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Investigations into the Genomes of Two Ascomyetes

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Fusarium verticillioides is a filamentous ascomycete that is both a plant endophyte and pathogen, causing disease during any life stage of the plant. When *F. verticillioides* grows in maize the fungus can synthesize a number of mycotoxins including the fumonisins, which have been linked with human esophageal cancer and neural tube associated birth defects. In an attempt to control fumonisin production my lab is searching the genome of *F. verticillioides* for a selfish genetic element known as *Spore killer*. The plan is simple; we envision creating a bio-control strain capable of harnessing the genetic transmission-distorting properties of *Spore killer* to modify the genetic structure of agricultural populations of fungi by linking the *Spore killer* element with the gene cluster responsible for fumonisin production that has been deleted or is deficient in the ability to synthesize the mycotoxins. In the case of *F. verticillioides*, this could allow us to target fumonisin synthesis in an agricultural population and limit the contamination of agricultural products. Here I present the necessary steps toward cloning and characterizing the locus that causes spore killing in *F. verticillioides*.

Neurospora crassa is a model organism that has long been a vital tool in the laboratory setting. *Neurspora crassa* is often used in research focusing on genome defense mechanisms. One such mechanism is Meiotic Silencing by Unpaired DNA (MSUD). MSUD identifies unpaired sequences in the genome during the fungus' brief diploid phase during meiosis. Any sequence detected as unpaired is then directed down an RNAi pathway. MSUD has only been shown to occur in the fungus *N. crassa* and the mechanism of how the fungus identifies unpaired DNA regions is, as of yet, still unknown. By demonstrating the presence of MSUD in a close relative, *N. intermedia,* this research is the first to show MSUD occurring outside of *N.* crassa and should provide new data to assist in better understanding the search mechanism of MSUD.

INVESTIGATIONS INTO THE GENOMES OF TWO ASCOMYETES

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CHAPTER I

THE PROBLEM AND ITS BACKGROUND

Statement of the Problem

The fungal endophyte *Fusarium verticillioides* (Teleomorphs: *Gibberella moniliformis* or *Gibberella fujikuroi*) is commonly known to colonize agricultural crops such as cereal grains and is a common maize endophyte. *F. verticillioides* colonization has shown to be useful to plants by preventing the colonization of the host by other competing fungal colonies which are often times more detrimental to the plant's development (Kensub, Pan, and May, 2009). *Fusarium* endophytes have also been shown to stave off infection of host plant tissues from bacterial invasion. In one study Bacon and colleagues found that established endophytes such as *Fusarium verticillioides* were able to greatly reduce colonization by the bacterial strain *Bacillus mojavensis,* via the production of Fusaric acid, which was being tested as a form of bio-control to prevent plant pathogen infections (Bacon Et al., 2004).

Despite the protective benefit of *Fusarium* strains there is a major down side to *Fusarium* infection which is that, given the right set of conditions, *Fusarium* colonization can switch from asymptomatic endophytic colonization to the necrotizing stage of infection during which hydrolytic enzymes and mycotoxins are produced (Oren Et al., 2003). One major class of mycotoxins produced is the Fumonisins, of which there are

more than 10 characterized. However the most prevalent form of Fumonisin is FB1 which also happens to be the most toxic form. Fumonisin class B toxins are composed of a linear 20-carbon backbone formed from a polyketide that has been substituted at various positions with an amine, from one to three hydroxyl groups, two methyl groups, and two tricarboxylic acid groups (Proctor et al., 2003).

FB1 is a relatively large molecule which undergoes multiple intermediate metabolic steps which are regulated by the FUM cluster which is a cluster of at least 15 genes directly regulating fumonisin production (Proctor et al., 2003). Currently one of the most common means of controlling infection and disease related to the Fusarium genus has been through the application of systemic fungicides. The majority of these fungicidal chemicals are known as demethylation inhibitors (DMIs) and act through the act through inhibition of ergosterol biosynthesis thereby decreasing membrane integrity. The use of DMIs has remained relatively high since their inception in the 1970s primarily due to the lack of effective competitors. DMIs have been used almost exclusively for fungal pathogen control. It is not surprising then that through excessive use there are now strains of pathogenic fungi which have reduced sensitivity to DMIs and even a few strains that are beginning to show resistance (Koch Et al., 2013). More modern attempts at controlling fumonisin production has been aimed at eliminating the fungus by using biological control by inoculating atoxigenic *Fusarium* strains, other fungi, or bacteria into the agricultural settings. These methods often have proven to not be completely effective at controlling *Fusarium* growth and even less efficient at reducing fumonisin production. These methods also may kill off or replace *Fusarium*

strains which prevent naturally occurring *Fusarium* strains from having beneficial effects that stall further colonization of the crops by other harmful species. As such, mycotoxin contamination, such as fumonisins, of food and feed crops remains a dramatic worldwide problem.

Significance

A study in 2003 indicated that mycotoxin derived losses from three crops (corn, wheat, and peanuts) results in a $932 million annual loss nationally. This figure does not even include the $436 million lost annually to contamination testing and regulation enforcement related to mycotoxin contamination of these same three crops (Dohlman, 2011). Currently there are relatively relaxed worldwide limitations on fumonisin levels in food products, but with an increasing concern for public safety other nations are adopting more stringent policies. As the number one corn exporting country in the world it would be highly detrimental to our economy if newer more strict standards were imposed on global corn commodities considering their current levels in our exports.

Even more important than financial losses, fumonisins have a global impact on health and has been connected with regions that have a high incidence rate of human esophageal cancer. Fumonisins, produced during the necrotizing stage of *Fusarium* pathogenesis, have been shown to cause liver and kidney cancer in laboratory rodents (Howard Et al., 2001). The consumption of fumonisin contaminated maize has also been linked to an increased rate of esophageal cancer incidence in areas with a high rate of

maize consumption (Marasas, 2001), and have been linked to neural tube defects and craniofacial defects in laboratory mouse embryos (Marasas, 2004).

Fusarium species have been isolated from corn, sorghum, rice, and other cereal grains from around the world. The carcinogenicity of fumonisins has also been linked with diet deficiencies such as folate and is also linked with other biological agents such as hepatitis and helicobacter (Marliere, Pimenta, and Cunha, 2009). These risk factors point towards a high occurrence bias in underdeveloped and developing countries. A better understanding the potentiation of mycotoxin production and the genetic regulation mechanisms would be of global economic and medical benefit.

Hypothesis

The overall hypothesis is that the placement of a *Sk* element within a defective/deficient fumonisin producing gene cluster will decrease the ratio of fumonisin producers compared to non-producers in populations of *Fusarium verticillioides* by driving the progeny towards the defective/deficient phenotype during meiosis by harnessing the transmission distorting properties of *Sk*.

While this is the working hypothesis for the primary goal of the laboratory I present here the hypotheses that represent milestones for our laboratory's research towards producing a biocontrol strain of *Fusarium verticillioides* capable of distorting fumonisin producing populations in the wild.

1- The endogenous *Sk* element is capable of generating meiotic drive sufficient to carry proximal genes through meiosis at a high rate in *Fusarium verticillioides*.

2- In-vitro sexual reproduction is dependent on media nutrient levels.

Definition of Terms

Fusarium verticillioides

Fusarium verticillioides, also known as *Gibberella moniliformis,* is a filamentous ascomycete which, like many other fungi, has two life cycles. The fungi can reproduce sexually through the production of ascospores within the perithecia, or clonally via asexual conidiation by means of either macroconidia or microconidia (Leslie and Summerell, 2006). *F. verticillioides* is a commonly identified inhabitant of soils and like other members of the *Fusarium* genus associate with plants, most notably maize. *F. verticillioides'* genome consists of 12 identifiable chromosomes while one of those is non-essential for healthy growth and development (Xu and Leslie, 1996).

Mycotoxins

Mycotoxins are relatively difficult to describe concisely because they belong to such a diverse group of compounds. Mycotoxins vary dramatically when looking at their structural and toxicological profiles. They typically exist as secondary metabolites, which mean that these molecules are not required for the viability, growth, or function of the organism producing it. The compounds are also most commonly small molecules with a low molecular weight. Simply put, mycotoxins are any chemical compound produced by filamentous fungi that are capable of causing disease and/or death in humans and animals. Beyond this is where confusion sets in. The confusion about what mycotoxins could be considered to begin with the ability for these molecules to be

advantageous for humans. Many modern chemicals that are still in use today are small, secondary metabolites produced by filamentous fungi. They just happen to be known by other names. For example, fungal toxins that are targeted towards killing bacteria are not considered mycotoxins. They are considered antibiotics. Also, small metabolites that are targeted towards plants are known as phytotoxins. Also adding to the confusion is dosage. Small molecule secondary metabolites produced by many fungi, such as ethanol, are only acutely toxic at high concentration and are commonly enjoyed for recreational consumption (Zain, 2011).

Meiotic Drive Elements

While considering genome biology we generally see that genomes are composed of groups of genes that work cooperatively toward the ultimate survival and passing on of genetic traits. As a whole these genes act selfishly for self-preservation of the whole group, and it is generally taught that each of these genes follow along with "dogmatic principles" and Mendelian Inheritance. However, there are those genes which independently increase the ratio of transmission into progeny and do not act for the overall organism's fitness. Examples of these meiotic drive elements have been described and are becoming increasingly better understood. Such elements include: Segregation Distorter (SD) in Drosophila, the t haplotype in mouse models, and *Spore killer* in Neurospora and *Fusarium* fungal species (Lyon, 2003; Kusano Et al., 2003; Turner, 2001). The lack of defenses for these types of genes can allow for the reduction and possibly even extinction of alternate alleles.

Spore killer

Spore killer (Sk) is a type of meiotic drive element present in the fungal taxa Neurospora and *Fusarium*. These genera are classified as Ascomycetes and as such reproduce sexually through production of ascospores. Typically, reproducing strains would produce 8 ascospores per ascus in a cross not containing killers, or in which both mating strains contain a copy of the killer element, of these eight spores that are produced in the ascus only four are produced from the nuclei of each parental strain. However, in a cross containing a single copy of the *Spore killer* only the four spores derived from the original parent containing the killer gene will reach maturity while the other four spores are prevented from maturing by the *Spore killer* element from the other parent, see Figure 1.

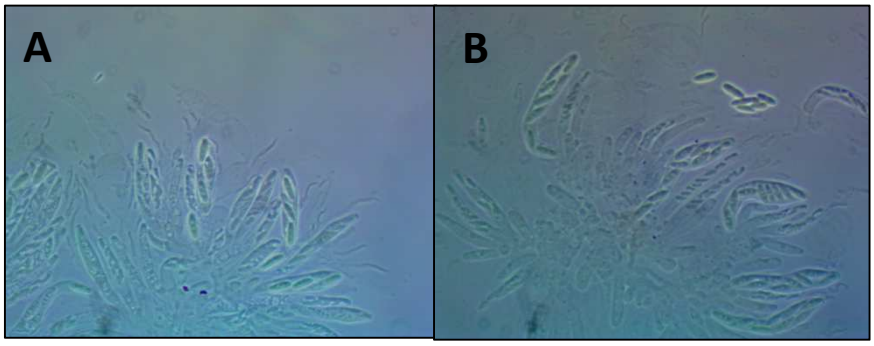

Figure 1. Illustration of Killing in *Spore killer* Strains of *Fusarium verticillioides*.*Sk* , *Spore killer*, Sk^{S} , *Spore killer* susceptible. A) Illustrates progeny from a cross containing a Sk^{S} strain and a *Sk* strain producing only 4 viable ascospores. B) Illustrates progeny from a cross between two *Sk* strains of *Fusarium verticillioides*, note the 8 matured spores.

CHAPTER II

REVIEW OF RELATED LITERATURE

Fusarium verticillioides

Fusarium verticillioides is a filamentous ascomycete that frequently colonizes maize crops and is prevalent worldwide with an expansive global distribution (Glenn Et al., 2007; Leslie, 1996; Nelson, 1992; Reid Et al., 2002; Brown Et al., 2007). *F. verticillioides* can infect maize at any stage during the plant's life cycle and has been identified as a causative agent for disease of the ears, stalks, and seedlings (Brown Et al., 2007). Contamination of human food and animal feed stocks by *Fusarium* is becoming a more highly recognized global health concern specifically due to the implications of the consumption of fumonisins. In livestock the adverse effects associated with the consumption of fumonisins well below the lethal dosage have been documented as contributing to a wide variety of damage at the cellular and molecular level. Fumonisin consumption has been linked to lesions of the liver, lungs, brain, and gastrointestinal tract in livestock ranging from porcine to poultry. There is also evidence to suggest a link between fumonisin consumption and onset of equine leukoencephamalacia (ELEM) and Porcine Pulmonary Edema (PPE) (Fazekas, 1998; Schumacher et al., 1995; Nelson et al., 1992).

Despite their toxic effects not being entirely understood fumonisins have been a concern since their identification in a South African research study from 1988 by Gelderblom, Et al. In 1970 a severe outbreak of ELEM led an investigative team to the identification of *F. verticillioides* growing in mold infested maize stores that served as feed stock for the affected animals. The infamous strain MRC 826 was isolated from these feed stores and was subsequently identified as the causative agent associated with the ELEM outbreak in 1971 by B.J. Wilson and colleagues (Gelderblom Et al., 1988 and Wilson Et al., 1972). At this point the study of disease outbreak was focused on these MRC 826 isolates and had not yet been refined to a single group of molecules. These fungal isolates, along with their growth medium, were used in studies that showed them to be able to cause the onset of ELEM and PPE and to also be toxic in both liver and heart tissue in pigs, sheep, and baboons (Kriek Et al., 1981).

