UNDERSTANDING THE BARRIERS TO INTERSPECIFIC HYBRIDIZATION IN NEUROSPORA

Honors Fellow Thesis

by

SAMEER RAJENDRA GAJJAR

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

HONORS UNDERGRADUATE RESEARCH FELLOW

May 2012

Major: Biochemistry
Genetics

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Approved by:

Research Advisor: Rodolfo Aramayo
Associate Director, Honors and Undergraduate Research: Duncan MacKenzie

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ABSTRACT

Understanding the Barriers to Interspecific Hybridization in Neurospora. (May 2012)

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Crosses between two species of Neurospora are typically sterile and result in the production of non-viable or few viable ascospores. It is unclear what prevents successful sexual reproduction and the viability of hybrid progeny, given that most species of Neurospora share high levels of genetic and phenotypic similarity. It has been hypothesized that genome defense and integrity checkpoints play a critical role in preventing successful crosses between two different species. In particular, we hypothesized that either meiotic silencing, DNA methylation, and/or DNA mismatch repair, or a combination of the above might pose a barrier to interspecific reproduction. To test this hypothesis, we have selected the species *Neurospora crassa* and *Neurospora tetrasperma*. We used loss-of-function mutations or deletions in key genes for meiotic silencing, DNA methylation, and/or DNA mismatch repair in *Neurospora crassa* strains and, using spore quantification assays, examined the effect of such mutations in crosses with *Neurospora tetrasperma.* A significant increase in ascospore production was only shown in crosses containing a deletion of the S*ad-1* gene in the *N. crassa* strain. In

contrast, no increase in ascospore production was observed by either deletion or mutagenesis of the *Sms-4* gene. Progeny obtained from crosses containing the deletion in *Sad-1* were germinated and analyzed based on genetic and phenotypic characteristic. Most of the progeny were classified as hybrid and inherited Linkage Group I containing the deletion of *Sad-1* from *N. crassa*. Our results suggest that meiotic silencing does not have a significant contribution to reproductive isolation; rather *Sad-1* contributes directly to reproductive isolation at the meiotic stage.

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NOMENCLATURE

N. crassa	*Neurospora crassa*
N. tetrasperma	*Neurospora tetrasperma*
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
psi	Pounds per square inch
dim-2	Defective in DNA methylation (DNA methyltransferase-2)
msh-2	Mismatch repair gene-2
Sad-1	Suppressor of ascus dominance-1
Sms-4	Suppressor of meiotic silencing-4
Δ	Deletion allele
fs	Frameshift allele
UV	UV-induced mutation
RIP	Repeat-induced point mutation
WT	Wild-type
fl	Fluffy (does not produce macroconidia)

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CHAPTER I

INTRODUCTION

In 1942, Ernst Mayr defined a biological species based on two criteria: a group that is able to interbreed and exhibits reproductive isolation with similar groups [1]. While it is often debated as to what is the most accurate criteria to define a species, most definitions are defined by the absence or presence of genetic exchange [2]. Reproductive isolation is the collection of behaviors and processes that prevents two species from being able to successfully interbreed with each other and poses as a major barrier of genetic exchange that leads to speciation.

Reproductive isolation is not a finite barrier of genetic exchange; both genetic and environmental factors play a key role in the extent to which reproductive isolation contributes as a barrier to genetic exchange and speciation. As a result, in nature, we often find varied degrees of interspecific hybridization. For instance, the barrier of reproductive isolation in plants tends to not be very rigid in comparison to more complex organisms such as animals. Crosses between two different sunflower species, *Helianthus annus* and *Helianthus petiolaris* only exhibited low levels of fertility and viability, but were also able to produce three stabilized hybrid species [3,4]. In another study with

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Rhagoletis fruitflies, genetic analysis of molecular markers of *Lonicera spp*, a species of flies that was found to be infesting an invasive honeysuckle, was determined to be a hybrid of the blueberry maggot *Rhagoletis mendax* and the snowberry maggot *Rhagoletis zephyria* [5]. While these two examples do not cover the breadth of hybridization that can occur, it demonstrates that interspecific hybridization can be both induced and occur naturally in most types of organisms. It is important to note that production of stabilized species through interspecific hybridization is very rare. In addition, for the hybrid species to thrive, it must have a significant evolutionary advantage over the parental species [6]. In the case of the sunflowers, the hybrid species demonstrated an increased vigor in growth [4]. In the fruit flies, the hybrid species was better adapted to a different host, the invasive honeysuckle [5]. Therefore, reproductive isolation poses a significant, but not impenetrable, barrier to genetic exchange that can lead towards speciation.

While it is commonly known that reproductive isolation has both an environmental and genetic components, most research has focused primarily on environmental aspects such as geographic barriers and physiological conditions. In this study, we are focusing on the genetic components to reproductive isolation using the filamentous fungus Neurospora as our model organism. Neurospora is an ideal organism for this purpose because its genetics have been studied extensively, it contains several species with varied abilities to interbreed, and has a well-studied sexual cycle that can be monitored at each stage. Therefore, by manipulating various genetic factors in interspecific crosses

between different species of Neurospora, it is possible to elucidate the genetic components that contribute to reproductive isolation.

Model of interspecific barriers

In general, there are four primary barriers that as a whole, determine the rigidity of reproductive isolation in interspecific crosses (Figure 1). These barriers are present from the initial fertilization between two different species up to the potential development and growth of a hybrid progeny, consisting of genome components from both parental species. The first or "geographical and/or anatomical" barrier acts at the physical level, preventing coupling of the organisms involved. In fungi, this barrier would prevent successful fertilization through mechanisms like geographical isolation. In animals, the same general mechanism could prevent hybridization through anatomical incompatibility of the organisms in question. In both cases, the effect is the same: reproductive isolation. The second or "cytogenetical and/or karyotipic block" barrier ensures that the chromosome number is compatible between the participating species and that karyogamy can successfully occur. The third or "genome defense" barrier ensures that the level of match/mismatch at the DNA level is acceptable. Insertions and/or deletions, or other minute differences in sequence can result in the activation of mismatch repair and/or genome defense mechanisms, like RNA silencing. The fourth or "molecular complex compatibility" barrier acts at all levels and is a major issue for the development and fertility of hybrid progeny. It is activated by the "mixing" of proteins of different origins into common molecular complexes, such that the resulting

complexes are inactive. These barriers are sequential and can be generalized to most other organisms that undergo a similar sexual life cycle.

