

Reinforced postmating reproductive isolation barriers in *Neurospora*, an Ascomycete microfungus

E. TURNER, D. J. JACOBSON & J. W. TAYLOR

Department of Plant and Microbial Biology, University of California, Berkeley, CA, USA

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Abstract

Maladaptive hybridization promotes reinforcement, selection for stringent reproductive isolation barriers during speciation. Reinforcement is suspected when barriers between sympatric populations are stronger than allopatric barriers, and particularly when stronger barriers evolve in the species and sex suffering the greatest costs of hybridization. Canonically, reinforcement involves premating barriers. Selection for postmating barriers is controversial, but theoretically possible. We examined geographical patterns in reproductive isolation barriers between *Neurospora crassa* and *Neurospora intermedia*, fungi with pheromone-mediated mate recognition and maternal care. We find that isolation is stronger between sympatric populations than allopatric populations, and stronger barriers are associated with the species (*N. crassa*) and mating role (maternal) suffering the greater costs of hybridization. Notably, reinforced isolation involves a postmating barrier, abortion of fruitbodies. We hypothesize that fruitbody abortion is selectively advantageous if it increases the likelihood that maternal *Neurospora* individuals successfully mate conspecifically after maladaptive hybrid fertilization.

Introduction

The origin of species is a fundamental process in biology, and understanding speciation is a basic goal of evolutionary biology. For sexual organisms, geographical or biological barriers to sexual reproduction are required for speciation, and also for the maintenance of species integrity. Biological barriers act throughout the lifecycle and are often classified according to the point in the life cycle that they are encountered (e.g. premating vs. postmating). Barriers at each stage can arise as byproducts of within lineage evolution as a result of natural or sexual selection or genetic drift, but natural selection against maladaptive hybridization itself can also drive evolution of reproductive isolation barriers (Wallace, 1889; Fisher, 1930; Dobzhansky, 1937). This process is usually termed reinforcement, and as the name implies, it requires the pre-existence of some degree of reproduc-

tive isolation, which is then 'reinforced' by the evolution of additional barriers.

Studies of reinforcement have focused overwhelmingly on premating barriers. The selective advantage of premating barriers is that they prevent the harm of maladaptive hybridization altogether. Additionally, postmating barriers risk reducing the fitness of hybridizing parents if, for example, hybrids are viable and partially fertile, and the likelihood of subsequently reproducing conspecifically is low. Nevertheless, selection on postmating barriers is at least theoretically possible (Wallace, 1889; Coyne, 1974). Wallace argued that selection among demes could drive hybrid inviability by reducing the negative impact of low-quality hybrids (Wallace, 1889). Theories that eschew group selection identify several factors that could make the evolution of postmating barriers selectively advantageous for parents (Coyne, 1974; Coyne & Orr, 2004). In Coyne's model, hybrid sterility or late hybrid lethality can select for early hybrid inviability if there is substantial parental investment in the production and care of progeny, if individuals can mate more than once, and if the temporal or energetic costs of bearing or caring for sterile hybrids makes subsequent reproduction less likely. Selection

Correspondence: Elizabeth Turner, Department of Plant & Microbial Biology, University of California, Berkeley, 111 Koshland Hall, Berkeley, CA 94709, USA. Tel.: 510 642 8441; fax: 510 642 4995; e-mail: eturner@berkeley.edu

should not favour the evolution of hybrid sterility or hybrid breakdown, and selection for postmating inviability should not occur when premating isolation is complete. In fact, reinforcement of postmating barriers is most likely to occur when there is little opportunity for premating barriers to evolve (Johnson & Wade, 1995). Selection for inviability will mainly occur in females, unless males provide parental care that prevents or delays further reproduction, and organisms that can abort hybrid offspring are considered more susceptible to selection for hybrid offspring than organisms that must lay eggs or bear young regardless of their viability. Earlier barriers are more efficient at reducing the cost of hybridization, so selection will favour inviability at the earliest stages of development (Coyne, 1974).

Selection against hybridization can only occur during sympatry, so a pattern of stronger differentiation of premating characters and enhanced premating isolation between sympatric taxa has been considered the hallmark of reinforcement, and this pattern has been found to exist in a wide diversity of animal and plant studies (Howard, 1993; Noor, 1997; Coyne & Orr, 2004; Van der Niet *et al.*, 2006) and some fungi (Burnett, 2003; Le Gac & Giraud, 2008), although not all studies find the pattern for all taxa (Moyle *et al.*, 2004; Le Gac & Giraud, 2008). As with premating reinforcement, the signature of postmating reinforcement would be a barrier that is stronger in zones of sympatry than in areas of allopatry. Although no examples of this pattern have been reported for animals, stronger postmating isolation in sympatry has been observed in the plant genera *Gossypium* (Stephens, 1946; Wallace, 1988), *Gilia* (Grant, 1966) and *Lycopersicon* (Rick & Lamm, 1955; Stadler *et al.*, 2005, 2008), as well as in species of the fungus *Neurospora* (Dettman *et al.*, 2003b). If the cost of hybridization is asymmetrical, theory predicts that enhanced sympatric isolation barriers are more likely to evolve in the species and sex that suffers the higher cost, because the strength of reinforcing selection is proportional to the cost of hybridization (Coyne & Orr, 2004).

Reproductive isolation and reinforcement between *Neurospora crassa* and *Neurospora intermedia*

In this study, we analyze reproductive isolation barriers between sympatric and allopatric populations of two *Neurospora* species, *Neurospora crassa* Shear & Dodge and *Neurospora intermedia* Tai (Sordariales, Ascomycota), and test whether observed patterns conform to those expected under reinforcement selection. These species have broadly overlapping ranges in tropical and subtropical regions spanning both the Eastern and Western Hemispheres, except that *N. crassa* is absent from East Asia and the Pacific Islands. Additionally, *N. crassa* is found in Europe, where *N. intermedia* is absent (Turner *et al.*, 2001; Jacobson *et al.*, 2006). Both species are adapted to growth on partially burnt vegetation, and

the two species cannot be reliably distinguished on the basis of morphology, physiology or ecology. Both species are frequently collected together at burn sites, and laboratory crosses between some strains of the species yield viable, fertile progeny, but hybrids are not known in nature (Dettman *et al.*, 2003a). Nevertheless, conflicts between certain gene trees and *Neurospora* species phylogenies suggest that a history exists of introgression among species (Strandberg *et al.*, 2009).

Neurospora possesses many of the characteristics that facilitate reinforcement selection on postmating barriers. The sequences of the peptide pheromones that mediate premating attraction between mating-type compatible individuals are so conserved that they have been found to be identical in *N. crassa* and *Sordaria fimicola*, a member of *Neurospora*'s sister genus (Poeggeler *et al.*, 2000). After fertilization, progeny are nurtured within fruitbodies composed entirely of maternal tissue, so that the costs of reproduction are borne overwhelmingly by the maternal strain. The period of maternal care is a time when postmating barriers can be selectively advantageous to maternal individuals. Maternal care is extensive in *Neurospora*. As in most Ascomycete and Basidiomycete fungi, after fertilization plasmogamy, the fusion of cells, is not followed immediately by karyogamy, the fusion of nuclei, but instead by a prolonged heterokaryotic ($n + n$) stage, culminating in the production of special cells (asci), where karyogamy occurs. The zygote immediately undergoes meiosis, and meiosis is followed by mitosis and then sporogenesis, the growth and development of the haploid progeny or ascospores. All of these stages – dikaryon, karyogamy, zygote, meiosis, sporogenesis – occur within the maternal tissues of the fruitbody (perithecia). Most *N. crassa* × *N. intermedia* laboratory crosses result in at least the initiation of fruitbody development, indicating that mating has occurred. Postmating isolation manifests in a variety of ways: fruitbodies may fail to develop fully or prove to be barren and fail to sporulate. When sporulation does occur, 98% of hybrid ascospores are inviable (Dettman *et al.*, 2003b). Ascospore inviability represents a post-meiotic defect and is genetically akin to hybrid sterility in plants and animals.