Around the same time as the studies of these veterinary disease outbreaks researchers began investigating the regional hotspots of esophageal cancer (EC) incidence of Transkei, South Africa. The investigation was an exemplary case study because the rate of EC in the Southern portion of Transkei was among the highest in the world while the Northern region of Transkei had relatively low levels of EC incidence. Interestingly, the second most prevalently reported cancer in the region was liver cancer in males (Jaskiewicz Et al., 1987 and Makaula Et al., 1996). These studies also identified locally grown maize as being a staple in the diet of people of the Transkei region, and they showed that the most prevalent contaminant in both maize crops was *Fusarium*

verticillioides isolates. The disparity of cancer incidence between the two regions along with the data associated with the animal studies came together driving the researchers' consensus to be that fungal contamination was not the direct causative agent, but that the causative agent must be something that is not constitutively produced by *F. verticillioides.*

While researchers at the timebegan to look for classes of compounds or even single molecules associated with the toxicity of certain *Fusarium verticillioides* cultures and culture media the task remained daunting. In the mid-1980s there was a large variety of already known mycotoxins produced by *Fusarium sp.* including: gibberellins, moniliformin, tricocethenes (specifically T-2 toxin, deoxyvalinol (DON), and diacetoxyscirpenol), Zearalenone, Fusaric Acid, and Fusarins A, B, C, and D to name a few (Marasas, 2001). After extensive testing it was revealed that the causative agent contained in the MRC 826 strain was not a known mycotoxin, so the next step in the research was to fractionate the toxic strain and isolate the carcinogen.

The first major result occurred in 1988 by Gelderblom and colleagues who isolated two unknown compounds, found primarily in aqueous methanol extracts of the growth medium, that were subsequently named Fumonisins (FB1 and FB2) based on elution order. These extracts were shown to promote tumorigenesis when present after diethylnitrosamine (DEN) initiation. Further analysis of FB1 in cancer promotion revealed that it was capable of promoting cancer in rats with oral dosage through feeding at concentrations of 0.1% in the diet, but there was also a highly significant

increase in cancer promotion when fed at the same concentration to rats previously initiated with DEN (Gelderblom, Et al., 1988).

The outbreak of ELEM in South Africa and the radically high incidence rates of EC in the Transkei region were both motivating factors for *Fusarium* research, but the field did not take off internationally until the identification of *Fusarium* related problems on this side of the Atlantic divide. Two major outbreaks of ELEM and PPE were identified in the United States in 1989 and 1990. The 1989 maize crop was heavily contaminated with fumonisins and subsequently used in the production of commercially mixed animal feeds which resulted in a high volume of horse and pig deaths over the course of the next two years. The deaths resulted in considerable losses for the United States economy which spurred a great increase in interest for *F. verticillioides* and fumonisins research within the U.S. and abroad (Marasas, 2001).

After these two U.S. incidences research and funding in regards to Fumonisins, *Fusarium verticillioides*, and mycotoxins in general began to rise dramatically. In the United States the first conference on fumonisins was held in Ames, Iowa in 1990 followed by other international conferences. The International Agency for Research on Cancer (IARC) in Lyon, France identified fumonisins (specifically FB1) as a Group 2B carcinogen, meaning possibly carcinogenic to humans (Marasas, 2001). Based on these, and many other studies, the FDA, in 2000, posted guidance for industry that included tolerance and action levels for fumonisin contamination of human food and animal feed crops based on a risk assessment study. These standards were soon adopted by USDA,

in 2001. The standards set limits of contamination in human food products to between 2 and 4ppm depending on the food product and between 5 and 100ppm in animal feed depending on the animal and its intended use (CFSAN, 2000). With multiple animal models confirming Fumonisins, FB1 specifically, as being a causative agent for so many varied diseases/toxicoses along with the possible link between FB1 and human associated conditions it appears logical to impose regulations or restrictions on their presence in food and feed.

Currently, in the food and feed production industries companies have protocols in place for screening samples for fumonisins to maintain reduced levels in their products, but due to the chemical nature of fumonisins they are difficult to eliminate. Fumonisins are also relatively stable in food processing at moderate temperatures. On average processing fumonisin contaminated food products at around 150^{o}C, on average, only resulted in ~20% reductions of FB1 content (Pietri, Zanetti, and Bertuzzi, 2009). It is difficult to process fumonisins out of food products and costly for maize processing facilities. Attempts have been made to reduce the production of fumonisins in the agricultural setting, but these attempts have not been highly effective. The primary focus of research for reducing FB1 levels in maize crops has been the production of cultivars with resistance to *Fusarium* infection, competitive inoculations of non-toxigenic strains, and production of biocontrol strains (Löffler et al., 2010; Small et al., 2012; Bacon et al., 2004).

In attempts to develop a biocontrol strain of *Bacillus mojavensis* to reduce or eliminate fumonisin infections in maize the researchers found that *F. verticillioides* was capable of eliminating the biocontrol strain via production of fusaric acid (Bacon et al., 2004). In another study simultaneous inoculation of maize with the smut fungus Ustilago maydis the researchers found that *F. verticillioides* also reduced infection and symptoms of the smut disease. They also found that inoculation of the plant with *F. verticillioides* had negligible effects on the plants growth when compared to controls, and that when the plant was simultaneously inoculated with both fungi the plants actually grew better than with *Ustilago* alone (Lee, Pan, and May, 2009). Taking into consideration that the association of *Fusarium verticillioides* with maize cultivars can have these beneficial relationships it only seems practical to develop methods of reducing fumonisin concentrations without adversely affecting the fungus itself.

CHAPTER III

MATERIALS AND METHODS

Strains and Media

Wild type strains M-3125, Fv149, Fv999, and FGSC 7603 were maintained on V8 juice agar. Temporary stocks were maintained on V-8 slants at 4 °C. To reduce the chance for loss of fertility in the these strains stocks were duplicated onto 100mm plates and allowed to grow until the mycelia nearly reached the edge of the plates. The plates were then parafilmed and stored at 4 °C in a climate controlled room. These stock plates were then used for inoculating experimental cultures. Cultures grown for the purpose of generating conidia were inoculated from the stock plates and grown on V8 juice agar, and cultures grown for the purpose of generating ascospores were inoculated onto 0.1X carrot agar for sexual crossing. Transformations were performed on regeneration media.

Table 1

Fusarium Strains Used in This Study

Strain	Mating Type	Other Designations	Killer Phenotype
M-3125	$matA^-$ / MATA-1	FGSC 7600	Sk^S
FGSC 7603	$matA^+$ / MATA-2	M-3703, ATCC 201261	Sk^K
Fv149	$matA^-$ / MATA-1	-	Sk^S
Fv999	$matA^+$ / MATA-2	-	Sk^K
FRC M-3120	$matA^+$ / MATA-2	-	Sk^K

(Table Continues)

Strain	Mating Type	Other Designations	Killer Phenotype
FRC M-3125	$matA^-$ / MATA-1	FGSC 7600	Sk^S
ISU 94040	$matA^+$ / MATA-2	-	Sk^S
21894	$matA^-$ / MATA-1	-	Sk^K
FRC M-7358	$matA^-$ / MATA-1	-	Sk^K
ISU-3	$matA^+$ / MATA-2	-	Sk^K
ISU-9	$matA^-$ / MATA-1	-	Sk^K
ISU-18b	$matA^-$ / MATA-1	-	Sk^K
ISU-28	$matA^+$ / MATA-2	-	Sk^K
ISU-58-1	$matA^-$ / MATA-1	-	Sk^K
ISU-67	$matA^-$ / MATA-1	-	Sk^K
ISU-80	$matA^-$ / MATA-1	-	Sk^K
ISU-82	$matA^+$ / MATA-2	-	Sk^K
ISU-94b	$matA^+$ / MATA-2	-	Sk^K
ISU-110	$matA^+$ / MATA-2	-	Sk^K
ISU-115	$matA^-$ / MATA-1	-	Sk^K
ISU-137	$matA^-$ / MATA-1	-	Sk^K
ISU-152	$matA^+$ / MATA-2	-	Sk^K
ISU-174	$matA^-$ / MATA-1	-	Sk^K
ISU-177	$matA^+$ / MATA-2	-	Sk^K
ISU-180	$matA^-$ / MATA-1	-	Sk^K
AMR F-2	$matA^-$ / MATA-1	-	Sk^K
AMR F-4	$matA^+$ / MATA-2	-	Sk^K
AMR F-5	$matA^-$ / MATA-1	-	Sk^K
AMR F-7	$matA^+$ / MATA-2	-	Sk^S
AMR F-8	$matA^+$ / MATA-2	-	Sk^S
AMR F-9	$matA^+$ / MATA-2	-	Sk^K
AMR F-11	$matA^+$ / MATA-2	-	Sk^K
AMR F-12	$matA^+$ / MATA-2	-	Sk^K
AMR F-13	$matA^+$ / MATA-2	-	Sk^K
AMR F-14	$matA^+$ / MATA-2	-	Sk^K
NRRL 13586	$matA^-$ / MATA-1	-	Sk^K
NRRL 25457	$matA^-$ / MATA-1	-	Sk^K
NRRL 26518	$matA^+$ / MATA-2	-	Sk^K
Fv152	$matA^+$ / MATA-2	-	Sk^K
FGSC 7415	$matA^-$ / MATA-1	-	Sk^S

Note. Table 1 shows *Fusarium verticillioides* strains used in this study.

Carrot Media

Originally Klittich and Leslie published their paper demonstrating that 30% w/v carrot agar was the optimal crossing media for sexual reproduction. However, during our initial investigations in *Fusarium verticillioides* the crosses were not effectively producing perithecia on 1.0X or 0.6X carrot puree. Knowing that our model organism, *Neurospora crassa*, requires a nitrate limiting media, Synthetic Crossing Media (Westergaard and Mitchell, 1947), to achieve optimal mating we ran an assay utilizing a gradient of carrot puree concentrations to assess mature perithecia production. Based on our data, we observed the highest rate of mature perithecia production utilizing 0.1X carrot media. Media was produced by autoclaving 200g frozen baby carrot with 200mL of distilled water and autoclaving for 20 minutes. The autoclaved carrots and water were then pureed and aliquoted into 30-33 mL aliquots. The puree was then either used for media production or frozen at -20 for future media production. Final media composition: 30mL carrot puree, 220mL distilled water, and 5g of agar to bring the final carrot concentration to approximately 0.1X.

V8 Media

V8 media is a commonly used media for growth of sporulating fungi. In this study V8 media was used for growing fungi for the generation of conidia and maintenance of stock strains. The protocol was produced by combining 100mL V8 with 400mL distilled water, 1g calcium carbonate, and 7.5g agar (Dhingra and Sinclair, 1985; Stevens, 1974).

Vogel's Liquid Media

Vogel's liquid media was used during growth of mycelia for genomic DNA isolation (Vogel, 1956). Vogel's media was prepared according to the 1956 paper except for: no agar was added to maintain a liquid media form and the prepared media was divided into 25mL aliquots and autoclaved in individual 125mL Erlenmeyer flasks.

Regeneration Media

0.1% Yeast Extract, 0.1% Casein-Enzyme Hydrolysate, 1.6% BactoAgar, and 0.8M Sucrose. Prepare by making a 2X sucrose solution, and a separate 2X solution with the Yeast Extract, Casein-Enzyme Hydrolysate, and BactoAgar. Autoclave separately and then combine. Then plate by pipetting 20mL/100mm petri plates. Save enough to use as overlay medium.

Hygromycin Overlay Media

Is used to overlay transformants in Regeneration media the day following transformation and is composed of 1% water agar containing 150µg/mL Hygromycin B.

Permanent Stocking

Permanent stocking of *Fusarium verticillioides* was achieved by growing strains on V8 slants for up to two weeks or the slant was covered with conidiating mycelia. Then 2mL of 15% glycerol was added using a 5mL pipette followed by light scrubbing of the slant surface with autoclave sterilized glass rods to dislodge conidia. 1-2mL of the resulting suspension was then transferred into cryogenic vials and immediately stored at -80^{o}C.

Genomic DNA Isolation

Samples were grown in 125mL Erlenmeyer flasks containing 25mL of Vogel's minimal medium for three days. Samples were shaken each day to prevent growth up the flask sides. The samples were then decanted of media and washed once with autoclave sterilized distilled water. After rinsing, the samples were pressed dry using flame sterilized spatulas and filter paper. The samples were then placed into microcentrifuge tubes and labeled. The microcentrifuge tubes were then punctured with sterile needles to allow for gas exchange and the tubes were placed into a lyophilyzation chamber and lyophilized for a period greater than four hours to ensure samples were thoroughly dry. Dehydrated samples were then weighed to ensure less than 100mg of sample were being extracted. Genomic DNA Extraction was performed using IBI's Genomic DNA Mini Kit (Plant) Protocol, solutions, and columns according to the manufacturer's protocols.