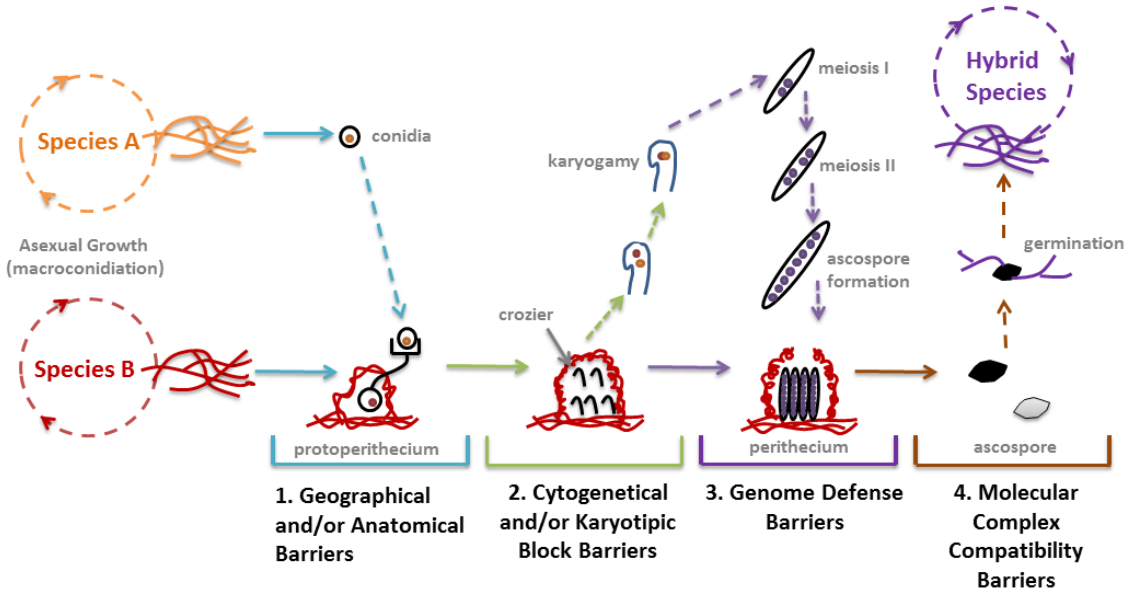

Figure 1. Barriers to interspecific hybridization in Neurospora. A conidia from species A fertilizes a protoperithecium from species B. This will result in the beginning of the sexual reproductive life cycle, generalized to most species of Neurospora. The brackets indicate the stages at which the barriers will have the greatest consequences. Sexual life cycle was adapted from Perkins (1988) [7].

In the model shown in Figure 1, we start with two independent species that are physically separated and undergo asexual growth. In Neurospora, the process of asexual growth is cyclical and is termed macroconidiation. In macroconidiation, a single conidium will grow into a tube-like branched hyphae structure. The hyphal structure will undergo segmentation and budding to form macroconidium that contain multiple nuclei and micronuclei that contain a single nuclei [8] . Both macronuclei and micronuclei are able to further asexual growth.

Geographical and/or anatomical barriers

To trigger the start of sexual growth, a microconidium will fertilize a protoperithecium of the opposite mating type. A protoperithecium is typically formed under conditions of reduced carbon and nitrogen and serves as the principle structure for sexual reproduction to occur [9]. Originating from the protoperithecium, a trichogyne structure will grow towards the conidia in response to pheromones that are emitted by the conidia [10]. Afterwards, the male conidia will fuse to the trichogyne structure (plasmogamy) and allow the nucleus to travel down the trichogyne into the ascogonial or ascus-mother cell containing the female nucleus.

At this stage in the sexual cycle, any factors that can prevent any of these processes from successfully occurring are considered to be barriers to coupling. The barriers to fertilization consist of geographic barrier, behavioral barriers, and anatomical barriers, and/or molecular complex compatibility barriers. Geographic barriers consist of primarily environmental factors such as location and physical conditions such as temperature, weather, and habitat requirements that would prevent two different species from coming into close enough contact for fertilization to be attempted. Geographical barriers were first observed by Charles Darwin, when he saw that finches had unique and distinguished characteristics depending on which of the Galápagos islands that they were located [11]. The finches had adapted unique characteristics, as observed by changes in the beak based on the environment of the individual islands. Since the islands posed a significant barrier to gene flow, this led to the speciation of the finch into multiple

species with unique characteristics. Furthermore, by a process called reinforcement (Wallace effect) after speciation has occurred, the new species, due to natural selection, will have a decreased ability to intercross with similar species or even their parental species, resulting in a greater amount of reproductive isolation [12].

Behavioral isolation is the result of differences in courtship and mating rituals that can prevent two different organisms from having the desire to undergo sexual reproduction. For instance, in Neurospora, the emission of a pheromone molecule by the male conidia to trigger the female trichogyne structure is largely a behavioral response. The absence of the pheromone is suggested to largely inhibit the further development of the perithecium, effectively reducing the efficacy of fertilization [10]. As a result, potential misrecognition of the pheromone structure by a different species due to genetic drift could prevent the appropriate pheromone response from occurring. From this example, it is clear that the appropriate behavioral conditions are necessary to ensure that both organisms have the desire to undergo sexual reproduction and also for organisms of different species to correctly recognize the behaviors and coordinate timing when attempting to undergo sexual reproduction.

Anatomical isolation is due to differences in physical structures that can prevent mating from occurring. For example, in most species of Neurospora, the trichogyne structure will respond to the pheromone signal of the male conidia and grow towards and fuse with the male conidia. If the trichogyne structure is unable to fuse with the male conidia,

the male nucleus will not be able to mix with female nuclei within ascogenous tissue. For instance, the species *Aspergillus nidulans*, which shares many similar characteristics to Neurospora, produces haploid conidia, but relies directly on cells whose mating identity is internally determined, instead of a perithecial structure to trigger the sexual reproductive cycle [13]. Therefore, it can be argued that anatomical differences between Neurospora and Aspergillus will prevent sexual reproduction from occurring.

Cytogenetical and/or karyotipic block barriers

If fertilization successfully occurs and male nuclei join the female nuclei inside the ascogenous tissue, then, assuming that the molecular complex compatibility barriers do not act at this level, karyogamy will occur. In Neurospora, the period immediately before and after karyogamy is critical for the preparation of the genome to undergo meiosis. Even small segment duplications within the genome will activate genome defense mechanisms like repeat-induced point mutations (RIP) and/or meiotic silencing [14,15]. Differences that arise due to differences in chromosome number immediately after karyogamy can arrest sexual development. The period before karyogamy serves as a time when the integrity of the genomes participating in meiosis is checked for integrity. Repeated sequences are either removed by recombination, mutated by repeat-induced point mutation and/or modified by DNA methylation [16]. As a result, the genome is tightly controlled and differences or favorable variation due to repeats could be destroyed. In addition, the two nuclei must be able to physically fuse. In *Candida albicans* it has been shown that specific proteins can directly impact nuclear fusion and

pose as a barrier to karyogamy. For example, Kar3, a microtubule-based kinesin motor protein is critical for karyogamy to occur during sexual reproduction [17]. The deletion of Kar3 sharply decreases the efficacy of karyogamy and results in a decreased rate of sexual reproduction. Polymorphism in the Kar3 protein between different species of Candida has the potential to serve as a significant barrier to karyogamy since it is directly responsible for nuclear fusion.

