Title
Genetic architecture of a reinforced, postmating, reproductive isolation barrier between Neurospora species indicates evolution via natural selection.

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Abstract

A role for natural selection in reinforcing premating barriers is recognized, but selection for reinforcement of postmating barriers remains controversial. Organisms lacking evolvable premating barriers can theoretically reinforce postmating isolation, but only under restrictive conditions: parental investment in hybrid progeny must inhibit subsequent reproduction, and selected postmating barriers must restore parents’ capacity to reproduce successfully. We show that reinforced postmating isolation markedly increases maternal fitness in the fungus Neurospora crassa, and we detect the evolutionary genetic signature of natural selection by quantitative trait locus (QTL) analysis of the reinforced barrier. Hybrid progeny of N. crassa and N. intermedia are highly inviable. Fertilization by local N. intermedia results in early abortion of hybrid fruitbodies, and we show that abortion is adaptive because only aborted maternal colonies remain fully receptive to future reproduction. In the first QTL analysis of postmating reinforcement in microbial eukaryotes, we identify 11 loci for abortive hybrid fruitbody development, including three major QTLs that together explain 30% of trait variance. One of the major QTLs and six QTLs of lesser effect are found on the mating-type determining chromosome of Neurospora. Several reinforcement QTLs are flanked by genetic markers showing either segregation distortion or non-random associations with alleles at other loci in a cross between N. crassa of different clades, suggesting that the loci also are associated with local effects on same-species reproduction. Statistical analysis of the allelic effects distribution for abortive hybrid fruitbody development indicates its evolution occurred under positive selection. Our results strongly support a role for natural selection in the evolution of reinforced postmating isolation in N. crassa.

Introduction

The evolution of reproductive isolation between diverging lineages is a critical step in speciation. Most reproductive isolation barriers between taxa evolve as side effects of changes resulting from within-population processes, including, as Darwin recognized, natural selection [1]. Wallace reasoned that natural selection against maladaptive hybridization itself could drive the evolution of reproductive isolation barriers when taxa co-occur geographically (are sympatric) [2]. This mechanism, known as ‘the Wallace effect’, is also termed reinforcement, because preexisting reproductive isolation is ‘reinforced’ by natural selection for stronger barriers. Following an extensive correspondence with Wallace on this matter, Darwin remained skeptical [1–3]. Today, reinforcement of premating barriers by natural selection is widely accepted, but natural selection for reinforced postmating isolation still remains controversial [4–6].

Theoretically, reinforcement of postmating barriers can occur when 1) evolution of premating barriers is constrained, 2) there is substantial parental investment in the production and care of progeny, 3) individuals that are capable of mating more than once are unable to do so because the energetic costs of nurturing the unfit hybrids make subsequent reproduction less likely, and 4) reinforcement of the postmating barrier restores parents’ capacity to successfully reproduce after hybridization [7–9].

After reinforcement, sympatric species or populations should show stronger barriers than those that are geographically separated (allopatric), because natural selection for stronger barriers only occurs when populations are overlapping. This biogeographic pattern has been observed for premating barriers in many animals, plants, and fungi where it has been investigated [6,10,11], but only a few instances of stronger postmating reinforcement in sympathy have been reported over the past 65 years [9,12–14], including a microbial example involving abortion of hybrid fruitbodies (perithecia) in matings between sympatric populations of the haploid fungal species N. crassa and N. intermedia [15,16].

Reproductive isolation of N. crassa and N. intermedia

The geographical ranges of N. crassa and N. intermedia are broadly overlapping, and individuals of both species can be collected from the same site [15,17,18]. Both species are largely outbreeding, and outbreeding is confirmed by population genetic analysis [19–22]. Hybrids of the two species can be
**Author Summary**

Although Darwin believed that natural selection could not drive intersterility between species, it is now well established that there is a role for natural selection in the evolution of premating discrimination that reinforces barriers to hybridization. However, natural selection for postmating barriers, like hybrid inviability, is still controversial, because it can only occur when overall maternal fitness is increased by the inviability of hybrid offspring. Constraint on adaptive evolution of postmating barriers poses a problem when organisms without premating preferences must adapt to the presence of related species and ensure that reproduction occurs only between members of the same species. We studied the evolutionary genetics of a reinforced, postmating barrier between two species of mold, *Neurospora crassa* and *N. intermedia*. Although hybrids have low fitness, *Neurospora* females do not discriminate against different-species sex partners before mating. Instead, *N. crassa* has adapted to the presence of the *N. intermedia* in its range by selectively aborting hybrid fruitbodies. We show that abortion increases maternal fitness because *N. crassa* can mate again after hybridization only if fruitbodies abort. Abortion is controlled by 11 loci, whose genetic effects are consistent with an adaptive evolution model, confirming that abortion evolved via natural selection against hybridization.

In *Neurospora*, mating can occur only between individuals having different alleles at the mating-type locus (*mat a* or *mat A*). Under nutrient limited conditions, a haploid colony of *Neurospora* differentiates female reproductive structures (protoperithecia). Fertilization occurs when a specialized hypha (trichogyne) growing from a protoperithecium fuses with a cell from a colony of the opposite mating type. The attraction of trichogynes to fertilizing cells is mediated by mating-type specific pheromones. After fertilization, nuclei from the fertilizing strain travel through the trichogyne to the protoperithecium, where karyogamy eventually occurs. A series of independent meiotic events give rise to the sexual progeny (ascospores), which develop within flask shaped fruitbodies (perithecia) on the maternal thallus. Upon maturity, the ascospores are forcibly ejected from the fruitbody.

