumigatus*, PSR with five regions flanking nuclear microsatellites found two global species; one had many more isolates than the other, but neither showed any hint of endemism (Pringle *et al*., 2005). This PSR study echoed a previous study that used DNA fingerprint data and a more limited sampling of isolates (Debeaupuis *et al*., 1997). In terms of its global geographic range,

A. fumigatus clearly stands in contrast to the other species examined to date. How this fungal species maintains a global geography is not yet understood.

Reproductive mode certainly plays a role in maintaining species. A strictly clonal species would have to experience periodic global sweeps of a single genotype to avoid fragmentation into endemic clades. In a recombining species, individuals with many different, recombined genotypes would have to be able to disperse throughout the globe and mate to avoid establishing endemic populations. As mentioned above, many socially important fungi, including species of *Aspergillus*, *Coccidioides* and *Fusarium*, are morphologically mitosporic and can reproduce clonally. Alas, morphology is not necessarily an accurate means of assessing reproductive mode. In many cases, analyses of association of alleles at the same loci used to recognize species has been used to reject the hypothesis of exclusively clonal reproduction for these fungi (Burt *et al*., 1996; Geiser *et al*., 1998b) and, therefore, indicate that genetic recombination also is occurring. *A. fumigatus* is among those fungi that are morphologically mitosporic but whose population genetics give evidence of recombination (Paoletti *et al*., 2005; Pringle *et al*., 2005). Very recent research with *A. fumigatus* has discovered individuals of both mating types (Pöggeler, 2002; Dyer *et al*., 2003), which are often in equal proportion at single geographic locations (Rydholm *et al*., 2004; Paoletti *et al*., 2005). This information, combined with the observation that many different genotypes of *A. fumigatus* can be found in the same geographic location (Pringle *et al*., 2005), suggests that *A. fumigatus* does not maintain its global species by worldwide sweeps of a clonal genotype. Instead, almost any individual of *A. fumigatus* must be capable of very long-distance travel. A combination of clonality and recombination does not, however, explain the difference between *A. fumigatus* and other fungi as regards endemism. For example, *Coccidioides*, *Histoplasma* and at least some *Fusarium* species are capable of both clonal and recombining reproduction, but lack the global reach of *A. fumigatus*. The explanation for the difference in biogeography between *A. fumigatus* and the other species undoubtedly lies in their ecology, again a subject in need of additional research.

Challenging phylogenetic species recognition with biological species recognition

PSR relies on genetic isolation for species identification, but much of the discussion on species recognition has focused on biological species

recognition (BSR) based on sexual compatibility within species and reproductive isolation between species. Much has been written about the relative merits of BSR and PSR but data comparing both methods in a single fungus were wanting. Unfortunately, in most cases where PSR had been used to recognize fungal species it was not been possible to compare PSR and BSR because the fungi were morphologically asexual or difficult to mate in the laboratory. Exceptions included the 'mating populations' of *Fusarium* species (O'Donnell *et al*., 1998), species of *Ceratocystis* (Baker *et al*., 2001, 2003; Engelbrecht & Harrington, 2005), and a number of Basidiomycota (Vilgalys & Sun, 1994; Petersen & Hughes, 1999; Burnett, 2003). Possibly the best fungus for a comparison of PSR and BSR is *Neurospora*, because it can complete its entire life cycle in cultivation, from meiospore to meiospore, in 14 days. This attribute caused it to be associated with elements of BSR from its description in 1927 (Shear & Dodge, 1927) and has made it the fungus most associated with BSR. Neurosporologists, principally Perkins and colleagues at Stanford University, have used mating testers to assign more than 6000 natural individuals to one of the five outbreeding (conidiating) species (*N. crassa, N. intermedia, N. sitophila, N. tetrasperma* and *N. discreta*) (Perkins & Turner, 1988; Turner *et al*., 2001). Knowing that these five outbreeding species formed a monophyletic clade with respect to homothallic species (Pöggeler, 1999; Dettman *et al*., 2001), PSR was applied to *c*. 150 individuals from all five outbreeding species, with the collection of individuals weighted toward what were thought to be the two most similar species, *N. crassa* and *N. intermedia*. With DNA sequences from four nuclear regions flanking microsatellites, and with three criteria for PSR (support from a majority of gene genealogies *or* significant support from one gene genealogy and no significant conflict from others *and* the rule that all individuals must belong to a species) eight species-level clades were defined, including three new phylogenetic species (Dettman *et al*., 2003a).

