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REPRODUCTIVE ISOLATION AND PHYLOGENETIC DIVERGENCE IN *NEUROSPORA*: COMPARING METHODS OF SPECIES RECOGNITION IN A MODEL EUKARYOTE

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Abstract.—We critically examined methods for recognizing species in the model filamentous fungal genus *Neurospora* by comparing traditional biological species recognition (BSR) with more comprehensive applications of both BSR and phylogenetic species recognition (PSR). Comprehensive BSR was applied to a set of 73 individuals by performing extensive crossing experiments and delineating biological species based on patterns of reproductive success. Within what were originally considered two species, *N. crassa* and *N. intermedia*, we recognized four reproductively isolated biological species. In a concurrent study (Dettman et al. 2003), we used genealogical concordance of four independent nuclear loci to recognize phylogenetic species in *Neurospora*. Overall, the groups of individuals identified as species were similar whether recognized by reproductive success or by phylogenetic criteria, and increased genetic distance between parents was associated with decreased reproductive success of crosses, suggesting that PSR using genealogical concordance can be used to reliably recognize species in organisms that are not candidates for BSR. In one case, two phylogenetic species were recognized as a single biological species, indicating that significant phylogenetic divergence preceded the development of reproductive isolation. However, multiple biological species were never recognized as a single phylogenetic species. Each of the putative *N. crassa* × *N. intermedia* hybrids included in this study was confidently assigned to a single species, using both PSR and BSR. As such, no evidence for a history of hybridization in nature among *Neurospora* species was observed. By performing reciprocal mating tests, we found that mating type, parental role, and species identity of parental individuals could all influence the reproductive success of matings. We also observed sympatry-associated sexual dysfunction in interspecific crosses, which was consistent with the existence of reinforcement mechanisms.

Key words.—Biological species, genealogical concordance, hybridization, phylogenetic species, reinforcement, reproductive success, species concepts.

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Species concepts and the criteria to recognize species are much discussed and controversial topics. These topics have taken on additional importance since the recent discovery that species defined by phenotypic characters (morphological species) or reproductive isolation (biological species) commonly harbor multiple genetically differentiated clades that qualify as phylogenetic species. The fact that different methods of species recognition may have different empirical consequences has been demonstrated in several groups of organisms (e.g., invertebrates: Hilton and Hey 1997; Gleason et al. 1998; van Oppen et al. 2000; angiosperms: Young 1998), and quite commonly in fungi (e.g., Vilgalys and Sun 1994; Hibbett et al. 1995; Aanen et al. 2000; Taylor et al. 2000; Harrington et al. 2002).

Morphological species recognition (MSR) is the dominant method of species recognition because it is an integral element of the description of every species, and can be applied to most eukaryotic organisms. If sexual reproduction can be assessed, biological species recognition (BSR) using mating tests may be used to designate reproductively isolated biological species sensu Mayr (1942). Biological species recognition has been readily accepted because it is based on sexual compatibility, which is clearly related to species cohesion and divergence. However, the relationship between mating behavior in the laboratory and the potential to interbreed in nature often is unclear, and sexual activity has not been observed in nature or the laboratory for approximately 20% of the fungal kingdom (Hawksworth et al. 1995). Phylogenetic species recognition (PSR) using genealogical concordance (Avise and Ball 1990; Baum and Shaw 1995; Taylor et al. 2000) can be applied to all organisms, even those that are asexual or uncultivatable, and is challenging MSR and BSR as the method of choice, especially among mycologists (Koufopanou et al. 1997; Geiser et al. 1998; Kasuga et al. 1999; O'Donnell et al. 2000a,b; Kroken and Taylor 2001; Cruse et al. 2002). However, a thorough comparison of PSR and BSR has not been performed.

We aimed to critically examine methods of recognizing species in the model filamentous fungal genus *Neurospora* (Sordariales, Ascomycota) by comparing the traditional BSR methods with more comprehensive applications of both BSR and PSR (Dettman et al. 2003). We chose to compare BSR and PSR in *Neurospora* because it is the fungus in which BSR has been most thoroughly applied (Perkins et al. 1976; Perkins and Turner 1988; Turner et al. 2001), and because its sexual cycle is easily manipulated and well characterized, due to *N. crassa*'s long history as model organism (Davis 2000; Davis and Perkins 2002). When Shear and Dodge (1927) first described *Neurospora* species, they had conducted mating tests to support their morphological species descriptions. This work preceded the formulation of the biological species concept by Dobzhansky (1937) and Mayr (1942). Today, all newly collected individuals are assigned to a biological species by mating tests with one or two pairs of tester strains from each known species. In this paper, we refer to this method as traditional biological species recog-

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TABLE 1. The design of the crossing experiments showing the numbers of intra- and interspecific crosses grouped as within or between geographic regions. The original species designations of individuals and the broad geographic regions, as defined in the text, were used in categorizing the crosses. For the purpose of hypothesis testing, the individuals originally identified as possible hybrids between *Neurospora crassa* and *N. intermedia* (Turner et al. 2001) were considered a separate group.

	N. crassa × *N. crassa*	*N. intermedia* × *N. intermedia*	Hybrids × hybrids	*N. crassa* × *N. intermedia*	*N. crassa* × hybrids	*N. intermedia* × hybrids	Total
Within region	61	96	5	54	40	47	303
Between regions	80	160	15	217	79	75	626
Total	141	256	20	271	119	122	929

nition (traditional BSR). Most individuals will form 50–90% mature progeny in crosses with one, and only one, of the species-specific testers. However, some irregularities in *Neurospora* BSR have been noted. In some cases, regional testers are needed to accommodate the variation found in a single geographically widespread biological species (Perkins and Turner 1988; Turner et al. 2001), suggesting possible local adaptation or cryptic speciation. In addition, individuals assigned to one species may, in some cases, be partially fertile with individuals from another species (Turner et al. 2001), a promiscuity noted by Shear and Dodge (1927) in their original description. In fact, *N. intermedia* was given its epithet because it was ''intermediate'' in both morphology and mating behavior between *N. crassa* and *N. sitophila* (Tai 1935). Although the majority of progeny produced in most *N. crassa* × *N. intermedia* crosses are aborted, in some crosses up to 10% of progeny are viable hybrids. Conversely, some individuals do not mate well with any of the testers (< 0.3% of those collected worldwide), and have been described as possible hybrids between *N. crassa* and *N. intermedia* (Turner et al. 2001). Earlier phylogenetic studies suggested that *N. crassa* and *N. intermedia* were not reciprocally monophyletic, but formed a species complex (Natvig et al. 1987; Taylor and Natvig 1989; Skupski et al. 1997). The phylogenetic data available at the time we began our study, the significant proportion of hybrid progeny seen in laboratory matings, and the putative hybrids collected from nature, raised the possibility of interspecific hybridization among natural populations of *N. crassa* and *N. intermedia*.

Here we apply comprehensive BSR to a set of 73 individuals that have been identified by traditional BSR as *N. crassa*, *N. intermedia*, or putative *N. crassa*/*N. intermedia* hybrids. We crossed these individuals, not just with testers but also amongst each other, and delineated biological species based on patterns of reproductive success. Because comprehensive BSR and PSR (Dettman et al. 2003) were independently implemented in parallel, we could examine the correspondence between these two methods of species recognition. We asked the following questions: Were the groups of individuals identified as species by phylogenetic or reproductive criteria equivalent, or were there discrepancies between the two methods? Did one method provide greater resolution than the other? Did genetic distance between parents, a measure for PSR, predict the reproductive success of crosses, a measure for BSR? Was there evidence for a history of hybridization in nature among *N. crassa* and *N. intermedia* individuals? Finally, we investigated how the reproductive success of crosses was influenced by other factors, such as the mating type, parental role, species identity, geographic separation, and sympatry or allopatry of parental individuals.

MATERIALS AND METHODS

Selection of Individuals and Geographic Sources

For this study, we chose almost half of the 147 individuals analyzed in the concurrent PSR study (Dettman et al. 2003): 73 individuals were selected, including 64 from *N. crassa* and *N. intermedia* and nine putative hybrids (according to the original species designations; see Appendix). These individuals were chosen prior to the application and results of PSR to maintain the independence of species recognition by the two approaches. Our collection included individuals from four well-separated geographic regions based on known distributions of *N. crassa* and *N. intermedia* (Turner et al. 2001): India, the Caribbean Basin, Africa, and East Asia (including the Pacific Islands). The entire collection of 147 individuals has been deposited into the Fungal Genetics Stock Center (FGSC, Department of Microbiology, University of Kansas Medical Center, Kansas City, KS). Approximate latitude and longitude coordinates of collection sites were obtained from online resources of the Getty Research Institute Thesaurus of Geographic Names (http://www.getty.edu/research/tools/vocabulary/tgn/index.html) and the Global Gazetteer (http://www.calle.com/world/). A website provided by the U.S. Department of Agriculture Agricultural Research Service (http://www.wcrl.ars.usda.gov/cec/java/lat-long.htm) was used to obtain surface distances in kilometers between collection sites.

Experimental Crossing Design and Mating of Strains

The experimental design included a subsample (929) of the possible crosses (1330) between the 73 selected strains (Table 1), covering a wide range of intraspecific and interspecific combinations. To provide a baseline for successful mating, all possible crosses involving the same species from the same geographic region were performed. To investigate isolation by geographic distance within a species, crosses between individuals from different regions were performed. Most of the possible crosses involving putative hybrids were performed. Interspecific crosses between individuals from the same geographic region were limited to India and the Caribbean Basin, whereas interspecific crosses between individuals from different geographic regions were performed for five of the six possible combinations of two regions, that is, all except Africa × East Asia.

Sexual reproduction in outbreeding *Neurospora* occurs be-

TABLE 2. The categories used to rate reproductive success of matings and delineate biological species within *Neurospora*. If a mating was rated as category 6, the two parents were deemed members of the same biological species.

Category	Description
0	sterile, no perithecia produced
1	barren perithecia, no ostiole developed
2	perithecia developed ostioles, no ascospores ejected
3	<1% of ejected ascospores were black
4	1–15% of ejected ascospores were black
5	15–50% of ejected ascospores were black
6	>50% of ejected ascospores were black

tween two haploid individuals of opposite mating type (*mat A* or *mat a*). Each individual is hermaphroditic and self-sterile, producing ''male'' fertilizing spores (conidia) and ''female'' receptive protoperithecia. Fertilized hyphae proliferate within the maturing protoperithecium, which develops into a fruiting body, or perithecium. Apical segments of the fertilized hyphae, asci, are the sites of karyogamy and meiosis, which is followed by one mitotic division, yielding eight haploid ascospores per ascus. Ascospores are forcibly ejected from asci and exit the perithecium through the open ostiole. Matings were performed as previously described (Perkins 1986; Jacobson 1995; Turner et al. 2001). For every cross, each haploid parental *mat A* and *mat a* strain was inoculated into a separate 13 × 100 mm-test tube that contained 2.5 ml of synthetic crossing medium (Westergaard and Mitchell 1947) with 1% sucrose. Strains were incubated for 4 days at 25°C to allow for the development of receptive protoperithecia. Fertilization was performed by transferring mitotic spores (conidia) from the *mat A* strain to the *mat a* strain, and vice versa, to produce two reciprocal matings per cross. After an additional 10–14 days of incubation, the reproductive success of each mating was evaluated and scored on a scale with seven rating categories (Table 2).

To summarize mating behavior among groups of individuals, we devised a reproductive isolation index, or RII = 1 − (RS_B / RS_W), where RS_B is the average reproductive success of between-group matings, and RS_W is the weighted average of reproductive success of within-group matings (RS_W = [RS_{W1} + RS_{W2}] / 2). The RII was calculated using results from both reciprocal matings, and values could range from 0 (no reproductive isolation) to 1 (complete reproductive isolation). Neighbor joining (Saitou and Nei 1987) was used to display the RII matrix as a phenogram (PAUP, ver. 4.0b8a; Swofford 2001).