In *Neurospora*, evolution of premating isolation is apparently constrained because the sequences of the mating-type–specific, peptide pheromones controlling attraction between trichogynes and fertilizing cells are conserved throughout the genus (as determined by BLAST analysis of the *N. crassa*, *N. tetrasperma*, and *N. discreta* genomes [24]) and even beyond [25]. Evolution of the extracellular, ligand-binding portions of mating-pheromone receptor proteins is also comparatively constrained [26]. In *Neurospora*, sex cells of mating-type-compatible partners usually fuse before incompatibilities are expressed, and incompatibility arises either prezygotically in the fusion cell and the subsequent dikaryotic cells that proliferate from it, or postzygotically during the meiosis that directly follows karyogamy and during the formation and development of the ascospores [17]. Since *Neurospora* progeny develop within fruitbodies composed entirely of maternal tissue, the maternal colony (mycelium) bears virtually the entire cost of reproduction. Because 90% of *N. crassa* × *N. intermedia* hybrid progeny are inviable [15], and because allocation of resources to developing fruitbodies on one part of the colony abolishes the fertility of uncrossed regions of the colony [27], hybridization is severely maladaptive.

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

| Strains   | FGSC¹ | D² | Mating type | Species³ | Geographic location |
|-----------|-------|----|-------------|----------|---------------------|
|           |       |    |             |          |                     |
| 8903⁴     | 143   | A  | *N. crassa* (NcA) | Carib. Basin | Marrero, Louisiana  |
| 8865⁵     | 105   | A  | *N. crassa* (NcC) | India      | Madurai, Tamil Nadu |
| 8866⁴     | 106   | a  | *N. crassa* (NcC) | India      | Rameshwaram, Tamil Nadu |
| 8833³     | 73    | a  | *N. intermedia* | Africa    | Adiopodoume, Ivory Coast |
| 8843¹     | 83    | A  | *N. intermedia* | Africa    | Makokou, Gabon     |
| 8825¹     | 65    | A  | *N. intermedia* | Africa    | Yopougon, Ivory Coast |
| 8824⁴     | 64    | A  | *N. intermedia* | Carib. Basin | Carrefour Mme. Gras, Haiti |
| 8786⁵     | 26    | A  | *N. intermedia* | Carib. Basin | Homestead, Florida |
| 8869⁵     | 109   | a  | *N. intermedia* | India     | Madurai, Tamil Nadu |
| 8861¹     | 101   | A  | *N. intermedia* | India     | Mailllintham, Tamil Nadu |
| 8808¹     | 48    | A  | *N. intermedia* | India     | Rameshwaram, Tamil Nadu |

*Neurospora* strains were used to study the genetics and evolution of postmating reproductive isolation between sympatric populations of *N. crassa* and *N. intermedia*.

¹FGSC strain numbers are from the Fungal Genetics Stock Center. Symbols after strain numbers are as follows:

²Parent of the *N. crassa* Nc × Nc QTL mapping population;

³Strain used in sequential mating experiments;

⁴*N. intermedia* strain crossed to the QTL mapping population.

⁵D numbers are as assigned in [16].

⁶As determined in [15], with infraspecific subgroups in parentheses.

⁷Carib. 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.

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Figure 1. Maternal fitness of sequentially mated Neurospora. To determine the effect of hybrid matings on subsequent conspecific matings, receptive N. crassa NcC-India cultures were initially fertilized in four different ways: distilled water as a negative control (A, E, K, O); N. crassa NcC-India as a conspecific positive control (B, F, I, L); N. intermedia allopatric to N. crassa NcC-India (C, G, J, M); N. intermedia sympatric to N. crassa NcC-India (D, H, N, P). In all experiments, the second fertilizing strain was an N. crassa NcC-India. The photographs show: whole plates (A–D), typical fruitbody development (E–H, K–N; bar = 500 μm) and ejected ascospores, if any (I, J, O, P; bar = 50 μm), resulting from the first and second fertilizations. Note that the conspecific second fertilizations resulted in ascospore production only when the initial heterospecific partner was a sympatric strain or when the initial fertilization was a water control (P and O, respectively). Second-fertilization sexual development was completely inhibited after initial fertilization by allopatric heterospecifics or by the conspecific positive control (M and L, respectively). The clear ascospores in J are inviable hybrid progeny typical of crosses between allopatric strains of N. crassa and N. intermedia.

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Table 2. The effect of initial fertilization on subsequent maternal fertility in sequentially fertilized Neurospora crassa (NcC-India) colonies.

| Species of initial fertilizing male (conspecific, allopatric or sympatric) | Strain (n) | Region | RSS\(^2\) of initial fertilization mean (SE) | RSS of subsequent fertilization mean (SE) |
|---|---|---|---|---|
| Water control | na (n = 3) | na | 0.00 (0.00) | 6.00 (0.00) |
| N. crassa (conspecific) | 8865 (n = 3) | India | 6.00 (0.00) | 0.00 (0.00) |
| N. intermedia (allopatric) | 8786 (n = 3) | Carib.\(^2\) | 5.00 (0.00) | 0.00 (0.00) |
| N. intermedia (allopatric) | 8824 (n = 3) | Carib. | 4.00 (0.00) | 0.00 (0.00) |
| N. intermedia (allopatric) | 8843 (n = 3) | Africa | 4.00 (0.00) | 0.00 (0.00) |
| N. intermedia (allopatric) | 8825 (n = 3) | Africa | 4.33 (0.33) | 0.00 (0.00) |
| N. intermedia (sympatric) | 8808 (n = 3) | India | 1.00 (0.00) | 6.00 (0.00) |
| N. intermedia (sympatric) | 8861 (n = 3) | India | 1.00 (0.00) | 6.00 (0.00) |

One half of a Neurospora crassa (NcC-India) colony was fertilized by a conspecific strain or by N. intermedia from sympatric of allopatric populations (initial fertilization). Five days later, the other half of the colony was fertilized by a conspecific strain (subsequent fertilization). The experiment shows the reproductive success of initial fertilizations and subsequent fertilizations.

\(^1\)Carib. = Caribbean Basin.