Neurospora crassa strains are most interfertile with *N. intermedia* from East Asia, the only portion of *N. intermedia*'s distribution where it is not sympatric with *N. crassa* (Perkins *et al.*, 1976; Perkins & Turner, 1988; Dettman *et al.*, 2003b). Sympatric crosses between *N. crassa* and *N. intermedia* from regions of overlap are more likely than allopatric crosses involving Asian *N. intermedia* to result in underdeveloped or nonsporulating fruitbodies (43% and 9% of crosses, respectively), whereas allopatric crosses are more likely than sympatric crosses to result in sporulation of mostly inviable ascospores (64% and 31% of crosses, respectively) (Dettman *et al.*, 2003b). The cost of hybridization is asymmetrical in the two species, because *N. crassa* individuals

invest significantly more in fruitbody development than *N. intermedia* strains after hybrid fertilization; 69% of *N. crassa* maternal crosses make well-developed fruitbodies, whereas only 33% of *N. intermedia* maternal crosses do so (Dettman *et al.*, 2003b). Given the extremely low viability of hybrid spores (2% on average), the species that invests more, *N. crassa*, suffers the greater burden of maladaptive hybridization.

The burden of hybridization falls almost entirely on maternal individuals and is exacerbated because maternal individuals cannot mate again after fruitbody development is completed (Howe & Prakash, 1969). In contrast, millions of conidia are released by a single thallus, and the potential for each of these to act as a fertilizing element is unaffected by the fate of any other conidia, so that male investment in any particular instance of maladaptive hybrid fertilization is negligible. Therefore, selection for reinforced barriers between sympatric *N. crassa* and *N. intermedia* would be strongest in *N. crassa* on barriers associated with the maternal role.

In this article, we analyze previously published data (Dettman *et al.*, 2003b) and present a model of the effects of geography (sympatric or allopatric), mating role (*N. crassa* or *N. intermedia* maternal) and parental populations on hybrid fruitbody development. We characterize variation in hybrid fruitbody development across seven populations of the two species, comparing reproductive isolation in allopatric and sympatric matings, and in *N. crassa* maternal and *N. intermedia* maternal matings. We determine using survival analysis whether particular isolation barriers are associated with sympatric matings for each population and mating role. Finally, we test whether patterns of reproductive isolation between *N. crassa* and *N. intermedia* conform to Coyne & Orr's (2004) prediction that under reinforcement selection, enhanced reproductive isolation barriers in sympatry will be associated with the species and sex suffering the greatest cost of hybridization.

We find that geography, maternal species, maternal population, paternal population and a geography \times maternal species interaction effect all significantly affect hybrid fruitbody development. Reproductive isolation is stronger in sympatry, and stronger barriers are particularly associated with *N. crassa* and the maternal role, as predicted. Across all populations, sympatry is associated with enhanced postmating barriers. These results support the evolution of postmating reproductive isolation barriers via reinforcement selection in *Neurospora*.

Methods

Neurospora reproductive biology

Neurospora is a saprobe adapted to cycles of fire and the sexual spores, known as ascospores, represent its resting stage. New haploid individuals colonizing incompletely

burnt vegetation arise from the germination of ascospores after wildfires or fires in agricultural settings, as during sugarcane harvest. The *Neurospora* lifecycle is shown in Fig. 1. *Neurospora* is a filamentous fungus. The vegetative thallus, known as a mycelium, is haploid and coenocytic (protoplasm within the filaments is multinucleate and undivided by cell walls). *Neurospora crassa* and *N. intermedia* are heterothallic (mating can only occur between genetically distinct individuals), and mating is controlled by a one-locus mating-type system, such that only strains with different mating types (*mat a* or *mat A*) can mate.

Neurospora is hermaphroditic, and under nutrient limited conditions, cultures of either mating type develop maternal structures called protoperithecia, which surround the female gametes (ascogonia). Fertilization occurs when a specialized receptive hypha (trichogyne) growing from an ascogonium fuses with a fertilizing element, such as a conidium (asexual spore) or any other cell from an individual of opposite mating type (Fig. 1, A–B). Trichogyne attraction to fertilizing elements is mediated by mating-type-specific expression of pheromones (Bistis, 1981). Upon fertilization, the protoperithecium grows into a perithecium (fruitbody) inside which the ascospores (sexual spores) will develop. There is an extended heterokaryotic stage, during which nuclei from the fertilizing male strain proliferate and travel through the trichogyne to the ascogonium. There, coordinated cell and nuclear divisions package pairs of male and female nuclei into special cells (asci), wherein karyogamy finally occurs, and meiosis follows immediately (Fig. 1, C–F). The four products of meiosis undergo a mitotic division, yielding eight haploid nuclei per ascus cell, and these nuclei give rise to ascospores, the sexual progeny (Fig. 1, G–I). Dozens of independent karyogamy/meiosis events within each fruitbody give rise to hundreds of different ascospore genotypes. A mycelium growing in a 9-cm Petri plate can support hundreds of fruitbodies, each of which arises from an independent fertilization event. At maturity, the flask-shaped fruitbodies have a 'beak' with an apical pore (ostiole) through which melanized, maturing ascospores will be forcibly ejected (Fig. 1, J).

Neurospora crassa and *N. intermedia* populations are largely outbreeding. Heterothallic fungi that have lost forcible discharge of sexual spores [e.g. *Saccharomyces* sp. (Tsai *et al.*, 2008) and *Microbotryum* sp. (Giraud *et al.*, 2008)] frequently inbreed by intersibling mating. Heterothallic *Neurospora* species, however, retain forcible discharge of ascospores, which, in combination with passive dispersal of dormant ascospores between fires and extensive vegetative growth of the haploid on burnt substrates prior to mating (Pandit & Maheshwari, 1996), greatly reduces the potential for inbreeding. Heterothallic *Neurospora* populations are highly genetically polymorphic and carry a high genetic load of deleterious diplophase recessive alleles, so that their population

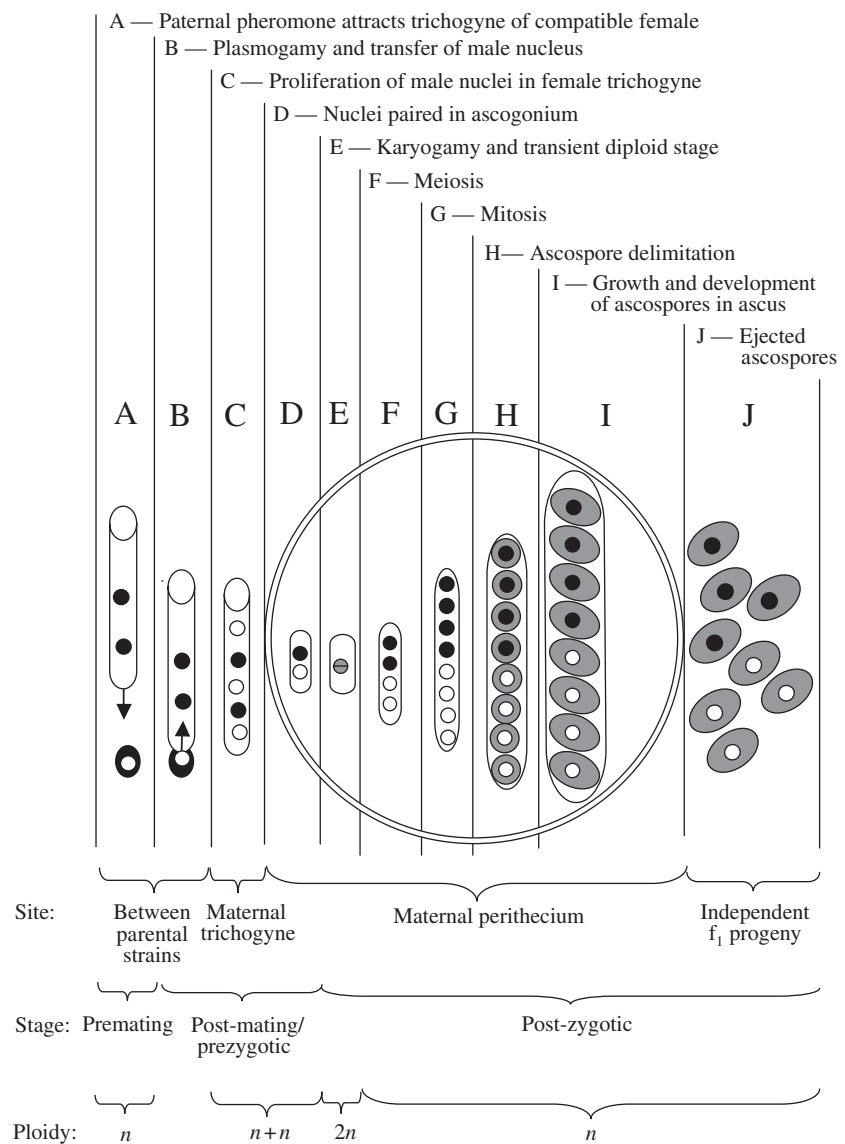


Fig. 1 Mating and reproduction in heterothallic *Neurospora*. Black and white nuclei represent nuclei with opposite mating types (*mat a* or *mat A*). Black, white and stippled cells represent paternal, maternal and progeny cells, respectively. Events illustrated in parts D, E, F, G, H and I on the figure occur within the developing fruitbody, as indicated by the enclosing circle. The site of potential reproductive isolation barriers, the stage in the sexual cycle, and the ploidy of the reproductive nuclei are indicated below the figure.

structures resemble those of obligately outbreeding animals and plants (Spieth, 1975; Leslie & Raju, 1985; Perkins & Turner, 1988; Dettman *et al.*, 2003a; Dettman & Taylor, 2004).