Genome defense barriers

If karyogamy is successful, the next step in the sexual cycle for Neurospora is meiosis. Meiosis occurs inside the perithecium and will result in the formation of eight highly ordered ascospores within the ascus. Once these ascospores fully mature, they are forcefully ejected from the perithecium [18]. Meiosis is a highly regulated process and requires several precise factors to be correct in order to proceed to completion. As a result, many potential barriers exist to meiosis; we hypothesized that DNA methylation, DNA mismatch repair, and meiotic silencing of unpaired DNA pose as three significant barriers to meiosis in interspecific crosses.

DNA methylation is a process by which specific genes or regions of the genome are densely methylated. DNA methylation occurs during all stages of the cell cycle and is a key epigenetic tool in regulating the expression of genes. In meiosis, DNA methylation can pose an issue if significant differences in the DNA sequences of two different species cause the methylation of key genes necessary for meiosis to occur. In addition,

DNA methylation is a key regulator of imprinting, selecting whether certain genes are expressed from the paternal strain versus the maternal strain when existing in the diploid state. In studies of interspecific hybridization between two species of Arabidopsis, it was determined that imprinting had a large impact on the overall "genomic strength" and the ability to hybridize [19]. Therefore, by manipulating the level of genetic methylation, it is possible to change how certain genes are expressed, potentially affecting which meiotic components are produced and have the greater capacity to deal with genome sequences of two different species.

In addition, DNA methylation is associated with repeat-induced point mutation (RIP), a process that silences repeated sequences or elements in DNA in haploid genomes by inserting GC-to-AT transition mutations [16,20,21]. Remaining unmutated cytosine bases are methylated by a cytosine DNA methyltransferase, *dim-2* [22]. Therefore, the DNA methyltransferase *dim-2* has an important function during the sexual cycle in Neurospora. The absence of *dim-2* could induce the expression of previously silent genes or alter the balance of transcription of different genes, which may help interspecific crosses overcome reproductive isolation due to barriers present during meiosis.

Chromosomes participating in interspecific crosses are expected to have significant DNA heterologies. DNA mismatch repair recognition and repair proteins act as a checkpoint in meiosis. When DNA heterology is high, meiosis is halted by the Pachytene checkpoint. Mutants in DNA mismatch repair systems are predicted to

overcome the Pachytene checkpoint in high levels of DNA heterology [23]. It was proposed that the mismatch repair system recognizes regions with homology, only allowing recombination to occur between homologous sequences in order to prevent lethal rearrangements and maintain the structural integrity of the chromosomes [24]. This system has been shown to have a significant impact in both prokaryotic and eukaryotic interspecific crosses. In particular, deletions of the mismatch repair gene *msh-2* in the yeast strains *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* was determined to greatly decrease meiotic sterility between these two species [24]. This suggests that *msh-2* has a significant role in genome stability, inhibiting recombination unless there is a high sequence identity in Saccharomyces [25]. In Neurospora, a similar orthologue of *msh-2* is present [26]. We hypothesized that *msh-2* may have a similar role in inhibiting recombination in Neurospora, thus proving to be a substantial barrier to interspecific hybridization between different species of Neurospora.

The third potential barrier to meiosis is meiotic silencing of unpaired DNA. Meiotic silencing is a process that occurs exclusively during meiosis and triggers the silencing of all unpaired genes and paired homologs of those genes [27]. As a result, if a gene necessary for meiosis and/or ascospore maturation is unpaired, it will block meiosis and/or ascospore maturation. Several suppressors of meiotic silencing have been identified. In this study we are focusing on *Sad-1* (*Suppressor of ascus-dominance-1*) and *Sms-4* (*Suppressor of meiotic silencing-4*). *Sad-1* mutants have been hypothesized to increase the fertility of crosses between different species of Neurospora [28].

Sad-1 is an RNA-dependent RNA polymerase (RdRP) that exists at the cytoplasmic face of the nuclear periphery [28,29]. Its primary role has been suggested to be the conversion of aberrant RNAs (aRNAs) triggered by unpaired DNA into double-stranded RNA (dsRNA) [30]. The dsRNA will later be processed into small RNAs which can be used for the post-transcriptional silencing of unpaired DNA [31]. In addition, *Sad-1* is essential for the completion of meiosis since crosses without *Sad-1* are barren [28]. Like *Sad-1, Sms*-4 is also a suppressor of meiotic silencing. *Sms-4* is a RNA binding protein with nuclear localization [32]. Unlike *Sad-1*, homozygous crosses between *Sms-4* mutants are fertile in the absence of meiotic silencing [32].

Molecular complex compatibility barriers

The viability of hybrid progeny is strictly dependent on any molecular complexes to perform their original functions. During evolutionary time, molecular complexes present inside a cell have been optimized for function. For example, the DNA replication machinery would be expected not only to perform its function but to do so at the correct growth temperature and rate of the organism in question. It is likely that key components of the system would be aided by ancillary proteins that as a whole, would ensure optimal performance. If we think of such a complex as a molecular machinery, the components of such machinery would be expected to be mixed or absent in interspecific crosses. The function or performance of such hybrid complexes is unpredictable. Only events that result in more robust complexes are expected to be fixated during evolution. These mechanisms are expected to be active in the hybrid ascospores of an interspecific cross.

Typically, the ascospores produced by interspecific crosses will either be black, indicative of a healthy ascospore, or white, indicative of an aborted ascospore. Only black ascospores will be able to successfully germinate. At this stage, the hybrid progeny will only be able to be viable and potentially be fertile if it contains all the necessary genetics components necessary for survival and reproduction. Hybrid ascospores are expected to contain a mixture of proteins from every single molecular complex in the cell, unless those complexes are essential and not functional. In addition, chromosomal translocations can affect the viability and fertility of hybrid progeny [33]. The challenge is to determine the extent to which molecular complex compatibility barriers affect interspecific hybridization.

The present study

This study aims to further understand the genome defense and molecular complex compatibility barriers that may exist in interspecific hybridization from a genetic stand point. It has been frequently established that two particular species of Neurospora, *N. crassa* and *N. tetrasperma* appear to exhibit fertility and undergo karyogamy, but fail to produce any viable progeny. Thus, by studying the interspecific crosses between these two species, we can focus specifically on the barriers to meiosis and further explore the potential development barriers that may arise. To understand the effects of the three potential barriers to meiosis discussed, knock-out mutants were utilized that would suppress DNA methylation, DNA mismatch repair, and meiotic silencing. We expected that suppression of these mechanisms would result in increased production of viable

ascospores. In addition, the progeny from successful interspecific crosses were obtained and analyzed to determine their identity.