\(^2\)RSS = Reproductive success score, based on a seven-stage scale where 0 represents no sexual response to fertilization, and 6 represents normal fertility.

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Abortion of hybrid fruitbodies is adaptive evolution by positive natural selection. The genetic architecture that we observe is consistent with the allelic effects distribution for the detected loci, we show that (QTL) analysis of hybrid fruitbody abortion and statistical analysis of the allelic effects distribution for the detected loci, we show that abortion of hybrid fruitbodies is adaptive, because it preserves the fertility of maternal *N. crassa*. Then by quantitative trait locus (QTL) analysis of hybrid fruitbody abortion and statistical analysis of the allelic effects distribution for the detected loci, we show that the genetic architecture that we observe is consistent with evolution by positive natural selection.

### Results

**Abortion of hybrid fruitbodies is adaptive**

We tested whether female fertility of colonies was preserved after abortion of sympatric hybrid fruitbodies in sequential mating experiments between the two Neurospora species. *N. crassa* colonies were grown in Petri plates on synthetic cross agar medium, which promotes sexual reproduction. One half of the receptive *N. crassa* colony was fertilized by either an allopatric or sympatric *N. intermedia* strain, and the effect of allopatric vs. sympatric fertilization on the reproductive success of subsequent conspecific fertilization on another portion of the maternal colony was assayed. Initial fertilizations by a conspecific strain or with water (pseudo-fertilizations) were also performed as controls. Each of the four initial-fertilization treatments (allopatric male, sympatric male, conspecific male, pseudo-fertilization) was replicated three times. Following Dettman, et al., reproductive success was scored on a seven-category scale incorporating fruitbody development and quality of ejected ascospores, if any [15]. The *N. crassa* and *N. intermedia* strains used are described in the Materials and Methods and listed in Table 1. In all sequential matings, both the maternal strain and conspecific fertilizing strains were *N. crassa* from the NeC clade endemic to India, hereafter referred to as NeC-India [15].

Fruitbody development on portions of the maternal colony fertilized by allopatric *N. intermedia* strains is normal and results in ascospore ejection, although a majority of the hybrid ascospores are unmelanized and inviable (reproductive success score (RSS) = 4.33±0.14, Figure 1). Following allopatric hybridization, response to conspecific fertilization at the second time point is completely inhibited (RSS = 0.00±0.00).

In contrast, fertilization by sympatric *N. intermedia* strains at the first time point yields only aborted fruitbodies (RSS = 1.00±0.00), but the subsequent conspecific matings are fully fertile (RSS = 6.00±0.00, Table 2). We performed semi-parametric regression using a proportional hazards survival model [20] to examine the effects of fertilization at the first time point (water control, conspecific control, or allopatric or sympatric heterospecific) on progression through the sexual cycle after fertilization at the second time point (measured as RSS). The first-time-point fertilization treatment had a significant effect (P<0.0028), whereas the nested effects of geographic origin and strain identity of the first-time-point male do not have significant effects (Table 3). We examined the effects of the type of first fertilization (pseudo-fertilization, conspecific, allopatric heterospecific, sympatric heterospecific) and the geographic origin and strain identity of the initial fertilizing strain on subsequent fertility of the maternal colony were assessed by a proportional hazards model.

The key questions are: 1) Does abortion of hybrid fruitbodies by *N. crassa* make subsequent reproduction possible for the maternal colony, thereby conferring a fitness advantage? and 2) Did this postmating barrier evolved by natural selection? First we show that hybrid fruitbody abortion is adaptive, because it preserves the fertility of maternal *N. crassa*. After abortion of sympatric hybrid fruitbodies in sequential mating experiments between the two Neurospora species, *N. intermedia* strains at the geographic origin and strain identity of the initial fertilizing strain were measured by a proportional hazards model.

### Table 3. Proportional hazards model of how initial fertilization affects subsequent maternal fertility of *Neurospora* colonies.

| Test | Source | −Log Likelihood | DF | Chi Square | Prob>Chi Square |
|------|--------|-----------------|----|------------|----------------|
| Whole model | Difference | 7.05 | 7 | 14.10 | 0.0494 |
| | Full | 60.40 |  |  |  |
| | Reduced | 67.45 |  |  |  |
| Effects | Type of first fertilization | 3 | 14.10 | 0.0028 | |
| | Geographic origin of first fertilizing strain | 1 | 0 | 1.0000 | |
| | First fertilizing strain identity | 3 | 0 | 1.0000 | |

The effects of the type of first fertilization (pseudo-fertilization, conspecific, allopatric heterospecific, sympatric heterospecific) and the geographic origin and strain identity of the initial fertilizing strain on subsequent fertility of the maternal colony were assessed by a proportional hazards model. The model incorporates the type of initial fertilization (water negative control, conspecific, allopatric heterospecific, or sympatric heterospecific), the geographic origin of the initial fertilizing strain, and the strain identity of the initial fertilizing strain as nested effects.

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### Table 4. Summary of hybrid fruitbody development phenotypes analyzed by QTL mapping.

| Trait symbol | Role of *N. crassa* mapping strains | Origin of *N. intermedia* tester strains | Sympatic or allopatric |
|--------------|-------------------------------------|----------------------------------------|-----------------------|
| A            | Maternal                            | India                                  | Sympatic              |
| B            | Paternal                            | India                                  | Sympatic              |
| C            | Maternal                            | Ivory Coast                            | Allopatric            |
| D            | Paternal                            | Ivory Coast                            | Allopatric            |

The *N. crassa* QTL mapping strains were crossed reciprocally to *N. intermedia* tester strains from India and Africa. Hybrid fruitbody development was measured in each of these four types of crosses.

The *N. crassa* mapping strains are derived from an inter-clade NcA-Louisiana × NeC-India f1 cross.

Crosses to Indian tester strains, which are sympatric to the NeC-India parent of the mapping population, are designated sympatric. African tester strains are allopatric to both parents.