Primer Design

Primers, excluding all primers designed to add sequence to constructs, were designed between 20 and 24 nucleotides with a TM of between 65 and 71. Optimal conditions were set to: maximum poly-X = 3, maximum self-complementarity = 2, and maximum 3' self-complementarity = 1. Adjustments were first made to maximum self-complementarity before 3' self-complementarity on an as needed basis up to 5 and 3 respectively before looking for new primer locations if needed. See Table 2 for primers used in this study.

Table 2

Primers Used in This Fusarium verticillioides Study

Primer	Oligo name	Sequence (5->3)	Notes
335	Fv-RFLP-3-F1	CCAGAATGGCTCTGACCTGGTGT	RFLP3 Amplification
336	Fv-RFLP-3-R1	GCGCAGATCATGGAGAGGGTATG	RFLP3 Amplification
425	Fv-RFLP1B-F	GCGATACAGAACCCCCATTCTCTT	RFLP1 Amplification
426	RFLP-2B-F1	TTATCTGCACACCTGGAGGA	RFLP2B Amplification
427	RFLP-2B-R1	CGGCTTCACCTGAGACATTT	RFLP2B Amplification
428	FvRFLP4B-F1	ATGTGTGCAGCTCGTTTTTG	RFLP4B Amplification
429	FvRFLP4B-R1	TCAACCCCGAGACTTTCATC	RFLP4B Amplification
430	FvRFLP5B-F1	GACCAGACCCGAAACCAAT	RFLP5B Amplification
431	FvRFLP5B-R1	CAGCCAAATCACGCTGTCT	RFLP5B Amplification
432	FvRFLP6B-F1	GGTCGAGAACAAAGGGGTTC	RFLP6B Amplification
433	FvRFLP6B-R1	CAAAGATGGGAGGAACATGG	RFLP6B Amplification
399	031314 RFLP9-F	TGGCTTGGGTCTGAAGGAGGTTC	RFLP9 Amplification
400	031314 RFLP9-R	GCTGTTTGATTGGCCGTTCTGG	RFLP9 Amplification
421	041014 RFLP10-F	TGCTGCCTCGATTCCTTCTTCC	RFLP10 Amplification
422	041014 RFLP10-R	CGGAGTACCATTGTTCGGGTGA	RFLP10 Amplification
423	041014 RFLP11-F	TGCACAGAAGCGAGACAAACATCC	RFLP11 Amplification
424	041014 RFLP11-R	TCGAGCATGACCAAGGCAGAAC	RFLP11 Amplification
458	MAT-1F	GTTCATCAAAGGGCAAGCG	Mat-1 detection

(Table Continues)

Primer	Oligo name	Sequence (5->3)	Notes
459	MAT1-R	TAAGCGCCCTCTTAACGCCTTC	Mat-1 detection
512	MAT-2F - F	ACCGCCCTCGTCGTCCTTCT	Mat-2 detection
513	MAT-2R - R	CCACGGGTATCGTCAAATCCACA	Mat-2 detection
577	NOTI-HPH-R-TH	TTTTGCGGCCGCAACTGGTTCCCGGTCGGCAT	Addition of NotI site
78	NOTI-HPH-F-TH	TTTTGCGGCCGCAACTGATATTGAAGGAGCAT	Addition of NotI site

Note. Table 2 lists all primers used in the study of *F. verticillioides' Spore killer*.

Sexual Crosses

For studies involving sexual crosses, females were inoculated to 60mm carrot agar plates and males were inoculated to 3mL of V8 agar poured into 16mm disposable culture slants. The female fungi were allowed to grow incubated at 27 degrees Celsius until the mycelia reached the edge of the plates (12-14 days). Carrot concentration was adjusted to 0.1X of the original concentration to optimize the generation of sexual reproductive structures. Male strains grown in slants were suspended in 2mL of Tween-20, and 1mL of the resulting suspension was transferred to the female plate. The spore suspension was spread using sterile glass rods. The plates were returned to the incubator. Dissections for phenotyping were performed from approximately 10 to 20 days post fertilization (DPF). Ascospores were collected from cirrhi 15-20 DPF.

Ascospore Collection

Ascospores were collected from cirrhi 15-20 DPF by harvesting Cirrhi with needles under a dissecting scope. Initially a heated needle was used to singe away

extra aerial hyphae to prevent contamination. A flame sterilized and cooled needle was then used to scrape away cirrhi that were then suspended in 300µL of sterile water. Ascospores were dispersed from cirrhi by vortexing the micro-centrifuge tube.

Isolating Sexual Progeny

Ascospores were isolated by spreading a dilution of ascospore suspensions to 100mm water agar plates at approximately 200-300 spores/plate. Ascospore concentrations were determined by counting the spores on a hemocytometer and diluting accordingly. Spreaders were flame sterilized and spreading was done in a laminar flow hood.

Single Spore Isolation of Conidial Strains

Conidia were isolated by spreading a dilution of conidial suspensions to 100mm water agar plates at approximately 200-300 spores/plate. Conidial suspension concentrations were determined by counting the spores on a hemocytometer and diluting accordingly. Spreaders were flame sterilized and spreading was done in a laminar flow hood. Individual germinating conidia were picked to V-8 slants using sterilized needles.

Carrot Agar Assay

Optimization of media for sexual crossing was performed to ensure sufficient perithecial development for analysis of crosses. Carrot agar was prepared in a gradient of carrot concentrations: 0.001X, 0.01X, 0.1X, 0.25X, 0.5X, and 1.0X. Twenty milliliters of the media was poured into 60mm disposable petri dishes. Crosses were

carried out as described above. To ensure accurate data collection male conidia were adjusted to 1 x 10^6 per mL fertilizing females. Perithecia developed 9 days post fertilization (DPF). Perithecia were counted over the course of 25 days using a light box as a backlight. Each cluster of touching perithecia was counted as one perithecium.

Restriction Fragment Length Polymorphism (RFLP) Generation

RFLP markers were generated by analyzing previously generated sequences for FGSC7603 fungal strain on Tablet analysis software (Milne 2013) which is used to analyze sequence assemblies. The sequence was aligned to M-3125, sequenced by the Broad Institute (*F. verticillioides* 7600 (FV3)), to select single nucleotide polymorphisms within restriction enzyme consensus sites for HaeIII (GGCC) on either one or the other of the parental strains resulting in a defective restriction site for that strain. Primers were designed to amplify a 200-400bp region around the RFLP locus. RFLP markers were designed across supercontig_3.3 of chromosome V to aid in mapping of the *Sk* locus and on separate chromosomes (supercontig_3.13 of chromosome I, supercontig_3.8 of chromosome VII, and supercontig_3.21 of chromosome XI) to be used in meiotic drive assays.

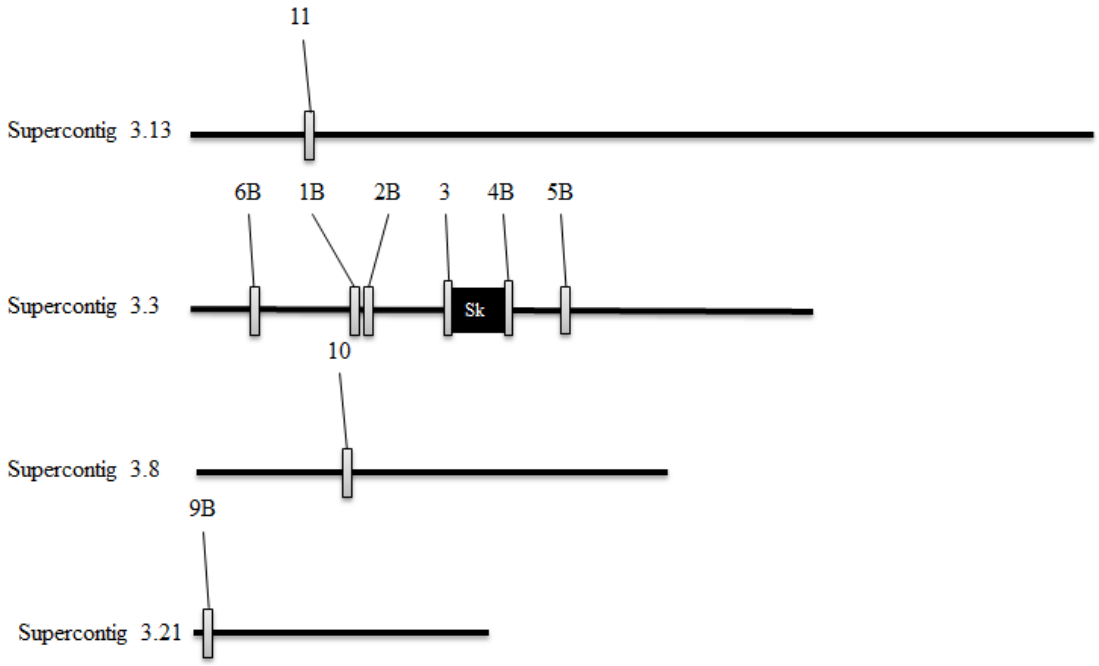

Figure 2. Map of RFLP Markers and Their Associated Supercontigs

Conidial DNA Preparation

Small amounts of conidia were transferred from young fungal cultures, no more than two weeks, into Micro-centrifuge Tubes containing 200µL of sterile TE Buffer pH 8.0 using sterile wooden applicators. Samples were then heated to 105°C for 12 minutes before being place on ice for 2 minutes. The samples were next vortexed for 5 seconds and then spun down at 21,000Xg for 10 minutes. Finally, 25µL of the supernatant was then transferred into clean MCTs and used as template DNA for PCR amplification or stored at -20°C.

RFLP PCR

RFLP loci were amplified from conidial DNA preparations using Bullseye Taq. PCR conditions were as follows: 1µL of template conidial DNA, 0.3µM of each primer, 1.5mM MgCl, 200mM dNTP, and 0.5µL of Bullseye Taq. The PCR settings were set to the manufacturer's specification using 40X cycles during the extension phase and the temperature for the annealing phase was set to -5^{o}C of the TM for the primer set.

Analysis of Meiotic Drive

Drive was analyzed by PCR amplification of RFLP loci followed by restriction digest from the 60 cultures isolated via ascospores isolation. RFLP digests were genotyped to either the killer or non-kill parent. The inheritance of genetic loci was analyzed as a ratio of killer genotype to total progeny.

Dissections

Mature perithecia were transferred to slides containing 50% glycerol and then dissected using flame sterilized needles under a dissecting scope. Rosettes from the dissected perithecia were transferred from the dissecting slide to a clean slide. Then, 50% glycerol was added to 10µL total volume. A coverslip was placed over the sample rosettes and any excess glycerol was absorbed from under the coverslip by placing a Kimwipe at the interface where excess glycerol was present. Final slides were visualized under a compound phase contrast microscope.

Spore killer Assay

Spore killing phenotypes were identified by crossing strains with unknown phenotypes to both *Sk* and Sk^{s}strains. These crosses were then dissected as described above after sufficient time for perithecial development. Typically, perithecia matured and began producing cirrhi around 19 DPF. The dissections were then imaged using phase contrast microscopy to obtain phenotypic data. Phenotypes were either killing (displaying 4 viable ascospores) or non-killing (displaying 8 viable ascospores).

Female Assay

Females were inoculated to 60mm carrot agar plates and males were inoculated to 3mL of V8 agar poured into 16mm disposable culture slants. The female fungi were allowed to grow, in the incubator, until the mycelia reached the edge of the plates (12-14 days). Carrot concentration was adjusted to 0.1X of the original concentration to optimize the generation of sexual reproductive structures. Male strains grown in slants were suspended in 2mL of Tween-20, and 1mL of the resulting suspension was transferred to the female plate. The spore suspension was spread using sterile glass rods. To ensure accurate data collection male conidia were adjusted to 1 x 10^6 per mL fertilizing females. The plates were returned to the incubator. Dissections for phenotyping can be performed from approximately 10 to 20 days post fertilization (DPF). Perithecia developed 9 days post fertilization (DPF). Perithecia were counted after of 25 days using a light box as a backlight. Each cluster of touching perithecia was counted as one perithecium. Female ability was inferred by the ability of crosses to produce mature ascospores and the number of viable perithecia produced per plate.

Protoplasting

Conidia were harvested from strain FGSC 7603 between 7 and 10 day old cultures by centrifugation and germinated in YEPD (0.3% yeast extract, 1% Bacto peptone, and 2% D-glucose) broth for 8 to 8.5 h at room temperature at 200 rpm. Isolation of protoplasts occurred in 25 mg/ml driselase (InterSpex Products, Inc. San Mateo, CA), 0.05 mg/ml chitinase (Sigma Chemical Co., St. Louis, MO), and 5 mg/ml lysing enzyme (Novozyme 234 - Sigma Chemical Co., St.Louis, MO) in a 0.7 M NaCl buffer. Protoplasts were collected by filtration through a 30-mm Nitex nylon membrane (Tetko Inc., Kansas City, MO) and washed three times in STC buffer (1.2 M sorbitol; 10 mM Tris-HCl, 50 mM CaCl2, pH 8.0). Protoplast cells were resuspended to a concentration of between 2-3*10^8. Protoplasts were stored by diluting to 1*10^8 in STC:SPTC:DMSO (8:2:0.1) and then storing at -80^{o}C

Transformation of Protoplasts

Transformation was performed in a 250µl volume with1 to 10µg of the disruption vector brought to 50µL with STC and then mixed 1:1 with 2X STC. This was then added to 100µL of the protoplast suspension and transformation was mediated by 50µL 30% polyethylene glycol solution (30% polyethylene glycol 8000, 10 mM Tris-HCl, 50 mM CaCl2, pH 8.0) for 20 minutes. Then 2mL of 30% PEG 8000 was mixed in by gently inverting the tube followed by another 5 minute incubation at room temperature. After incubation 4mL of 1X STC was added and mixed in by inversion. The resulting suspension was added to 45mL of molten regeneration media and 5mL was

overlayed per plate. Cultures were incubated for 15 h in the dark and then overlaid with 10 ml water agar amended with 150g/ml hygromycin B (Calbiochem-Novabiochem Corp., San Diego, CA) to recover transformants. Putative transformants were selected within 4 to 10 days and hygromycin resistance was confirmed by growth on V8 juice slants amended with 450g/ml hygromycin B.