CHAPTER II

METHODS

This study consisted of two stages: interspecific crosses between *N. crassa* and *N. tetrasperma* and the analysis of the potentially hybrid progeny that resulted from these crosses. In the first stage, we used mutants of *N. crassa* that would suppress DNA methylation, DNA mismatch repair, or meiotic silencing and crossed these strains to *N. tetrasperma*. The spores from these crosses were harvested and quantified. In the second stage, the spores obtained from these crosses were germinated and a set of genetic and phenotypic analysis was performed to determine the identity and genetic composition of the potentially hybrid progeny.

Stage 1: Interspecific crosses between N. crassa and N. tetrasperma

Preparation of media

Synthetic crossing media was prepared in a 1 liter volume according to the directions prescribed by Westergaard and Mitchell [34] and supplemented with 1 mM inositol, 1 mM histidine, 1% sucrose, and 1.5% Bacto Agar (BD). The media was autoclaved for 20 minutes (260°F, 18 psi) and approximately 30 ml was poured into several sterile petri dishes.

Liquid media was used to activate the strains. Vogel's media [35] supplemented with 2% sucrose, 1mM inositol, and 1 mM histidine were prepared and mixed in a beaker.

This media was used to fill 1 ml of media into several 1 mm glass test tubes. These test tubes were then plugged with cotton and autoclaved for 20 minutes (260°F, 18 psi).

Strains

The strains that were utilized for the interspecific crosses are shown in Table 1.

Strain	Genotype	Species
FGSC 1270	wild-type *A*	*N. tetrasperma*
FGSC 1271	wild-type *a*	*N. tetrasperma*
74 ORS 23A	wild-type *A*	*N. crassa*
ORS a	wild-type *a*	*N. crassa*
FGSC 8740	*Sad-1*$^{\Delta}$::*hph*$^{+}$ *A*	*N. crassa*
FGSC 8741	*Sad-1*$^{\Delta}$::*hph*$^{+}$ *a*	*N. crassa*
RPNCR 202	*his-3*; *Sms-4*$^{\Delta}$::*hph*$^{+}$::*npt*; *inl A*	*N. crassa*
RPNCR 203	*his-3*; *Sms-4*$^{\Delta}$::*hph*$^{+}$::*npt*; *inl a*	*N. crassa*
RPNCR 335	*rid-1*^{RIP245}, *his-3*$^{RA+}$::*Sms-4*$^{W97*fs[BamHI-HinDIII]}$; *Sms-4*^{UV} *A*	*N. crassa*
RPNCR 336	*rid-1*^{RIP245}, *his-3*$^{RA+}$::*Sms-4*$^{W97*fs[BamHI-HinDIII]}$; *Sms-4*^{UV} *a*	*N. crassa*
RPNCR 77	*his-3*$^{-}$; *dim-2 A*	*N. crassa*
RPNCR 75	*his-3*$^{-}$; *dim-2 a*	*N. crassa*
RPNCR 192	*his-3*; *msh-2*^{RIP172} *A*	*N. crassa*
RPNCR 193	*his-3*; *inl* ; *msh-2*^{RIP172} *a*	*N. crassa*

Table 1. Strains utilized for interspecific crosses. Important abbreviations: "*A*" and "*a*" refer to the mating type of the strain. *Hph*$^{+}$ indicates that the strain possesses hygromycin resistance. *inl* (89601), *his-3* indicates that inositol or histidine supplements will be required for the strain to grow.

Parental Strain 1	Parental Strain 2
FGSC 1270	FGSC 1271
ORS a	ORS A
FGSC 1271	ORS A
FGSC 1271	FGSC 8740
FGSC 1271	RPNCR 202
FGSC 1271	RPNCR 335
FGSC 1271	RPNCR 77
FGSC 1271	RPNCR 192
ORS a	FGSC 1270
FGSC 8741	FGSC 1270
RPNCR 203	FGSC 1270
RPNCR 336	FGSC 1270
RPNCR 75	FGSC 1270
RPNCR 193	FGSC 1270

Table 2. Interspecific crosses.

Experimental procedure

Liquid media in 1 mm glass tubes was inoculated with small portions of the conidia from frozen stock using a wet sterile cotton applicator to transfer the conidia. The inoculated media was incubated for two days at 35°C and one day at room temperature. Afterwards, the tubes were vortexed. Small portions of the activated conidia were used to point inoculate the petri dishes containing synthetic crossing media on opposite sides of the dish to set up the crosses listed in Table 2. All crosses were performed in triplicates. After inoculation, the plates were places inside a sterile translucent box and placed in the room temperature (25°C) incubator. Light was present for the crosses at all times during experimentation. Eight days post inoculation, the lids of the petri dishes were replaced with new sterile lids. Thirty-two days post inoculation, ejected ascospores were harvested from the lid of the petri dish with 100µl of 0.5 M EDTA and stored in a 1.5

mL Eppendorf tube. Spores were quantified under a light microscope using a hemacytometer. Figure 2 shows an illustration of the basic experimental scheme.

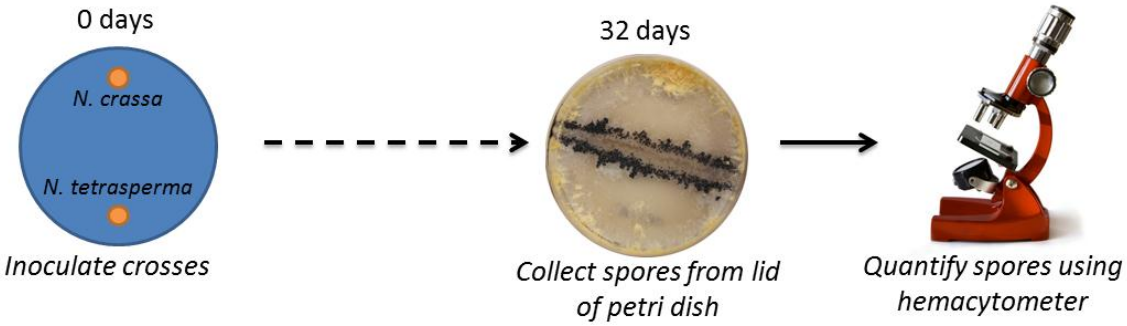

Figure 2. **Experimental schematic of interspecific crosses.**

Stage 2: Analysis of hybrid progeny

Media preparation

The media used to germinate the ascospores was prepared using Vogel's media supplemented with 1 mM inositol, 1 mM D-panthotheonic acid, 1 mM Histidine, 0.125% N-Z-Amine (Sigma), 2% Difco Agar (BD), and Brockman and De Serres (BdeS) sugar (2% sorbose, 0.05% glucose, 0.05% fructose) in a 0.5 Liter volume. Media was autoclaved for 20 minutes (260°F, 18 psi), after which sterile BdeS was added. The media was poured into sterile petri dishes at an approximate volume of 30 ml.

Liquid media in 1 mm glass tubes was prepared identically to stage one to grow progeny after they germinated.