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conclude that abortion of hybrid fruitbodies is selectively advantageous because abortion preserves maternal fertility of the colony after hybridization.

Design of QTL mapping experiments

Previous research on the genetics of reinforcement focused on premating barriers in animals [29–31]. Here we investigate whether the genetic architecture of a reinforced, microbial, postmating barrier is consistent with evolution by directional natural selection [32,33]. A 500-member, N. crassa mapping population was derived from an intraspecific, inter-clade cross between the NcC-India strain FGSC 8866, and a Louisiana, USA, strain, FGSC 9903, a member of the NcA clade, hereafter referred to as NcA-Louisiana. Neurospora are hermaphroditic so that the parental strains could be mated reciprocally. Based on the identity of the maternal parent we can infer that the mapping population contains both individuals with NcA-Louisiana cytoplasm (57%) and individuals with NcC-India cytoplasm (43%).

The N. crassa mapping strains were crossed maternally and paternally to N. intermedia strains from Tamil Nadu, India, which are sympatric to the NcC-India parent and allopatric to the NcA-Louisiana parent. NcC-India aborts fruitbodies after fertilization by N. intermedia from India, but NcA-Louisiana does not [15,16]. The mapping strains were also crossed maternally and paternally to African N. intermedia strains, which are allopatric to both the NcC-India and NcA-Louisiana parents. Neither the NcA-Louisiana parent nor the NcC-India parent aborts fruitbodies after fertilization by the African N. intermedia strain. The four traits that we studied were fruitbody development in the four different types of matings. The four types of matings are defined by the parental role of the mapping strain (maternal or paternal) and the geographic relationship of the N. intermedia strain (Indian and therefore sympatric to the NcC-India parent of the mapping population, hereafter termed “sympatric”; or African, and therefore allopatric to both the NcC-India parent and the NcA-Louisiana parent, hereafter termed “allopatric”); see Table 4). Therefore each member of the mapping population was crossed twice to an Indian N. intermedia strain and twice to an African N. intermedia strain, once with the mapping strain in the maternal role and once with the mapping strain in the paternal role, for a total of four crosses per mapping strain. We examined fruitbody development in each cross, recording its fruitbody development score (FDS) 10 days after fertilization (see Materials and Methods).

Linkage and segregation analysis

The mapping strains were genotyped at 69 AFLP (Table 5 and Table 6) and 28 microsatellite (Table 7) markers as well as the mat locus. A genetic map containing seven linkage groups (LG) reflecting the seven chromosomes of Neurospora was estimated, with a total map length of 837.9 cM and an average intermarker distance of 9.2 cM (Figure 2).

Non-Mendelian segregation and non-random associations (NRA) among alleles at multiple loci can reflect genetic incompatibilities between the NcC and NcA genomes. Individuals in the mapping population inherited 53.6% of genotyped alleles from the NcA-Louisiana parent, and this is significantly higher than the 50% expected under Mendelian segregation (t Test, \( t = 6.8721, \text{DF} = 93, p < 0.0001 \)). The proportion of NcA-Louisiana alleles inherited varied across linkage groups (ANOVA, \( P_{r} = 23.34, \text{DF} = 93, p < 0.0001 \)), with linkage groups II, VI, and VII showing below 50% inheritance of NcA-Louisiana alleles (Figure 3). The skew towards NcA alleles was strongest on linkage group I, with 59.0% of marker alleles inherited from that parent. Of 22 markers showing significant segregation distortion (\( \chi^2, P < 0.05 \)), only one marker on LG IV (nc4L4) was significantly skewed in favor of the NcC-India allele, while the 21 other significantly distorted markers favored the NcA-Louisiana and were located on LG I (16 markers) and LG IV (5 markers).

Seven marker pairs representing four pairs of linkage groups exhibit significant non-random associations (Table 8). Positive non-random associations, reflecting an overrepresentation of parental haplotypes, are consistent with the existence of negative epistasis among clade specific alleles, as predicted by the Dobzhansky-Muller (D-M) model of the evolution of genetic incompatibilities first articulated by Bateson [34]. Per this model, incompatibilities between two loci, \( X \) and \( Y \), must arise in a two-step fashion, as follows: Consider that at the time that the NcA and NcC clades diverged, both populations contained ancestral \( (\text{anc}) \) alleles at every locus. The first fixation of a derived allele, e.g., \( X_{\text{anc}} \rightarrow X_{\text{NcA}} \), will not cause incompatibility, because \( X_{\text{NcA}} \)

\[
\begin{table}
\centering
\caption{Primer sequences for preselective and selective AFLP markers.}
\begin{tabular}{|c|c|}
\hline
\textbf{Primer} & \textbf{Sequence (5' to 3')} \\
\hline
E-00 & GA\textit{ATT}G\textit{GTT}C\textit{ACC}AA\textit{TTC}C \\
E-AA & GA\textit{ATT}G\textit{GTT}C\textit{ACC}AA\textit{TTC}A \\
E-TA & GA\textit{ATT}G\textit{GTT}C\textit{ACC}AA\textit{TCTA} \\
E-TC & GA\textit{ATT}G\textit{GTT}C\textit{ACC}AA\textit{TCTC} \\
M-00 & GA\textit{TGT}G\textit{TCT}C\textit{GATGAA} \\
M-AA & GA\textit{TGT}G\textit{TCT}C\textit{GATGAA}A \\
M-AC & GA\textit{TGT}G\textit{TCT}C\textit{GATGAA}A \\
M-AG & GA\textit{TGT}G\textit{TCT}C\textit{GATGAA}G \\
M-C\textit{A} & GA\textit{TGT}G\textit{TCT}C\textit{GATGAA}A \\
M-C\textit{G} & GA\textit{TGT}G\textit{TCT}C\textit{GATGAA}G \\
M-G\textit{C} & GA\textit{TGT}G\textit{TCT}C\textit{GATGAA}G \\
\hline
\end{tabular}
\end{table}

\begin{table}
\centering
\caption{Primer pairs for preselective and selective AFLP reactions.}
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{E-primer} & \textbf{M-primer} & \textbf{Primer pair type} & \textbf{Letter code}^3 \\
\hline
E-00 & M-00 & preselective & — \\
E-AA & M-AC & selective & a \\
E-AA & M-AA & selective & b \\
E-AA & M-AG & selective & c \\
E-TA & M-CG & selective & d \\
E-TA & M-CA & selective & e \\
E-TC & M-CG & selective & f \\
E-TC & M-GC & selective & g \\
\hline
\end{tabular}
\end{table}

The AFLP markers incorporated into the N. crassa linkage map were obtained with seven different primer-pair combinations. The preselective primer pair used was the same in each case.