Data Analysis

The data analysis for this paper was generated using SAS software. Copyright, SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA.

CHAPTER IV

RESULTS

Carrot Agar Assay

The optimal medium for the production of viable perithecia is still a matter of debate (Choi Et al., 2009); however sources indicate that the use of carrot agar produces sufficient viable perithecia (Klittich and Leslie, 1988). While the Choi paper appeared to show V8 could be a better media the analysis was not quantitative, and the difference was only marginal. Their further study only tested for concentration of V8 and ignored carrot. Without attempting to optimize carrot it was not an accurate comparison. So studies in this paper used the formerly cited method of carrot agar for sexual crossing. In spite of those results, the initial crosses on carrot agar produced minimal perithecia. In our studies using *Neurospora crassa* it is optimal to use a Westergaard medium which is nitrate limiting to induce perithecial development, for sexual crosses. To test if nutrient limitation may be a factor for the production of sexual structures in *Fusarium verticillioides,* triplicate sexual crosses of FGSC 7603 X M-3125 were set up using FGSC 7603 and M-3125 to serve both as the male and the female strains. Crosses were inoculated to a gradient of carrot dilutions (1.0X, 0.50X, 0.25X, 0.10X, 0.01X, 0.001X). Perithecial development was noticed 9DPF and total perithecia per plate was counted at

least twice weekly. Counting continued until the majority of perithecia were excreting cirrhi, which is an indication of mature perithecia, which took 25 days.

0.1X to 0.25X serves as an optimal concentration of carrot puree for sexual crossing media. Analysis of the crosses over the course of the experiment revealed that the optimal carrot concentration was approximately 0.1-0.25X for crosses utilizing FGSC 7603 as female. The FGSC 7603 female crosses on plates containing above 0.25X and below 0.1X concentration of carrot puree in the media produced well below an optimal number of perithecia to be used in future assays. All crosses using M-3125 as females produced negligible quantities of perithecia. The data for perithecial production was statistically analyzed using SAS software to perform ANOVA analysis. ANOVA analysis revealed no statistical difference across the range of carrot concentrations used for the M-3125 assays with a p-value of P= 0.1011. This supports the hypothesis that our M-3125 strain was significantly decreased in viability for sexual crossing. This event has been previously suggested in another ascomycete, *Magnaporthe oryzae* (Saleh Et al., 2012). Analysis performed using SAS software to perform ANOVA testing also revealed significant differences in crosses using FGSC 7603 females with a P value of P= 0.0003. The replicates crossing on both 0.1X and 0.25X carrot agar were grouped by the post hoc Tukey's studentized ranged test. The other four concentrations grouped together and had far fewer mature perithecia produced during the cross. Data are shown in Figures 3 and 4.

Carrot Agar Female Assay

The carrot agar assay served a dual purpose. The first was the analysis of the concentration of carrot puree in the crossing medium, and the second purpose was to assay the viability of both the M-3125 strain and FGSC 7603 for being used as the female in future crosses. After the 25 day crossing period the triplicate plates were counted following the protocol for perithecial counts. The plates using strain M-3125 as female produced dramatically less perithecia than 7603. M-3125 data is shown in Figure 3, and FGSC 7603 data is shown in Figure 4. In fact, it appears that the M-3125 produced so few perithecia that the strain has lost the ability to proficiently perform sexual crossing as a female. The SAS ANOVA analysis revealed no significant differences for M-3125 females across the entire range of media being tested while the FGSC 7603 had strong significant differences.

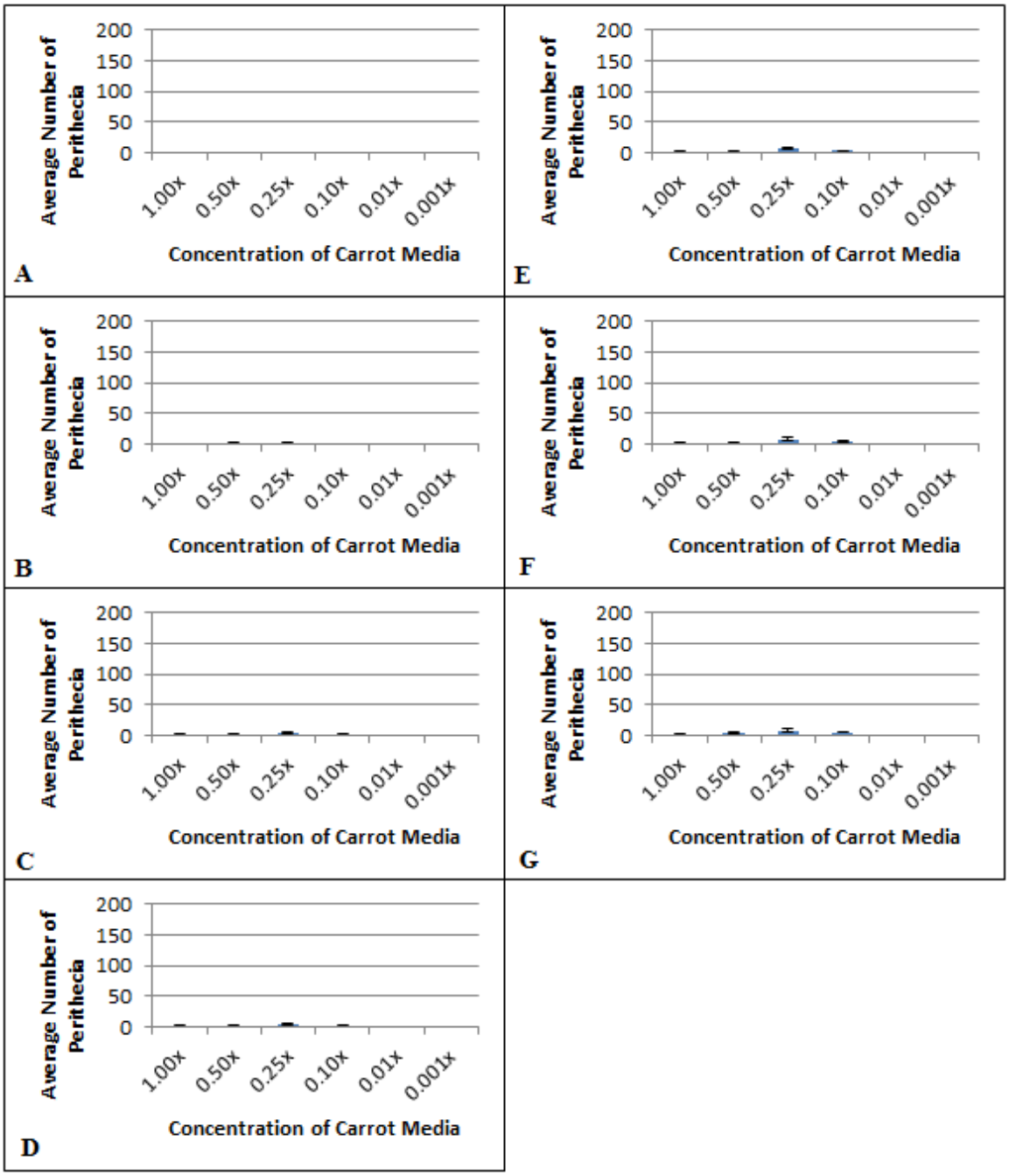

Figure 3. M-3125 Female Cross Perithecia Counts for Carrot Agar Assay. Shown are the average number of Perithecia produced at: **A.)** 9 DPF **B.)** 13 DPF **C.)** 15 DPF **D.)** 19 DPF **E.)** 21 DPF **F.)** 23 DPF **G.)** 25 DPF. Error bar represents standard deviation.

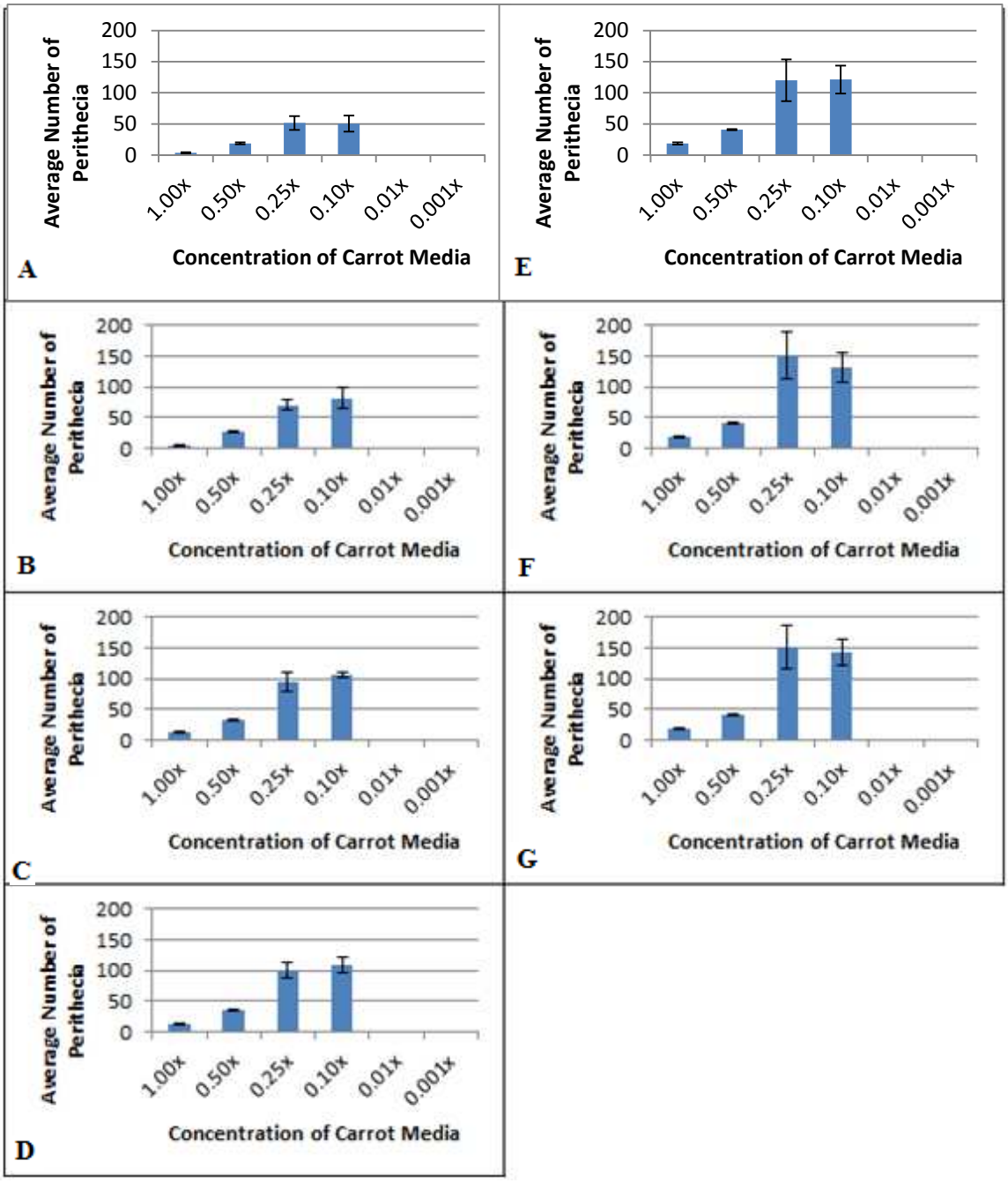

Figure 4. FGSC 7603 Female Cross Perithecial Counts for Carrot Agar Assay. Shown are the average number of Perithecia produced at: **A.)** 9 DPF **B.)** 13 DPF **C.)** 15 DPF **D.)** 19 DPF **E.)** 21 DPF **F.)** 23 DPF **G.)** 25 DPF. Error bars represent standard deviation.

Meiotic Drive Assay

Meiotic drive associated with Supercontig 3.3 appears to be sufficient for driving linked genes into future generations. The hypothesis that the *Sk* element within the Fusarium genome can suppress functional fumonisin producing FUM gene clusters within a population must undergo multiple determinations of efficacy. The first analysis was performed to determine if the *Sk* element is capable of effectively driving linked segments of DNA into the genomes of progeny at an effective rate. To determine if this was possible RFLP markers were designed by comparing available FGSC7600 (M-3125) sequence from the Broad institute with recent sequencing data compiled by the USDA-NCAUR-ARS center in Peoria, IL for the opposite mating type, FGSC 7603. The RFLP map is shown in Figure 2.