Sexual crossing media was prepared using the same method in stage one, but was poured into 50 mm square petri dishes at an approximate volume of 50 ml.

Strains

Strain	Genotype	Species
RANCR 49	wild-type *fl A*	*N. crassa*
RANCR 50	wild-type *fl a*	*N. crassa*
FGSC 1271	Wild-type *a*	*N. tetrasperma*

Table 3. Strains utilized in stage 2. Important abbreviations: "fl" indicates that the strain is a fluffy mutant that does not produce macroconidia.

Experimental procedure

In stage 2, hybrid progeny are germinated and assessed for their genotypic and phenotypic characteristics (Figure 3). 0.5µl of spores are added to a germination plate along with 100 µl water. The ascospores were spread evenly on the plate. The plate was then placed in a 60°C incubator for 45 minutes to activate the ascospores. Afterwards, the plate was removed allowed to sit at room temperature overnight. The following day, the percent germination rate was determined by counting the number of spores on the plate that successfully germinated and counting the number of spores on the plate that were unable to successfully germinate. The number of spores germinated divided by the total number of spores counted gives the germination rate. Then the germinated spores were transferred individually using a sterile flat needle tip into the 1 mm tube contain Vogel's media supplemented with 1 mM inositol and 1 mM histidine. The inoculated media was incubated at 35°C for two days and at room temperature for two days. The

formation of perithecia or false perithecia at the bottom of the test tube was noted. In the meantime, a wedge of the wild type fluffy *N. crassa* strains (Table 3) was used to inoculate the sexual media. After approximately 5 days of growth, a uniform orange mycelial mat appears. A small portion of the conidia from the baby tubes was used to fertilize a portion of this mycelial mat. The crosses were grown for three to four days and the level of fertility and mating type were recorded. Only strains of the opposite mating type of the mycelial mat will be able to form perithicia. Also, it is important to note that *N. tetrasperma* will have significantly reduced fertility with the WT *N. crassa* mycelial mat.

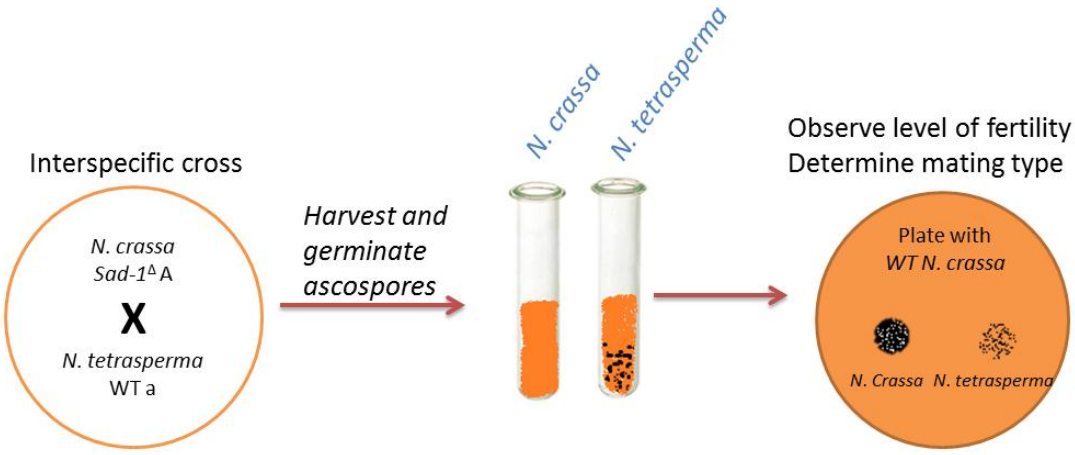

Figure 3. Basic schematic of phase 2. In this scheme, spores are harvested from the interspecific crosses are germinated and used to inoculate liquid media. The physical absence or presence of perithecia is a key differentiator between *N. crassa* and *N. tetrasperma*. Conidia from the liquid media is used to fertilized a wild-type *N. crassa* mycelia mat of both mating types determine mating type and the level of fertility the hybrid progeny has with *N. crassa*.

Once the characteristics of the progeny were determined, progeny were selected representing each set of genotypic and phenotypic characteristics. These progeny were then crossed back to *N. tetrasperma* (Table 3) using point inoculation on synthetic crossing media. The experimental set-up for these crosses is identical to that of the interspecific crosses. After 32 days, the ascospores were harvested and quantified using a hemacytometer.

CHAPTER III

RESULTS

In the first part of the experiment, we quantified the ascospore production from the interspecific crosses and compared them to intraspecific crosses. It is clear that the number of progeny produced from interspecific crosses between wild-type *N. crassa* and wild-type *N. tetrasperma* is insignificant compared to the progeny produced by intraspecific crosses under wild-type conditions using the same strains (Figure 4). Intraspecific wild-type crosses grew at a much faster rate and produced 4000 to 5000 healthy ascospores per petri dish. In contrast, the wild-type interspecific cross produced less than 10 ascospores, the majority of which appeared inviable. This result was expected since it was commonly known that most interspecific crosses between *N. crassa* and *N. tetrasperma* are largely barren.

While observing the effects of having loss of function mutations in interspecific crosses, an unexpected outcome appeared (Figure 5). The mutation of *dim-2*, a DNA methyltransferase required for DNA methylation produced no significant change or difference in outcome. The mutation in *msh-2*, a necessary component of the DNA mismatch repair machinery, tended to cause a slight increase in the production of black ascospores. Statistical analysis suggested that this increase was significant only at a p-value less than 0.1, providing a 90% confidence that this increase in ascospore

production is statistically significant. Further verification and repetitions will be necessary to ensure that this increase is truly statistically significant.