\( ^3 \)in the names of mapped AFLP markers this letter code is the third letter of the three-letter prefix.

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## Table 7. Microsatellite targeted markers on the *Neurospora crassa* linkage map.

| Marker | Position (bp) | µsat | Fragment size (bp) | Primers (5’ to 3’) | Forward | Reverse |
|--------|---------------|------|--------------------|--------------------|---------|---------|
| nc1L2b | 293960 +      | 12 aga | 103 170            | tacattccccctgacccctgg | gttctccgggccggtgag |
| nc1L3  | 788170 +      | 10 tgt | 177 163            | ggggaaccaaaaacggaagaag | ctagaagcataacagtacatc |
| nc1R2b | 9103466 +     | 17 caa | 276 313            | cgtctctctctctctctgcttgcttg | agttcagggggctggtgcttg |
| nc1R2e | 9208198 +     | 12 ttc | 245 259            | gcgcgtctgaaatgaaagcact | tcacatcaccctctctctctc |
| nc1Rf  | 9646074 –     | 11 gct | 282 263            | gacgcacccagacagagg | ccctcttcgacaacctctt |
| nc2L1  | 59713 –       | 8 aag | 414 404            | gaggaagagaaaaggttgtg | ccaggttctcaatgctgtc |
| nc2R4a | 3472577 +     | 12 ctt | 192 178            | cccattacctctgaccaagca | tccacctcttttcacctc |
| nc3L1b | 1222692 –     | 8 gaa | 210 198            | rtcctcgggctgtatctgctg | tgtggaagggggttgagag |
| nc3L9a | 1411723 +     | 12 caa | 456 455            | ccgccagttgctttgaaagc | agggtggggaggtttag |
| nc3R1  | 2989674 +     | 19 gac | 216 182            | aggactgggacagagagaga | atcccatcaccctctcaca |
| nc3R9a | 3754818 +     | 41 tgt | 401 466            | gcacacagggctctctctc | tccacctcttttcacctc |
| nc3R8  | 4883113 +     | 9 gat | 226 233            | gcagctttggggcgggtag | gcgaatggagagggggtg |
| nc4L4  | 58410 –       | 16 aca | 231 219            | tctctgtgctgtgctgtgc | ggctaggctgcagggag |
| nc4R3c | 495719 +      | 14 tgt | 201 194            | ggccttgtgagaaagagttg | tgtgcgttggtgattagttc |
| nc4R1a | 590576 –      | 15 aca | 124 95             | gggggaaacaatcactcatttt | aatgtggcagctgaagag |
| nc5L1a | 113742 –      | 9 ctt | 301 308            | agggtggcttttcttagtgcg | ccctcagttccatatcagaa |
| nc5L3a | 363346 –      | 12 caa | 373 411            | gcctggctttctctctcttca | acctcctcttttcctctgctt |
| nc5R4  | 5641416 –     | 9 tgt | 265 267            | tgtggggttggtgttggttg | tgtggcagctttctctcctc |
| nc6L10 | 302027 +      | 8 gat | 201 195            | tgggtgtcatactctcttcg | ctaggtggctggctggctt |
| nc6L13 | 350117 +      | 7 tgt | 229 222            | aacgtgcctgtgctgcttc | agttaaacgctgggagag |
| nc6L2  | 425652 +      | 7 ggt | 318 306            | tcggagttgctttgcttttcc | aaggtggcagctggagatc |
| nc6L15 | 495534 +      | 12 gaa | 169 143            | tcgtaaaggaaggttgggag | ctccaaggtccggtggagag |
| nc6L16 | 631408 +      | 8 tcc | 213 210            | tcggggagaaaaagaacaaagagaa | atagagttgctgggagag |
| nc6L6  | 1569043 +     | 8 ctc | 326 332            | gaggagaaacagcaggggactgaa | tgggggcttggtgattagtg |
| nc6R4  | 3762945 +     | 16 gaa | 183 153            | tttgtgacagccagatgctc | ttcctgaagattcagagag |
| nc6R2  | 4094250 +     | 24 cat | 194 144            | gagggtgggtgtgggtggag | agagttgaggaggtattcagcag |
| nc7R5  | 3383817 –     | 14 aga | 240 180            | tgtgtgtgtgtgtgtggg | cggcgggtgtgtgtgtgg |
| nc7R4  | 4190212 –     | 16 caa | 220 180            | ggtggaagagacgaccggagag | acgacacacaggttaccc |

The linkage group, chromosome position, repeat number, and primer sequences are given for all of the microsatellite markers incorporated into the *N. crassa* linkage map.

1. Each microsatellite marker name consist of the prefix ‘nc’ followed by a numeral indicating linkage group, then the letter ‘R’ or ‘L’ indicating right or left arm of the chromosome, and then an alpha-numeric identifier.

2. Supercontigs refer to assembly 10 of the *Neurospora crassa* genome by the Broad Institute. The position of the targeted repeat is given.

3. µsat is microsatellite.