Alignments were made using Tablet graphical viewer software (Milne, 2010). Genomes were searched for HaeIII restriction endonuclease consensus sites in which a single nucleotide polymorphism, SNP, was present in one strain of the mating pair. Primers were designed to amplify a region surrounding the SNP of approximately 400 base pairs (bp). Progeny were collected from sexual crosses via ascospore isolation. The resulting progeny were then used to isolate conidial DNA to be analyzed via PCR and restriction digest for RFLP inheritance patterns.

The markers that were designed to amplify DNA segments from along supercontig_3.3 of chromosome V which is the region that Sk has formerly mapped to (Jurgenson, Zeller, and Leslie, 2002) appeared to be distorted toward the Sk genotype

with the most skewed region being across RFLP1B, RFLP2B, and RFLP3 showing 96.5% probability of inheriting the Sk parent's allele for the corresponding loci (see Figure 5 and corresponding Table 3). This data along with the data suggesting that RFLP5B is heritable similar to RFLP markers from across the other chromosomes, following Mendelian inheritance ratios, suggests that the *Sk* element might be located 5' of the original placement in the genome and is not linked with RFLP5B. While the rate is slightly lower than expected, the results still look promising considering that the marker location is such a great distance from the predicted location of the *Sk* element.

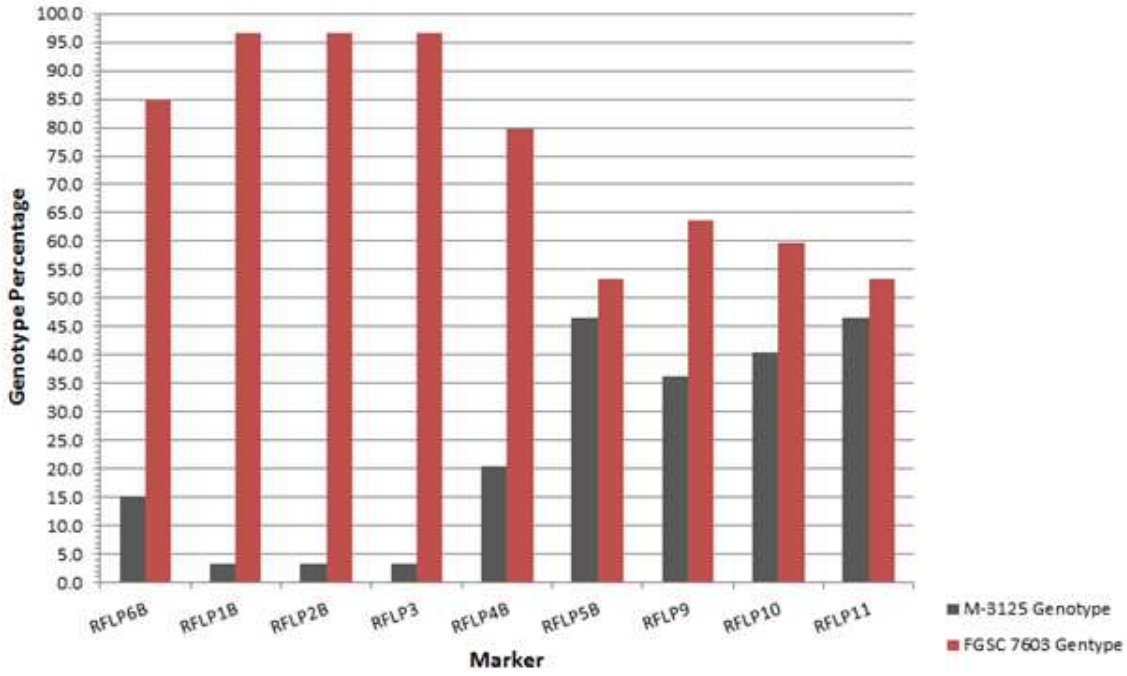

Figure 5. Restriction Digest Analysis of RFLP Inheritance Patterns in Progeny From Cross RJP98 (FGSC 7603 ♀ x M-3125 ♂). (represented as a percentage of progeny analyzed) RFLP markers from along chromosome 5, specifically those from 1B to 3, displayed an un-proportionate inheritance of the sequence from the killer parent strain.

Table 3

RFLP Inheritance Patterns

Marker	% 3125 Genotype	% 7603 Genotype
RFLP6B	15.3	84.7
RFLP1B	3.3	96.7
RFLP2B	3.4	96.6
RFLP3	3.4	96.6
RFLP4B	20.3	79.7
RFLP5B	46.7	53.3
RFLP9	36.2	63.8
RFLP10	40.4	59.6
RFLP11	46.6	53.4

Note. Table 3 shows the percentage of RFLP inheritance genotyped to parental strains from along chromosome 5 (in grey) and from chromosomes not associated with *Spore killer* (in blue).

New Strain Screen

Due to M-3125's female sterility we assayed new strains to serve as replacement females. Strains Fv149 and Fv999 (Listed in Table 1) were tested for mating type, *Spore killer* phenotype, and female fertility via sexual crosses. Each strain was set up as females being crossed to both M-3125 and FGSC 7603 along with being crossed to each other. Fv149 produced plentiful perithecia while serving as female to both Fv999 and FGSC 7603. Fv149 displayed MAT1-1 mating patterns and phenotyped Sk^{S}. Fv999 also produced plentiful mature perithecia when crossed to both Fv149 and M-3125. Fv999 displayed MAT1-2 mating patterns and phenotyped Sk^{K}. These data showed Fv149 to have the same mating type and Sk status as M-3125 while Fv999 has the same mating type and Sk status as FGSC 7603. The strains Fv149 and Fv999 also readily perform sexual crosses with each other as either the male or the female and can be used as controls.

Sk Assay

Sk demonstrate the killer status providing phenotypic evidence that can be compared to the genotypic evidence from RFLP markers. *Spore killer* assays were performed by crossing the progeny from RJP98 to Fv149 (MAT1-1)—used as replacement for M-3125 which is female infertile, Fv999 (MAT1-2), and FGSC 7603 (MAT1-2). The resulting perithecia were then dissected for phenotyping which was performed on a compound microscope as described in the methods. By assessing the *Sk* phenotype and comparing that data to the RFLP data sets we expected to see a coupling of the RFLP profile from the original Sk strain to the progeny of the cross correlating with Sk phenotype in the progeny. Theoretically, this should be the case 100% of the time because Sk should kill off any progeny that was non killer. If the RFLP markers are linked as they appeared to be in the Meiotic Drive analysis then the opposite should be true. Progeny displaying RFLP patterns similar to FGSC 7603 should also exhibit spore killing while progeny displaying RFLP patterns similar to M-3125 should not exhibit spore killing. Theoretically this would be because the recombination that carried over the RFLP from FGSC 7603 should also carry over the *Sk* sequence as well. The data from Figure 5 showed that there was the highest amount of drive equivalently associated with RFLP1, RFLP2, and RFLP3. We focused the Sk assay on progeny from RJP98 that displayed interesting crossover events near or within this region. As expected all of the progeny assayed showed killing which is what would be expected by the presence of the Sk element; however the RFLP data suggested that some of the strains would not likely display killing (Figure 6).

Spore Killer Data						RFLP Data					
Fv149	3125	Fv999	7603	Sk Status	RJP98 Strain	RFLP6b	RFLP1	RFLP2	RFLP3	RFLP4b	RFLP5b
4	X	X	X	SkK	75	7	7	7	7	3	3
X	X	8	8	SkK	77	7	7	7	7	7	3
X	X	8	8	SkK	95	7	7	7	7	7	7
X	X	8	8	SkK	99	7	7	7	7	7	7
4	X	X	X	SkK	111	3	3	3	3	3	7
4	X	X	X	SkK	116	7	7	7	7	3	3
4	4	X	X	SkK	118	3	3	3	7	7	7

X	Represents no crossing
8 or 4	Represents # of viable ascospores/asci

3	Represents inheritance of M-3125 genotype
7	Represents inheritance of FGSC 7603 genotype

Figure 6. Comparison of Selected *Spore killer* Data to RFLP Data. The RFLP data suggest possible positioning of the Sk element within the range of RFLP2, RFLP3, and RFLP4. However, when looking at RJP98.111 the expected outcome is loss of killing according to RFLP data which is not seen in the *Spore killer* data.

Single Spore Isolation Assay

To ensure that the unexpected results that we were seeing in the *Spore killer* assay were accurate conidial suspensions of RJP98.75, RJP98.111, and RJP98.118 were prepared for conidial isolation. Isolates were then replated for a second round of isolation. Isolate cultures from both rounds were grown and retested for RFLP genotypes at RFLP2 and RFLP3 loci and Sk phenotype. The isolates' genotypes matched back to the original progeny, and the phenotypic data segregated accordingly as shown in Figure 7 and Table 4.

Retested RFLP Results			Original RFLP Results		
Strain	**RFLP2**	**RFLP3**	**RJP98 Strain**	**RFLP2**	**RFLP3**
RJP98.75A	7	7	**75**	7	7
RJP98.75D	7	7	**111**	3	3
RJP98.75D1	7	7	**118**	3	7
RJP98.75D2	7	7			
RJP98.75D3	7	7			
RJP98.75D4	7	7			
RJP98.111A	3	3			
RJP98.111D	3	3			
RJP98.111D1	3	3			
RJP98.111D2	3	3			
RJP98111D3	3	3			
RJP98.111D4	3	3			
RJP98.118A	3	7			
RJP98.118D	3	7			
RJP98.118D1	3	7			
RJP98.118D2	3	7			
RJP98118D3	3	7			
RJP98.118D4	3	7			

3	Represents inheritance of M-3125 genotype
7	Represents inheritance of FGSC 7603 genotype

Figure 7. Retest of *Spore killer* in Isolates From RJP98 Progeny. Retest of RFLP Inheritance at RFLP2 and RFLP Loci in single spore isolates from RJP98 progeny. Data corresponded with the original RFFLP profiles in both the single and double isolation groups.

Table 4

Retest of Spore killer Data

RJP98 Strain	Fv149	Fv999	Sk Status	DPF
75A	4	X	SK^K	28 DPF
75D	4	X	SK^K	28 DPF
75D1	4	X	SK^K	28 DPF
75D2	4	X	SK^K	28 DPF
75D3	4	X	SK^K	28 DPF
75D4	4	X	SK^K	28 DPF
111A	4	X	SK^K	28 DPF
111 D	4	X	SK^K	28 DPF
111D1	4	X	SK^K	28 DPF
111D2	4	X	SK^K	28 DPF
111D3	4	X	SK^K	28 DPF
111D4	4	X	SK^K	28 DPF
118A	4	X	SK^K	28 DPF
118D	4	X	SK^K	28 DPF
118D1	4	X	SK^K	28 DPF
118D2	4	X	SK^K	28 DPF
118D3	4	X	SK^K	28 DPF
118D4	4	X	SK^K	28 DPF

Note. Table 4 shows the results from the retest of spore killer assays of single spore isolates which confirmed the SK phenotype of the original culture. All cultures were confirmed as *Spore killer* based on phenotype results from crossing to Fv149

USDA Strain Screen

Since both the results from the *Spore killer* assay and the single spore isolation *Spore killer* assay were counter intuitive based on the results from the RFLP analysis of meiotic drive we began to search for duplicate strains to test *Sk* phenotypes to ensure that the Sk phenotype was not simply poor mating with the original parent strains. To test this and to confirm *Sk* status we were looking for strains in each mating type with

the opposite Sk phenotype than the original parent of the same mating type. We were given 39 isolates from our collaborators at the USDA-ARS. These strains were tested for mating type and spore killing as described in the methods. From the 39 strains 2 strains stood as having the proper mating type/*Sk* phenotype combination while still maintaining strong ability to form perithecia in sexual crosses. The strains selected were strain 21984 which was MAT1-1/ Sk^S and strain AMR F-7 which was MAT1-2/Sk^K.