Sequence Data and Phylogenetic Species Recognition

In our companion study (Dettman et al. 2003), PSR using genealogical concordance of four anonymous unlinked nuclear loci was performed on 147 *Neurospora* individuals. Briefly, a clade was recognized as an independent evolutionary lineage if it satisfied either of two criteria: (1) Genealogical concordance: the clade was present in the majority (3/4) of the single-locus genealogies, as revealed by a majority-rule consensus tree. (2) Genealogical nondiscordance: the clade was well supported in at least one single-locus genealogy, as judged by both maximum parsimony (MP) bootstrap proportions (Hillis and Bull 1993) and Bayesian posterior probabilities (Rannala and Yang 1996), and was not contradicted in any other single-locus genealogy at the same level of support. To identify such clades, a tree possessing only branches that received MP bootstrap proportions ≥70% and Bayesian posterior probabilities ≥0.95 was chosen to represent each of the four loci, then a semistrict consensus tree (combinable component) was produced from these four trees. This criterion prohibited poorly supported nonmonophyly at one locus from undermining well-supported monophyly at another locus. When deciding which independent evolutionary lineages represented phylogenetic species, two additional ranking criteria were applied: (1) Genetic differentiation: to prevent minor tip clades from being recognized, phylogenetic species had to be relatively distinct and well differentiated from other species. (2) Exhaustive subdivision: all individuals had to be placed within a phylogenetic species, and no individuals were to be left unclassified.

To determine the phylogenetic relationships among individuals, the four-locus combined dataset (2141 aligned nucleotides, TREEBASE accessions S950 and M1574) was pruned to contain only the 73 individuals used in the crossing experiments, and one individual of *N. discreta* (D17, FGSC8777) to represent the outgroup. Insertions/deletions (indels) were treated as missing data unless they were consistently alignable across all taxa, in which case they were recoded as single phylogenetically informative characters for MP analyses. Flat-weighted MP analyses and Kimura two-parameter genetic distance (Kimura 1980) calculations were performed using PAUP. One hundred replications of random stepwise addition MP heuristic searches (nearest-neighbor interchange [NNI] branch-swapping, maximum of 10,000 trees retained [maxtrees]) were performed, and the shortest resulting trees were subjected to further tree bisection-reconnection branch swapping (maxtrees = 5000). Of the multiple MP trees produced, the tree with the greatest likelihood was chosen for display (as determined using maximum likelihood with mean parameter values estimated from the data in Bayesian analyses). Branch support was assessed by MP bootstrapping (2000 replications, simple stepwise addition, NNI branch-swapping, maxtrees = 1000) and Bayesian posterior probabilities. Bayesian analyses were performed using MrBayes (ver. 3.0; Huelsenbeck and Ronquist 2001) with the following parameters free to vary: six substitution rates, four base frequencies, proportion of invariable sites, and alpha value of gamma distribution. Two independent runs were performed, each with four incrementally heated Markov chains run simultaneously, and samples were taken every 100th generation for 500,000 generations. Likelihood values were plotted against generation number and all samples taken

prior to ''burn-in'' were discarded. Samples taken after the stationary phase had been reached in each run (3561 and 2501 samples) were used to determine the posterior probability distributions, and the posterior probabilities of branches reflect the mean of the two runs.

Statistical Analyses

We examined the relationship between PSR and BSR by asking what effect genetic distance had on reproductive success. The measure of reproductive success was the only dependent variable, and was considered continuous because it was simply a code for recording the underlying continuum (Sokal and Rohlf 1995). The Kimura two-parameter genetic distance between parents (hereafter called genetic distance) served as a measure of phylogenetic divergence, and was an independent variable. Other independent variables included geographic distance in kilometers between parental collection sites, and categorical dummy variables used to identify the strains that participated in each cross. These dummy variables allowed strain identities to be used as covariates, thus controlling for the nonindependence of the data and strain-specific effects. The influence on reproductive success of genetic distance, geographic distance, their interaction, and strain-specific effects was evaluated using analyses of covariance (ANCOVA). Although some strains had significant effects in the various classes of crosses examined (all crosses, 23 of 73 strains; *N. crassa* × *N. crassa*, 3 of 25; *N. intermedia* × *N. intermedia*, 6 of 35; *N. crassa* × *N. intermedia*, 8 of 60), they were not considered further because no meaningful patterns were apparent among the strain effects. Reproductive success, and genetic and geographic distances were $\log_{10}$-transformed to improve normality, and *F*-statistic calculations were based on type III sums of squares.

We investigated the differences between sympatric and allopatric interspecific crosses by comparing both genetic distance between parents and reproductive success of matings using Mann-Whitney *U* tests. The proportions of allopatric and sympatric interspecific matings successfully progressing through each stage of sexual development were compared with Fisher exact tests (computed at http://www.matforsk.no/ola/fisher.htm). Log-rank tests (Peto and Peto 1972) were used to determine whether interspecific allopatric matings were different from interspecific sympatric matings in the overall progression through the sexual cycle. Chi-square tests with Yates continuity correction for bias in tests of two categories (Zar 1984) were used to compare the frequency of perithecial superiority of *mat a* versus *mat A* strains. We investigated the differences between matings in which perithecial parents were of different mating type, and, in the case of interspecific matings, different species, by comparing reproductive success using Mann-Whitney *U* and Wilcoxon paired sample tests. Statistical analyses were performed using PROC GLM of SAS, version 6.12, or JMPin, version 3.2.6, software (SAS Institute 1996, 1999, respectively).

RESULTS

Biological Species Recognition

Perithecial development and ascospore production were evaluated and reproductive success was rated for 929 (70%) of the 1330 possible crosses among the 38 *mat A* and 35 *mat a Neurospora* strains (Table 1). Matings were scored on a scale with seven rating categories (Table 2) that represent natural stages in reproductive development (Jacobson 1995). Two ratings of reproductive success were available for each cross because reciprocal matings were performed with each strain as the perithecial parent. Figure 1 displays the crossing matrix and reproductive success for all 1858 matings.

To group individuals with the most similar reproductive behavior, the rows and columns of the crossing matrix were arranged such that the most successful crosses were clustered along the diagonal (Fig. 1). The ratings for reciprocal matings were not necessarily equal, and unless otherwise noted, the most successful of the two reciprocal matings was chosen to represent the cross in statistical analyses because it better described reproductive potential, typically the criterion used for BSR sensu Mayr. Using only the higher rating compensated for any strain-specific reproductive deficiencies, such as low female fertility, that could mask the higher potential for successful reproduction revealed in the reciprocal mating.

When two individuals from the same *Neurospora* species are mated, typically over 50% of the ejected ascospores are black, that is, mature progeny (Turner et al. 2001). Previous studies of the reproductive behavior of *Neurospora* (Perkins et al. 1976; Perkins and Turner 1988; Jacobson 1995; Turner et al. 2001) noted a natural gap in the continuum of percent ascospore maturation, which marked the difference between intraspecific and interspecific matings. This gap was confirmed by our study, so the criterion of >50% black ascospores, which is equivalent to our reproductive success category 6 (Table 2), appeared to be the proper threshold for conspecificity.

We used the qualitatively distinct category 6 to delimit mutually exclusive, reproductively isolated groups, that is, biological species. Because losing the ability to complete a complex sexual cycle is significantly easier than gaining that same ability, we did not require that all individuals within a biological species achieve category 6 matings with all other individuals in the same biological species. Instead, our main guideline was that an individual from one biological species could not achieve a category 6 mating with an individual from a different biological species.

The four biological species recognized by our comprehensive BSR method corresponded well with previous species assignments based on traditional BSR (Appendix, Fig. 1). Sixty-three of the 73 strains fell into two species that corresponded to *N. intermedia* (35) and *N. crassa* (28). All of the strains in these two species had been identified as *N. intermedia* or *N. crassa* by traditional BSR, except three strains that had been described as a putative hybrids. The remaining ten strains formed two additional species, Biological Species 1 (BS1) and 2 (BS2). BS1 and BS2 were composed of three and seven strains, respectively, all of which had been identified as *N. intermedia* or putative hybrids by traditional BSR. Thus, within what was originally considered two species, *N. crassa* and *N. intermedia*, our comprehensive application of BSR methods identified four biological species.

Nine *N. crassa* individuals from Tamil Nadu, India, formed a subgroup that generally had greater reproductive success

when crossed amongst themselves than when crossed with other *N. crassa* individuals (Fig. 1). However, these individuals were reproductively compatible (category 6) with some other *N. crassa* individuals, so we did not consider them a distinct biological species.

Based on the data from multiple matings, all nine putative hybrid strains could be unequivocally assigned to one, and only one, biological species, providing no evidence of true hybrid individuals. Six putative hybrids strains (D57, D58, D92, D93, D120, D121) were assigned to BS1 or BS2, and the remaining three putative hybrids (D51, D42, D100) were assigned to *N. intermedia* or *N. crassa*.

Different crosses involving the same strain clearly displayed different levels of reproductive success, and some strains were more variable than others (Fig. 1). Even when restricted to intraspecific crosses, ratings that ranged from complete sterility (category 0) through full fertility and fecundity (category 6) were observed for some strains. One strain, D8, did not achieve a rating of 6 in any of its crosses, perhaps due to an abnormal growth phenotype (see Dettman et al. 2003) that reduced its capacity to mate. In an effort to assign all strains to a biological species, D8 was included in *N. intermedia* because it mated best with members of this group, a placement confirmed later by independent phylogenetic analysis (Dettman et al. 2003). Another unusual strain, D86, proved to be consistently sterile when acting as the perithecial parent, but was competent when functioning as the fertilizing parent. All other strains were competent as the perithecial parent in at least some of their crosses. Reproductive success was clearly influenced by the interaction of both parental genotypes, that is, one parent was not solely responsible for the observed phenotype.

Ascospores that have matured and developed black pigmentation have the possibility to germinate and form new haploid individuals, whereas ascospores that have aborted before becoming pigmented are inviable and cannot germinate. To assess the viability of black ascospores, we isolated 100 of them from each of 41 intraspecific and 6 interspecific matings and subjected them to standard germination-inducing conditions (Perkins 1986). A mean of 55.7% (SE = 2.3) and 18.3% (SE = 1.5) of the black ascospores from intraspecific and interspecific matings, respectively, germinated to form sustainable growing colonies. Therefore, black ascospores from interspecific matings (categories 4 and 5) had significantly lower viability than those from intraspecific matings (category 6; Mann-Whitney U test, $z = -3.80$, $P < 0.0001$).

Phylogenetic Species Recognition

The phylogenetic species designations of all 73 individuals were taken directly from Dettman et al. (2003), which should be consulted for additional information. Briefly, PSR using genealogical concordance of four anonymous unlinked nuclear loci was performed, and a phylogenetic species was a well-supported monophyletic group that was concordantly supported by the majority of the loci, or was well supported by at least one locus but not significantly contradicted by any other locus.

Five of the eight phylogenetic species delineated by Dettman et al. (2003) were present in our sample of 73 strains. These species were *N. intermedia* (35 strains, most from the NiA subgroup), *N. crassa* (25 strains from the NcA and NcC subgroups), Phylogenetic Species 1 (PS1; three strains), Phylogenetic Species 2 (PS2; seven strains), and Phylogenetic Species 3 (PS3; three strains). Thus, within what were originally considered two species, *N. crassa* and *N. intermedia*, PSR identified five phylogenetic species.

The phylogram in Figure 2 displays the phylogenetic relationships among the crossing strains based on combined DNA sequence data from the four unlinked nuclear loci. The overall topology of the tree was congruent with the trees constructed from the larger collection of 147 strains (Dettman et al. 2003).