Strains, culturing and crossing conditions

Strains are listed in Table 1. These 53 strains, a subset of those used in Dettman *et al.* (2003a,b), comprise members of two *N. crassa* clades (NcA, from Africa and the Caribbean Basin; and NcC, from India) and one *N. intermedia* clade (NiA, from East Asia, India, and the Caribbean Basin). Strains were obtained from either the Fungal Genetics Stock Center (FGSC) or the Perkins Strain Collection, which is now housed by the FGSC. Culture and crossing conditions are described in detail in Dettman *et al.* (2003b). Briefly, strain stocks were stored

as frozen, conidiated cultures in minimal medium slants (Vogel, 1964). Conidiating asexual cultures were grown from conidia transferred from frozen stocks to fresh minimal medium slants and incubated at 34 °C.

Crosses were performed at 25 °C in synthetic cross medium slants (Westergaard & Mitchell, 1947) to promote protoperithecial (female sexual) development as well as conidiation. For each cross, an *N. crassa* and an *N. intermedia* strain were cultured separately from conidia collected from growing, asexual cultures. After 5 days, protoperithecial development was confirmed, and cultures were cross-fertilized with conidia collected via a wetted loop from one partner and transferred to the surface of the other culture. Each cross was performed reciprocally, testing both parents in male (fertilizing) and female (protoperithecial) roles. Two hundred thirty-two (232) of the possible 344 mating-type compatible

Table 1 Strains of *Neurospora* used in this study.*

Strain numbers†						Geographic location	
D	FGSC	Old FGSC	Perkins	Mating type	Species‡	Region§	Collection site
140	8900	430		A	<i>Neurospora crassa</i> (NcA)	Africa	Adiopodoume, Ivory Coast
68	8828	4825	3681	A	<i>N. crassa</i> (NcA)	Africa	Tiassale, Ivory Coast
69	8829	4826	3684	a	<i>N. crassa</i> (NcA)	Africa	Tiassale, Ivory Coast
59	8819	3427		a	<i>N. crassa</i> (NcA)	Carib. Basin	Carrefour Dufort, Haiti
62	8822	4824	3491	A	<i>N. crassa</i> (NcA)	Carib. Basin	Carrefour Mme. Gras, Haiti
144	8904	1131		A	<i>N. crassa</i> (NcA)	Carib. Basin	Cristobal, Panama
115	8875	4480		A	<i>N. crassa</i> (NcA)	Carib. Basin	Franklin, Louisiana
116	8876	4481		a	<i>N. crassa</i> (NcA)	Carib. Basin	Franklin, Louisiana
23	8783	3970	1409	A	<i>N. crassa</i> (NcA)	Carib. Basin	Homestead, Florida
27	8787	1417		A	<i>N. crassa</i> (NcA)	Carib. Basin	Homestead, Florida
85	8845	4130		a	<i>N. crassa</i> (NcA)	Carib. Basin	Kabah, Yucatan, Mexico
60	8820	4712	3433	a	<i>N. crassa</i> (NcA)	Carib. Basin	Leogane, Haiti
143	2489, 8903	987		A	<i>N. crassa</i> (NcA)	Carib. Basin	Marrero, Louisiana
88	8848	4150		a	<i>N. crassa</i> (NcA)	Carib. Basin	Sayil, Yucatan, Mexico
91	8851	4155		A	<i>N. crassa</i> (NcA)	Carib. Basin	Uman, Yucatan, Mexico
90	8850	4154		A	<i>N. crassa</i> (NcA)	Carib. Basin	Uxmal, Yucatan, Mexico
42	8802	8198	2543	a	<i>N. crassa</i> (NcC)	India	Madurai, Tamil Nadu
104	8864	4359		a	<i>N. crassa</i> (NcC)	India	Madurai, Tamil Nadu
105	8865	4360		A	<i>N. crassa</i> (NcC)	India	Madurai, Tamil Nadu
98	8858	4333		A	<i>N. crassa</i> (NcC)	India	Mallilnatham, Tamil Nadu
99	8859	4334		A	<i>N. crassa</i> (NcC)	India	Mallilnatham, Tamil Nadu
100	8860	8203	4335	a	<i>N. crassa</i> (NcC)	India	Mallilnatham, Tamil Nadu
103	8863	4358		a	<i>N. crassa</i> (NcC)	India	Mallilnatham, Tamil Nadu
106	8866	4361		a	<i>N. crassa</i> (NcC)	India	Rameshwaram, Tamil Nadu
107	8867	4362		A	<i>N. crassa</i> (NcC)	India	Rameshwaram, Tamil Nadu
73	8833	6263	3770	a	<i>Neurospora intermedia</i>	Africa	Adiopodoume, Ivory Coast
81	8841	6276	3852	a	<i>N. intermedia</i>	Africa	Bouanza, Congo
79	8839	4274	3839	a	<i>N. intermedia</i>	Africa	Madingo, Congo
83	8843	6286	3932	A	<i>N. intermedia</i>	Africa	Makokou, Gabon
141	8901	434		A	<i>N. intermedia</i>	Africa	Monrovia, Liberia
65	8825	6254	3540	A	<i>N. intermedia</i>	Africa	Yopougon, Ivory Coast
66	8826	6255	3543	a	<i>N. intermedia</i>	Africa	Yopougon, Ivory Coast
64	8824	6251	3495	A	<i>N. intermedia</i>	Carib. Basin	Carrefour Mme. Gras, Haiti
16	8776	3213	831	a	<i>N. intermedia</i>	Carib. Basin	Fred, Texas
22	8782	1408		a	<i>N. intermedia</i>	Carib. Basin	Homestead, Florida
25	8785	1413		a	<i>N. intermedia</i>	Carib. Basin	Homestead, Florida
26	8786	1415		A	<i>N. intermedia</i>	Carib. Basin	Homestead, Florida
122	8882	1543	8045	A	<i>N. intermedia</i>	Carib. Basin	Puerto Cortes, Honduras
52	8812	5369	2938	A	<i>N. intermedia</i>	East Asia	Ban Khao Yai, Thailand
7	8767	1792	142	A	<i>N. intermedia</i>	East Asia	Bogor, Java
51	8811	8199	2632	A	<i>N. intermedia</i>	East Asia	Georgetown, Malaya (Penang)
142	8902	435		A	<i>N. intermedia</i>	East Asia	Levuka, Fiji
43	8803	2544		a	<i>N. intermedia</i>	India	Konappatti, Tamil Nadu
44	8804	5344	2546	A	<i>N. intermedia</i>	India	Konappatti, Tamil Nadu
108	8868	4363		A	<i>N. intermedia</i>	India	Madurai, Tamil Nadu
109	8869	4364		a	<i>N. intermedia</i>	India	Madurai, Tamil Nadu
127	8887	M105		a	<i>N. intermedia</i>	India	Maddur, Karnataka
128	8888	M110		A	<i>N. intermedia</i>	India	Maddur, Karnataka
129	8889	M14		A	<i>N. intermedia</i>	India	Maddur, Karnataka
130	8890	M17		a	<i>N. intermedia</i>	India	Maddur, Karnataka
101	8861	4336		A	<i>N. intermedia</i>	India	Mallilnatham, Tamil Nadu
47	8807	5345	2552	a	<i>N. intermedia</i>	India	Rameshwaram, Tamil Nadu

Table 1 (Continued)

Strain numbers†						Geographic location	
D	FGSC	Old FGSC	Perkins	Mating type	Species‡	Region§	Collection site
48	8808	2554		A	<i>N. intermedia</i>	India	Rameshwaram, Tamil Nadu

FGSC, Fungal Genetics Stock Center.

*Table adapted from Dettman *et al.*, 2003b.

†Cross reference of strain numbers from different collections. D numbers are as assigned in Dettman *et al.*, 2003a. Old FGSC numbers are for the progenitors of the strains, which have been subcultured from a single conidium to obtain the strains that we used. Perkins numbers are for the progenitors in the Perkins collection (now curated by FGSC).