Table 5

Results of USDA Strain Analysis

Strain	ISU Stock #	Mating Type	Sk Phenotype
FRC M-3120	ISU 3386	$matA^+$ / MAT1-2	Sk^K
FRC M-3125	ISU 3387	$matA^-$ / MAT1-1	Sk^S
ISU 94040	ISU 3388	$matA^+$ / MAT1-2	Sk^S
21894	ISU 3389	$matA^-$ / MAT1-1	Sk^K
FRC M-7358	ISU 3391	$matA^-$ / MAT1-1	Sk^K
ISU-3	ISU 3392	$matA^+$ / MAT1-2	Sk^K
ISU-9	ISU 3393	$matA^-$ / MAT1-1	Sk^K
ISU-18a	ISU 3394	$matA^+$ / MAT1-2	Sk^K
ISU-18b	ISU 3395	$matA^-$ / MAT1-1	Sk^K
ISU-28	ISU 3396	$matA^+$ / MAT1-2	Sk^K
ISU-58-1	ISU 3397	$matA^-$ / MAT1-1	Sk^K
ISU-67	ISU 3398	$matA^-$ / MAT1-1	Sk^K
ISU-80	ISU 3399	$matA^-$ / MAT1-1	Sk^K
ISU-82	ISU3411	$matA^+$ / MAT1-2	Sk^K
ISU-94b	ISU 3412	$matA^+$ / MAT1-2	Sk^K
ISU-115	ISU 3414	$matA^-$ / MAT1-1	Sk^K
ISU-137	ISU 3415	$matA^-$ / MAT1-1	Sk^K
ISU-152	ISU 3416	$matA^+$ / MAT1-2	Sk^K
ISU-174	ISU 3417	$matA^-$ / MAT1-1	Sk^K
ISU-177	ISU 3418	$matA^+$ / MAT1-2	Sk^K

(Table Continues)

Strain	ISU Stock #	Mating Type	*Sk* Phenotype
ISU-180	ISU 3419	$matA^-$ / MAT1-1	Sk^K
AMR F-2	ISU 3421	$matA^-$ / MAT1-1	Sk^K
AMR F-4	ISU 3422	$matA^+$ / MAT1-2	Sk^K
AMR F-5	ISU 3423	$matA^-$ / MAT1-1	Sk^K
AMR F-7	ISU 3424	$matA^+$ / MAT1-2	Sk^S
AMR F-8	ISU 3425	$matA^+$ / MAT1-2	Sk^S
AMR F-9	ISU 3426	$matA^+$ / MAT1-2	Sk^K
AMR F-10b	ISU 3427	$matA^+$ / MAT1-2	Sk^K
AMR F-11	ISU 3428	$matA^+$ / MAT1-2	Sk^K
AMR F-12	ISU 3429	$matA^+$ / MAT1-2	Sk^K
AMR F-13	ISU 3431	$matA^+$ / MAT1-2	Sk^K
AMR F-14	ISU 3432	$matA^+$ / MAT1-2	Sk^K
NRRL 13586	ISU 3433	$matA^-$ / MAT1-1	Sk^K
NRRL 20984	ISU 3434	$matA^-$ / MAT1-1	Sk^S
NRRL 25457	ISU 3435	$matA^-$ / MAT1-1	Sk^K
NRRL 26518	ISU 3436	$matA^+$ / MAT1-2	Sk^K
Fv152	ISU 3437	$matA^+$ / MAT1-2	Sk^K
FGSC 7415	ISU 3438	$matA^-$ / MAT1-1	Sk^S

Note. Table 5 shows all the strains obtained from the USDA.

USDA Female Assay

After the mating types and phenotypes were characterized in the 39 USDA strains the next important characteristic to be identified was the ability of the strains to mate as female. The initial characterization crosses of the USDA strains were carried out using the 39 strains as female. All of the crosses resulted in negligible perithecia production and we were unable to characterize the mating type and *Sk* phenotypes from those crosses. Therefore the initial assays were carried out using the strains as males in the test crosses. To obtain the proper strains with the adequate ability to cross as female progeny were obtained from the initial crosses via ascospore collection and

germination. The resulting strains derived from the cross were then screened for isolates that maintained the same mating type/Sk status as the original parent strain identified from the USDA crosses. The isolates that fit the criteria and were selected for further study were RBM26.10 (AMR F-7), RBM43.31 and RBM43.34 (21894).

CHAPTER V

DISCUSSION

The investigations in *Fusarium verticillioides* presented in this paper posed a variety of challenges. *F. verticillioides* is not nearly as well-developed of a laboratory strain as other genera of fungi such as *Neurospora*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Coprinopsis*, or *Ustilago*, but considering that *Fusarium* research did not really take off until around the 1970s that is no great surprise. While the literature pointed towards using V8 media as a nutrient source for sexual crosses it became apparent that concentrated media was not an acceptable growth medium. During these initial crosses it also became apparent that while the perithecial production was not acceptable in either strain there appeared to be a bias for a greater production of perithecia in the strains utilizing FGSC 7603 as the female. To ensure an optimal environment for sexual crossing of our labs strains of *F. verticillioides* the strains were ran through an assay to evaluate concentrations of V8 in the crossing medium and to assess the ability of both lab strains as females.

It became readily apparent after the initial perithecia count that there were less perithecia, on average, produced from M-3125 females when compared to the FGSC 7603 females. It also became apparent after the 19DPF measurement that both 0.1X and 0.25X carrot media were dramatically better at promoting sexual crosses since at

this point both concentrations were producing more than double the perithecia than the other concentrations. The data for perithecial production was statistically analyzed using SAS software to perform ANOVA analysis. ANOVA analysis revealed no statistical difference across the range of carrot concentrations used for the M-3125 assays with a p-value of P= 0.1011. The same analysis of the crossing data used for ANOVA testing revealed significant differences in crosses using FGSC 7603 females with a P value of P= 0.0003. The replicates crossing on both 0.1X and 0.25X carrot agar were grouped by the post hoc Tukey's studentized ranged test. The other four concentrations grouped together and had far fewer mature perithecia produced during the cross. This data supports the idea that the mating of *Fusarium verticillioides* may be dependent upon nutrient limitation forcing the culture away from asexual reproduction. At the same time it shows that a certain level of nutrient availability is required for sexual reproduction to occur since there was insignificant mating occurring on media below 0.01X carrot.

The M-3125 mating insufficiency was definitely a cause for concern. It was a concern because it would be necessary to have both mating types able to be used both as female and as male. The second cause for concern is that this strain was a well-used strain making it imperative to better understand what would have caused the loss of female fertility in this strain to prevent any further losses in the future.

Magnaporthe oryzae, like *Fusarium verticillioides*, is capable of both sexual and asexual reproduction. In nature clonal, asexual, reproduction is common place while sexual reproduction occurs sporadically. In vitro sexual reproduction in *Magnaporthe*

oryzae is heterothallic, meaning that it has two different mating types, and bipolar, meaning that the variance between mating types is determined by different alleles of the same locus. Also required is the ability for one of the two strains to be used as "female" meaning that the strain is inoculated first to the media and allowed to produce female sexual structures, perithecia. It was determined that after approximately 10-19 rounds of selection under asexual reproduction female-sterile mutations affected the genome at a high rate. This mutation was segregated into the progeny in accordance with the mating type resulting in progeny incapable of acting as a female strain. The strains were also subjected to a variety of stressors that are often known to induce sexual reproduction, but none of the strains reacted to any of the stressors. These findings suggest a genetic mechanism behind female sterility after in-vitro propagation (Saleh Et al., 2012).

This evidence suggests a further need for developing a better understanding of *Fusarium verticillioides* strains used in the lab and for population genetics studies of wild *Fusarium* populations. The understanding as to whether this phenomenon affects wild populations would be essential to predicting outcomes of the possible use of biocontrol strains *of Fusarium* that utilize *Spore killer* elements. If this characteristic is common in wild populations it could greatly reduce, possibly even eliminate, the efficacy of using *Spore killers* due to the lack of sexual reproduction. Currently I have a project going in which I have been sequentially isolating cultures of asexually propagated Fv149 and Fv999. The cultures are isolated via single spore isolations and transferred to V8 slants

that are then incubated for 7 days. After sufficient growth a conidial suspension is then made from the asexually cloned strain and the whole process repeated. Currently five generations of asexual clones have been collected and the work should continue through at least 25 generations and then all generations should be crossed back to the parental stocks of the opposite mating type to assay for female insufficiency. This data would be important when planning future manipulations of strains. It may also become pertinent to ensure that future strains generated are female fertile.

With a basic understanding of the optimal conditions to induce sexual crossing the next step was to analyze the ability of *F. verticillioides* containing Sk to drive linked DNA through meiosis. As indicated in the methods and results we designed primers for the amplification of RFLP containing loci across chromosome V, which contains the Sk element, and on 3 independent chromosomes to compare drive results both within Chr. V and between Chr. 5 and Chrs. I, VII, and XI. By determining the pattern of inheritance, whether they are from FGSC 7603 or M-3125, at each RFLP loci we can compare if they are being inherited in the traditional Mendelian pattern or if the genotypes at specific loci are being skewed in favor of the *Spore killer* parent's genotype, FGSC 7603.

Going into the assay blind we hypothesized that the markers along Chr. V would show meiotic drive skewing the progeny to the Sk genotype. We predicted that this would be the case because to produce viable spores in a cross with a killer the resulting progeny must contain a copy of the killer resistance. If recombination were to take place around the location of the Sk element then it must transfer the Sk in its entirety.

Otherwise, if any other spore in the ascus retains a functional copy of the killer element then all ascospores lacking the *Sk* element or retaining a partial copy would be susceptible to killing. This would result in a reduction of the probability for a successful recombination around the killer locus.

What was observed when we analyzed progeny from RJP98 (FGSC 7603xM-3125) was that the strongest linkage between any two RFLP loci was between RFLP1B and RFLP2B. In this assay these two loci co-segregated 100% of the time that RFLPs were characterized. In one strain the PCR reaction failed to amplify the RFLP2B locus (strain RJP98.98); therefore, this data point was culled when considering successful co-segregation because the data was unavailable. RFLP1B and RFLP2B only displayed two successful recombinant events out of the 59 strains analyzed. The two strains that were identified recombinants at RFLP1B and RFLP2B simultaneously were RJP98.111 and RJP118. This is what was expected for regions within close proximity of the killing locus.

RFLP6B was located approximately 650 kb 5' of RFLP1B and RFLP3 was located 641 kb 3' of RFLP2B. With such a close similarity in distance between the markers it would be reasonable to expect that they would have an equivalent ability to segregate with their neighbor (RFLP6B with RFLP1B and RFLP2B with RFLP3). However, a disproportionate segregation rate was evident. Of the 59 strains assayed RFLP6B segregated with RFLP1B only 88.1% of the time while the 58 strains analyzed for co segregation of RFLP2B segregated with RFLP3 96.6% of the time. Based on the observed segregation pattern I assessed its inverse relationship of transmission with distance to

establish a theoretical direct correlation between distance and segregation. Based on this modeling the locus with the strongest drive would be one nucleotide 3' of RFLP2B. Following this assumption an RFLP designed 701 kb 3' of the center point should achieve the same percent inheritance of RFLP6B in relation to RFLP1B. In the same regard, an RFLP designed 640 kb 5' of this center point should achieve the same percent inheritance as RFLP3 in relation to RFLP2B.

Following this hypothesis, future work could conceivably utilize a series of knockouts working towards this center point to establish the locus of the *Sk* element. Again, this is assuming that the *Sk* element exerts drive equally in both the 3' and 5' directions originating from the derived center point.

The *Spore killer* assay was designed to compare phenotypic evidence from sexual crosses of the progeny of cross RJP98 to the RFLP results for the progeny used in each corresponding cross. This allowed for a direct comparison of phenotypic outcome relative to the genotype inherited by that strain. In this manner if a strain genotyped to M-3125 at each RFLP along chromosome V the assumption would be that homologous recombination had occurred such that the *Sk* had been recombined out of the strain. This would mean that when looking at the phenotype the killing phenotype would be expected; however, this was not the case. In all of the strains, with interesting crossovers, that were selected for *Sk* analysis the observed phenotype was Sk^K. The RFLP results pointed to a region around RFLP1B, RFLP2B, and RFLP3 as hosting the *Sk* locus.

Often secondary metabolites, mycotoxins, are essential for the onset or perpetuation of the disease symptoms associated with phytopathological disorders (Bennett and Klich, 2003). In the case of fumonisin production by *Fusarium verticillioides* naturally occurring strains have been identified in nature (Desjardins et al., 1992). This observation might suggest that fumonisins may not be essential for pathogenesis. Keeping in mind that the relationship between maize and *F. verticillioides* is so intimate that practically every plant contain fungal inoculum it would be of great importance to assess the possible role of fumonisins in the plant fungus interaction or in the ability of the fungus to maintain its defenses against bacterial and fungal invasion of the plant. It could also be important to consider the targeting of genes that would not exclude the production of all fumonisins.

Other researchers have shown that the Sk^K allele is much more prevalent than other known meiotic drive elements. Their research showed the presence of killer alleles in more than 80% of the isolates they screened. They also showed that killer phenotypes were more prevalently associated with isolates from Europe and America (Kathariou and Spieth, 1982). In the event that populations of *Spore killers* are too prevalent for a *F. verticillioides* Sk strain to overcome the naturally occurring strains it is still possible to utilize an *Sk* element introgressed into *Fusarium verticillioides* from another genus of fungus such as *Neurospora crassa* or *Podospora anserine*. In this case, it would still be essential for the preservation of the biocontrol strain to have identified and included a copy of the *Fusarium verticillioides Sk* along with the transgenic *Sk.*

CHAPTER VI

MSUD AND NEUROSPORA INTERMEDIA

Introduction

The process of Meiotic Silencing by Unpaired DNA (MSUD) is a recently discovered phenomenon in which the filamentous ascomycete Neurospora crassa has been shown to silence unpaired DNA. It has been proposed that MSUD may act as a genome defense mechanism to preserve the structure and integrity of the genome against invading selfish DNA segments such as: transposable elements, viruses, and viroids. However, little is known about the inner workings of MSUD within the nucleus, the degree to which MSUD has been spread across species, what sparked the evolution of such a mechanism, and the possible functions of MSUD associated with developing genomes and evolution. This research is aimed at identifying MSUD activity across the species barrier within the Neurospora genera. If this research is successful it would be the first findings to identify MSUD in another species outside of N. crassa.