Congruence of Phylogenetic and Biological Species Recognition

When our phylogenetic species designations were superimposed upon the crossing matrix (Fig. 1), or our biological species designations were mapped onto the phylogenetic tree (Fig. 2), the congruence of BSR and PSR results was apparent. Overall, strains fell into very similar groups whether recognized by reproductive success or phylogenetic criteria, indicating that both methods were reliable ways to recognize species of *Neurospora*. Three biological species, *N. intermedia*, BS1, and BS2, corresponded exactly with the phylogenetic species *N. intermedia*, PS1, and PS2, respectively. The one case in which BSR and PSR did not correspond concerned the two phylogenetic species, *N. crassa* and PS3, which collectively constituted just one biological species, *N. crassa*. The three PS3 individuals were the only ones assigned to different species by the two recognition methods, and this inconsistency represented a difference in resolution between PSR and BSR rather than direct conflict.

To summarize levels of reproductive isolation, we calculated the reproductive isolation index (RII) among the six main phylogenetic groups (*N. intermedia*, NcA, NcC, PS1, PS2, and PS3). The neighbor-joining tree (Fig. 3) tree constructed from the RII matrix was topologically similar to the phylogram constructed from the sequence data (Fig. 2), except for the branching order of NcC and PS3. Overall, the relative levels of reproductive isolation among groups were consistent with the relative levels of phylogenetic divergence.

Very little reproductive isolation was evident between NcA and PS3, as indicated by the short branch length in Figure 3. However, PS3 formed a distinct phylogenetic species that was separate from, though sister to, phylogenetic species *N. crassa* (Fig. 2). This discrepancy indicated that significant phylogenetic divergence can precede the development of reproductive isolation in *Neurospora*.

The NcC subgroup of *N. crassa* was equivalent to the partially reproductively isolated subgroup of nine *N. crassa* individuals from Tamil Nadu, India (Fig. 1). Although NcC was phylogenetically distinguishable from NcA and PS3 (Fig. 2), it was not recognized as a distinct phylogenetic species because its monophyly did not receive significant support in any of the four single-locus genealogies. However, there was a general reduction in reproductive success between NcC and NcA, and to a lesser extent, between NcC and PS3 (Fig. 3). NcC may be an incipient species with incomplete and pos-

Phylogenetic species →				int	int	int	int	int	int	int	int	int	int*	int	int	int	int	int	int	int	int
↓ Biological species →				int	int	int	int	int	int	int	int	int	int*	int	int	int	int	int	int	int	int
↓ Original sp. →				int	int	int	int	int	int	int	int	int	hyb*	int	int	int	int	int	int	int	int
↓ Region →				India	India	India	India	India	India	Carib	Carib	Carib	Asia	Asia	Asia	Asia	Asia	Asia	Asia	Asia	Africa
↓ a|A→				D44	D48	D101	D108	D128	D129	D26	D64	D122	D51	D142	D7	D32	D36	D52	D1	D141	
int	int	int	India	D43	6/6	6/6	6/6	6/6	6/6	6/6	6/2	6/6	6/6	1/0	6/0	5/0	6/6	6/6	6/0	5/5	2/2
int	int	int	India	D47	5/5	6/6	6/6	5/6	6/6	6/6	5/0	5/5	1/1	0/0	6/1	1/1	6/6	6/6	6/1	5/5	5/1
int	int	int	India	D109	6/6	6/6	6/6	6/6	6/6	6/6	6/6	5/5	6/0	0/0	6/0	4/1	6/6	6/6	0/0	5/5	4/1
int	int	int	India	D127	6/6	5/6	5/6	6/6	6/6	6/6	5/5	5/5	5/6	4/0	6/0	4/4	6/6	6/6	6/6	5/5	4/0
int	int	int	India	D130	5/6	4/6	6/6	6/6	6/6	6/6	6/6	6/6	6/1	6/6	6/5	5/0	6/6	6/6	6/6	5/5	5/5
int	int	int	Carib	D22	0/0	6/6	0/5	6/6	1/0	6/6	6/6	6/6	6/6	6/6	6/6	0/0	2/6	5/5	6/0	6/6	6/1
int	int	int	Carib	D16	6/6	6/6	6/6	6/6	6/6	6/6	6/6	5/1	4/6	0/0							
int	int	int	Carib	D25	6/6	6/6	6/6	6/6	0/6	6/6	6/6	6/5	6/6	4/0		6/1					
int	int	int	Africa	D66							0/0	0/0	0/6		0/0	6/0					0/5
int	int	int	Africa	D81							6/0	0/0	6/6		0/0	0/0					6/6
int	int	int	Africa	D73							6/6	0/0	6/6		6/6	6/0					6/6
int	int	int	Africa	D79							6/6	6/0	6/6		6/6	6/0					6/6
int	int	int	Asia	D2							6/6	6/0	6/6	1/2	5/0	5/0	6/6	6/6	6/0	5/6	6/0
int	int	int	Asia	D3							0/0	0/0	0/5	5/5	4/4	5/5	6/6	6/6	6/6	6/6	1/1
int	int	int	Asia	D35							6/1	5/1	1/5	1/0	5/0	1/1	6/6	6/6	6/0	5/5	5/2
int	int	int	Asia	D8	2/1	2/5	2/2	2/1	1/1	2/4	4/0	3/0	1/1	2/0	4/4	1/3	5/5	5/5	5/0	2/2	2/5
cra	cra	cra	India	D103							5/5	5/0	1/1	4/1							
cra	cra	cra	India	D104							4/1	4/4	4/2	4/3							
cra	cra	cra	India	D106							4/3	4/0	3/1	4/3							
cra*	cra*	hyb*	India	D42	2/2	2/2	2/2	2/1	3/3	2/1	5/5	5/5	5/0	3/0	4/3						3/0
cra*	cra*	hyb*	India	D100	2/2	0/1	0/1	1/1	1/0	1/1	4/4	4/2	3/2	3/3	4/0						4/0
cra	cra	cra	Carib	D116	5/0	3/3	3/3	3/2	3/0	3/3	3/1	3/0	0/0	3/0	3/0	3/0	3/3	3/3	3/0	3/3	3/0
cra	cra	cra	Carib	D85	1/1	3/3	3/3	3/3	3/0	1/3	1/0	3/0	0/0	3/0	2/0	3/0	3/3	3/3	1/0	3/3	2/0
cra	cra	cra	Carib	D59	2/0	3/3	3/1	2/1	3/3	3/3	2/1	3/1	1/3	2/0	3/0	3/0	3/3	3/3	3/0	3/3	2/0
cra	cra	cra	Carib	D60	2/1	3/3	3/3	3/1	3/0	3/3	1/1	1/0	0/0	3/0	3/0	3/0	3/3	3/3	3/0	3/3	3/0
cra	cra	cra	Carib	D88	3/0	3/3	2/2	3/1	3/0	3/3	0/1	3/0	3/0	0/0	1/0	3/0	3/3	3/3	3/0	3/3	1/0
cra	cra	cra	Africa	D69							1/1	1/1	1/0	3/0							
PS3*	cra*	cra*	Africa	D77							3/3	3/3	3/0	3/3							
PS3*	cra*	cra*	Africa	D75							4/1	4/0	3/0	3/0							5/0
PS2*	BS2*	int*	Carib	D86	0/0	0/0	0/4	0/0	0/0	0/0	0/4	0/0	0/2	0/0							0/0
PS2*	BS2*	int*	Carib	D89	1/3	3/4	3/0	3/0	2/0	1/1	4/3	4/3	3/1	3/0							
PS2*	BS2*	hyb*	Carib	D92	1/1	1/3	3/1	1/0	1/3	2/1	3/4	4/4	4/0	3/3	3/0						3/0
PS2*	BS2*	hyb*	Carib	D93	3/0	1/5	3/1	3/0	4/0	2/1	4/4	4/4	3/3	3/0	4/0						3/0
PS2*	BS2*	hyb*	Africa	D121	5/4	4/4	3/3	4/4	5/0	4/1	5/5	5/4	4/0	3/0	4/3						3/0
PS1*	BS1*	int*	Carib	D55	0/0	0/1	0/1	0/0	0/0	0/0	0/1	0/0	0/0	3/1							

Category of Reproductive Success	
6	>50% black ascospores
5	15-50% black ascospores
3 & 4	<1% black ascospores; & 1-15% black ascospores
2	perithecia developed ostioles, no ascospores ejected
0 &1	sterile, no perithecia produced; & barren perithecia, no ostiole developed

FIG. 1. Matrix displaying the reproductive success of 929 crosses (1858 matings). 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 (Table 2) 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 matings were not performed for that cross. Additional row and column headings indicate the phylogenetic species designation (see Fig. 2), 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.