4. doi:10.1371/journal.pgen.1002204.t007
We used composite interval mapping to identify QTLs for fruitbody development. The genetic basis of postmating reinforcement was revealed by mapping loci for maternal influence on sympatric fruitbody development (trait A, see Table 4). We identified 11 additive-effect QTLs for this trait (Figure 4a and Table 9; complete CIM scans for all traits are in Figure 5). Seven of the QTLs are located on LG I, including one of large effect, while LG II and V each contain a single broad QTL region of weak effect, and the left arm of LG VI harbors two other QTLs of large effect. The detected QTLs account for roughly 61% of trait variance, with the three loci of large effect accounting together for roughly 30% of trait variance. The inferred cytoplasm type (NcA-Louisiana or NcA-India) of the mapping strains did not significantly affect this trait.

For 10 of the 11 QTLs, the allele from the sympatric NcC-India parent has a negative effect on sympatric fruitbody development. Only the weak QTL on LG II has the opposite effect. The prevalence of negative alleles in the NcC-India background is consistent with evolution of abortion via directional natural selection. This inference can be statistically tested by the QTL sign test, which tests the null hypothesis that the observed genetic architecture was generated during neutral trait evolution, i.e., without selective advantage for negative alleles [32].

The QTL sign test assumes that all QTL effects are additive. We note that the accepted model of intrinsic, postzygotic isolation barriers involves negative among species-specific alleles in hybrids. However, our experiments were not designed to, nor can they, interrogate the genetics of hybrid dysfunction, but rather the genetics of evolutionary response to maladaptive hybridization. The genetic architecture of reinforcement may or may not involve epistatic effects. However, in contrast to the case of hybrid dysfunction, epistasis would involve within-genome, interaction effects among loci contributing to the reinforced barrier. To determine whether or not epistasis plays a role in the genetics of reinforced sympatric barriers in N. crassa, and to determine whether or not the genetic data conform to the assumptions of the QTL sign test, we performed a two-dimensional genome scan for interacting QTLs.

No significant interaction effect was detected. Moreover, the genome-wide maximum LOD score for any interaction effect was determined to be 16.7, well below the critical LOD score of 37.4 (for a Type I error of 0.05), which was estimated from 1000 permutations of the data. Because the two-way scan for genetic interactions among loci failed to find any significant or marginally significant interaction effects, the data are consistent with an additive genetic model and conform to the assumption of additivity required for the QTL sign test.
Given the observed number of positive and negative QTLs and the distribution of their effect magnitudes (gamma $= 0.034$, $c = 13.5$), and conditioned on the parental difference in fruitbody development after sympatric fertilization (F.D.S = 2), the null hypothesis of neutral trait evolution for abortion in NcC-India is rejected (QTL sign test, $P = 0.0099$) (Figure 4b). This result implies that fruitbody abortion in sympatric NcC-India–maternal $N. intermedia$–paternal $N. crassa$ crosses (trait A) evolved under positive natural selection via a reinforcement mechanism.

One of the major QTLs on LG VI is flanked by two microsatellite loci, nc6L15 and nc6L16, and can therefore be physically located to a 135,874 bp region of the $N. crassa$ genome containing 24 ORFs (Table S1) [36].

**Genetics of fruitbody development in the absence of reinforced barriers**

We also investigated the genetics of fruitbody development in crosses not showing reinforced isolation (Traits B, C, and D, see Table 4). Note that NcC-Indian strains show enhanced isolation from Indian $N. intermedia$ only when the NcC-Indian strain performs the maternal role [16]. We did not detect any QTLs for paternal influence on the development of sympatric fruitbodies (trait B), but in crosses to allopatric $N. intermedia$ strains we detected two QTLs affecting maternal influence and three QTLs affecting paternal influence on fruitbody development (traits C and D, respectively; Figure 4a). All five of these loci are located on LG I. Four of the five allopatric QTLs co-localize with three of the sympatric QTLs on LG I, which could either indicate the presence of genes with pleiotropic effects or linked genes with trait-specific effects.

**Discussion**

**Reinforcement alleles and segregation distortion on Linkage Group I**

Linkage group I represents less than 24% of the genome, and it is striking that 75% of our QTLs map here. Interestingly, 73% of loci showing non-Mendelian segregation also map to linkage group I, so that every QTL on this linkage group is flanked by at least one marker showing segregation distortion. In all cases, the NcC alleles of the linkage group I QTLs have a negative effect on sympatric hybrid fruitbody development, and the NcC alleles of the linkage group I markers are underrepresented in the NcA×NcC $N. crassa$ mapping population.

It is not known why one-fifth of genetic markers are distorted in favor of the NcA allele. It is possible that, because laboratory strains of $N. crassa$ have historically been derived from the NcA clade, our crossing and progeny-isolation methods, which were developed for NcA-clade $N. crassa$, have inadvertently created a selective environment favoring NcA alleles at loci linked to distorted markers. It is also possible that distorting loci present in the NcA background are normally repressed through the action of NcA modifier loci, but become unpressed and active in some recombinant NcA×NcC genotypes. Although segregation distortion can be caused by nuclear-cytoplasm incompatibilities, it is unlikely to be the cause in this case. The $Neurospora$ mapping population comprises a mixture of individuals with NcA-Louisiana cytoplasm and NcC-India cytoplasm. Moreover, even in individuals with NcC-India cytoplasm, linkage group I markers are distorted in favor of the NcA allele, with an overall NcA allele frequency of 0.61 for markers on this linkage group.

Another hypothesis is that reinforcement alleles themselves can pleiotropically cause ascospore inviability in conspecific, interclade crosses. Laboratory crosses between members of the NcA and NcC clades are partially intersterile, usually resulting in <50% ascospore viability [15]. In Neurospora, all products of meiosis contribute to the ascospore cohort, so segregation distortion most likely results from inviability of hybrid ascospores carrying the disfavored NcC allele(s).