‡As determined in Dettman *et al.*, 2003a; with infraspecific subgroups in parentheses. All *N. intermedia* are in clade NiA.

§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.

N. crassa × *N. intermedia* pairs (67%) were mated reciprocally for a total of 464 reciprocal crosses (Fig. 2). Hybrid fruitbody development was evaluated 10 days after fertilization.

Biogeography of reproductive isolation

Crossing data are from Dettman *et al.* (2003b). Crosses were considered sympatric if parental strains were obtained from the same regional population (i.e. East Asia, India, Africa or the Caribbean Basin). A previous comparison of reproductive isolation in allopatry and sympatry among five phylogenetic species of *Neurospora* investigated three different scales of sympatry: regional, subregional and local (Dettman *et al.*, 2003b). Evidence for significantly increased reproductive isolation in sympatry was observed at all three scales. Given this result, we analyze regional sympatry to maximize the number of crosses categorized as sympatric and permit allopatric/sympatric contrasts of appropriately large samples.

Hybrid fruitbody development was coded on a four-category scale: 0, sterile, no perithecia produced; 1, incompletely developed perithecia, no beak or ostiole present; 2, well-developed perithecia, but no ascospores ejected; and 3, ascospores ejected. Fruitbody development score was the response variable in all of our analyses and was considered continuous because it reflects an underlying developmental continuum (Sokal & Rohlf, 1995).

The influence of geography (sympatric or allopatric), maternal species (*N. crassa* or *N. intermedia*), the interaction of geography and maternal species, and *N. crassa* and *N. intermedia* populations on fruitbody development were evaluated using analysis of covariance (ANCOVA) in a model that also incorporated *N. crassa* and *N. intermedia* strain identities as random variables to control for strain specific effects.

To illustrate the effect of sympatry on *N. crassa* maternal and *N. intermedia* maternal crosses, we compared mean fruitbody development in the four geography/mating-role classes (allopatric *N. crassa* maternal,

sympatric *N. crassa* maternal, allopatric *N. intermedia* maternal, and sympatric *N. intermedia* maternal) using Wilcoxon two-sample tests.

To assess among population variation of hybrid inter-fertility, fruitbody development in allopatric crosses was compared across populations of both species and in both mating roles. Fruitbody development of sympatric crosses was also compared across populations of both species and in both mating roles. Then, for each population and mating role, fruitbody development in allopatric and sympatric crosses was compared using a Wilcoxon two-sample test to test the hypothesis that sympatric development is lower than allopatric development.

We tested the hypothesis that enhanced sympatric barriers would be more strongly associated with *N. crassa* and the maternal role. First, for each population and mating role, we performed a series of pairwise contrasts of fruitbody development in sympatric crosses to fruitbody development in the population's crosses with strains from each different allopatric population (Wilcoxon two-sample tests). Based on the results of these pairwise comparisons, we used the Fisher exact test to determine whether diminished sympatric fruitbody development is significantly associated with the mating role (maternal) and species (*N. crassa*) suffering the greatest costs during allopatric (baseline) hybridization.

To investigate the relationship between a strain's allopatric and sympatric interfertility, relative sympatric fruitbody development was regressed against mean allopatric fruitbody development for each of 34 *Neurospora* strains involved in sympatric hybrid matings. Relative sympatric fruitbody development was calculated as follows: (mean sympatric fruitbody score + 1)/(mean allopatric fruitbody score + 1).

Finally, we tested whether sympatric matings were associated with each of three the barriers: 0, no fruitbody development; 1, no ostiole; 2, failure to sporulate. Survival analysis using Fisher exact tests was used to compare numbers of matings arrested at or proceeding through each isolation barrier for each population and mating role. Fisher exact tests were computed at <http://www.langsrud.com/fisher.htm>. All other statistics were

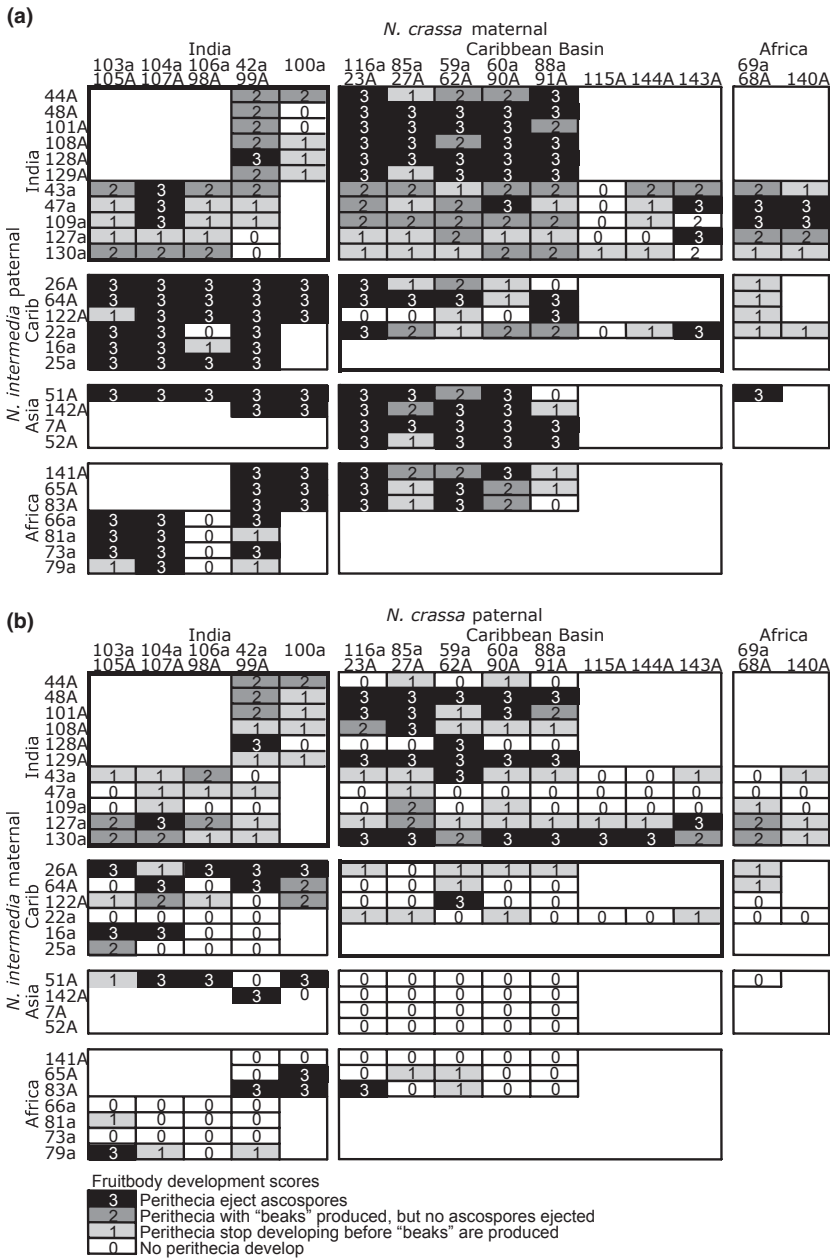


Fig. 2 Matrices displaying the fruitbody development scores of 232 *Neurospora crassa* × *Neurospora intermedia* crosses (464 reciprocal matings). This figure is adapted from Dettman *et al.*, 2003b, where these data were originally published. Rows represent the 28 *N. intermedia* strains, and columns represent the 25 *N. crassa* strains, with strain numbers and mating type (A or a) along the row and column headings of the matrices. Each number within a matrix cell indicates the fruitbody development score for the matings. Although, columns can contain data for two *N. crassa* strains of opposite mating type, each cell only contains the datum for a single mating because the *N. intermedia* strain on the corresponding row can only mate with the *N. crassa* strain of the opposite mating type. The matrix cells have been shaded in proportion to the fruitbody development score. Additional row and column headings indicate the species, mating role and geographical source of the strains. Sympatric matings are outlined with a bold border. Only data for interspecific crosses is presented. An interspecific matrix cell without entry indicates that the cross was not performed. (a) *N. crassa* maternal × *N. intermedia* paternal; (b) *N. crassa* paternal × *N. intermedia* maternal.

performed using JMP, version 5.0.1a (SAS Institute, Cary, NC, USA). Throughout, the false discovery rate for multiple tests was controlled using the Benjamini–Hochberg method, and adjusted *P* values were calculated using the R package multtest.