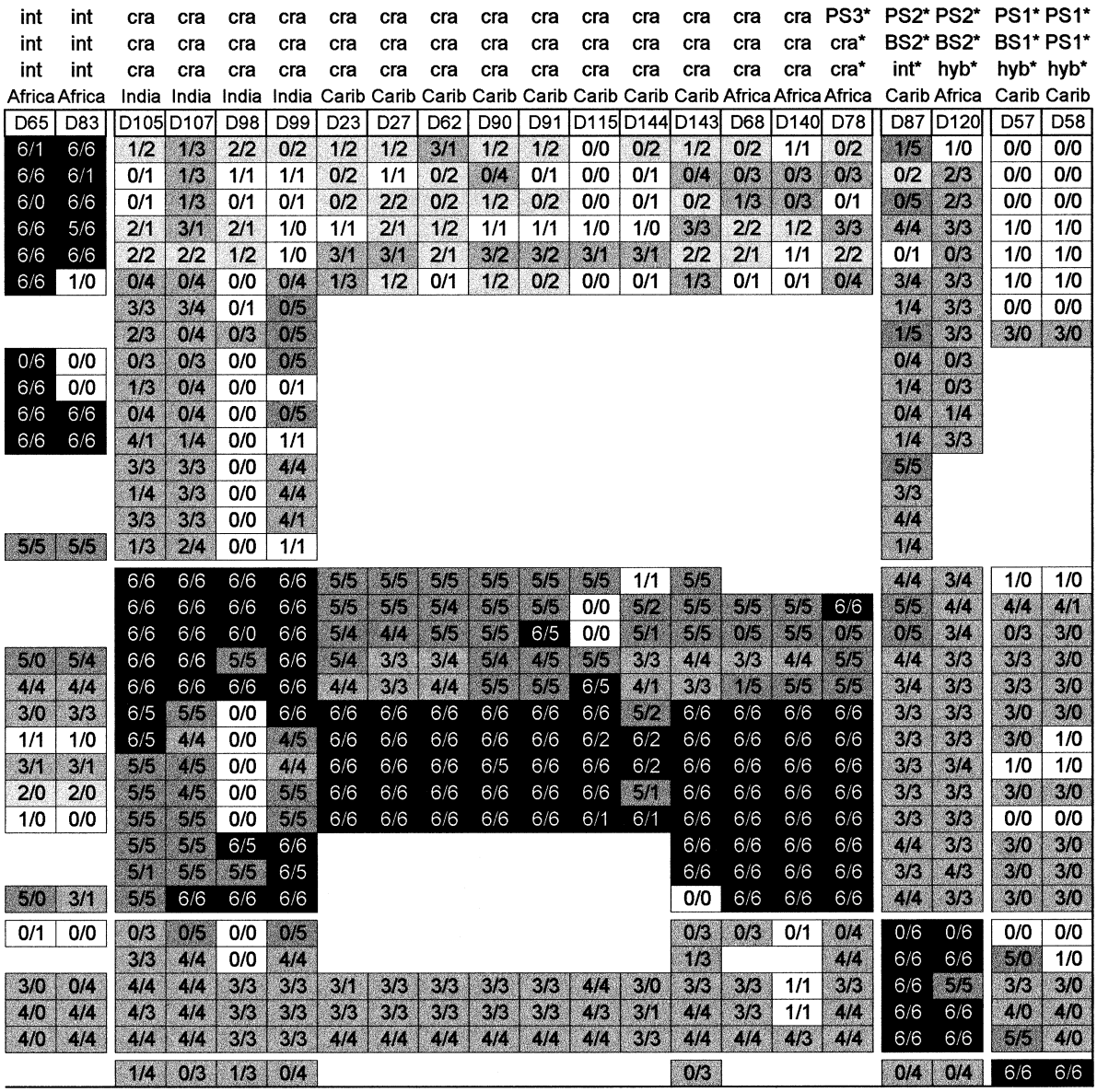
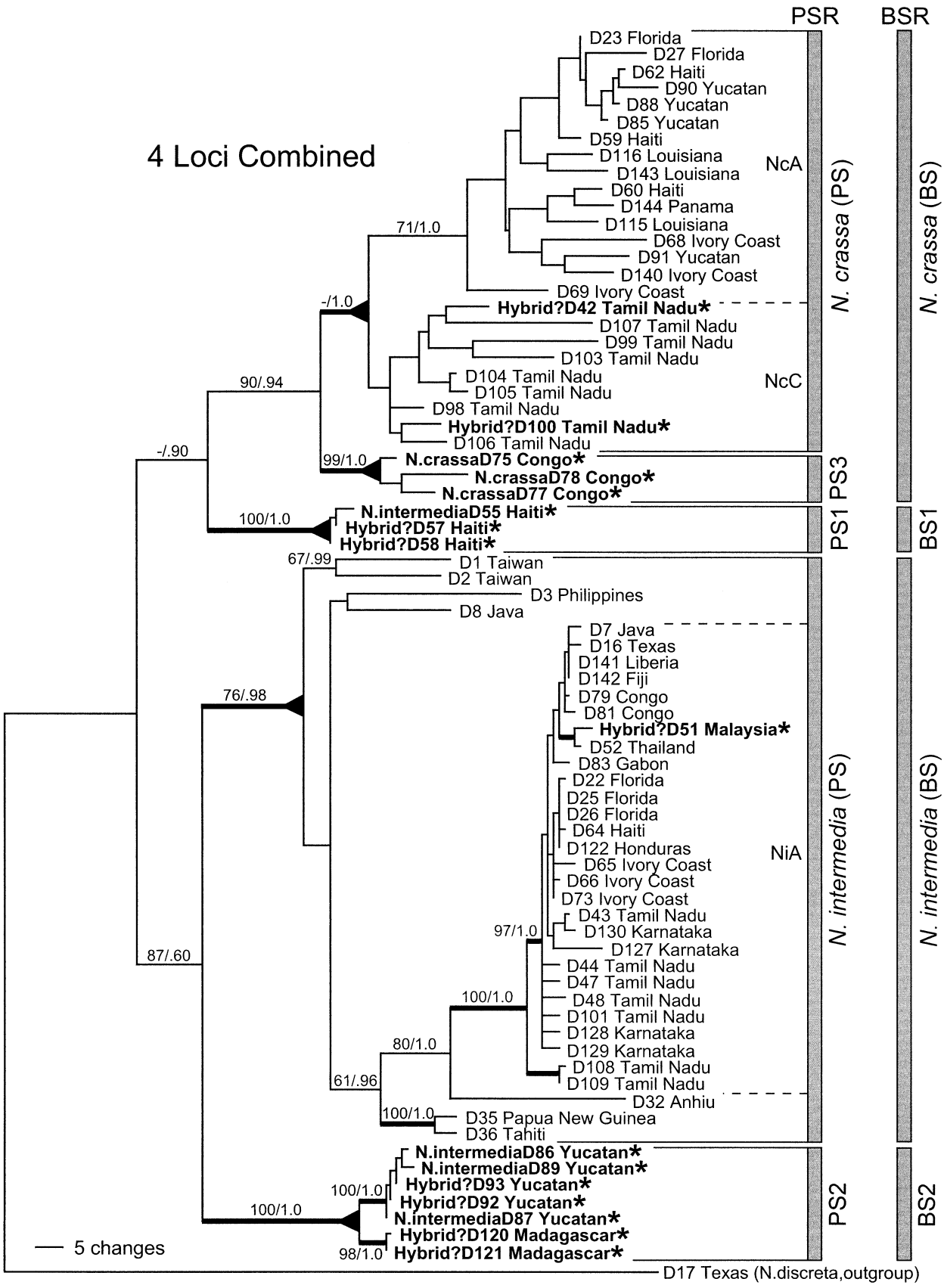


FIG. 1. Continued.

sibly ongoing phylogenetic divergence and reproductive isolation from NcA and PS3.

Statistical Comparison of Measures Used for Phylogenetic and Biological Species Recognition

To go beyond visual comparison of PSR and BSR, we used statistics to examine the relationship between genetic distance, a measure for PSR, and reproductive success, a measure for BSR. The combined influence on reproductive success of genetic distance, geographic distance, their interaction, and strain effects was modeled by ANCOVA for all 73 strains and 929 crosses (Table 3). Together, these factors explained 47% of the variation in reproductive success ($R^2 = 0.47$, $P < 0.0001$). The individual components of genetic and geographic distance also had significant effects on reproductive success ($P < 0.0001$ and $P < 0.0007$, respectively, Table 3), that is, reproductive success decreased as genetic or geographic distance increased. The significant relationship between genetic distance and reproductive success reflected the fact that intraspecific crosses typically had low genetic distance and high reproductive success, whereas interspecific crosses had high genetic distance and low reproductive success. Similarly, the fact that strains from the same species tended to be sampled from the same location may explain why geographic distance was also a significant predictor of reproductive success. The interaction between genetic and geographic distance was significant as well ($P < 0.0007$). A factor that likely contributed to this interaction was that both intra- and interspecific crosses were included in these analyses, and genetic and geographic distance could have different influences on each of these cross types (see below). The overall correspondence between genetic distance and re-


PSR
BSR
4 Loci Combined
D23 Florida
D27 Florida
D62 Haiti
D90 Yucatan
D88 Yucatan
D85 Yucatan
D59 Haiti
D116 Louisiana
D143 Louisiana
NcA
D60 Haiti
D144 Panama
D115 Louisiana
D68 Ivory Coast
D91 Yucatan
D140 Ivory Coast
D69 Ivory Coast
Hybrid?D42 Tamil Nadu*
D107 Tamil Nadu
D99 Tamil Nadu
D103 Tamil Nadu
D104 Tamil Nadu
D105 Tamil Nadu
NcC
D98 Tamil Nadu
Hybrid?D100 Tamil Nadu*
D106 Tamil Nadu
N.crassaD75 Congo*
N.crassaD78 Congo*
N.crassaD77 Congo*
N.intermediaD55 Haiti*
Hybrid?D57 Haiti*
Hybrid?D58 Haiti*
D1 Taiwan
D2 Taiwan
D3 Philippines
D8 Java
D7 Java
D16 Texas
D141 Liberia
D142 Fiji
D79 Congo
D81 Congo
Hybrid?D51 Malaysia*
D52 Thailand
D83 Gabon
D22 Florida
D25 Florida
D26 Florida
D64 Haiti
D122 Honduras
NiA
D65 Ivory Coast
D66 Ivory Coast
D73 Ivory Coast
D43 Tamil Nadu
D130 Karnataka
D127 Karnataka
D44 Tamil Nadu
D47 Tamil Nadu
D48 Tamil Nadu
D101 Tamil Nadu
D128 Karnataka
D129 Karnataka
D108 Tamil Nadu
D109 Tamil Nadu
D32 Anhiu
D35 Papua New Guinea
D36 Tahiti
N.intermediaD86 Yucatan*
N.intermediaD89 Yucatan*
Hybrid?D93 Yucatan*
Hybrid?D92 Yucatan*
N.intermediaD87 Yucatan*
Hybrid?D120 Madagascar*
Hybrid?D121 Madagascar*
D17 Texas (N.discreta,outgroup)
71/1.0
-/1.0
90/.94
-/.90
99/1.0
100/1.0
67/.99
76/.98
87/.60
97/1.0
100/1.0
80/1.0
61/.96
100/1.0
100/1.0
100/1.0
98/1.0
5 changes
N. crassa (PS)
N. crassa (BS)
PS1 PS3
BS1
N. intermedia (PS)
N. intermedia (BS)
PS2
BS2

productive success (Fig. 4) illustrated why PSR and BSR could be expected to identify similar species. The most salient conclusion was that increased phylogenetic divergence, the measure used for PSR, predicted increased reproductive isolation, the measure used for BSR.

Having performed the global analysis of all crosses together, we separated the intraspecific and interspecific crosses to explore how genetic and geographic distance influenced reproductive success in each cross type. We restricted analyses to those crosses involving strains from phylogenetic species *N. crassa* and *N. intermedia*, which had sufficient sample sizes (25 and 35 strains, respectively). The combined variables explained 47% and 37% of the variation in reproductive success of intraspecific *N. crassa* and *N. intermedia* crosses (both $P < 0.0001$, Table 3). For *N. crassa* × *N. crassa* crosses, genetic distance between parental strains had a significant effect ($P < 0.04$), that is, increased genetic distance alone predicted decreased reproductive success. This result reflected the correspondence between genetic distance and reproductive isolation for the incipiently speciating NcA and NcC subgroups, because when restricted to within-NcA or within-NcC comparisons, the relationship was not significant. For *N. intermedia* × *N. intermedia* crosses, neither genetic nor geographic distance had a significant effect on reproductive success.

To investigate the effect of genetic and geographic distance on reproductive success of interspecific crosses, we analyzed the 263 *N. crassa* × *N. intermedia* crosses (Table 3). The combined variables accounted for 55% of the variation in reproductive success of interspecific crosses ($P < 0.0001$), but in contrast to the intraspecific results, geographic distance had a significant effect ($P < 0.04$). Interestingly, the positive slope (0.18) of this relationship showed that reproductive success in interspecific crosses increased as geographic distance increased.

Sympatric and Allopatric Effects on Interspecific Matings

Analysis of covariance suggested that interspecific crosses involving sympatric strains would have lower reproductive success than those involving allopatric strains, so we tested whether any form of sympatry-associated sexual dysfunction was evident in *Neurospora*. Since neither the scale of local variation nor the geographic dimensions of breeding populations of *Neurospora* spp. are known, we investigated the effects of sympatry at three different geographic scales: regional, subregional, and local. Sympatry at the regional scale was defined as strains isolated from the same large geographic region: India, the Caribbean Basin, Africa, and East Asia.

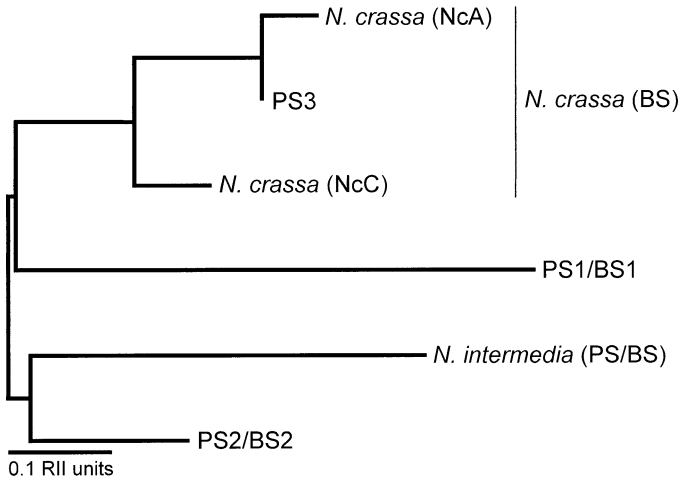


FIG. 3. Neighbor-joining tree constructed from the reproductive isolation indices (RII; see Materials and Methods). Branch lengths are proportional to RII; a short branch indicates that little reproductive isolation was observed between the two groups of individuals. For the sake of comparison, the RII tree is rooted in the same fashion as the phylogram in Figure 2.