Pleiotropy for reinforcement and reproductive isolation between allopatric conspecifics has previously been observed in animals [37,38]. Reduced conspecific fertility can present a challenge to the evolution of reinforced barriers, since the fitness advantage of
avoiding hybridization must outweigh the cost of lower conspecific fertility. However, restricted migration between conspecific populations should reduce the incidence of interpopulation mating and reduce the fitness costs associated with pleiotropic effects on conspecific fertility. The NeC and NeA clades are geographically separated, so limited interclade migration would reduce the fitness cost to NeC of lower fertility with NeA and facilitate the spread of reinforcement alleles in the NeC clade. If the pleiotropy hypothesis is correct, the evolution of reinforcement QTL on linkage group I could be a partial explanation for the evolution of incomplete reproductive isolation between the NeC and NeA clades.

Notably, QTLs on other linkage groups (II, V and VI) are flanked by markers showing Mendelian segregation, so that for these QTLs there is no suggestion of pleiotropic negative effects on within-species, inter-clade reproduction. Moreover, the two reinforcement QTLs on linkage group VI lie in the vicinity of three markers (nc808, nc6L13, nc6L2), which participate in non-random associations with loci on other linkage groups, such that recombinant, non-parental haplotypes are overrepresented in the mapping population. Therefore the patterns of marker inheritance near QTLs on these other linkage groups do not suggest any connection between reinforcement QTLs and isolation of conspecific allopatric N. crassa populations.

We note that linkage group I contains the mating-type locus of Neurospora, and that some studies have found that reproductive isolation loci are more prevalent on sex-determining chromosomes than on autosomes [39,40]. It is true that in a closely related species, N. tetrasperma, recombination is suppressed over a large region of Linkage Group I in a process considered analogous to an early stage of sex-chromosome evolution [41]. However, no recombination block exists on linkage group I of N. crassa. Additionally, Neurospora species are hermaphroditic, so the mating-type locus determines mating compatibility, rather than sexual role. We therefore consider it unlikely that the same forces that cause reproductive isolation loci to preferentially accumulate on sex chromosomes can account for the prevalence of the observed QTLs on linkage group I.

A previously identified reproductive isolation QTL on linkage group I

Earlier genetic studies of reproductive isolation in Neurospora identified a QTL on linkage group I as the N. crassa member of a Dobzhansky-Muller incompatibility locus-pair responsible for a severe defect in hybrid perithecial development between allopatric N. crassa and N. intermedia when N. crassa acts as the male partner [42,43]. These incompatibility loci were first identified in populations of N. crassa × N. intermedia hybrids evolved under divergent environmental conditions in a test of the hypothesis that ecological adaptation can incidentally drive reproductive isolation [42]. Subsequent mapping determined that the incompatibility was caused by interactions between an N. crassa locus (dna on linkage group I) and an N. intermedia locus (dsf on linkage group V) [43]. Considering that both the geographic relationship of the species and gender role of N. crassa differ between this study and ours, it is very interesting that the N. crassa dna locus maps to a region of linkage group I that potentially coincides with the locations of QTLs identified in our study. Direct comparison between mapping results is prevented by the absence of sequence anchored, microsatellite markers in this region of our map.

Conclusions

Sexual microbes are likely to have simple premating recognition mechanisms, but will nevertheless experience selective pressure to avoid maladaptive hybridization. When evolution of premating barriers is constrained, microbial reinforcement may be more likely to involve non-premating-recognition mechanisms, including differentiated substrate or host fidelities [10] or evolution of divergent mating kinetics [44]. Here we have shown that selective abortion of hybrid fruitbodies by N. crassa fertilized by sympatric N. intermedia had the potential to evolve by natural selection by demonstrating that maternal colonies that abort hybrid fruitbodies are capable of undergoing reinforcement selection for selective abortion of hybrid offspring. Further studies on the evolution and genetics of reproductive isolation in microbial eukaryotes will be needed to challenge this hypothesis.
Materials and Methods

Neurospora strains and culture conditions

The biology of Neurospora, the evolutionary relationships among species and clades, and the biogeography of reproductive isolation between N. crassa and N. intermedia have been described previously [15,16]. Culturing, crossing, and isolation of ascospore progeny were performed as previously described [15,17]. Table S1 lists the wild-collected Neurospora strains used in this study. The QTL mapping population created for this study has been deposited with the Fungal Genetics Stock Center, Kansas City, Missouri.

Sequential fertilization

Sequential fertilization was performed according to the methods of Howe and Prakash [27], except that at the first mating time point (5 days after inoculation of the NcC-India maternal strain), the conidial suspension of one fertilizing strain (either an NcC-India strain as conspecific positive control; an allopatric N. intermedia (African (n = 2) or Caribbean (n = 2)); sympatric N. intermedia (Indian (n = 2)); or water negative control) was applied to 50% of the plate, while at the second time point (10 days after maternal inoculation), the fertilizing suspension of NcC-India was applied to two 1 cm² spots located 1.5 cm–2 cm from the edge of the first fertilization. Three replicates were performed for a total of 24 plates. Fertility was scored 20 days after maternal inoculation using a 0–6 reproductive success scale (RSS) [15]. The effects of cross type and geographic origin and strain identity of the first–time-point fertilizing males on reproductive success of the second–time-point crosses were analyzed using a semi-parametric, proportional hazards model, with nested effects, as implemented by JMP 5.0.1a.

Genotyping

We obtained genomic DNA for each member of the QTL mapping population following the protocol of Dettman et al. [15]. AFLP and microsatellite primer sequences are shown in Table 5, Table 6, and Table 7. The mat-a1 and mat-A1 loci were amplified by multiplex PCR with the following primers: Ba1-5, AAGAAGGTTCAACCGGATTCATG; Ba1-3, CCAGAGCCATGT-TCTAGGAATCATT; Sa1-5, CGTCGATGGCAATCGTTT-
amplification of AFLP loci with the Invitrogen AFLP Core agarose gel electrophoresis. Genomic DNA was prepared for SSR in some ends and QTL regions) were selected from a published list of Carlsbad, CA).