The process of MSUD has only been identified in *N. crassa*, and has yet to be identified outside of the species. Currently the proposed mechanism behind MSUD is that there is a physical homology search between homologous chromosomes in the zygote of sexually reproducing *N. crassa* post karyogomy. This searching is believed to

function by the production of aberrant single stranded aRNA molecules which are then directed to a perinuclear RNAi pathway. The aRNA molecules are believed to be modified in a way that allows them to be recognized by the RISC machinery. This pathway has been identified as a RNA-induced silencing complex (RISC) composed of: an RNA dependent RNA polymerase known as SAD-1 (Shiu et al. 2001), an argonaute class protein, SMS-2(Lee et al. 2003), and a Dicer protein recognized as DCL-1 (Alexander et al. 2008). Along with these three proteins are four more putative proteins which form the perinuclear RNA-induced silencing complex (RISC) which include: a scaffolding protein SAD-2 (Shiu et al. 2006), a helicase, SAD-3,(Hammond et al. 2013), an exonuclease, QIP, (Xiao et al. 2010), and a protein required for the generation of masiRNAs, SAD-4, (Hammond et al. 2013 a,b).

It has been proposed that the process of MSUD acts as a genome defense mechanism, but it is possible that given more information there could be other important processes associated with MSUD. The process of MSUD may have acted as a catalyst for speciation in the early evolution of fungal species.

Hypothesis

MSUD activity is present in other species of fungus other than *N. crassa* species.

Objective

The purpose of this research is to identify MSUD activity outside of the *N. crassa* species. To simplify this research we will be screening another species within the *Neurospora* genera for MSUD activity.

Materials and Methods

*Standard Neurospora Laboratory Procedures were performed in all work according to Davis and de Serres 1970, unless otherwise noted.

Strains and Media

Strains were maintained and manipulated using Vogel's media with the appropriate supplements (Vogel, 1956). Crossing was carried out using Synthetic Crossing Medium developed by Westergaard and Mitchell (1947). Strains were cultured at 31^{o}C while sexual crosses were performed at room temperature. Strains shown in Table 6.

Culture Conditions

Strains were cultured in an incubator at 28 degrees Celsius while experimental crosses were performed at room temperature.

Transformation at the his-3 Locus

Transformations were performed using linearized plasmid clones to target the his-3 locus through homologous flanks, and these constructs will be introduced by the electroporation of asexual spores as performed by Margolin et al. 1997. The his-3 locus has a mutation that prevents the biosynthesis of histidine, and the subsequent transformation at this locus introduces new his-3 sequence that repair the mutation restoring the biosynthetic pathway for histidine while targeting the insertion vector to that locus. This allows us to use media not supplemented with histidine as a selection criteria. Transformants also contain DNA that confers resistance to the antibiotic

hygromycin; therefore, transformants are also selected by transferring cultures that grew on minimal media onto slants containing hygromycin (Carroll et al. 1994).

Modification: Asexual spores were purified from vegetative tissues by filtration through 100µm nylon filters (Steriflip®, Millipore, Billerica, MA).

Transformation at the csr-1 Locus

Transformations were performed by electroporation of washed conidia (asexual spores) as described by Margolin et al. (1997). The gene Cyclosporine-resistance-1 (Csr-1) in the model fungus Neurospora crassa encodes a cyclophilin protein (Cyp20) which plays a critical role for sensitivity to cyclosporine A, an antifungal compound. Cyclosporine A, together with Cyp20, inhibits the ability for fungi to generate transcription factors that are essential for hyphal growth. Therefore, a targeted knockout to the csr -1 locus would result in the negative effects on growth in fungal cultures grown in the presence of cyclosporine-A. Sensitivity to cyclosporine-A would allow for a specific approach for transforming constructs that would also allow the selection for desensitization to cyclosporine-A. Primers used are included in Table 6.

Modification: Asexual spores were purified from vegetative tissues by filtration through 100µm nylon filters (Steriflip®, Millipore,Billerica, MA).

Table 6

Key Strains Used in the Neurospora intermedia Study

Strain	Mating Type	Genotype	Origin
FGSC 3417	a	Wt	f5 isolates from P13A (FGSC1766) x P17a (FGSC 1767): Taiwan
FGSC 3416	A	Wt	f5 isolates from P13A (FGSC1766) x P17a (FGSC 1767): Taiwan
TJP24.1	A	csr-1 Δ::hph::asm-1(Het)	Transformed with csr-1 vector
TJP24.10	A	csr-1 Δ::hph::asm-1(Het)	Transformed with csr-1 vector
TJP25.7	A	csr-1 Δ::hph::asm-1(Het)	Transformed with csr-1 vector
P8-65	a	csr-1 Δ::hph (N. crassa)	
P9-42	a	Oak Ridge WT (N. crassa)	Oak Ridge
TJP10.8	A	his-3 Δ::hph::asm-1(Het)	pJP14

Note. Table 6 displays the key *Neurospora crassa* strains used the research for this paper.

Vogel's Liquid Media

Vogel's liquid media was used during growth of mycelia for genomic DNA isolation (Vogel, 1956). Vogel's media was prepared according to the 1956 paper except for: no agar was added to maintain a liquid media form and the prepared media was divided into 25mL aliquots and autoclaved in individual 125mL Erlenmeyer flasks.

Sexual Crosses

For studies involving sexual crosses the strains were simulateously inoculated at opposite ends of a 100mm plate containing 45mL of Westergaards Synthetic Crossing Media. Crosses were maintained at room temperature. Crosses using *Neurospora intermedia* took about a month to reach maturity.

Primer Design

Primers, excluding all primers designed to add sequence to constructs, were designed between 20 and 24 nucleotides with a TM of between 65 and 71. Optimal conditions were set to: maximum poly-X = 3, maximum self-complementarity = 2, and maximum 3' self-complementarity = 1. Adjustments were first made to maximum self-complementarity before 3' self-complementarity on an as needed basis up to 5 and 3 respectively before looking for new primer locations if needed. See Table 2 for primers used in this study. The primers used in this study are listed in Table-7.

Table 7

Primers Used in Neurospora intermedia Studies

Primer #	Primer Name	Sequence	Notes
185	NotI-asm1-F	AAAAGCGGCCGCTCCCACTCCCATTACGCTTCTCA	Asm-1 amplification + NotI
186	NotI-asm1-R	AAAAGAATTCATCATCGTCCCGTCCTCGCTTC	Asm-1 amplification + NotI
297	his3-102113F	GAGGGAGTGTGGGAAATGGTGTC	Sequencing at the his-3 locus
408	seq-frm-hph-r	ATGTCCTCGTTCCTGTCTGCT	Sequencing at the his-3 locus
579	csr-1A	GCCTTGATGTCGTAGAGTGTCAGGT	Csr-1 Left Flank
580	csr-1B	CTATAGTGAGTCGTATTAAGGGCGTGGACGGATGTGGGCTTCGGATTGGTTCGT	Csr-1 Left Flank
581	csr-1C	GTTTCAGGGGTTTCGTTCGCTGTCAATCAAGCACCCACCCTTCTTCACTGCATC	Csr-1 Right Flank
582	csr-1D	GCATCTGGAACCGGACCATCAA	Csr-1 Right Flank
583	csr-1E	ACGTGTCTATCGCCGGAGTTGCT	Csr-1 KO Nested
584	csr-1F	GGCCTTCATCAACGAGCTACAGG	Csr-1 KO Nested
585	DJ-1152-2-F	CCGTCCACGCCCTTAATACGACT	Center Product
586	DJ-1152-2R	CTTGATTGACAGCGAACGAAACC	Center Product
683	Csr-KO-Chk-F	AATCATAACGCCCGCACCACTC	Conidial PCR
684	Csr-KO-Chk-R	GCCGTAGATGGACTTGCCACCA	Conidial PCR

Note. Table 7 lists all of the primers used in this study of *Neurospora intermedia.*

Backcrosses

Strains of opposite mating type, FGSC 3417 and FGSC 3416, were simultaneously inoculated to SCM media. Once spores were shot to the lids the ascospores were then collected, germinated, and phenotyped for mating type. FGSC 3416 mating types were continuously selected for and back crosses to FGSC 3417 to reduce homology differences between the mating strains. Over time successive generations of FGSC 3416 mating typed progeny were continuously back crossed to FGSC 3417 and selected for.

Test Crosses

After sufficient generations of FGSC 3416 mating typed backcrossed strains are collected they were then crossed to a FGSC 3417 containing an ectopic copy of Asm-1 for analysis of MSUD activity. The back crossed strains and Asm-1^{ef} strains were inoculated simultaneously from opposite ends of 100mm Westergaard Synthetic Crossing medium plates with 45mL of medium.

Genomic DNA Isolation

Samples were grown in 125mL Erlenmeyer flasks containing 25mL of Vogel's minimal medium for three days. Samples were shaken each day to prevent growth up the flask sides. The samples were then decanted of media and washed once with autoclave sterilized distilled water. After rinsing, the samples were pressed dry using flame sterilized spatulas and filter paper. The samples were then placed into microcentrifuge tubes and labeled. The microcentrifuge tubes were then puncture with sterile needles to allow for gas exchange and the tubes were placed into a lyophilyzation

chamber and lyophilized for a period greater than four hours to ensure samples were thoroughly dry. Dehydrated samples were then weighed to unsure less than 100mg of sample were being extracted. Genomic DNA Extraction was performed using IBI's Genomic DNA Mini Kit (Plant) Protocol, solutions, and columns according to the manufacturer's protocols.

Permanent Stocking

Permanent stocking of *Neurospora intermedia* was achieved by growing strains in snap cap tubes containing 2mL of Vogel's minimum liquid media. The strains were incubated for one week at 31^{o}C followed by a one week incubation at room temperature. Then 2mL of 14% sterile reconstituted milk was added using a 5mL pipette followed by light vortexing of the culture. 750µl of the resulting suspension was then transferred into cryogenic vials containing 750µl, vortexed, and immediately stored at -80^{o}C. 200µl of the suspension were then transferred to screw cap vials filled with silica beads. These vials were capped and left to dry overnight at room temperature. The following day vials were parafilmed and stored in a dry box at 4^{o}C.

Conidial DNA Preparation

Small amounts of conidia were transferred from young fungal cultures, no more than two weeks, into micro-centrifuge tubes containing 200µL of sterile TE Buffer pH 8.0 using sterile wooden applicators. Samples were then heated to 105°C for 12 minutes before being place on ice for 2 minutes. The samples were next vortexed for 5 seconds and then spun down at 21,000Xg for 10 minutes. Finally, 25µL of the supernatant was

then transferred into clean MCTs and used as template DNA for PCR amplification or stored at -20°C.

Dissections

Mature perithecia were transferred to slides containing 50% glycerol and then dissected using flame sterilized needles under a dissecting scope. Rosettes from the dissected perithecia were transferred from the dissecting slide to a clean slide. Then, 50% glycerol was added to 10µL total volume. A coverslip was placed over the sample rosettes and any excess glycerol was absorbed from under the coverslip by placing a Kimwipe at the interface where excess glycerol was present. Final slides were visualized under a compound phase contrast microscope.

Polymerase Chain Reaction (PCR)

PCR was performed using Phusion High Fidelity DNA Polymerase from NEB Inc. PCR reactions were performed in 20µL volumes according to the manufacturers protocol. The conditions were as follows: 0.5µL of template conidial DNA, 10µM of forward primer, 10µM of reverse primer, 10mM dNTPs, 0.2µL of Phusion HF Pol, and sterile water to 20µL. The PCR settings were set to the manufacturer's specification with the temperature for the annealing phase was set to -5^{o}C of the TM for the primer set.

Double Joint-PCR (DJ-PCR)

Double joint constructs were assembled by double-joint PCR (DJ-PCR) (Yu et al. 2004). Primers were designed to amplify two flanking sequences for targeting of the

ectopic DNA and the center fragment that contained the ectopic DNA. The primers were designed with some overlap between each flank and its corresponding side of the center fragment to allow fusion of each section to one another. The first three reactions amplify the three fragments. A fourth reaction is used without primers to facilitate the fusion of the fragments, and a fifth reaction with the nested primers amplifies the final construct to be used for transformation

Results

Initially, transformants of Asm-1 were targeted to the his-3 locus by forced cloning the *N. crassa* Asm-1 marker into the NotI-EcoRI site of pTH1152.2 which contains adjacent his-3 flanking sequences. This plasmid was then renamed pJP14. The vector, pJP14, was linearized with SspI and then transformed into the Wild Type *Neurospora intermedia* strain FGSC 3417 which was renamed TJP10.8. TJP10.8 was then set up for sexual crossing on Westergaard Synthetic crossing medium crossed to Wild Type FGSC 3416 of the opposite mating type. Examination of the rosettes revealed that even after 27DPF there did not appear to be a majority of silencing of the Asm-1 gene, and there were still underdeveloped ascospores present. This made it more difficult to identiy white ascospores produced by Asm-1 silenced spores when compared to underdeveloped ones. There were also a large volume of spores that were deformed or irregularly shaped. (Data shown in Figure 8) The perithecia also never shot spores to the lids of the crossing plate to be quantitatively analyzed under a hemocytometer.