The parallel and independent BSR study (Dettman *et al*., 2003b), which employed almost half of the individuals in the PSR study, focused on *N. crassa, N. intermedia* and the nine individuals that were thought to be their hybrids. This collection included individuals that had previously been identified as *N. crassa, N. intermedia* or putative hybrids, but that proved to be members of the three new phylogenetic species. The many reciprocal matings (more than 1800 in all) were evaluated in a seven-level scale of reproductive success based on methods developed by Perkins and colleagues (Perkins & Turner, 1988; Turner *et al*., 2001). When crosses were arranged to consolidate matings with the highest success (production of at

least 50% viable ascospores), four biological species were recognized (Fig. 15.4), that is, all of those recognized by PSR, save one (Dettman *et al*., 2003b). The remarkable agreement of PSR and BSR (Fig. 15.3) further supports the use of PSR in the many fungi for which BSR is either impossible or prohibitively arduous.

Speciation processes and the maintenance of species

We already have noted that a multilocus phylogenetic analysis of *Coccidioides* and *Uncinocarpus* species suggested that far more species are initiated than persist. Newly diverged species can be lost by chance events (especially if they originate as a numerically small population) or because individuals compete poorly with individuals from genetically similar sympatric populations, or because the two novel species reticulate. Newly or recently diverged species may not be kept separate if hybridization and introgression facilitate enough gene flow to reverse or stall genetic differentiation. As noted above, PSR for *Neurospora* included nine natural isolates thought to be hybrids because each of the individuals did not mate well with testers for any of the original five outbreeding species. Comparison of multiple gene genealogies can identify individuals as hybrids because hybrids will fall into different clades in different gene genealogies. In *Neurospora*, by PSR, all putative hybrids were found to be members of not more than one of the eight phylogenetic species by comparison of the four sequenced regions. Most putative hybrids proved to be members of new phylogenetic species (Fig. 15.3), but two belonged to *N. crassa* and another to *N. intermedia* (Dettman *et al*., 2003b). The inability to recognize the true affinities of the putative hybrids when mating testers were available for only five species is easy to understand. We now know that they mate with other members of their species, and testers have been proposed for the new biological species. As regards hybridization, *Neurospora* stands in contrast to other genera associated with agriculture or forestry that are known to form hybrids, for example, *Fusarium* (O'Donnell *et al*., 2000, 2004), *Botrytis* (Nielsen & Yohalem, 2001), *Neotyphodium* (Schardl & Craven, 2003; Moon *et al*., 2004), *Ophiostoma* (Brasier *et al*., 1998; Konrad *et al*., 2002), *Melampsora* (Newcombe *et al*., 2001) or *Heterobasidion* (Garbelotto *et al*., 2004), and also to the clade of *Saccharomyces* yeasts whose ancestor arose from hybridization followed by allopolyploidy (Wolfe & Shields, 1997; Wong *et al*., 2002).