- **Amplification:** Amplification were electrophoretically separated on an ABI 3100 Genetic analyzer, and data were collected and analyzed with the ABI software GeneScan and Genotyper (Applied Biosystems, Inc., Carlsbad, CA).

- **Fluorescent Dyes:** Amplification were fluorescently labeled with NED, 6-FAM, or HEX dyes, and size data were collected as for AFLP markers, above.

- **Microsatellite Loci:** Microsatellite loci in targeted genomic regions (e.g., chromosome ends and QTL regions) were selected from a published list of SSR in N. crassa [47], and primers were designed with Primer3 on the web [48]. Forward primers were 5’-fluorescently labeled with NED, 6-FAM, or HEX dyes, and size data were collected as for AFLP markers, above.

- **Microsatellite Genotyping:** Microsatellite markers targeted to that chromosome.

- **Genetic Architecture:** The genetic architecture of hybrid fruitbody development in four types of N. crassa x N. intermedia crosses was investigated by composite interval mapping. The positions and effects of significant QTLs are listed in the table, and co-localization of QTLs for different cross types is noted.

| Trait | LG | Position (cM) | 1-LOD Confidence Interval (cM) | Additive Effect | P.V.E. | LR |
|-------|----|---------------|-------------------------------|----------------|-------|----|
| **A)** Female effect; sympatric (n = 493) | 1 | 42.5 | 37.6–47.3 | -0.0552 | 0.0621 | 28.3 |
| | 1 | 52.9 | 49.9–56.4 | -0.5174 | 0.0596 | 30.9 |
| | 1 | 60.3 | 56.8–61.9 | -0.4912 | 0.0462 | 29.6 |
| | 1 | 66.3 | 64.3–71.1 | -0.5098 | 0.0471 | 29.6 |
| | 1 | 77.6 | 75.6–80.7 | -0.4209 | 0.0223 | 13.4 |
| | 1 | 84.3 | 84.2–87.1 | -0.6069 | 0.1102 | 61.3 |
| | 1 | 104.5 | 99.5–115.0 | -0.3162 | 0.0186 | 12.2 |
| | 2 | 56.8 | 33.7–75.1 | 0.2543 | 0.0201 | 11.9 |
| | 2 | 22.1 | 8.6–34.5 | -0.2896 | 0.0259 | 11.4 |
| | 6 | 5.5 | 2.0–8.3 | -0.5333 | 0.0866 | 49.1 |
| | 6 | 20.6 | 16.8–25.2 | -0.5928 | 0.1079 | 61.2 |

- **QTL Analysis:** QTL analysis was performed with R/qtl [50] with the “geno.table” command. Linkage disequilibrium in pairs of physically unlinked markers was tested in Genepop 3.4 [51], using option 2, which uses a Fisher exact test implementing a Markov chain to estimate an unbiased P-value. Experiment wide significance threshold (Type I error α = 0.05) was determined by Bonferroni correction for the 21 non-identical linkage-group pairs.

- **Microsatellite Genotyping:** Microsatellite markers targeted to that chromosome.

- **Genetic Architecture:** The genetic architecture of hybrid fruitbody development in four types of N. crassa x N. intermedia crosses was investigated by composite interval mapping. The positions and effects of significant QTLs are listed in the table, and co-localization of QTLs for different cross types is noted.

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and a window size of 20 cM using a 1 cM walk speed. At each step the likelihood ratio statistic (LR) testing the hypothesis that a QTL exists versus the null hypothesis that no QTL exists was determined. For each trait, a critical LR threshold reflecting a Type I error of 0.05 was estimated by permuting the data 1000 times. Significant QTLs were CIM maxima whose LR exceeded the critical threshold and whose 95% confidence intervals were discontinuous with those of other CIM maxima. Ninety-five percent support intervals were estimated as the area bounded by 1-LOD drops in the LR where LOD = log10(LR/2 ln 10).

The null hypothesis of neutral trait evolution for sympatric hybrid fruitbody abortion (the reinforcement trait) was tested by subjecting the genetic effects data to a QTL sign test [32], as implemented by the QTLsign test software provided by H. A. Orr. Because the QTL sign test assumes an additive genetic model, we first scanned for epistatic loci using “scantwo” of R/qtl using the expectation-maximization, interval mapping algorithm and multi-point genotype probabilities calculated using the “calc.genoprobb” command with a step size of 2.5 and an error probability of 0.01. For each chromosome position the likelihood ratio statistic comparing the full epistatic model to the two-locus additive model was determined. For each trait, a critical likelihood ratio statistic threshold reflecting a Type I error of 0.05 was estimated by permuting the data set 1000 times.

QTLsign test determines how likely the proportion of loci with positive vs. negative additive effects is under a neutral model of complex trait evolution, when conditioned on the magnitude of the trait difference in the parent strains, the number of detected QTLs, the threshold of detection, and distribution of the absolute value of additive effects, which are all empirically determined. QTLsign test was parameterized as follows: Parental RSS difference = 2; number of QTLs = 11; detection threshold = 0.25; effects distribution gamma (shape = 13.5, scale = 0.034).

Supporting Information

Table S1 Candidate genes for postmating reinforcement in *N. crassa*. The ORFs found between microsatellite markers nc6L15
and ncsL16 on linkage group VI, which flank a major female-ferility QTL affecting sympatric hybrid fruitbody development, are listed. The presence of these ORFs in publicly available reproductive EST libraries is noted. The Sexual, Perithecial, and Westergaard EST libraries were constructed from cDNA harvested from mycelia undergoing sexual development. All data are from the Broad Institute Neurospora crassa database.

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Author Contributions

Conceived and designed the experiments: ET DJJ JWT. Performed the experiments: ET DJJ. Analyzed the data: ET. Wrote the paper: ET DJJ JWT.

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