Subregional sympatry was defined as strains isolated from the same state or country within a region, and sympatry at the local scale was defined as strains isolated from the same collection site (see Appendix). Unlike the previous analyses, in which only the most successful mating was included to represent reproductive potential, data from both reciprocal matings were used to address the full range of variation in interspecific reproductive success among all five phylogenetic species. To allow for more direct comparison, we included only those allopatric crosses involving strains that also were involved in sympatric crosses at the relevant geographic scale.

The reproductive success of sympatric matings was significantly lower than that of allopatric matings at the subregional and regional geographic scales ($P < 0.015$ and <0.029, respectively), and nearly significantly lower at the local scale ($P < 0.053$, Table 4). The mean reproductive success of sympatric matings decreased with the scale of sympatry, so that the lowest mean reproductive success occurred between strains sympatric at the local scale. Genetic distance between strains in sympatric crosses was not significantly lower than that in allopatric crosses at any geographic scale (Table 4), so differences in genetic distance played no role in the reduction of reproductive success in sympatry.

←

FIG. 2. Maximum parsimony (MP) phylogram produced from the combined analysis of DNA sequences from four anonymous nuclear loci (TMI, DMG, TML, and QMA loci, total of 2141 aligned nucleotides; see Dettman et al. 2003 for details). 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 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) it 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 face. 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).

TABLE 3. Analysis of covariance for the influence of genetic distance and geographic distance between individuals on the reproductive success of crosses within and between *Neurospora* species. Significant probability values are shown in bold.

Cross type	Source	df	*F*-ratio	*P*	R^2
All crosses ($N = 929$)					
	Whole model	73	10.30	<**0.0001**	0.47
	Genetic distance	1	67.99	<**0.0001**	
	Geographic distance	1	11.69	<**0.0007**	
	Genetic × geographic distance	1	11.49	<**0.0007**	
	Error	855			
N. crassa × *N. crassa* ($N = 145$)					
	Whole model	26	3.98	<**0.0001**	0.47
	Genetic distance	1	4.40	<**0.04**	
	Geographic distance	1	1.02	<0.3	
	Genetic × geographic distance	1	0.75	<0.4	
	Error	118			
N. intermedia × *N. intermedia* ($N = 219$)					
	Whole model	36	3.02	<**0.0001**	0.37
	Genetic distance	1	0.27	<0.6	
	Geographic distance	1	1.51	<0.2	
	Genetic × geographic distance	1	0.25	<0.6	
	Error	182			
N. crassa × *N. intermedia* ($N = 263$)					
	Whole model	60	4.16	<**0.0001**	0.55
	Genetic distance	1	2.67	<0.1	
	Geographic distance	1	4.31	<**0.04**	
	Genetic × geographic distance	1	3.06	<0.08	
	Error	202			

Sexual reproduction can fail at any of several sequential stages in the sexual cycle (Table 2). We examined whether sympatric reproductive isolation was preferentially associated with particular types of developmental defects by comparing the proportions of allopatric and sympatric matings that arrested at the different categories of reproductive success (Table 5). For interspecific matings, at all three geographic scales, sympatric matings were significantly more likely than allopatric matings to arrest in category 1, that is, more likely to produce incompletely developed, barren perithecia. Sympatric matings were more likely than allopatric matings to arrest in categories 2 and 3 as well, but the dif-

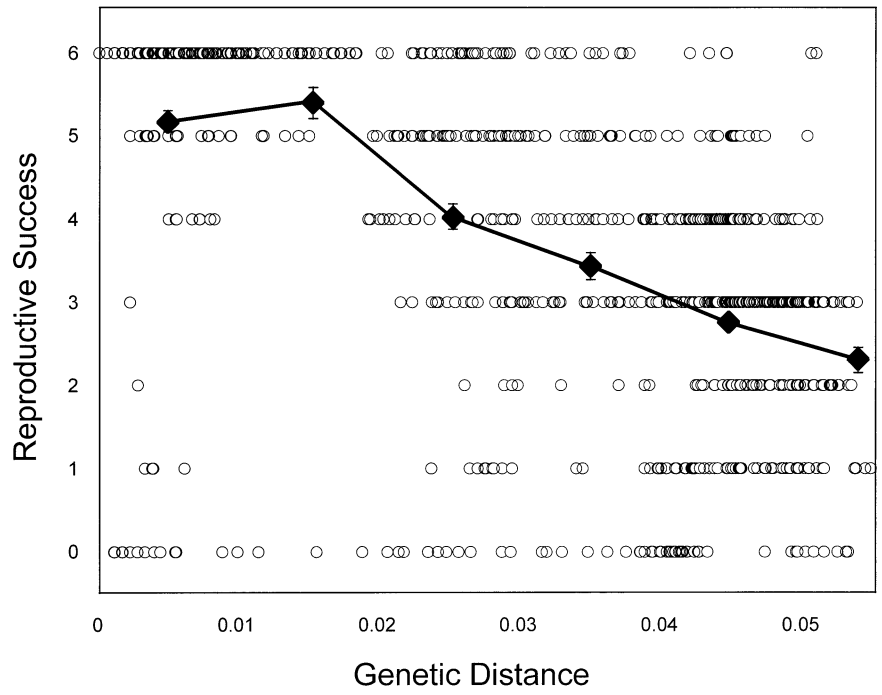


FIG. 4. Reproductive success plotted against genetic distance between individuals for all 929 crosses. Open circles represent the best of two reciprocal matings for each cross. Crosses were binned into categories for a range of genetic distance (i.e., 0—0.01, 0.01—0.02, etc. . .), and closed diamonds indicate mean reproductive success (vertical bars are ± standard error) of each genetic distance category.

TABLE 4. Summary of reproductive success, genetic distance, and geographic distance between individuals for interspecific crosses among all five phylogenetic species. Crosses were classified as sympatric or allopatric at three geographic scales (see Results). Genetic distance or reproductive success between sympatric and allopatric categories was compared using Mann-Whitney *U* tests. The reproductive success of both reciprocal matings from each cross was included. Significant probability values are shown in bold.

Geographic scale	Sympatric	Local allopatric	Statistic	Sympatric	Subregional allopatric	Statistic	Sympatric	Regional allopatric	Statistic
Range of distances between collection sites, km (median)	0–0 (0)	17–18,669 (9758)		0–383 (104)	322–18,669 (12,531)		0–6251 (1100)	2368–18,669 (15,112)	
Number of crosses	8	225		35	407		143	405	
Genetic distance between strains									
Mean (SE)	0.0399 (0.0029)	0.0413 (0.0005)		0.0425 (0.0011)	0.0426 (0.0004)		0.0423 (0.0006)	0.0427 (0.0004)	
z			−0.77			−0.76			−0.98
P			0.4			0.5			0.3
Reproductive success of matings									
Mean (SE)	1.13 (0.24)	1.99 (0.08)		1.54 (0.13)	2.08 (0.06)		1.93 (0.10)	2.18 (0.06)	
z			−1.93			−2.44			−2.18
P			0.053			**0.015**			**0.029**

ferences were significant only at some geographic scales (Table 5). No locally or subregionally sympatric matings progressed past category 3, but many allopatric matings did. As displayed in Figure 5, the discrepancy between allopatric and sympatric matings became more evident as the geographic scale of sympatry decreased. At all geographic scales, allopatric matings were significantly more likely than sympatric matings to proceed through the consecutive stages of the sexual cycle (regional, $P = 0.021$; subregional, $P < 0.0001$; local, $P = 0.007$; Fig. 5). Overall, interspecific matings between sympatric individuals were more likely to experience reproductive defects in the earlier stages of sexual development and less likely to achieve ascospore maturation than were matings between allopatric individuals.

Neurospora intermedia has a broad geographic range and has been collected from all four regions sampled in this study. The range of *N. crassa* overlaps with that of *N. intermedia* except that *N. crassa* has not been found in East Asia (Turner et al. 2001). Using the same tests described above, we found that the reproductive success of *N. crassa* × East Asian *N. intermedia* matings was significantly higher than that of *N. crassa* × non-East Asian *N. intermedia* matings (mean reproductive success = 2.13 and 1.66, respectively; $z = 3.28$, $P = 0.001$). In addition, *N. crassa* × non-East Asian *N. intermedia* matings were more likely than *N. crassa* × East Asian *N. intermedia* matings to arrest in reproductive success categories 1 and 2 (both $P < 0.0001$). In general, *N. crassa* strains mated better with *N. intermedia* strains that were collected from regions where the two species do not coexist.

Asymmetrical Reproductive Success between Reciprocal Matings

Two reciprocal matings were performed for each cross because outbreeding *Neurospora* individuals are hermaphroditic. In 419 of the total 929 crosses (45%), the two reciprocal matings achieved different categories of reproductive success, that is, asymmetrical reproductive success. In 261 (28%) of the total crosses, one mating produced at least some progeny (categories 3–6), whereas the reciprocal mating produced none (categories 0–2). For intraspecific crosses, asymmetrical reproductive success was more common in *N. intermedia* than in *N. crassa* (34% and 20%, respectively, Table 6). Interspecific *N. crassa* × *N. intermedia* crosses were more than twice as likely as intraspecific crosses to exhibit asymmetrical reproductive success.

In asymmetrical crosses, the strain that was the perithecial parent in the more successful mating was said to exhibit ''perithecial superiority.'' In both intraspecific and interspecific crosses, *mat a* strains displayed perithecial superiority more frequently than *mat A* strains (significant for intraspecific comparisons only, both $P < 0.01$; Table 6). Furthermore, in both intra- and interspecific crosses, the reproductive success of matings with *mat a* perithecial parents was significantly higher than that of matings with *mat A* perithecial parents (all $P < 0.022$; Table 6).

To test the hypothesis that both mating type and species identity influenced the reproductive success of interspecific crosses, all *N. crassa* × *N. intermedia* matings were divided into four classes based upon the mating type and species

TABLE 5. Numbers of interspecific matings that arrested at or proceeded through the consecutive reproductive success categories. Matings were classified as sympatric or allopatric, at three geographic scales, and were compared using Fisher exact tests. Significant probability values are shown in bold. na, comparison not applicable.

Category of reproductive success	Geographic scale	Allopatric matings		Sympatric matings		
		Arrested	Proceeded	Arrested	Proceeded	*P*
0 (sterile, no perithecia produced)						
	local	127	323	4	12	0.70
	subregional	234	580	14	56	0.96
	regional	217	593	78	208	0.47
1 (barren perithecia, no ostiole developed)						
	local	71	252	8	4	**0.002**
	subregional	119	461	23	33	**0.0007**
	regional	114	479	57	151	**0.010**
2 (perithecia developed ostioles, no ascospores ejected)						
	local	44	208	2	2	0.15
	subregional	53	408	14	19	**0.00002**
	regional	54	425	26	125	**0.04**
3 (<1% of ejected ascospores were black)						
	local	125	83	2	0	0.37
	subregional	233	175	19	0	**0.00003**
	regional	240	185	80	45	0.08
4 (1–15% of ejected ascospores were black)						
	local	58	25	0	0	na
	subregional	119	56	0	0	na
	regional	119	66	31	14	0.35
5 (15–50% of ejected ascospores were black)						
	local	21	4	0	0	na
	subregional	41	15	0	0	na
	regional	45	21	4	10	0.99

identity of the perithecial parent. With respect to species identity, mean reproductive success was significantly greater when *N. crassa* rather than *N. intermedia* acted as the perithecial parent ($P < 0.0005$, Fig. 6). When *N. crassa* was the perithecial parent, matings with *mat a* perithecia showed significantly greater reproductive success ($P < 0.0005$, Fig. 6), a pattern similar to that displayed in intraspecific matings (Table 6). However, when *N. intermedia* was the perithecial parent, matings with *mat A* perithecia showed greater reproductive success, although not significantly so. Overall, the most successful interspecific matings involved *N. crassa mat a* strains as the perithecial parent.