Surprisingly, BSR provides more information about hybrid matings than it does about conspecific matings, because most of the crosses are

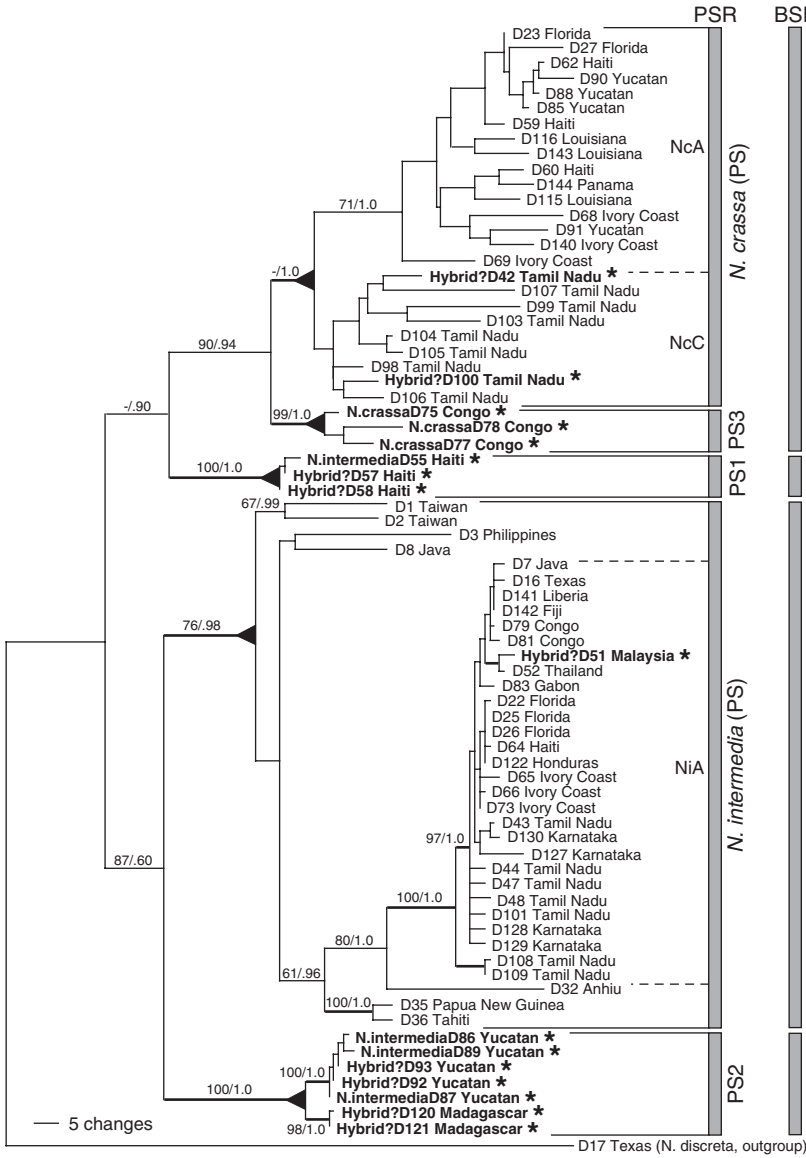

Fig. 15.3. Phylogenetic species recognition of *Neurospora* species and a graphic comparison to biological species recognized in the same species. Maximum parsimony (MP) phylogram produced from the combined analysis of DNA sequences from four anonymous nuclear loci (TMI, DMG, TML and QMA loci, a total of 2141 aligned nucleotides). Tree length = 916 steps. Consistency index = 0.651. Labels to the right of the phylogram indicate groups identified by phylogenetic species recognition and biological species recognition. Bold branches were concordantly

between parents from different species (Fig. 15.4). BSR focuses on the conspecific crosses that delimit species, but scrutiny of the hybrid or heterospecific crosses is informative about species maintenance. With *Neurospora*, there was a large variation in mating success (Fig. 15.4), from crosses that were almost as successful as conspecific matings (level 5, with more than 15% viable ascospores) to those at level 1 (abortion of incipient perithecial development) and those at level 0 (a complete absence of mating effort with no hint of perithecial development) (Dettman *et al*., 2003b). Remarkably, this variation was correlated with the geographic origin of mating partners: hybrid matings of sympatric *Neurospora* pairs were significantly less successful than those of allopatric pairs (Fig. 15.4). In other words, species are kept reproductively isolated either by geographic distance in allopatry or by genetic reinforcement of the species barrier in sympatry. Our inability to find natural hybrids implies that hybrid matings do not occur or do not produce successful progeny under natural conditions. The laboratory observation that hybrid matings produce fewer viable progeny than conspecific matings, coupled with the aforementioned inability to find natural hybrids, suggest that there is selection against hybridization. Individuals that can recognize and abort hybrid matings would be favoured, and this would select for the reinforcement of reproductive isolation in sympatry over geologic time scales. However, when allopatric individuals are brought together over human time scales their unreinforced reproductive isolation can be overcome, particularly in disturbed agricultural settings that may be permissive to the survival of hybrid progeny. This scenario may explain the conflicting