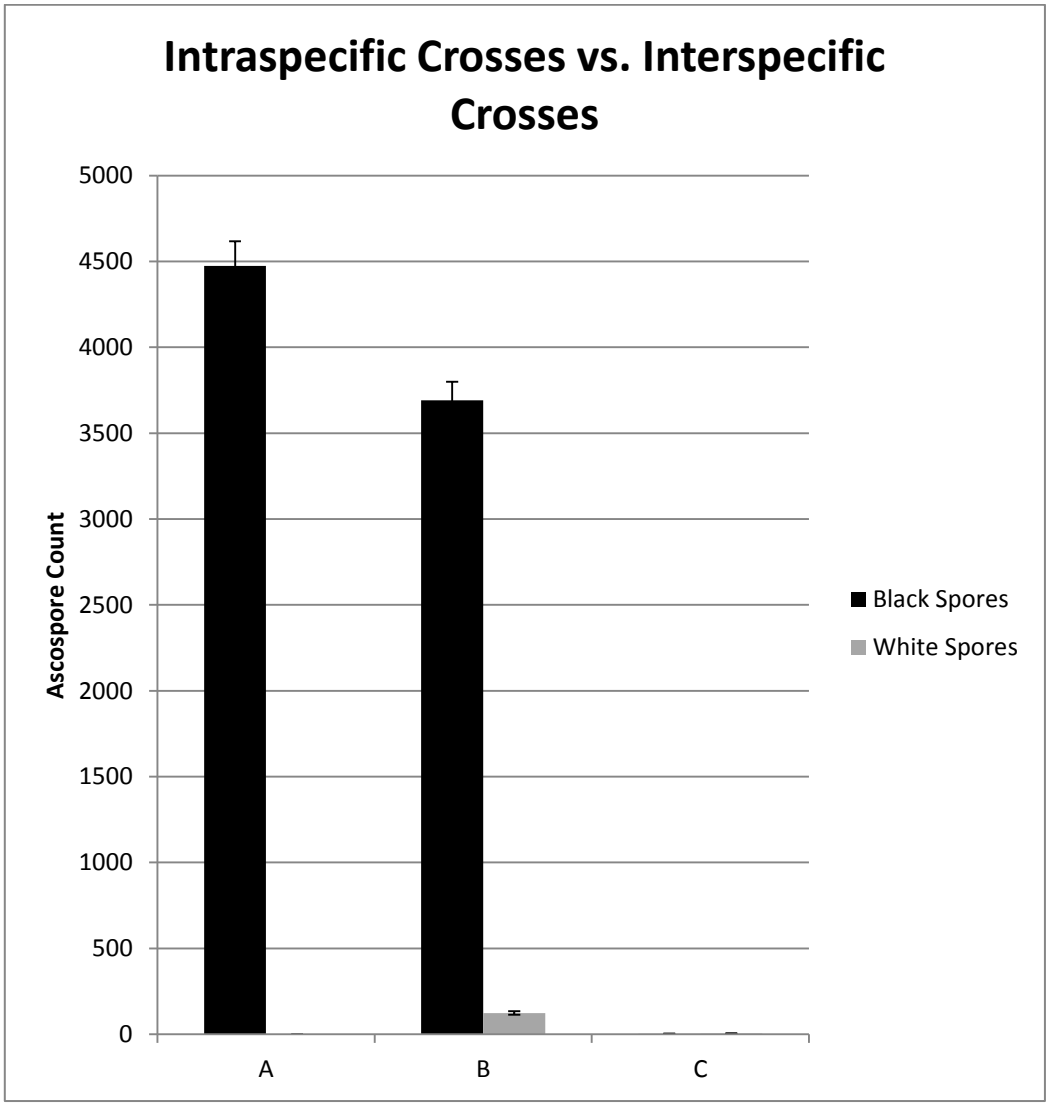

Figure 4. Comparison of intraspecific crosses to interspecific crosses. Order of bars: (A) WT *N. tetrasperma* X WT *N. tetrasperma*, (B) WT *N. crassa* X WT *N. crassa*, and (C) WT *N. crassa* X WT *N. tetrasperma.* The interspecific cross between wild-type *N. crassa* and wild-type *N. tetrasperma* had a negligible production of ascospores (<10) in contrast to the wild-type intraspecific crosses. WT interspecific cross is between ORS A and FGSC 1271. Similar results were observed with the reciprocal interspecific cross. These results are the average of two separate experiments with each cross performed in triplicate.