Results

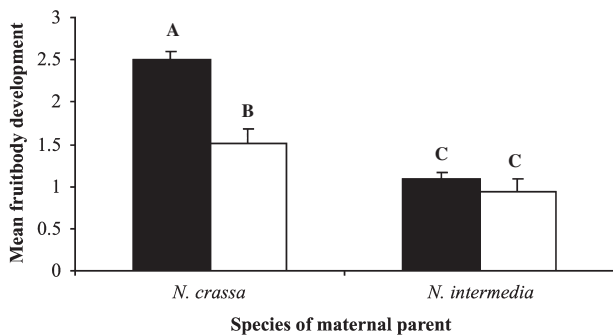
Geography, maternal species and parental population affect hybrid fruitbody development

Using ANCOVA to analyze *N. crassa* × *N. intermedia* interfertility data from Dettman *et al.* (2003b), we model the

effects of geography, mating role and parental population on hybrid fruitbody development (Table 2). The model is significant and explains 40% of variance in fruitbody development ($F_{53,410} = 5.65$, $P < 0.0001$, $R^2 = 0.40$). *Neurospora crassa* population, *N. intermedia* population, geography, maternal species and the interaction of geography and maternal species all significantly influence fruitbody development. Comparison of mean fruitbody development across the four mating-role/geography classes (allopatric *N. crassa* maternal, sympatric *N. crassa* maternal, allopatric *N. intermedia* maternal, and sympatric *N. intermedia* maternal) shows the asymmetry of reciprocal *N. crassa* × *N. intermedia* crosses

Table 2 Analysis of covariance for the influence of parental strain populations, the allopatry or sympatry of crosses, and the species performing the maternal role in *Neurospora crassa* × *Neurospora intermedia* crosses.

Cross type	Source	DF	SS	MS	F Ratio	Prob > F	R ²
All crosses (N = 464)	Model	53	271.46	5.12	5.65	< 0.0001	0.40
	Error	410	371.51	0.91			
	Geography	1	27.73		30.60	< 0.0001	
	Maternal species	1	67.71		74.73	< 0.0001	
	Geography × maternal species	1	8.40		9.27	0.0025	
	<i>N. intermedia</i> population	3	15.21		5.59	0.0009	
	<i>N. crassa</i> population	2	9.39		5.18	0.006	

**Fig. 3** Fruitbody development in reciprocal allopatric (black bars, $N = 177$) and sympatric (white bars, $N = 55$) *Neurospora crassa* × *Neurospora intermedia* matings. Mean fruitbody development scores that do not significantly differ from each other share the same letter code (A, B or C).

and the effect of geography (Fig. 3). Crosses with *N. crassa* maternal yield significantly greater fruitbody development. Sympatry has a significant negative effect on hybrid fruitbody development in *N. crassa* maternal crosses ($W = 6741.5$, $P = 0.000040$), but in *N. intermedia* maternal crosses, fruitbody development is not significantly different for sympatric and allopatric crosses ($W = 4650$, $P = 0.59$).

To investigate patterns of reproductive isolation among different regional populations of *N. crassa* and *N. intermedia*, we assess baseline, allopatric, reproductive isolation barriers to hybrid fruitbody development, across mating roles and across populations of the two species using the Wilcoxon two-sample test (Table 3). Allopatric crosses in which *N. crassa* performed the maternal role and *N. intermedia* performed the paternal role consistently have higher mean fruitbody development scores than

Table 3 Interfertility of allopatric and sympatric *Neurospora crassa* × *Neurospora intermedia* crosses by population.

Species	Population	No. of allopatric pairs	No. of sympatric pairs	Mating role	Allopatric interfertility mean ± SE*	Sympatric interfertility mean ± SE†	W‡	P
<i>N. crassa</i>	Africa	16	nd	Female	1.81 ± 0.23 ^{CD}			
				Male	0.63 ± 0.18 ^{FG}			
	Carib.	105	23	Female	2.11 ± 0.09 ^C	1.52 ± 0.25 ^W	1547	0.026
				Male	1.01 ± 0.12 ^F	0.52 ± 0.15 ^X	1401.5	0.13
	India	56	32	Female	2.55 ± 0.13 ^A	1.50 ± 0.16 ^W	1440	0.000060
				Male	1.13 ± 0.18 ^{EF}	1.22 ± 0.15 ^W	799.5	0.86
<i>N. intermedia</i>	Africa	37	nd	Female	0.57 ± 0.17 ^{FG}			
				Male	2.11 ± 0.19 ^{BC}			
	Carib.	32	23	Female	1.16 ± 0.23 ^{DEF}	0.52 ± 0.15 ^X	453	0.094
				Male	2.47 ± 0.17 ^{AB}	1.52 ± 0.25 ^W	534	0.0033
	East Asia	28	nd	Female	0.46 ± 0.20 ^G			
				Male	2.68 ± 0.15 ^A			
	India	80	32	Female	1.35 ± 0.14 ^{DE}	1.22 ± 0.15 ^W	1305.5	0.50
				Male	2.03 ± 0.11 ^C	1.50 ± 0.16 ^W	1680	0.0094

Carib., Caribbean Basin.

*Allopatric means that share any letter code (A, B, C, D, E, F or G) do not differ significantly, as determined by two-tailed Wilcoxon two-sample tests using the normal approximation ($P > 0.05$ after Benjamini–Hochberg correction for multiple hypothesis testing).

†Sympatric means that share any letter code (W or X) do not differ significantly, as determined by two-tailed Wilcoxon two-sample tests using the normal approximation ($P > 0.05$ after Benjamini–Hochberg correction).

‡One-tailed Wilcoxon two-sample tests using the normal approximation were performed to test the hypothesis that allopatric interfertility is greater than sympatric interfertility. P values reflect Benjamini–Hochberg correction. P values < 0.05 are bold.

nd, No sympatric matings were performed for the population.

crosses with reciprocal mating roles, and this result is generally significant except that African *N. crassa* maternal crosses (1.81) do not differ significantly from Caribbean *N. intermedia* maternal crosses (1.16) and Indian *N. intermedia* maternal crosses (1.35) (Table 3). Higher barriers in *N. intermedia* maternal crosses indicate that *N. crassa* invests more in hybrid fruitbodies. There are significant differences among populations. For example, the highest mean fruitbody development score for allopatric *N. crassa* maternal crosses is seen for the Indian *N. crassa* population (2.55), which is significantly greater than those of African and Caribbean *N. crassa* populations (1.81 and 2.11, respectively, Table 3).

We next investigated population effects in sympatric crosses involving strains from the two regions that contributed sympatric crosses, Caribbean Basin or India. Mean fruitbody development scores for sympatric crosses range from 0.52 to 1.52. Caribbean *N. intermedia* maternal crosses, with the lowest score of 0.52, show significantly worse fruitbody development than Caribbean *N. crassa* maternal crosses, but sympatric fruitbody development in Indian *N. crassa* maternal crosses and Indian *N. intermedia* maternal crosses do not differ significantly from each other, or from the sympatric Caribbean *N. crassa* maternal crosses (Table 3).

We compare fruitbody development in allopatric and sympatric matings for the two regions that contributed sympatric crosses (Caribbean Basin and India) using

Wilcoxon two-sample tests. In *N. crassa* maternal and *N. intermedia* paternal matings, sympatric crosses show significantly worse fruitbody development than allopatric crosses (Table 3). In contrast, there is no significant difference between sympatric and allopatric crosses when *N. crassa* acts paternally or *N. intermedia* acts maternally.

Sympatry-associated reinforcement and the cost of hybridization

Coyne & Orr (2004) predict that reinforcement of reproductive isolation barriers in sympatry should be greatest in the sex (or mating role in hermaphrodites) and species suffering the greatest cost to hybridization. In *Neurospora*, maternal individuals bear almost all of the cost of hybridization. Patterns of baseline allopatric reproductive isolation reveal that in *N. crassa* × *N. intermedia* crosses, hybridization costs are highest for *N. crassa* maternal individuals, because these individuals invest substantially in the production of predominantly inviable progeny. Therefore, we predict that reinforcement of reproductive isolation barriers should be expressed more strongly by *N. crassa* in the maternal role.

To test this prediction, we determine how often allopatric fruitbody development is significantly better than sympatric fruitbody development and whether the tendency towards sympatric deficiency is more strongly associated with *N. crassa* and the maternal role. First, for

Table 4 Interfertility of sympatric and allopatric *Neurospora crassa* × *Neurospora intermedia* crosses by population.