Fig. 15.3. (cont.)
supported by the majority of the loci, or were well supported by at least one locus but not contradicted by any other locus. Triangles at nodes indicate that all taxa united by (or distal to) a node belong to the same phylogenetic species. Taxon labels indicate strain number and geographic source. If a strain was originally identified by traditional mating tests to a species that did not match the phylogenetic or biological species identification, the original species name is listed before the strain number and is followed by an asterisk, all in bold type. If the original species identification matched both the phylogenetic and biological species identification, no name appears before the strain number. Branch support values for major branches with significant support are indicated by numbers above or below branches (MP bootstrap proportions/Bayesian posterior probabilities). Figure and legend from Dettman *et al*. (2003b) with permission of the authors and publisher.

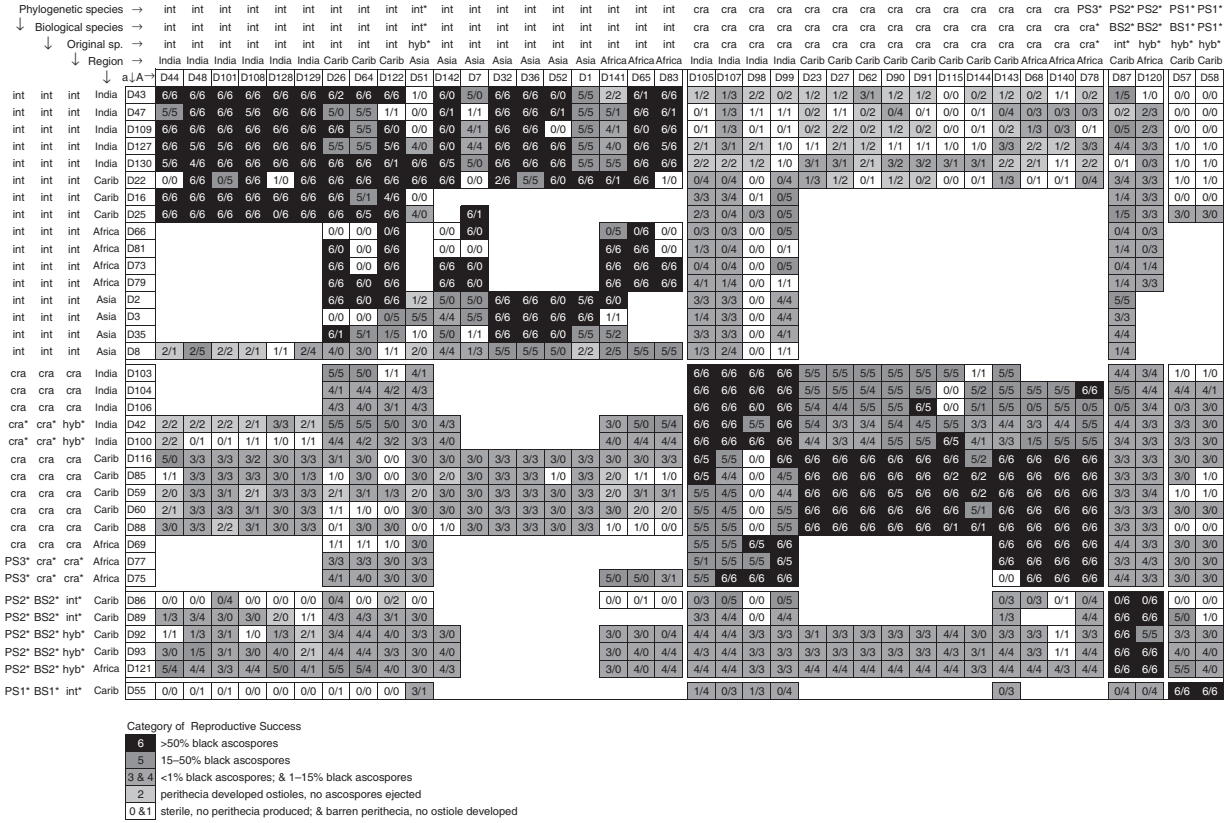

Fig. 15.4. Matrix displaying the reproductive success of 929 crosses (1858 matings) of outbreeding *Neurospora* species. Columns represent the 38 *mat A* strains and rows represent the 35 *mat a* strains, with strain numbers along the row and column headings of the matrix. Numbers within matrix cells indicate the reproductive success ratings (see inset) of the two reciprocal matings of the cross between the corresponding strains (*mat a* strain as the perithecial parent/*mat A* strain as the perithecial parent). For example, the top and leftmost cell of the matrix indicates that both matings of the cross between *mat a* strain D43 and *mat A* strain D44 received a rating of 6. The matrix cells have been shaded in proportion to the reproductive success of the best mating. A reproductive success rating of 6 was used to delineate biological species; and crosses satisfying that criterion have been filled black. Matrix cells without entries indicate that matings were not performed for that cross. Additional row and column headings indicate the phylogenetic species designation (see Fig. 15.3), biological species designation, original species designation and geographic source of the