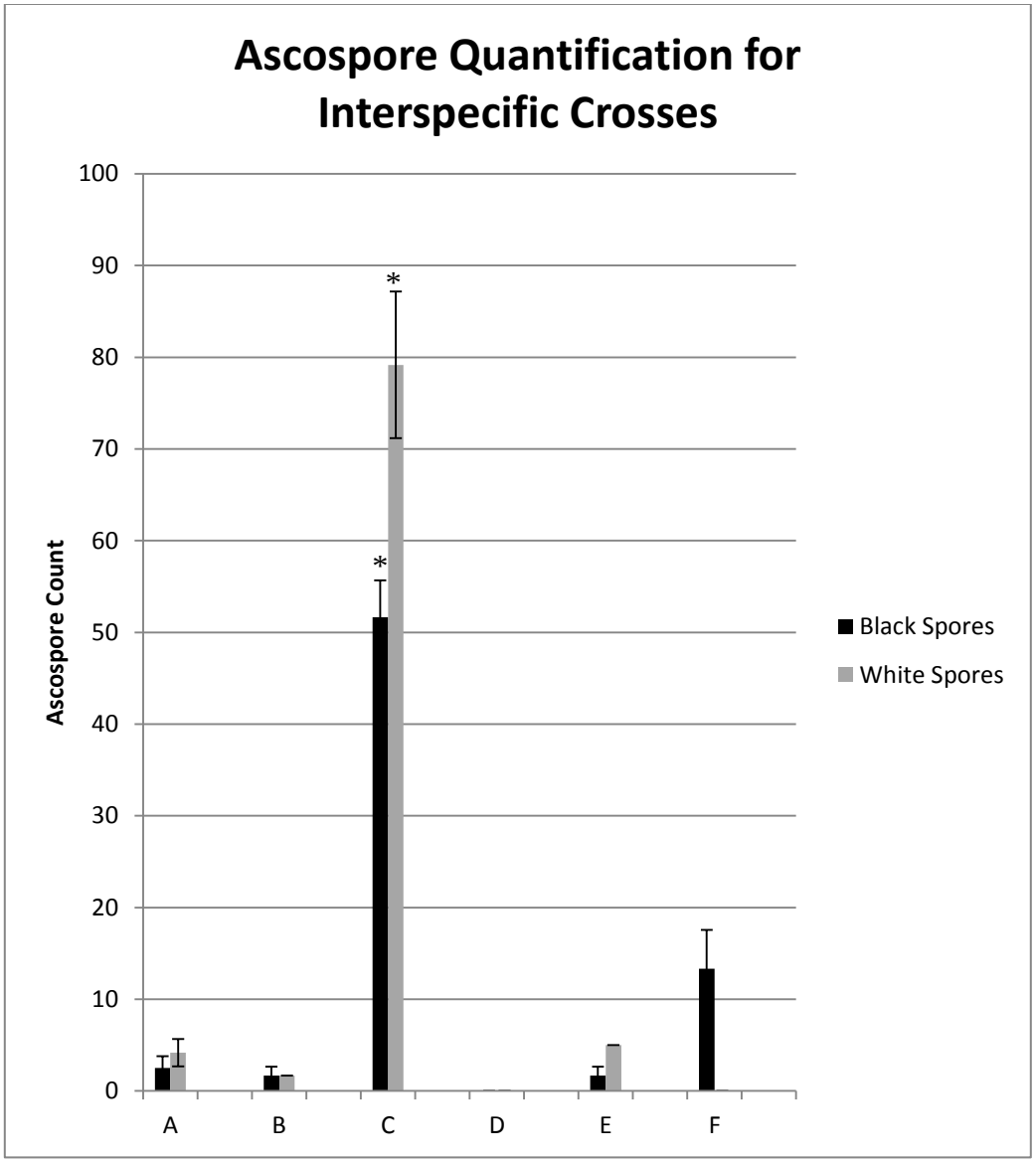

Figure 5. Comparison of wild-type interspecific crosses to interspecific crosses utilizing *N. crassa* loss of function mutants. *N. crassa* strains utilized and crossed to *N. tetrasperma*: (A) WT *N. crassa*, (B) *dim-2*, (C) *Sad-1*$^{\Delta}$, (D) *Sms-4*$^{\Delta}$, (E) *Sms-4*$^{fs/UV}$, *and* (F) *msh-2*^{RIP172}. * indicates that the increase is significant to a $p < 0.0001$ compared to the WT cross. Data is only shown for *A* mating type *N. crassa* knock-out strains crossed to FGSC 1271. Similar results were observed for the reciprocal cross. These results, except for *msh-2*^{RIP172}, is the average of two separate experiments with each cross performed in triplicate. The cross with *msh-2*^{RIP172} only shows the result for one experiment with each cross performed in triplicate.

A conflicting result appeared for the deletion of the meiotic silencing components, *Sad-1* and *Sms-4*. Deletion of *Sad-1* resulted in over a ten-fold increase in ascospore production, producing on average 50 black ascospores and 75 white ascospores. This increase is significant to a p-value of less than 0.0001, providing a 99.999% confidence. In contrast, neither deletion of *Sms-4* nor mutation in *Sms-4* produced any notable increase in ascospore production compared to wild-type interspecific crosses. Also, it is important to note that no significant difference was observed between reciprocal crosses, performed using strains of the opposite mating types.

In the second stage of the experiment, the progeny from the interspecific cross containing the *Sad-1* deletion were selected and germinated. Only this cross was selected since it was the only one the produce a sufficient number of progeny that could be analyzed. After germination, it was determined that the progeny of both reciprocal crosses containing the *Sad-1* deletion had an approximately 8% germination rate in contrast to intraspecific crosses which have a germination rate near 100%. The progeny that germinated were then analyzed based on the criteria presented in Table 4a.
In this table, any progeny that assumes characteristics from both columns was labeled as a “hybrid.” In addition, if a novel phenotype was visible, this was sufficient enough to be able to label the progeny as a hybrid.

A.

Neurospora crassa	***Neurospora tetrasperma***
Mating type: *A*	Mating type: *a*
High fertility with WT N. crassa	Low fertility with WT N. crassa
Forms no perithecia at room temperature	Forms false perithecia at room temperature or exhibits self-fertility

B.

Mating Type	Fertility	Formation of Perithecia in Liquid Media	Hybrid	Number of germinated ascospores
A	Low	No	Yes	2
A	Low	Yes	Yes	13
A	High	No	Undetermined	3
A	High	No	Yes	2*
A	High	Yes	Yes	8
a	High	Yes	Yes	1
a	High	No	Yes	1

Table 4. Identity of hybrid progeny. A.) Characteristics distinguishing *N. crassa* from *N. tetrasperma*. Any progeny that shares characteristic of both *N. crassa* and *N. tetrasperma* is classified as a hybrid. B.) Characterization of progeny germinated from a cross between FGSC 8740 and FGSC 1271. *These progeny had an unusual phenotype in which they did not produce macrocondia ("fluffy"). This was sufficient to conclude that these two progeny must by hybrids. A total of 27 germinated progeny were analyzed from this cross.

Using these criteria, I was able to determine that at least 24 of 27 progeny that I was successfully able to germinate were hybrids (Table 4b) from the cross between FGSC 8740 and FGSC 1271. Similarly, five of seven germinated progeny were determined to be hybrids (data not shown) for the reciprocal cross, FGSC 8741 crossed to FGSC 1270. The full identity profiles of the progeny are shown in Table 4b. The progeny that are classified as undetermined exhibited characteristics only of *N. crassa* or *N. tetrasperma*

based on the criteria in Table 4a**.** Further analysis will be required to determine whether these progeny are fully identical to the parental strains. In addition, it was interesting to note that over 90% of the progeny that were fully classified as hybrids from both crosses inherited the mating type allele from the *N. crassa* parental strain. This was highly unexpected since in normal intraspecific crosses, the probability of inherited either of the mating type alleles is 50%.

The ascospore quantification from the backcross of the hybrid to *N. tetrasperma* allowed us to assess whether the progeny had an improved or reduced level of fertility with *N. tetrasperma*. From our ascospore quantification (Figure 6), we were able to see large variations in the level of ascospore production, ranging from completely barren to restoring intraspecific levels of fertility. While the variability in fertility was not unexpected, it was surprising to see that select progeny had an extremely high level of fertility with *N. tetrasperma*. It was also interesting to note that some of the hybrid progeny had a reduced level of fertility than the parental interspecific cross even though they were higher in identity to *N. tetrasperma* than the initial *N. crassa* parental strain. This suggests that certain recombination favors inheriting genes that allow for greater viability with *N. tetrasperma*.