Reference species	Population	Mating role of reference species	Mean ± SE sympatric interfertility (number of crosses)	Allopatric population	Mean ± SE allopatric interfertility (number of crosses)	W	<i>P</i> *
<i>N. crassa</i>	Carib.	Female	1.52 ± 0.25 (23)	Africa	2.00 ± 0.26 (15)	212	0.19
				East Asia	2.55 ± 0.20 (20)	341.5	0.0069
				India	2.01 ± 0.11 (70)	993.5	0.084
		Male	0.52 ± 0.15 (23)	Africa	0.40 ± 0.21 (15)	146.5	0.97
				East Asia	0.00 ± 0.00 (20)	130	> 0.99
				India	1.43 ± 0.15 (70)	1125	0.0069
	India	Female	1.50 ± 0.16 (32)	Africa	2.18 ± 0.27 (22)	488	0.018
				East Asia	3.00 ± 0.00 (7)	210	0.00066
		Male	1.22 ± 0.15 (32)	Carib. Basin	2.74 ± 0.15 (27)	742	0.0000045
				Africa	0.68 ± 0.25 (22)	209.5	> 0.99
<i>N. intermedia</i>	Carib.	Female	0.52 ± 0.15 (23)	East Asia	1.86 ± 0.55 (7)	143.5	0.19
				Carib. Basin	1.30 ± 0.25 (27)	426.5	0.83
				Africa	0.40 ± 0.25 (5)	54.5	0.85
		Male	1.52 ± 0.25 (23)	India	1.30 ± 0.25 (27)	398.5	0.082
				Africa	1.00 ± 0.00 (5)	45	0.97
				India	2.74 ± 0.15 (27)	489	0.00033
	India	Female	1.22 ± 0.15 (32)	Africa	0.80 ± 0.25 (10)	117	> 0.99
				Carib. basin	1.43 ± 0.15 (70)	1188.5	0.93
		Male	1.50 ± 0.16 (32)	Africa	2.10 ± 0.28 (10)	216	0.084
				Carib. basin	2.01 ± 0.11 (70)	1464	0.016

Carib., Caribbean Basin.

*One-tailed Wilcoxon tests using the normal approximation test the hypothesis that allopatric interfertility is greater than sympatric interfertility. *P* values reflect Benjamini–Hochberg correction for multiple hypothesis testing. *P* values < 0.05 are bold.

each population and mating role, we perform a series of Wilcoxon two-sample tests of whether fruitbody development in its sympatric crosses is lower than fruitbody development in its crosses with each allopatric population (Table 4). Then, we determine whether or not significant contrasts (from Table 4) between sympatric and allopatric fruitbody development are associated with (1) maternal vs. paternal mating roles and (2) isolation barriers in *N. crassa* vs. *N. intermedia* (Table 5).

For the *N. crassa* India population, fruitbody development in sympatric matings is significantly worse than in crosses to each of three allopatric populations when *N. crassa* is the maternal parent, but not significantly worse with any allopatric population when *N. crassa* acts paternally (Table 5). This observation represents an association between strong sympatric isolation barriers and the maternal role for *N. crassa* India. This association, which is only marginally significant after Benjamini–Hochberg correction for multiple hypothesis testing (Fisher exact test, $P = 0.07$, Table 5), is consistent with one of our two main predictions. However, there is no association between reinforced barriers and the maternal role in the Caribbean *N. crassa* population or in either *N. intermedia* population (Table 5).

We also test for an association between enhanced sympatric barriers and *N. crassa*, the species bearing the greatest cost of hybridization. During the maternal role, sympatric defects are markedly pronounced in *N. crassa* (four of six contrasts are significant), but absent in *N. intermedia* (zero of four contrasts), so that the association of strong sympatric barriers with *N. crassa* is marginally significant (Fisher exact test, $P = 0.07$, Table 5). This finding is consistent with our second main prediction. However, an association between strong

sympatric barriers and *N. crassa* is not seen for the paternal role.

Linear regression of relative sympatric maternal fruitbody development, $(\text{mean sympatric} + 1)/(\text{mean allopatric} + 1)$, on mean allopatric fruitbody development for 34 *N. crassa* and *N. intermedia* strains yields a significant, negative relationship ($F_{1,32} = 27.57$, $P < 0.0001$, $R^2 = 0.46$, Fig. 4), demonstrating that as baseline maternal investment in allopatric crosses increases, relative sympatric interfertility decreases.

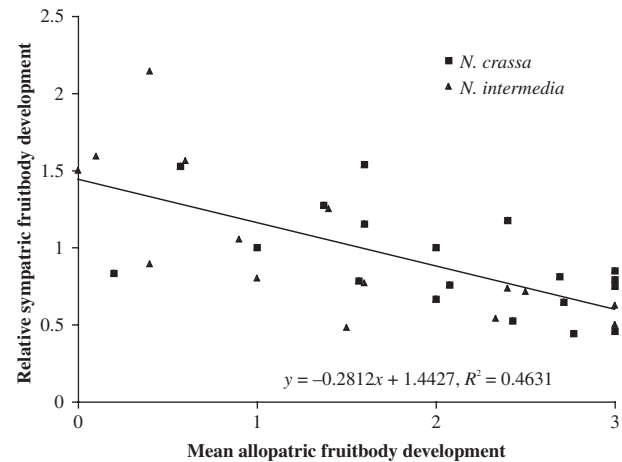


Fig. 4 Mean relative sympatric fruitbody development regressed against mean allopatric fruitbody scores for 34 *Neurospora crassa* (black squares) and *Neurospora intermedia* (black triangles) strains in *N. crassa* maternal \times *N. intermedia* paternal crosses. Overlapping points have been jittered. Relative sympatric fruitbody development was calculated as $(\text{mean sympatric fruitbody development score} + 1)/(\text{mean allopatric fruitbody development score} + 1)$.

Table 5 Asymmetry of significantly reinforced sympatric reproductive isolation in *Neurospora crassa* \times *Neurospora intermedia* crosses and the association of reinforcement with female vs. male interfertility and *N. crassa* vs. *N. intermedia* interfertility.

Reference population	Female inter-fertility lower in sympatry*		Male inter-fertility lower in sympatry		Reference mating role	<i>N. crassa</i> inter-fertility lower in sympatry		<i>N. intermedia</i> inter-fertility lower in sympatry		Predicted association observed? [†]	P [‡]
	Yes	No	Yes	No		Yes	No	Yes	No		
<i>N. crassa</i> Carib.	1	2	1	2						No	
<i>N. crassa</i> India	3	0	0	3						Yes	0.07
<i>N. intermedia</i> Carib.	0	2	1	1						No	
<i>N. intermedia</i> India	0	2	1	1						No	
					Female	4	2	0	4	Yes	0.07
					Male	1	5	2	2	No	

Carib., Caribbean Basin.

*Throughout, numbers of significant and nonsignificant contrasts are tallies of the one-tailed Wilcoxon two-sample tests presented in Table 4.

[†]Theory predicts that reinforcement selection should be stronger for the mating role and species that invests the most during maladaptive hybridization. The association of significantly reinforced sympatric reproductive isolation with the female role and with *N. crassa* inter-fertility is predicted.

[‡]When the predicted association is present, significance was calculated by the one-tailed Fisher exact test. P values reflect Benjamini–Hochberg correction for multiple hypothesis testing.

Table 6 Allopatric and sympatric *Neurospora crassa* × *Neurospora intermedia* crosses arresting at or proceeding through three stages of fruitbody development.

Species	Population	Mating role	Reproductive isolation category	Allopatric		Sympatric		<i>P</i> *
				Arrested	Proceeded	Arrested	Proceeded	
<i>N. crassa</i>	Carib.	Female	0 (no fruitbody development)	7	98	6	17	0.039
			1 (no ostiole)	22	76	6	11	0.34
			2 (failure to sporulate)	28	48	4	7	0.76
		Male	0 (no fruitbody development)	53	52	13	10	0.51
			1 (no ostiole)	22	30	9	1	0.022
			2 (failure to sporulate)	6	24	0	1	1.00
	India	Female	0 (no fruitbody development)	5	51	4	28	0.53
			1 (no ostiole)	5	46	12	16	0.011
			2 (failure to sporulate)	0	46	12	4	0.0000002
		Male	0 (no fruitbody development)	29	27	6	26	1.00
			1 (no ostiole)	7	20	15	11	0.045
			2 (failure to sporulate)	4	16	9	2	0.011
<i>N. intermedia</i>	Carib.	Female	0 (no fruitbody development)	15	17	13	10	0.47
			1 (no ostiole)	5	12	9	1	0.015
			2 (failure to sporulate)	4	8	0	1	1.0000
		Male	0 (no fruitbody development)	1	31	6	17	0.045
			1 (no ostiole)	7	24	6	11	0.40
			2 (failure to sporulate)	0	24	4	7	0.022
	India	Female	0 (no fruitbody development)	5	75	4	28	0.37
			1 (no ostiole)	19	56	12	16	0.14
			2 (failure to sporulate)	25	31	12	4	0.066
		Male	0 (no fruitbody development)	26	54	6	26	1.00
			1 (no ostiole)	23	31	15	11	0.28
			2 (failure to sporulate)	8	23	9	2	0.011

*The significance of an association between arrested development and cross sympatry was calculated via one-tailed Fisher exact tests. *P* values reflect Benjamini–Hochberg correction. *P* values < 0.05 are bold. Carib., Caribbean Basin.