strains (int, *N. intermedia*; cra, *N. crassa*; PS, phylogenetic species; BS, biological species; hyb, possible hybrid between *N. crassa* and *N. intermedia*; Carib, Caribbean Basin). Asterisks indicate that the phylogenetic or biological species designation differed from the original species identification. Figure and legend from Dettman *et al.* (2003b) with permission of the authors and publisher.

observations of an absence of hybrids in *Neurospora* compared with the aforementioned cases of hybridization in agriculturally important fungi (Schardl & Craven, 2003).

Burnett has opined that the failure to produce hybrid progeny occurs late in the mating pathway of Ascomycota and early in the mating pathway in Basidiomycota (Burnett, 2003). In the ascomycete *Neurospora*, however, hybrid mating success varies across mated pairs and reproductive failure is not confined to the later stages (Dettman *et al.*, 2003b). In a comparison of hybrid or heterospecific mating success between allopatric and sympatric partners (Fig. 15.5) only 70% of both allopatric and sympatric heterospecific matings make any sort of perithecium, only 55% of allopatric and 25% of sympatric crosses proceeded to make well-developed perithecia, and only 48% of allopatric and 13% of sympatric matings produced any ascospores. Although it is true that mating success is indistinguishable between allopatric and sympatric heterospecific partners in the very earliest stages of mating, it is not true that the barriers to

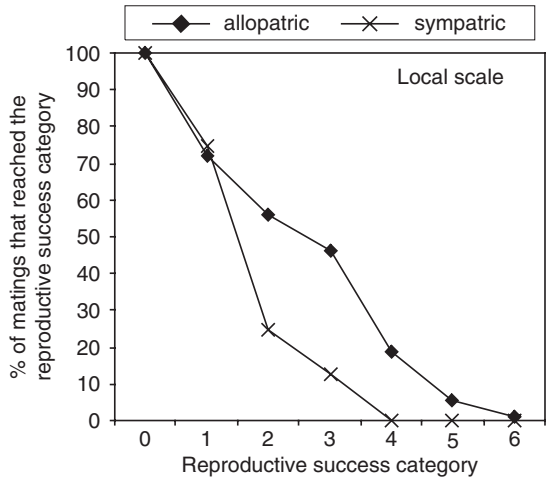

Fig. 15.5. Mating success for sympatric and allopatric heterospecific pairings: the percentage of heterospecific matings between allopatric or sympatric individuals that reached the successive categories of reproductive success. At all geographic scales (regional, sub-regional and local), allopatric matings were significantly more likely than sympatric matings to proceed through the consecutive stages of the sexual cycle. The discrepancy between the allopatric and sympatric curves increases as the scale of sympatry decreases and is most pronounced for local sympatry, shown here. Figure and legend modified from Dettman *et al.* (2003b) with permission of the authors and publisher.