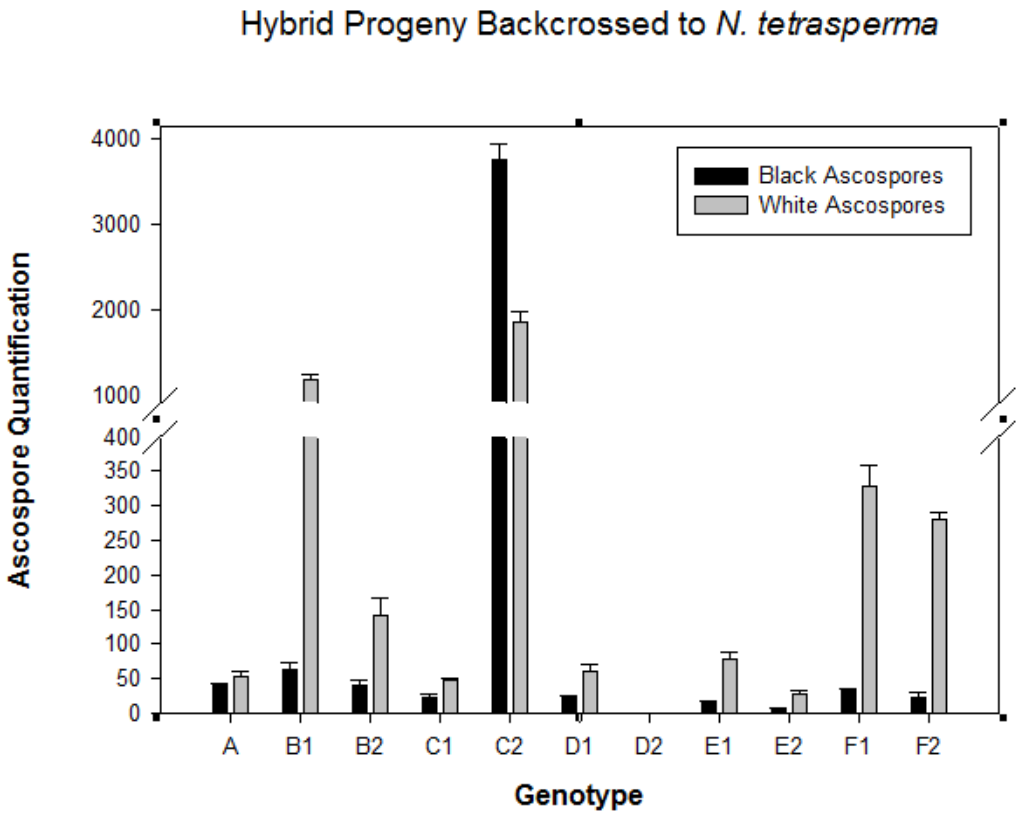

Figure 6. Ascospore quantification of backcross between hybrid progeny and *N. tetrasperma*. Spores harvested from select hybrid strains were quantified 32 days post inoculation. Two progeny were randomly selected from each category in Table 4b that were definitively concluded as a hybrid, indicated at 1 and 2 in the genotype. The genotypes of the hybrid strains are: (A) *Sad-1*$^{\Delta}$ WT *N. crassa*, (B) low fertility, no perithecia, (C) low fertility, perithecia, (D) low fertility, no perithecia, fluffy, (E) high fertility, no perithecia, and (F) high fertility, perithecia.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Our results reinforce the notion that *N. crassa* and *N. tetrasperma* and reproductively isolated. However, we observed that this barrier can be overcome with the use of mutants.

Of the mutants studied during this experimentation, only *Sad-1* and *msh-2* produced a noticeable increase in ascospore production. An increase in ascospore production suggests that the interspecific cross was able to proceed to completion to a larger extent than if the knock-out mutations were not present. *Sad-1* behavior was not unexpected since a similar result had been previously demonstrated [24,28]. Our *msh-2* observations were similar to that observed previously in Saccharamyces [24].

Surprisingly, neither *dim-2* nor *Sms-4* had any effect. We hypothesized that *Sms-4* would also have increased ascospore production since it was shown that the deletion of either *Sad-1* or *Sms-4* both resulted in the suppression of meiotic silencing [32]. Since both *Sad-1* and *Sms-4* are crucial components of the meiotic silencing pathway, this either challenges the notion that meiotic silencing serves directly as a barrier to the successful completion of meiosis and contributes to increased reproductive isolation between species or suggests that meiotic silencing is a more complex pathway. A further analysis of the activity of *Sad-1* and the testing of suppressors that act in the same stage

as *Sad-1* will be required to elucidate whether the greater interspecific fertility is due to the absence of meiotic silencing or due to the absence of *Sad-1*.

The only other mutant strain that showed an increase in ascospore production in interspecific crosses was an *msh-2* mutant, which suppressed DNA mismatch repair. The increase was only significant at a 90% confidence level; therefore further testing is necessary to clarify this result. It is important to note that any increase resulting due to a mutation in *msh-2* is much less than that resulting from the deletion of *Sad-1*. Therefore, these results are compatible with the hypothesis that *msh-2* plays a much weaker role as a barrier to meiosis in Neurospora than previously shown in interspecific crosses of others organisms such as those in the genus Saccharomyces [24]. This is most likely due to differences in the genomic architecture of Neurospora, which is highly regulated and controlled by several different genome defense mechanisms. In addition, it has been shown that *msh-2* in Neurospora is not as stringent in checking homology during recombination compared to Saccharomyces [36,37]. *msh-2* may not have as significant a role during the sexual life cycle of Neurospora, therefore having a less significant contribution to the overall barrier of reproductive isolation.

Hybrid progeny from an interspecific cross will typically demonstrate characteristic of both parents. Of the progeny that we were able to obtain and germinate, most of which we were able to conclude are true hybrids from the interspecific crosses. The major implication of this is that this proves recombination occurred at least on the

chromosomal level. This implies that given random segregation of chromosomes, no barrier exists that favors combining only chromosomes that would produce progeny identical to the parents. The methods used in this experimentation were unable to determine the full extent of chromosomal recombination. This can be further studied by using PCR to trace unique markers found on each of the chromosomes that differ between the two species or by whole genome sequencing. These are more expensive and time consuming techniques, but would have allowed us to understand the full breadth of chromosomal recombination that may have occurred.

The other unique finding is that over 90% of the germinated progeny inherited the mating type from the *N. crassa* strain. Both the *Sad-1* gene and the mating type gene are both present on linkage group I. Previous research has suggested that a significant inversion is present in linkage group I of *N. tetrasperma*, preventing recombination with linkage group I of *N. crassa* [38]. Therefore, it is likely that the viability of hybrid progeny favors the absence of the *Sad-1* gene or that some other element in the *N. crassa* Linkage Group I favors the development of the hybrid progeny. Further analysis will be required to ensure that the *Sad-1* allele is not present and to determine which elements specifically helps favors the development of the hybrid progeny. We are able to establish that an element of Linkage Group I greatly assists the viability of the hybrid progeny. Identification of the element that allows for hybrid progeny formation can be useful in understanding how to create stabilized hybrid species.

In the last stage of the project, we determined that hybrid progeny had a high level of variability in the level of fertility when backcrosses to *N. tetrasperma*. Given that some of the strains were less fertile when backcrossed with *N. tetrasperma* and some were more fertile, this suggests that many factors affect the fertility of the hybrid progeny. This is further emphasized by the fact that some progeny had reduced fertility even though we knew that they were hybrids, and thus had a greater percentage of the genome of *N. tetrasperma* than the parental *N. crassa* strain. Also, a few progeny had an unusual high level of fertility. These progeny either had an extremely high percentage of the genome inherited from *N. tetrasperma* or due to recombination and mutations inherited the right combination of genes that allowed for the high levels of fertility. The decreased levels of fertility are most likely the result of molecular complex compatibility barriers. By whole genome sequencing, we could potentially identify which factors in these hybrid progeny led to the unusually high level of fertility with *N. tetrasperma.*

The data from this study implies that singular factors can potentially have a larger contribution to reproductive isolation than complex processes such as DNA methylation, DNA mismatch repair, and meiotic silencing. The suppressor, *Sad-1*, which has a major role in meiotic silencing, was implicated in contributing significantly to reproductive isolation, but given the results of the *Sms-4* suppressor, this does not suggest that the role of *Sad-1* in reproductive isolation is due to meiotic silencing. Further research into how *Sad-1* functions may allow us to understand and identify other proteins whose presence or absence caused speciation of an organism into multiple species. By identifying such

factors, we can learn how to induce novel phenotypes more efficiently, which has its implications in both agriculture and understanding recombination in more complex organisms.

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