DISCUSSION

Phylogenetic and Biological Species Recognition

Independent implementation of phylogenetic species recognition (PSR) and biological species recognition (BSR) in the model filamentous fungal genus *Neurospora* showed that the groups of individuals identified as species by phylogenetic or reproductive criteria were nearly equivalent. The congruence between PSR and BSR was plainly seen (Figs. 1–3), and increased phylogenetic divergence predicted increased reproductive isolation. The overall agreement between the two methods examined in our study suggested that species recognized by either PSR or BSR are, in nature, on a trajectory toward both phylogenetic divergence and reproductive isolation. We therefore predict that PSR using genealogical concordance should reliably delineate species in organisms that are not candidates for BSR, such as those organisms for which sexual reproduction cannot be easily induced in the laboratory.

The results of BSR and PSR, however, were not identical: BSR identified four reproductively isolated species (*N. crassa*, *N. intermedia*, BS1, and BS2), whereas PSR identified five genetically differentiated species (*N. crassa*, *N. intermedia*, PS1, PS2, and PS3). The discrepancy involved the two phylogenetic species, *N. crassa* and PS3, that were recognized as a single biological species, *N. crassa*. PS3 was phylogenetically most closely related to *N. crassa*; fixed genetic differences between these two species were observed at only one of four loci, but fixed genetic differences between PS3 and the main *N. crassa* subgroup, NcA, existed at three of the four loci (Dettman et al. 2003). At this time, we are not proposing any taxonomic or nomenclatural revisions to the genus *Neurospora*. The newly identified phylogenetic and biological species (PS/BS1, PS/BS2, and PS3) are currently composed of only a small number of individuals. Before formally describing and naming these species, we plan to characterize them further and identify more individuals from each.

Nine individuals included in this study had been considered putative hybrids between *N. crassa* and *N. intermedia* (Turner et al. 2001), however, each was assigned confidently to a single biological species by our comprehensive BSR. The placement of all these putative hybrids in their respective biological species was fully corroborated by phylogenetic analyses of single-locus and combined datasets (Dettman et al. 2003). As such, these strains are likely not true hybrids, and by extension, no hybrids between any of the *Neurospora* species have yet been isolated from nature. This conclusion, along with the recognition of *N. crassa* and *N. intermedia* as two distinct species using both reproductive and phylogenetic criteria, requires that they no longer be considered a complex

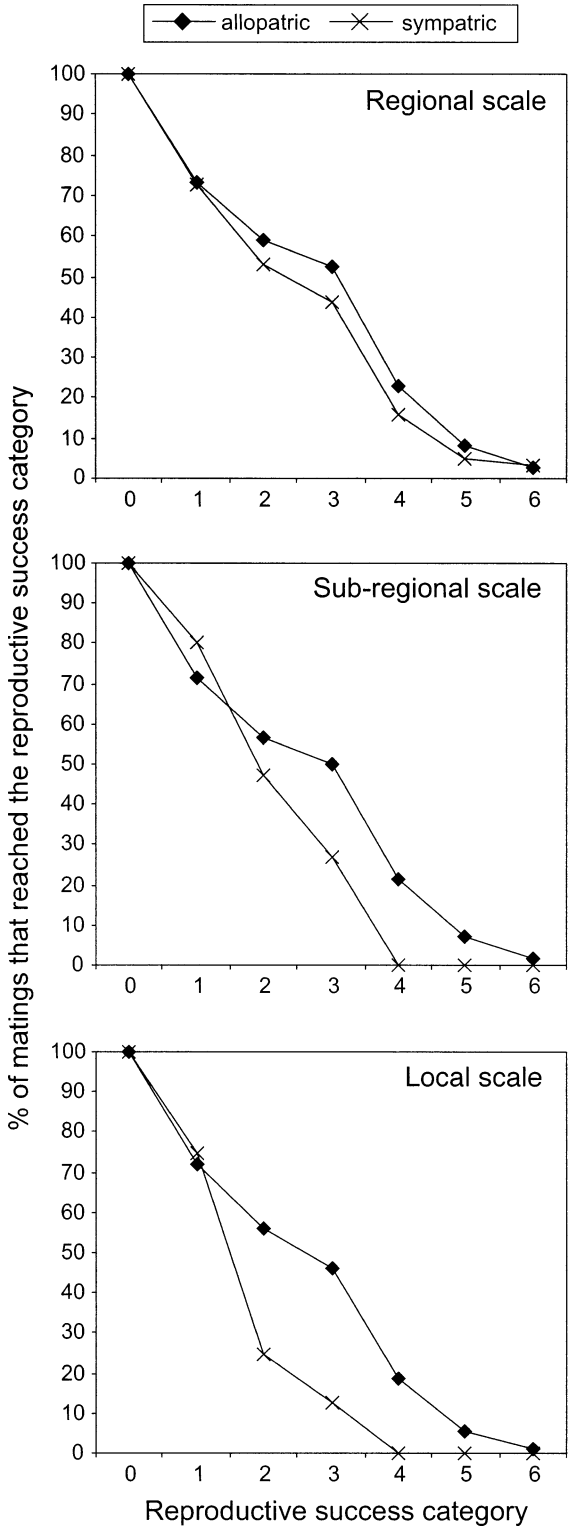


FIG. 5. Graphs displaying the percentage of interspecific matings between allopatric or sympatric individuals that reached the successive categories of reproductive success. At all three geographic scales, allopatric matings were significantly more likely than sympatric matings to proceed through the consecutive stages of the sexual cycle (log-rank tests, df = 1, regional: $\chi^2 = 5.35$, $P = 0.021$; subregional: $\chi^2 = 16.43$, $P < 0.0001$; local: $\chi^2 = 7.24$, $P = 0.007$). The discrepancy between the allopatric and sympatric curves increases as the scale of sympatry decreases.

of hybridizing sibling species, as previous authors have speculated (Natvig et al. 1987; Taylor and Natvig 1989; Skupski et al. 1997).

This is the first study, that we are aware of, to explicitly compare the thorough and detailed application of PSR, using genealogical concordance, and BSR, using mating tests, to the same large collection of individuals. Although other studies have investigated the relationship between PSR and BSR in fungi (e.g., Vilgalys and Sun 1994; Hibbett et al. 1995; Leuchtmann and Schardl 1998; Piercey-Normore et al. 1998; Aanen et al. 2000; O'Donnell et al. 2000b; Harrington et al. 2002), our study has the advantages of (1) true independence of methods, (2) large sample size, (3) sequence data from multiple loci, and (4) comprehensive crossing design. Similar to the results reported here, other studies have also discovered multiple cryptic phylogenetic species within a single biological species (e.g., Vilgalys and Sun 1994; Hibbett et al. 1995).

The superior resolution of PSR compared to BSR could be explained in two ways. The most likely explanation is that BSR failed to distinguish among phylogenetic species because reproductive compatibility was a retained, ancestral trait (Rosen 1979), and the ability for *N. crassa* and PS3 to mate has persisted despite significant genetic differentiation. Alternatively, BSR may have failed to recognize reproductively isolated species because the phenotypic measures of reproductive success overestimated the true potential for mating in nature. First, performing crosses under ideal conditions in the laboratory may artificially improve interspecies fertility. Additionally, with Basidiomycete fungi, matings typically are considered successful if the early stages of sexual reproduction have been initiated (see Petersen and Hughes 1999), however, the development of mature fruiting bodies and viable progeny is difficult to verify. As such, overly inclusive biological species, and the common occurrence of partial mating compatibility among biological species in Basidiomycetes (e.g., Petersen and Ridley 1996; Garbelotto et al. 1998; Aanen and Kuyper 1999), may be due to the overestimation of reproductive success. With Ascomycete fungi, the sexual cycle can usually be followed through to progeny production. Consequently, the correspondence between PSR and BSR generally is better in Ascomycetes (e.g., Leuchtmann and Schardl 1998; O'Donnell et al. 2000b; Harrington et al. 2002) than in Basidiomycetes, suggesting that ascospore production is a good indicator of reproductive success in nature.

Traditional versus Comprehensive Biological Species Recognition

Having performed most of the possible crosses among the 73 individuals, we could evaluate the accuracy of the traditional practice of assigning unknown individuals to biological species using matings to just a few tester strains (i.e., traditional BSR). Although there was general agreement between the results of both BSR methods, our comprehensive BSR outperformed traditional BSR in discovering new biological species (BS1 and BS2) and subgroups within species (NcA and NcC of *N. crassa*). Using only a few representatives of the existing species makes it difficult to discover new species, but not impossible, as shown by the most recently

TABLE 6. The effect of the mating type of the perithecial parent on reproductive success of reciprocal matings in intraspecific and interspecific crosses involving *Neurospora crassa* and *N. intermedia*. Chi-square tests with Yates correction were used to compare the numbers of crosses showing perithecial superiority of the *mat a* versus *mat A* parent for each cross type. The reproductive success of matings with *mat a* versus *mat A* perithecial parents was compared using Wilcoxon paired sample tests. Significant probability values are shown in bold.

Cross type	*N. crassa* × *N. crassa*	*N. intermedia* × *N. intermedia*	*N. crassa* × *N. intermedia*
Total number of crosses	145	219	263
Number that exhibited asymmetrical reproductive success	29 (20.0%)	75 (34.3%)	167 (63.5%)
Number for which *mat a* strain was superior perithecial parent	22	50	95
Number for which *mat A* strain was superior perithecial parent	7	25	72
χ^2	6.76	7.68	2.90
P	**<0.01**	**<0.01**	<0.10
Reproductive success			
Mean reproductive success (SE) when *mat a* strain was perithecial parent	5.03 (0.13)	4.55 (0.15)	1.87 (0.09)
Mean reproductive success (SE) when *mat A* strain was perithecial parent	4.71 (0.15)	3.92 (0.18)	1.65 (0.09)
Signed-rank	130	694.5	1243
P	**0.001**	**<0.001**	**0.022**

described outbreeding species of *Neurospora*, *N. discreta* (Perkins and Raju 1986).

Comprehensive BSR also outperformed traditional BSR in correctly assigning problematic individuals to biological species. Ten of the misidentifications by traditional BSR involved individuals that belong to PS/BS1 or PS/BS2, an understandable error considering that tester strains did not exist for these previously unknown species. Four of the ten misidentified individuals had been assigned to *N. intermedia*, and the other six had been considered putative hybrids. Another three individuals had been mistakenly identified as putative hybrids by traditional BSR, even though they belong to well-known species for which tester strains were available. Two of these putative hybrids belong to the NcC subgroup of *N. crassa*, and were misidentified probably because the *N. crassa* tester strains are from the NcA subgroup, which is partially reproductively isolated from NcC. The third putative hybrid belongs to *N. intermedia*, and was misidentified probably because it mated poorly with the *N. intermedia* testers and was fully fertile with only a limited number of other *N. intermedia* isolates (Fig. 1).