success are all late in the process. The application of BSR in Basidiomycota typically assesses mating success only to the point where clamp connections are formed, for example as reported for the recent studies of the *Hebeloma crustuliniforme* complex (Aanen & Kuyper, 1999) and those of other mushrooms (Petersen & Hughes, 1999). There may also be significant reductions in mating success subsequent to the production of clamp connections; additional studies should be made with this fungus or another where the complete reproductive cycle could be assessed. *Schizophyllum commune*, in which population structure has recently been studied by enzyme electrophoresis (James *et al*., 1999) and which completes the life cycle from meiospore to meiospore in cultivation, would seem to be an ideal candidate.

Selection

There is a debate about whether sympatric species need to exploit different niches to avoid competition for exactly the same resources and rapid extinction of one of the pair (Coyne & Orr, 2004). However, there is little debate that selection, along with drift, contributes to the genetic differentiation that supports the persistence of newly diverged species. Even when selection cannot act directly on reproductive isolation barriers (as in allopatry), selection can drive genetic divergence that results in genomic incompatibilities between new species, and also drive phenotypic differentiation that renders hybrids with intermediate phenotypes less fit than either parent.

The molecular signatures of directional and purifying selection, that is, the excess or deficit, respectively, of nucleotide substitutions causing amino acid replacements (non-synonymous substitutions, Ka) compared to those that do not (synonymous substitutions, Ks), are typically detected by comparing within- and between-species polymorphisms (McDonald & Kreitman, 1991). To pursue these analyses, *a priori* one must be able to place individuals in phylogenetic species. With knowledge of PSR, the effects of selection can be studied for any gene by using this and other population genetic approaches initially developed from studies of flies or mammals (McDonald & Kreitman, 1991; Yang & Bielawski, 2000; Yang & Nielsen, 2002). The first such studies in fungi focused on genes that, curiously enough, may be little affected by speciation, those genes responsible for mating recognition and regulation in basidiomycetes (May *et al*., 1999). This is not to say that mating genes evolve slowly (Pöggeler, 1999; Swanson & Vacquier, 2002) or that specificity is not involved in recognizing potential mates (Casselton & Olesnicky, 1998). However, fungi

transformed with the mating type allele of a close relative can mate with conspecific partners (Sharon *et al.*, 1996; Turgeon, 1998), suggesting that mating loci are not involved with reproductive isolation. May and colleagues (May *et al.*, 1999) showed that the diversity of mating alleles present early in the evolutionary history of a group of *Coprinus* species has been maintained through speciation events. These studies were followed by research on more genes with transspecies polymorphisms, i.e., vegetative self- and non-self-recognition genes in Ascomycota (Leslie, 1993). With these heterokaryon incompatibility loci, Glass and colleagues showed that more than two alleles are maintained in populations at a single locus by balancing selection (Wu *et al.*, 1998). Most recently, and still with genes that maintain diversity through speciation events, O'Donnell and colleagues found in a very thorough study of PSR in *Fusarium graminearum*, *sensu lato*, that clusters of genes responsible for synthesis of secondary products (trichothecene toxins) produced gene genealogies in strong conflict with those for six other regions (O'Donnell *et al.*, 2000). Subsequent analysis showed that individuals of a single phylogenetic species were capable of making one of three different trichothecenes found in the species because each individual possessed genes for only one of the three toxins (Ward *et al.*, 2002). Again, the maintenance of intraspecific diversity through speciation events could be explained by balancing or frequency-dependent selection (O'Donnell *et al.*, 2004).