Postmating barriers are stronger in sympatry

Survival analysis of sympatric vs. allopatric crosses shows that postmating barriers are significantly enhanced in sympatry for six of the eight population/mating roles examined ($P < 0.05$, Fisher exact tests, Table 6). Fruitbody development was more likely to arrest before ostiole development and/or before sporulation during sympatric matings in all populations and mating roles except maternal Caribbean *N. crassa* and maternal Indian *N. intermedia*. For maternal Caribbean *N. crassa* crosses, sympatry was significantly associated only with the absence of detectable fruitbody development (Fisher exact test, $P = 0.039$), which can either represent a premating barrier or a very early postmating barrier. Sympatric Caribbean *N. intermedia* paternal matings were also associated with failure before observable fruitbody development, as well as postmating arrest before sporulation (Fisher exact tests, $P = 0.045$ and $P = 0.0022$, respectively).

Discussion

Biogeographic evidence for reinforcement

Reinforcement selection should drive evolution of barriers more strongly in species and sexes that suffer

fitness burdens from hybridization (Coyne & Orr, 2004). *Neurospora* offspring are nurtured in fruitbodies that develop supported entirely by the female thallus, and a maternal thallus cannot mate again after an initial round of fruitbody development has occurred (Howe & Prakash, 1969). Although a particular fertilizing element (a single conidium for example) can perform the paternal function only once, the fact that many millions of conidia can be released by a single thallus shows that the male investment in any instance of reproduction is miniscule.

Maternal investment in allopatric hybrid fruitbodies differs between reciprocal crosses. Hybrid fruitbody development is significantly greater on *N. crassa*. Asymmetry in maternal vs. paternal investment and in reciprocal matings mean that allopatric hybridization, which results in mostly inviable offspring, would be particularly costly to *N. crassa* strains performing the maternal role. Therefore, we expect enhanced sympatric barriers to evolve more strongly in *N. crassa* and to be expressed maternally. In this article, we present a detailed dissection of the best available data (Dettman *et al.*, 2003b) on *N. crassa* × *N. intermedia* interfertility to test a hypothesis of reinforced reproductive isolation. Focusing on premating and early postmating barriers, which are most likely to be under reinforcement

selection, we analyzed maternally vs. paternally expressed barriers in reciprocal crosses to test this hypothesis.

Consistent with our predictions, we found that the effect of geography on hybrid fruitbody development depends on the mating roles of the parents, so that significant barriers to sympatric fruitbody development are most often seen when *N. crassa* is maternal and *N. intermedia* is paternal. The negative relationship between mean allopatric female interfertility and relative sympatric interfertility in 34 *N. crassa* and *N. intermedia* strains also suggests that the intensity of reinforcement selection has been greater when the burden of hybridization is greater. The expected association between enhanced sympatric isolation and *N. crassa* barriers was observed for female fertility in pairwise comparisons of sympatric crosses vs. crosses to each allopatric population, whereas for paternal fertility, no such association was expected or observed. Similarly, the expected association of enhanced sympatric isolation and maternal mating role was observed for one of the two *N. crassa* populations (India), but not for any of the *N. intermedia* populations. Taken together, these results support the hypothesis that reinforcement selection on isolation barriers between sympatric populations of *N. crassa* and *N. intermedia* has occurred.

Evidence for reinforcement selection is stronger for the Indian *N. crassa* population, because the Caribbean *N. crassa* population failed to show the expected association between enhanced sympatric isolation and female barriers. Differences between the Caribbean and Indian populations of *N. crassa* could contribute to this discrepancy. First, mean fruitbody development scores for allopatric *N. crassa* Caribbean Basin maternal crosses are lower than for allopatric *N. crassa* Indian maternal crosses (2.11 and 2.55, respectively), and it is possible that stronger baseline barriers in the Caribbean *N. crassa* population has reduced the intensity of reinforcement selection. Second, the Indian *N. crassa* population constitutes a partially phylogenetically differentiated clade (the NcC clade of Dettman *et al.*, 2003b), whose range is limited to Tamil Nadu state in southern India, whereas the Caribbean Basin population is part of the trans-Atlantic NcA clade. Inasmuch as reinforcement selection represents a mode of local adaptation, its force will be opposed by gene flow. Migration between African and Caribbean populations could impede local adaptation to local populations of *N. intermedia*, whereas the endemism and partial genetic isolation of the NcC/Indian population should promote local adaptation. Third, the Indian population's sympatric crosses are actually more 'local', because the Indian region is geographically smaller than the Caribbean Basin region. Mean distance between collection sites of sympatric Indian parents is only 267 km, but 1087 km for Caribbean parents (mean distance between collection sites of parents in allopatric crosses is 13 168 km). We note that our experimental design will miss certain types of reinforceable barriers

(e.g. the timing of gamete production and receptivity on natural substrates or even cryptic substrate preferences) that could conceivably separate sympatric populations.

Reinforcement of postmating isolation barriers in *Neurospora*

Survival analysis of sympatric and allopatric *N. crassa* × *N. intermedia* matings shows that sympatric crosses encounter reinforced postmating isolation barriers. Postmating reinforcement is more likely to occur where there are constraints on the evolution of premating barriers (Coyne, 1974; Coyne & Orr, 2004). We know of no ecological or temporal barriers that isolate the *Neurospora* populations in this study, so mating pheromones would seem to be the logical target for reinforcement. However, fungi in *Sordaria*, a genus sister to *Neurospora*, produce pheromones with sequences identical to those in the *Neurospora* genus (Poeggeler *et al.*, 2000). We hypothesize that conservation of pheromone sequences has impeded evolution of premating isolation in *Neurospora*.

Reinforcement of postmating barriers increases parental fitness when there is substantial parental investment in the production and care of progeny and if the postmating barrier makes subsequent mating more likely (Coyne, 1974; Coyne & Orr, 2004). Significantly, fully fruited *Neurospora* individuals cannot be fertilized a second time (Howe & Prakash, 1969), but the early abortion of fruitbodies could preserve 'female' fertility of previously fertilized individuals. The inhibitory effect of fertilization at one portion of a maternal *Neurospora tetrasperma* mycelium was shown to increase with time so that 100% inhibition of sexual development is only achieved 2–2.5 days after fertilization (Howe & Prakash, 1969). This observation suggests that abortion during the earlier states of fruitbody development could preserve fertility of the maternal mycelium after hybridization, potentially permitting a subsequent conspecific mating.

It is generally assumed that reinforcement selection cannot drive evolution of hybrid sterility because once hybrid individuals reach sexual maturity and are out of parental care, increased hybrid fertility should increase the fitness of parents (Coyne, 1974; Coyne & Orr, 2004). However, reinforcement selection for hybrid sterility could occur in organisms like *Neurospora*, where maternal fruiting bodies nurture not only hybrid diploids but also their meiotic products, the haploid progeny. In *Neurospora*, reinforcement selection could drive the evolution of hybrid sterility if meiotic failure or the early inviability of meiotic products results in early abortion of fruitbodies so that female receptivity is preserved.

Reproductive success categories 1 and 2, involving incompletely developed fruitbodies and nonsporulating fruitbodies, respectively, are both postmating barriers, but we do not know whether the barriers occur during the heterokaryotic stage, karyogamy, the diploid stage,

meiosis, or ascospore development. We also do not know whether abnormal fruitbody development and sporulation is a byproduct of genomic incompatibilities and inviability in the heterokaryon, zygote or ascospores, or is because of a controlled disinvestment by maternal strains. In either case, the abortion of fruitbodies would be selectively advantageous if it preserves the fertility and fecundity of maternal individuals, allowing them to successfully mate subsequently with conspecifics (Coyne, 1974; Coyne & Orr, 2004).