The genetic diversity (Fig. 2) and variation in reproductive success (Fig. 1) found within some species explains why a few tester strains cannot be expected to assign all newly collected individuals to the correct species. To accommodate intraspecific variation, the traditional BSR framework needs to be expanded to include additional tester strains. For instance, we recommend strains D106 (*mat a*) and D107 (*mat*

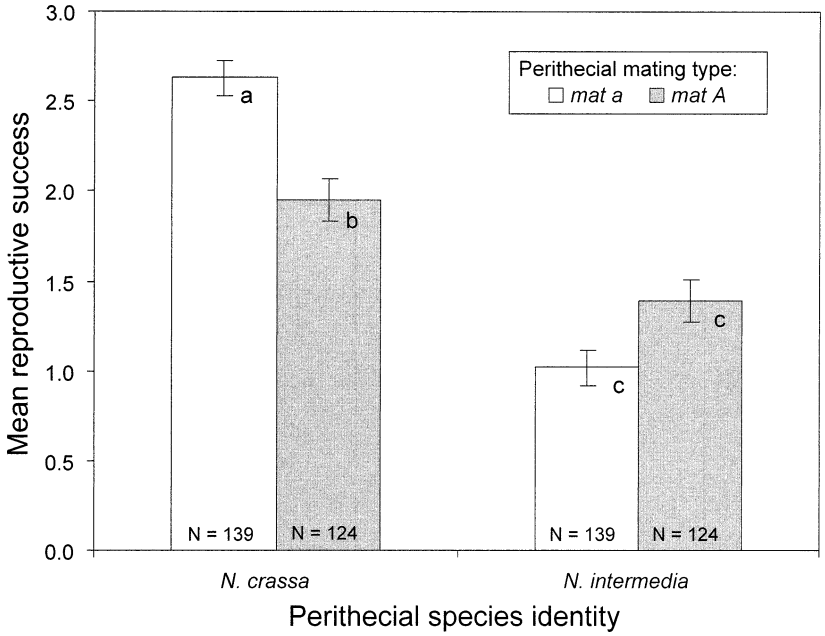


FIG. 6. Interaction of mating type and species identity of perithecial parent on reproductive success in the four types of interspecific *Neurospora crassa* × *N. intermedia* matings. Significant differences in reproductive success (vertical bars are ± standard error) are indicated by letters (a, b, c) as determined by Mann-Whitney *U* tests ($P < 0.0005$). Matings with *N. crassa* as the perithecial parent had a greater mean reproductive success, regardless of mating type, and mating type had different influences on the two perithecial species identities.

A) as supplementary *N. crassa* testers for the NcC subgroup. To identify individuals belonging to PS/BS1 and PS/BS2, we tentatively recommend strains D55 (*a*) and D57 (*A*), and strains D93 (*a*) and D87 (*A*), respectively. The problem of intraspecific variation has been previously realized and the use of ''local'' tester strains was recommended for some species (Perkins and Turner 1988; Turner et al. 2001). Despite its disadvantages, and the complexity of reproductive success as a trait, traditional BSR has performed very well in assigning unknown individuals to species, with very few false positives (4 of 73) or false negatives (3 of 73).

Speciation in Neurospora

Under all scenarios of speciation, the correspondence between phylogenetic divergence and reproductive isolation is predicted after species have been separated for long periods. For recently separated species pairs, however, these measures may not be correlated. Whether phylogenetic divergence or reproductive isolation developed first, and whether the species occur in sympatry or allopatry, may shed light upon the dynamics of speciation in *Neurospora*.

The results of this study demonstrated that significant phylogenetic divergence can precede the development of reproductive isolation. For example, PS3 and *N. crassa* were phylogenetically distinct yet not reproductively isolated from each other. A similar pattern was found within *N. intermedia*: the strains that formed the long-branched basal lineages could still mate relatively well with most other *N. intermedia* strains. Of equal importance was the fact that multiple biological species were never discovered within a single phylogenetic species. Thus, no examples were found in which reproductive isolation had developed prior to significant phylogenetic divergence, either in sympatry or allopatry.

The results also suggested that allopatric speciation was predominant among the species of *Neurospora* studied here. Phylogenetic Species 1, PS2, and PS3 were composed of strains that were geographically restricted, and each was allopatric with their sister species (e.g., PS2 vs. *N. intermedia*, or PS3 vs. *N. crassa*; Fig. 2). The same was true for the two subgroups within *N. crassa*, NcA and NcC, which may represent incipient species. The NcC strains all were collected from a unique location, Tamil Nadu, India, and showed both partial reproductive isolation and phylogenetic divergence from NcA. These observations were consistent with previous reports that many *N. crassa* strains from India had reduced reproductive success when crossed with *N. crassa* testers (Perkins et al. 1976).

Geography and Reinforcement

Reinforcement is selection for increased sexual reproductive isolation between species in response to the production of less fit hybrids (Dobzhansky 1937). We found multiple lines of evidence that were consistent with reinforcement in *Neurospora*. The mean reproductive success of sympatric interspecific crosses was lower than that of allopatric interspecific crosses at all three geographic scales (local, subregional, and regional; Table 4), and increased geographic distance was a significant predictor of increased reproductive success (Table 3). Moreover, the decreased reproductive success of sympatric interspecific crosses became more apparent as the scale of sympatry decreased (Table 3, Fig. 5). Sympatric and allopatric crosses did not differ significantly in terms of phylogenetic differentiation at neutral markers, which indicated that increased genome-wide divergence was not responsible for the observed sympatry-associated sexual dysfunction. Given that reinforcement selection can only occur where individuals encounter one another and have the opportunity to hybridize, finding greater reproductive isolation in sympatry than allopatry is support for reinforcement. Such a pattern has only rarely been reported in fungi (Capretti et al. 1990; Stenlid and Karlsson 1991).

Further evidence for reinforcement in *Neurospora* was found in the reduced fitness of hybrid progeny. Ascospores from interspecific matings were significantly less likely to reach maturity than ascospores from intraspecific matings. Furthermore, mature ascospores from interspecific matings were significantly less viable than ascospores from intraspecific matings. True hybrids have not been found in nature, despite the fact that species have broadly overlapping ranges and may be collected from the same site, just centimeters from each other on the same substrate (Powell et al. 2003). If interspecific matings do occur in nature, the absence of hybrid collections also suggests that hybrid *Neurospora* progeny have reduced fitness.

The disadvantage of the reduced potential of hybrid establishment in nature may be compounded because fertilization substantially inhibits initiation of new protoperithecia and represses perithecial development in subsequent matings (Howe and Prakash 1969). Not only does fertilization by the incorrect species waste reproductive effort, but it also reduces the likelihood of future matings with the correct species. A reinforcement mechanism that selectively prevented initiation of mating or allowed early abortion of meiotic products in interspecific *Neurospora* matings, thereby preserving subsequent fertility, would be favored by selection under conditions of sympatry. Consistent with such a mechanism, the increased reproductive isolation in interspecific crosses between sympatric strains involved arrest during the early stages of sexual development, prior to completion of perithecial development and ascospore ejection (e.g., categories 1 and 2; Table 5). In *N. crassa*, crosses that yield barren perithecia due to genetic incompatibilities usually arrest just before or at karyogamy (Raju and Perkins 1978). We did not examine the contents of perithecia that failed to eject ascospores, so the presence of asci containing diploid cells or meiotic products was not determined. It is unknown, therefore, whether developmental arrest experienced by immature and barren perithecia was prezygotic or postzygotic.

Hermaphroditism and Asymmetrical Reproductive Success

Reciprocal matings of the same cross achieved different categories of reproductive success in almost half of the total crosses performed in this study, which is similar to findings in *N. tetrasperma* (Jacobson 1995). The significant effects of mating type and species identity on asymmetrical matings underscore the complexity of reproductive success as a trait. They also suggest that when studying the reproductive biology of any hermaphroditic organism, reciprocal matings

should be performed not only between species, but also between individuals, if possible. This is an important aspect of natural variation in reproductive success that is often overlooked.

The perithecial superiority of *mat a* mating-type strains was expressed in both intraspecific and interspecific crosses (Table 6, Fig. 6). The genetic mechanism of *mat a* perithecial superiority in *Neurospora* remains unknown. The two *mat* idiomorphs each contain multiple genes without homologues in the alternative idiomorph. Some of these genes are required for mating-type identity and fertilization (Saupe et al. 1996), and some are involved in increasing the efficiency of post-fertilization sporogenesis (Ferreira et al. 1998). Thus, asymmetry of the two mating-type idiomorphs could explain asymmetry in perithecial superiority via any of the several developmental steps, from mating-type specific pheromone signaling (Pöggeler and Kuck 2001; Bobrowicz et al. 2002) to ascospore maturation. Fungal mating type has generally been considered not to affect fitness of individuals (Brasier 1999), and studies of a wide variety of fungal systems have supported this assertion (e.g., Dudzinski et al. 1993; Ahmed et al. 1996; Bardin et al. 1997). However, some studies have found an association between mating type and virulence in various pathogenic fungi (Fagan 1988; Kolmer and Ellingboe 1988; Kwon-Chung et al. 1992; Funnell et al. 2001).

The species identity of the perithecial parent had a marked effect on reproductive success in *N. crassa* × *N. intermedia* matings. *Neurospora crassa* strains displayed clear superiority as perithecial parents, especially when they were *mat a* mating type (Fig. 6). Perkins et al. (1976) also reported that certain *N. crassa* × *N. intermedia* crosses produced abundant ascospores when *N. crassa* was the perithecial parent, but were barren when *N. intermedia* was the perithecial parent. The asymmetrical reproductive isolation observed between *N. crassa* and *N. intermedia* could result from a number of factors, including interactions between nuclear and mitochondrial genomes, as has been implicated in asymmetrical reproductive isolation in a number of other organisms (Arnold 1993; Tiffin et al. 2001).

Neurospora as a Model Organism for the Study of Evolutionary Biology

We hope that this study, together with the companion study also published in this issue (Dettman et al. 2003), will provide a benchmark for the comparison of species recognition methods. Our reports build upon the fundamental resources of a historical biological species concept in *Neurospora* and a magnificent collection in which all individuals have been identified to species by reproductive ability (Perkins et al. 1976; Perkins and Turner 1988; Turner et al. 2001). Our comparison of phylogenetic divergence and reproductive isolation among species has added another layer to this foundation, bringing *Neurospora* closer to becoming a premier system for the investigation of evolution of species and speciation mechanisms in fungi, and beyond. *Neurospora* is one of the small set of organisms that can bridge the gap between genetics, molecular biology, and evolutionary biology. With an easily manipulated sexual cycle, a long history of genetic study, and the recently completed genome sequence of *N. crassa* (Galagan et al. 2003), it is poised to become a model organism for the study of species recognition, speciation, and the genetics of reproductive isolation.

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APPENDIX

Strains of *Neurospora* used to compare biological species recognition (BSR) to phylogenetic species recognition (PSR). BS1–2, Biological Species 1–2; PS1–3, Phylogenetic Species 1–3.