Most genes, however, are not under balancing or frequency-dependent selection and their variation is very much related to the species in which they are found. Recently, selection was studied in genes from *Coccidioides* species that produce proteins that have been shown to elicit an immune response in mice. In the first of these studies, Johannesson and colleagues found that a fungal gene product that stimulates a protective acquired immune response in mice (that is, a good vaccine candidate) was under strong positive selection in contrast to housekeeping genes (Johannesson *et al.*, 2004). However, the selection did not correlate with pathogenicity or virulence because it could not be demonstrated to be stronger in *Coccidioides* species than in *Uncinocarpus* species, their close, non-pathogenic relatives (Johannesson *et al.*, 2004). In the second study, a *Coccidioides* gene known to produce a protein that is a poor vaccine candidate (because it elicits a non-protective immunological response from the host) was found to have different numbers of repeats that influence the host immune response. The evolution of the repeated regions was shown by phylogenetic analysis to be concerted (Johannesson *et al.*, 2005), as with ribosomal repeat units (Dover, 1982); there is a possibility

that the diversity of repeat number is maintained in this species by a conflict between greater numbers of repeats promoting virulence and lesser numbers favouring the function of the protein as an adhesion factor (Hung *et al*., 2002). The existing studies of selection for individual genes, and individual nucleotide positions therein, are merely a prelude to the coming wave of whole-genome examination of selection and of accelerated nucleotide substitution by comparative genomics (Clark *et al*., 2003; Cliften *et al*., 2003; Kellis *et al*., 2003). Here, the many genomes sequenced for socially important fungi should prove extremely valuable. However, knowing which genes are under strong selection without understanding how the gene products relate to the natural history or ecology of the fungus will be less than satisfying and will leave us short of the goal of understanding ecologically relevant adaptation at the genetic level. Studies of adaptation also will require a thorough understanding of how to measure the fitness of filamentous fungi (Pringle & Taylor, 2002).

We have attempted to contribute to the discussion on identifying fungal species and to the discussion of the evolutionary processes whose study depends on accurate species recognition: species formation and persistence, species maintenance by geographic distance in allopatry and by reinforcement in sympatry, and natural selection. Like Brasier, we have left discussion of the genetic systems of mating compatibility to others (Glass & Kuldau, 1992; Fraser *et al*., 2004; Fraser & Heitman, 2004). Most of our comments address the pattern of speciation, but the new challenge facing mycology is to better understand the processes of speciation and adaptation. Rapid speciation certainly can be achieved by a change in the mating system, for example, from homothallism to heterothallism or back (Geiser *et al*., 1998a; Turgeon, 1998; Yun *et al*., 1999; Lee *et al*., 2003). Likewise, hybridization events, sometimes associated with polyploidy, as in yeast (Wong *et al*., 2002; Dujon *et al*., 2004), *Botrytis* (Nielsen & Yohalem, 2001) or the asexual *Epichloë* relatives in the genus *Neotyphodium* elegantly unravelled by Schardl and colleagues (Moon *et al*., 2004) can produce instant speciation. Mycologists also accord species status to clonal lineages that probably emerge from sexual species (Summerbell, 2002; Kaszubiak *et al*., 2004) and that are probably evolutionary dead ends (LoBuglio *et al*., 1993), although the argument has been made that such lineages are better considered as individuals (Janzen, 1977). However, less well studied is the more common speciation process, whereby geographic distance, or adaptation to heterogenous environments in the same geographic location [which might have to very small to be considered sympatric for a fungus (Le Gac & Giraud, 2004)], contribute to a diminution of gene flow between populations of a

single species and lead to speciation. We hope that studies of 'normal' speciation, of the adaptation of newly diverged species to different microhabitats, and of reinforcement of the species barrier in sympatry, will become the subject of sustained study, at least in species for which phylogenetic species have been recognized.

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