Reinforcement in Ascomycete fungi

A recent comprehensive survey of the literature on reproductive isolation across 430 fungi concluded that

sympatric species show enhanced reproductive isolation in the phylum Basidiomycota, but not in the Ascomycota (Le Gac & Giraud, 2008). Noting the prevalence of pathogenic Ascomycetes in nature and in their data set, the authors propose that host specialization can permit sympatry of Ascomycete sibling species in the absence of strong reproductive isolation barriers (Giraud, 2006; Le Gac & Giraud, 2008). *Neurospora* species are not pathogenic and generally lack habitat specialization, and it is reasonable to speculate that sympatric selection on reproductive isolation barriers is stronger because of the absence of ecological barriers. However, examples of reinforcement-like reproductive isolation patterns in pathogenic Ascomycete fungi have also been reported (Harrington & McNew, 1998; Harrington & Wingfield,

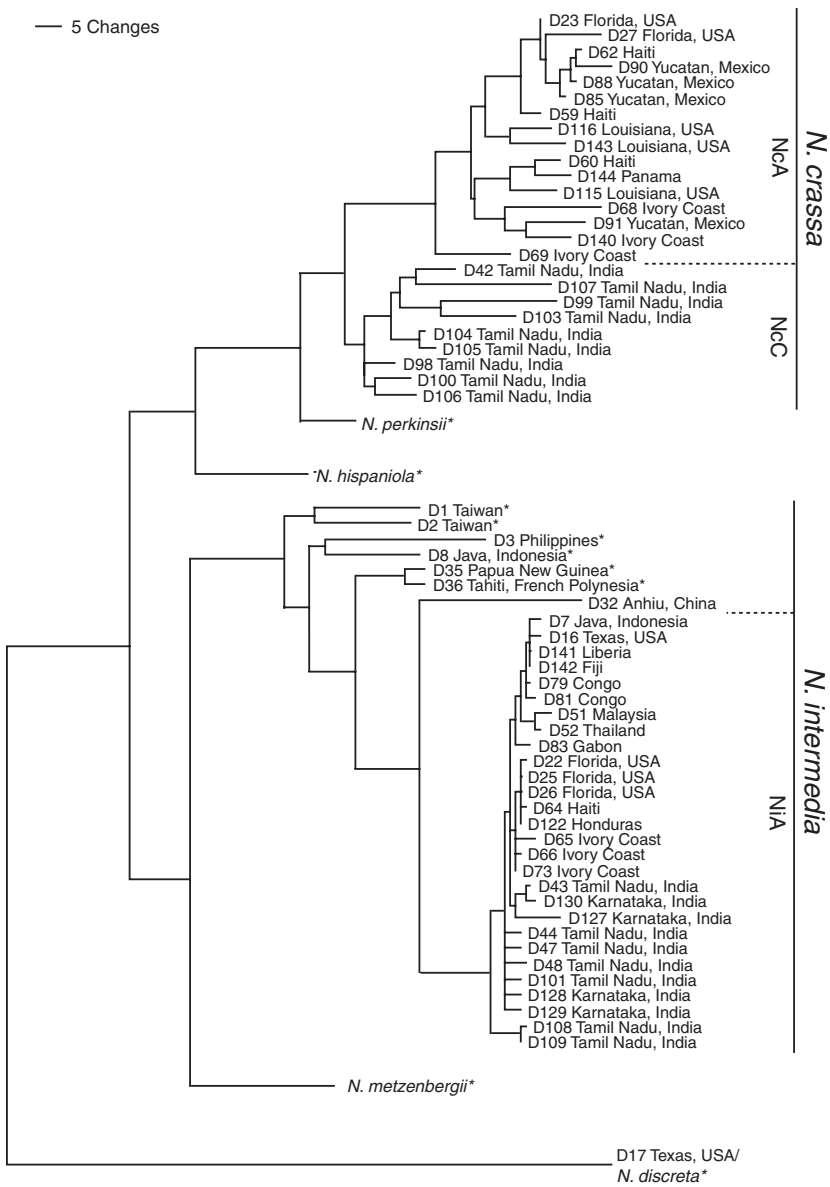


Fig. 5 Maximum parsimony phylogram adapted from Dettman *et al.*, 2003b. Phylogram was produced from the combined analysis of DNA sequences from four anonymous nuclear loci for a total of 2141 aligned nucleotides. Taxon labels at branch tips indicate strain number and geographical source or phylogenetic species, if italicized. Taxa with asterisks were not included in the present study. Sequence from multiple strains was analyzed for the asterisked species *Neurospora perkinsii*, *Neurospora hispaniola* and *Neurospora metzenbergii*, but the species have each been collapsed to a single branch for simplicity. NcA = *Neurospora crassa* clade A; NcC = *N. crassa* clade C; NiA = *Neurospora intermedia* clade A.

1998; Burnett, 2003). We propose that the absence of stronger sympatric barriers in Ascomycete, but not Basidiomycete, fungi reported by Le Gac and Giraud, arises, in part, from other differences in the reproductive biology of Basidiomycota and Ascomycota that have led researchers to use different kinds of mating assays for the two phyla.

In Ascomycota, the period between mating of haploid individuals and zygote formation is relatively short, and compatible laboratory matings of Ascomycota typically complete the reproductive cycle and yield sexual spores. In Basidiomycota, the period between fusion and zygote formation can be measured in years or even centuries (Smith *et al.*, 1992), a period in most cases too long for laboratory investigation. Therefore, although for Ascomycota both prezygotic and postzygotic reproductive barriers are readily observed in the laboratory, in Basidiomycota only the earliest, prezygotic barriers are typically assessed.

Reflecting this difference, Le Gac and Giraud's index of reproductive isolation for Basidiomycota was $1 - p$, where p is the proportion of strain pairs that show evidence of fusion of the mated partners. For Ascomycota, a five-category scale was used: '...1, 0.75, 0.5, 0.25 and 0 indicate, respectively, lack of ascus formation, asci without ascospores, abnormal ascospores, viable but sterile ascospores and fertile ascospores' (Le Gac & Giraud, 2008). Three of the five categories (0.5, 0.25 and 0) express ascospore quality, reflecting late postpopulation barriers, including fertility of the free-living, hybrid progeny, which are not likely to be the target of reinforcement. In fact, when we focus on early, presporulation barriers only – 'lack of ascus formation' and 'asci without ascospores' (categories 1 and 0.75, respectively) – we find that 42/94 (45%) sympatric vs. 7/27 (26%) allopatric species pairs exhibit early reproductive isolation barriers. The result that barriers most susceptible to reinforcement selection are in fact more prevalent than expected in sympatry ($\chi^2_1 = 17.218$, $P = 0.000033$), supports the hypothesis that reinforcement selection is a factor in the evolution of reproductive isolation barriers in the Ascomycota.

Phylogeography and evolution of reinforced barriers in Indian *Neurospora crassa*

We hypothesize that the common ancestor of *N. intermedia* and *N. crassa* was located in East Asia, because the most basal, extant strains of *N. intermedia* are from this region (Fig. 5). We propose that an early migration event to India permitted initial allopatric differentiation. Given the basal location of the *N. crassa* Indian clade within *N. crassa*, we propose that the establishment of an Indian population gave rise to modern *N. crassa*. Subsequent migrations of *N. crassa* from India to Africa and the Caribbean Basin permitted differentiation of Indian (NcC) and Afro-Caribbean (NcA) *N. crassa* clades. A later

migration of *N. intermedia* from East Asia to India, rapidly followed by range expansion to Africa and the Caribbean Basin, marked the beginning of secondary contact between *N. intermedia* and *N. crassa* in these regions, and the beginning of reinforcing selection.

Conclusion

Reproductive isolation is stronger between sympatric populations of *N. crassa* and *N. intermedia*. Enhanced sympatric reproductive isolation is marked by stronger *N. crassa* barriers and, in Indian populations, barriers that are expressed maternally. This finding is consistent with the prediction that evolution of barriers under reinforcing selection should occur in the species and sex suffering the burdens of hybridization. Reinforced isolation in these species occurs *via* defects in hybrid fruitbody development, suggesting that, although premating barriers are conventionally studied, postmating barriers can be susceptible to reinforcement selection in organisms with constrained evolution of premating mechanisms and significant parental investment. Questions on the prevalence and importance of reinforcement selection will best be answered by considering reinforcement's role in a wide diversity of organisms, and it is important to identify which traits are likely to be under reinforcing selection. We demonstrate this point by revisiting the data on reproductive isolation in Ascomycete fungi surveyed by Le Gac & Giraud (2008) and showing that presporulation barriers, in particular, are more common in sympatric species pairs vs. allopatric species pairs.

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