Strain numbers[1]				Mating type	Original species[2]	Biological species	Phylogenetic species[3]	Geographic location			
D	FGSC	Old FGSC	Other					Region[4]	Collection site	Latitude	Longitude
140	8900	430		*A*	*N. crassa*	*N. crassa*	*N. crassa* (NcA)	Africa	Adiopodoume, Ivory Coast*	5°20′N	4°08′W
68	8828	4825	3681	*A*	*N. crassa*	*N. crassa*	*N. crassa* (NcA)	Africa	Tiassale, Ivory Coast	5°53′N	4°57′W
69	8829	4826	3684	*a*	*N. crassa*	*N. crassa*	*N. crassa* (NcA)	Africa	Tiassale, Ivory Coast	5°53′N	4°57′W
59	8819		3427	*a*	*N. crassa*	*N. crassa*	*N. crassa* (NcA)	Carib. Basin	Carrefour Dufort, Haiti*	18°27′N	72°38′W
62	8822	4824	3491	*A*	*N. crassa*	*N. crassa*	*N. crassa* (NcA)	Carib. Basin	Carrefour Mme. Gras, Haiti*	18°22′N	73°37′W
144	8904	1131		*A*	*N. crassa*	*N. crassa*	*N. crassa* (NcA)	Carib. Basin	Cristobal, Panama	9°21′N	79°54′W
115	8875		4480	*A*	*N. crassa*	*N. crassa*	*N. crassa* (NcA)	Carib. Basin	Franklin, Louisiana	29°48′N	91°31′W
116	8876		4481	*a*	*N. crassa*	*N. crassa*	*N. crassa* (NcA)	Carib. Basin	Franklin, Louisiana	29°48′N	91°31′W
23	8783	3970	1409	*A*	*N. crassa*	*N. crassa*	*N. crassa* (NcA)	Carib. Basin	Homestead, Florida*	25°32′N	80°28′W
27	8787		1417	*A*	*N. crassa*	*N. crassa*	*N. crassa* (NcA)	Carib. Basin	Homestead, Florida*	25°32′N	80°28′W
85	8845		4130	*a*	*N. crassa*	*N. crassa*	*N. crassa* (NcA)	Carib. Basin	Kabah, Yucatan, Mexico*	20°07′N	89°29′W
60	8820	4712	3433	*a*	*N. crassa*	*N. crassa*	*N. crassa* (NcA)	Carib. Basin	Leogane, Haiti	18°38′N	72°37′W
143	8903	987		*A*	*N. crassa*	*N. crassa*	*N. crassa* (NcA)	Carib. Basin	Marrero, Louisiana	30°00′N	90°03′W
88	8848		4150	*a*	*N. crassa*	*N. crassa*	*N. crassa* (NcA)	Carib. Basin	Sayil, Yucatan, Mexico	20°16′N	89°42′W
91	8851		4155	*A*	*N. crassa*	*N. crassa*	*N. crassa* (NcA)	Carib. Basin	Uman, Yucatan, Mexico	20°51′N	89°43′W
90	8850		4154	*A*	*N. crassa*	*N. crassa*	*N. crassa* (NcA)	Carib. Basin	Uxmal, Yucatan, Mexico	20°21′N	89°46′W
77	8837	4820	3826	*a*	*N. crassa*	*N. crassa*	PS3	Africa	Loubomo, Congo	4°09′S	12°47′E
78	8838	4822	3838	*A*	*N. crassa*	*N. crassa*	PS3	Africa	Madingo, Congo*	4°05′S	11°24′E
75	8835	4821	3816	*a*	*N. crassa*	*N. crassa*	PS3	Africa	Makaba, Congo	4°11′S	12°41′E
42	8802	8198	2543	*a*	putative hybrid	*N. crassa*	*N. crassa* (NcC)	India	Madaurai, Tamil Nadu*	9°55′N	78°07′E
104	8864		4359	*a*	*N. crassa*	*N. crassa*	*N. crassa* (NcC)	India	Madaurai, Tamil Nadu*	9°55′N	78°07′E
105	8865		4360	*A*	*N. crassa*	*N. crassa*	*N. crassa* (NcC)	India	Madaurai, Tamil Nadu*	9°55′N	78°07′E
98	8858		4333	*A*	*N. crassa*	*N. crassa*	*N. crassa* (NcC)	India	Mallilinatham, Tamil Nadu*	12°40′N	80°05′E
99	8859		4334	*A*	*N. crassa*	*N. crassa*	*N. crassa* (NcC)	India	Mallilinatham, Tamil Nadu*	12°40′N	80°05′E
100	8860	8203	4335	*a*	putative hybrid	*N. crassa*	*N. crassa* (NcC)	India	Mallilinatham, Tamil Nadu*	12°40′N	80°05′E
103	8863		4358	*a*	*N. crassa*	*N. crassa*	*N. crassa* (NcC)	India	Mallilinatham, Tamil Nadu*	12°40′N	80°05′E
106	8866		4361	*a*	*N. crassa*	*N. crassa*	*N. crassa* (NcC)	India	Rameshwaram, Tamil Nadu*	9°18′N	79°19′E
107	8867		4362	*A*	*N. crassa*	*N. crassa*	*N. crassa* (NcC)	India	Rameshwaram, Tamil Nadu*	9°18′N	79°19′E
36	8796	6582	2362	*A*	*N. intermedia*	*N. intermedia*	*N. intermedia*	East Asia	Arue, Tahiti (Moorea)	17°30′S	149°30′W
32	8792	3989	1526	*A*	*N. intermedia*	*N. intermedia*	*N. intermedia*	East Asia	Hefei, China	31°55′N	117°18′E
3	8763	1763	25	*a*	*N. intermedia*	*N. intermedia*	*N. intermedia*	East Asia	Manila, Philippines	14°35′N	120°59′E
1	8761	1766	13	*A*	*N. intermedia*	*N. intermedia*	*N. intermedia*	East Asia	Taipei, Taiwan	25°5′N	121°32′E
2	8762	1767	17	*a*	*N. intermedia*	*N. intermedia*	*N. intermedia*	East Asia	Taipei, Taiwan	25°5′N	121°32′E
35	8795	4876	1878	*a*	*N. intermedia*	*N. intermedia*	*N. intermedia*	East Asia	Tiaba, Papua New Guinea	9°27′S	147°27′E

APPENDIX. Continued.

Strain numbers[1]				Mating type	Original species[2]	Biological species	Phylogenetic species[3]	Geographic location			
D	FGSC	Old FGSC	Other					Region[4]	Collection site	Latitude	Longitude
73	8833	6263	3770	*a*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	Africa	Adiopodoume, Ivory Coast*	5°20′N	4°08′W
81	8841	6276	3852	*a*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	Africa	Bouanza, Congo	4°11′S	13°07′E
79	8839	4274	3839	*a*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	Africa	Madingo, Congo*	4°05′S	11°24′E
83	8843	6286	3932	*A*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	Africa	Makokou, Gabon	0°38′N	12°47′E
141	8901	434		*A*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	Africa	Monrovia, Liberia	6°20′N	10°46′W
65	8825	6254	3540	*A*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	Africa	Yopougon, Ivory Coast	5°16′N	4°02′W
66	8826	6255	3543	*a*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	Africa	Yopougon, Ivory Coast	5°16′N	4°02′W
64	8824	6251	3495	*A*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	Carib. Basin	Carrefour Mme. Gras, Haiti*	18°22′N	73°37′W
16	8776	3213	831	*a*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	Carib. Basin	Fred, Texas	30°34′N	94°10′W
22	8782		1408	*a*	*N. intermedia*[5]	*N. intermedia*	*N. intermedia* (NiA)	Carib. Basin	Homestead, Florida*	25°32′N	80°28′W
25	8785		1413	*a*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	Carib. Basin	Homestead, Florida*	25°32′N	80°28′W
26	8786		1415	*A*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	Carib. Basin	Homestead, Florida*	25°32′N	80°28′W
122	8882	1543	8045	*A*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	Carib. Basin	Puerto Cortes, Honduras	15°50′N	87°55′W
52	8812	5369	2938	*A*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	East Asia	Ban Khao Yai, Thailand	13°15′N	99°53′E
7	8767	1792	142	*A*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	East Asia	Bogor, Java	6°34′S	106°45′E
51	8811	8199	2632	*A*	putative hybrid	*N. intermedia*	*N. intermedia* (NiA)	East Asia	Georgetown, Malaya (Penang)	5°30′N	100°28′E
142	8902	435		*A*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	East Asia	Levuka, Fiji	17°42′S	178°50′E
43	8803		2544	*a*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	India	Konappatti, Tamil Nadu	9°50′N	78°15′E
44	8804	5344	2546	*A*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	India	Konappatti, Tamil Nadu	9°50′N	78°15′E
108	8868		4363	*A*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	India	Madaurai, Tamil Nadu*	9°55′N	78°07′E
109	8869		4364	*a*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	India	Madaurai, Tamil Nadu*	9°55′N	78°07′E
127	8887		M105	*a*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	India	Maddur, Karnataka	12°36′N	77°00′E
128	8888		M110	*A*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	India	Maddur, Karnataka	12°36′N	77°00′E
129	8889		M14	*A*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	India	Maddur, Karnataka	12°36′N	77°00′E
130	8890		M17	*a*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	India	Maddur, Karnataka	12°36′N	77°00′E
101	8861		4336	*A*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	India	Mallilinatham, Tamil Nadu*	12°40′N	80°05′E

APPENDIX. Continued.

Strain numbers[1]				Mating type	Original species[2]	Biological species	Phylogenetic species[3]	Geographic location			
D	FGSC	Old FGSC	Other					Region[4]	Collection site	Latitude	Longitude
47	8807	5345	2552	*a*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	India	Rameshwaram, Tamil Nadu*	9°18′N	79°19′E
48	8808		2554	*A*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiA)	India	Rameshwaram, Tamil Nadu*	9°18′N	79°19′E
8	8768	2215	147	*a*	*N. intermedia*	*N. intermedia*	*N. intermedia* (NiB/yellow)	East Asia	Bogor, Java	6°34′S	106°45′E
55	8815		3423	*a*	*N. intermedia*?	BS1	PS1	Carib. Basin	Carrefour Dufort, Haiti*	18°27′N	72°38′W
57	8817	8200	3425	*A*	putative hybrid	BS1	PS1	Carib. Basin	Carrefour Dufort, Haiti*	18°27′N	72°38′W
58	8818	8225	3426	*A*	putative hybrid	BS1	PS1	Carib. Basin	Carrefour Dufort, Haiti*	18°27′N	72°38′W
120	8880	7847	4576	*A*	putative hybrid	BS2	PS2	Africa	Nosy Be, Madagascar	13°24′S	48°17′E
121	8881	7848	4578	*a*	putative hybrid	BS2	PS2	Africa	Nosy Be, Madagascar	13°24′S	48°17′E
89	8849		4151	*a*	*N. intermedia*?	BS2	PS2	Carib. Basin	Kabah, Yucatan, Mexico*	20°07′N	89°29′W
86	8846		4146	*a*	*N. intermedia*	BS2	PS2	Carib. Basin	Merida, Yucatan, Mexico	20°59′N	89°39′W
87	8847		4148	*A*	*N. intermedia*	BS2	PS2	Carib. Basin	Merida, Yucatan, Mexico*	20°59′N	89°39′W
92	8852	8201	4157	*a*	putative hybrid	BS2	PS2	Carib. Basin	Merida, Yucatan, Mexico	20°59′N	89°39′W
93	8853	8202	4158	*a*	putative hybrid	BS2	PS2	Carib. Basin	Merida, Yucatan, Mexico	20°59′N	89°39′W

[1] Cross reference of strain numbers from different collections. Consecutive D numbers were assigned as a convenient label for the independent BSR and PSR portions of this study (see also Dettman et al. 2003). The entire collection was deposited in the Fungal Genetics Stock Center (FGSC). Some of the progenitors of these strains, prior to single conidium subculturing, were previously deposited in FGSC (Old FGSC). Other numbers (Other) refer to progenitors in the Perkins collection (now curated by FGSC), except M14, M17, M105, and M110, which are in the personal Jacobson collection.

[2] Species designation originally assigned by traditional BSR (Perkins et al. 1976; Perkins and Turner 1988; Turner et al. 2001). Putative hybrid = strain originally designated as possible hybrid between *N. crassa* and *N. intermedia.* A question mark indicates species identification was regarded as questionable.

[3] As determined in Dettman et al. 2003, with intraspecific subgroups in parentheses.

[4] Carib. Basin, Caribbean Basin, which includes the coastal areas along the Gulf of Mexico and Caribbean Sea and the islands within. East Asia includes east of India and the Pacific islands.

[5] The progenitor of strain D22 was listed as *N. crassa* in the Perkins database. Based on preliminary crosses with testers strains, this identification was proven incorrect and classification was adjusted accordingly.

* Collection sites from which we selected both *N. crassa* and *N. intermedia* strains (based on original species designations).