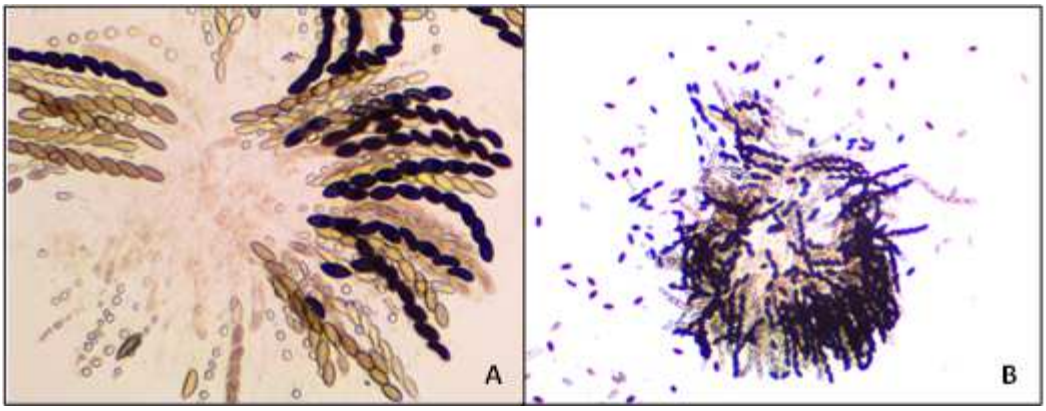

Figure 8. Asm-1 Crosses for Detecting MSUD in *N. intermedia*. **A.)** Representative from first set of crosses using TJP10.8.1X3416. **B.)** Representative rosette from the second round of crosses using TJP10.8.1.1.1X3416.

To test if the Asm-1 marker amplified from *N. crassa* was not homologous enough to the *N. intermedia* copy of Asm-1 to induce silencing by MSUD the *N. intermedia* copy was sent off for sequencing. Sequencing revealed greater than 96% sequence homology between the *N. crassa* and *N. intermedia* copies of Asm-1. With the sequence homology of the two strains' Asm-1 copies confirmed the next step was to establish that the mating pair of *N. intermedia* strains did not contain heterologous genomes.

If sufficient heterology were present it could result in the MSUD machinery being localized to multiple loci in the genome resulting in insufficient signal production to induce silencing from each independent locus. To ensure genome homology of the mating pair FGSC 3416 and FGSC 3417 were subsequently backcrossed through multiple generations for testing of MSUD activity at each generation. Strain information is shown in Table 8. To ensure the ability of the strain to mate with the transformed

strains (FGSC 3417) backcrossing was focused on identifying strains of the FGSC3416 mating type. Ascospores were collected from the lids of backcrosses and germinated on vogel's minimum media, but only FGSC 3416 mating type strains were kept for further back crossing. Crossing began to fail at the fourth backcross in which the F3 progeny were crossed back to the parental strain. The repeat of the fourth round of backcrosses weakly produced perithecia and very few ascospores were shot to the lid.

Table 8

N. intermedia Backcrosses

Cross #	♂ Strain	♀ Strain	Notes
RJP43	3416	3417	First Back cross
RJP172	RJP43.3	3417	2nd back cross
RJP173	RJP43.6	3417	2nd back cross
RJP204	172.1	3417	Third Back Cross
RJP205	172.6	3417	Third Back Cross
RJP206	172.7	3417	Third Back Cross
RJP207	172.8	3417	Third Back Cross
RJP208	172.9	3417	Third Back Cross
RJP209	173.1	3417	Third Back Cross
RJP210	173.2	3417	Third Back Cross
RJP212	173.5	3417	Third Back Cross
RJP213	173.6	3417	Third Back Cross
RJP370	RJP206.3	3417	Fourth Back Cross--Fail
RJp371	RJP207.2	3417	Fourth Back Cross--Fail
RJP372	RJP208.2	3417	Fourth Back Cross--Fail
RJP373	RJP209.2	3417	Fourth Back Cross--Fail
RJP374	RJP212.2	3417	Fourth Back Cross--Fail
RJP375	RJP206.1	3417	Fourth Back Cross--Fail
RJP376	RJP207.1	3417	Fourth Back Cross--Fail
RJP377	RJP208.1	3417	Fourth Back Cross--Fail
RJP378	RJP209.1	3417	Fourth Back Cross--Fail
RJP379	RJP212.1	3417	Fourth Back Cross--Fail
RJP605	RJP209.1	3417	Fourth Back Cross--Weak
RJP606	RJP209.2	3417	Fourth Back Cross--Weak

Note. Table 8 shows the backcrosses used to generate strains with increasing homology to test if background MSUD levels are preventing recognition of introduced unpaired DNA.

The development of a new vector for transformation of the Asm-1 marker was designed around the Csr-1 locus for a targeted approach to transforming *N. intermedia.* Vector construction was performed by using double joint PCR as described in the methods. Primers 579x580 were used to amplify the left flanking sequence and

581x582 were used to amplify the right flanking sequence from the Csr-1 locus of *N. intermedia*. These two flanks have sequences that are complementary to overhangs added onto the center fragment by primers (585x586). Primers 585x586 were used to amplify the center product, which contains the ectopic fragment of Asm-1 and the transgenic HPH gene, from pJP21. The center product plasmid was generated by force cloning the EcoRI/NotI double digests of the Asm-1 amplicon and pTH1152. The three PCR products were gel purified using the IBI PCR clean up kit, and then the purified DNA fragments were used in the DJ PCR reaction in which the melted fragments serve as templates for fusion and amplification of the combined vector now composed of all three fragments. Then the fusion PCR reaction was used as template for the amplification of the final vector using the nested primers 583x584 in triplicate reactions. These reactions were then column purified into a single concentrated sample which was used to transform *N. intermedia* conidia via electroporation.

The transformants were selected on Vogel's plates with hygromycin and were then subsequently transferred onto Vogel's slants containing hygromycin. The putative transformants were then screened for cyclosporine resistance using the cyclosporine resistance assay as described in the methods. The initial results revealed three putative transformants (TJP24.1, TJP24.10, and TJP25.7); however, the screen showed that our negative control strain (P9-42) was showing resistance to cyclosporine as well. To confirm transformants the cyclosporine was rerun this time inoculating the three putative transformants, three apparent non-transformants, and the controls at 1X and

0.5X conidial suspension concentrations. The data was collected at 2DPI and 3DPI. During the second round of assays the original predictions were confirmed and both controls displayed proper phenotypes. Data is shown in Table 9.

Table 9

Cyclosporine Assay Results for Screening Transformants

2 DPI				3 DPI			
Dilution	Strain	Min.	Cyclo.	Dilution	Strain	Min.	Cyclo.
1X	TJP24.1	3	3	1X	TJP24.1	3	3
1X	TJP24.10	3	1	1X	TJP24.10	3	1
1X	TJP25.7	2	2	1X	TJP25.7	2	2
1X	TJP24.2	3	0	1X	TJP24.2	3	0
1X	TJP24.3	3	0	1X	TJP24.3	3	0
1X	TJP24.4	3	0	1X	TJP24.4	3	0
1X	P8-65	3	3	1X	P8-65	3	3
1X	P9-42	3	0	1X	P9-42	3	0
0.1X	TJP24.1	2	1	0.1X	TJP24.1	2	1
0.1X	TJP24.10	2	1	0.1X	TJP24.10	2	1
0.1X	TJP25.7	2	1	0.1X	TJP25.7	2	1
0.1X	TJP24.2	2	0	0.1X	TJP24.2	2	0
0.1X	TJP24.3	2	0	0.1X	TJP24.3	2	0
0.1X	TJP24.4	2	0	0.1X	TJP24.4	2	0
0.1X	P8-65	3	2	0.1X	P8-65	3	2
0.1X	P9-42	0	0	0.1X	P9-42	0	0

Note. Table 9 shows the cyclsporin assay results for putative Asm-1 transformants at the CSR-1 locus. 3 represents maximum growth of the + control strain (P8-65) for the sampling day at 1X cyclosporine. 2 represents less than the maximum growth compared to the + control strain for the sampling day at 1X cyclosporine. 1 represents germinating conidia with minimal growth when compared to the + control strain for the sampling day at 1X cyclosporine. 0 represents no growth.

To confirm the putative transformants, the strains TJP24.1, TJP24.10, and TJP25.7 along with negative transformants (TJP24.2, TJP24.3, and TJP24.4) were inoculated to

125mL Erlenmeyer flasks with 25mL Vogel's minimum media and incubated for three days at 31°C. Genomic DNA was isolated using IBI's Genomic DNA Mini Kit (Plant) according to the manufacturers protocol. The genomic DNA was used as the template for a PCR reaction confirming DNA transformation with the nested primers for the DJ-PCR reaction (583x584). The resulting products were run in a 1% TAE gel for one hour. Data are shown in Figure 9.

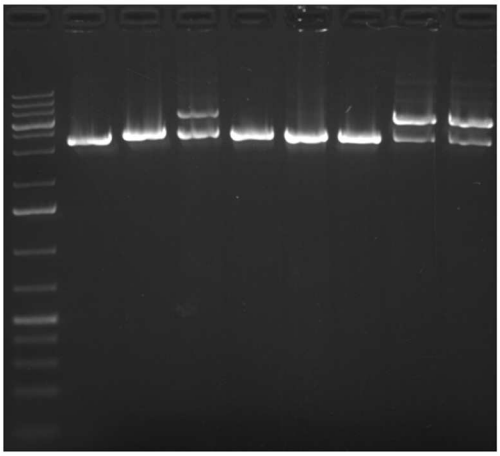

Figure 9. Screening Putative csr-1 Knockouts. This figure shows the results from the nested PCR amplification of the csr-1 locus. The putative transformants in lanes 4, 8, and 9 appeared to be heterokaryons amplifying both the wild type and transformed bands. (Expected transformant band was 4.9 kb and the expected Wild Type band was 3.7 kb)

The next step after identification of heterokaryons was to isolate homokaryons. Homokaryon isolations were attempted by repeat single spore isolations of conidia produced from the heterokaryons strains. Conidial suspensions were spread on Vogel's media containing cyclosporine overnight. Individual germinating conidia were then transferred to new Vogel's slants containing cyclosporine. Due to time constraints these

cultures, RJP25.7A2 and RJP24.1A, were then set up in crosses to controls and the back crossed strains: FGSC 3416, RJP43.3, RJP172.7, RJP207.2, and RJP605.3.

Table 10

N. intermedia Crosses Used to Assay MSUD Activity

Cross Number	P1	P2
607	FGSC 3417	FGSC 3416
608	RJP25.7A2	FGSC 3416
609	RJP25.7A2	RJP43.3
610	RJP25.7A2	RJP172.2
611	RJP25.7A2	RJP207.2
612	RJP25.7A2	RJP605.3
613	RJP24.1A	FGSC 3416
614	RJP24.1A	RJP43.3
615	RJP24.1A	RJP172.2
616	RJP24.1A	RJP207.2
617	RJP24.1A	RJP605.3

Note. Table 10 shows all of the crosses used to asses MSUD activity in this study.

The strains were dissected and the lids were checked for spores to phenotype. Phenotyping under the microscope revealed only white spores in crosses containing both RJP25.62A and RJP24.1A, while the wild type control cross (FGSC 3416 x FGSC 2417) produced mature black ascospores. When screening the plate lids insufficient ascospores for counting were observed on the lids of crosses containing a transformant in the crosses while black spores were visible on the lids of the control cross. Due to the lack of spores shot in the transformant crosses counts were not taken. To ensure that spores were not shot and then dropped off of the lid plates were scanned under a dissecting scope. There were no visible spores on the mycelia of transformant crosses

while the control crosses had many mature black spores on the plate. Dissection results are shown in Figure 10 and Figure 11.

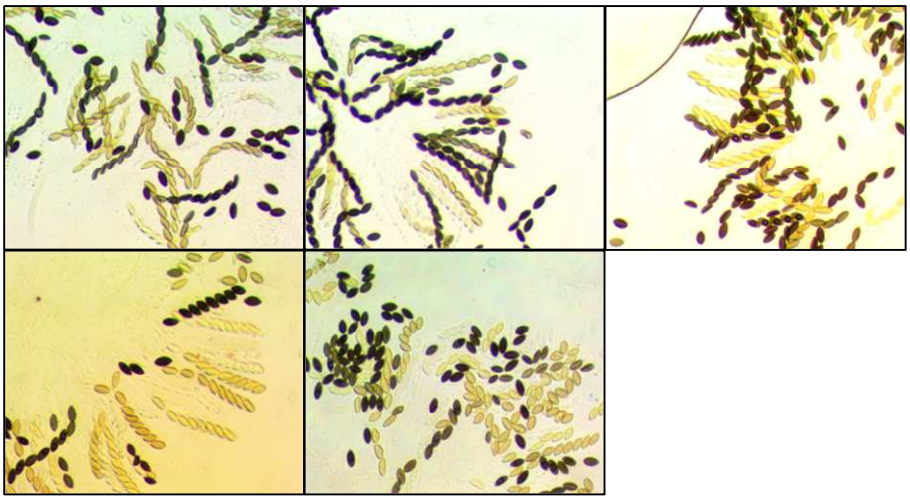

Figure 10. Dissection Results from Control Crosses for *N. intermedia* MSUD Screen. This figure shows rosettes dissected from 5 individual perithecia taken from the wild type control cross RJP607.

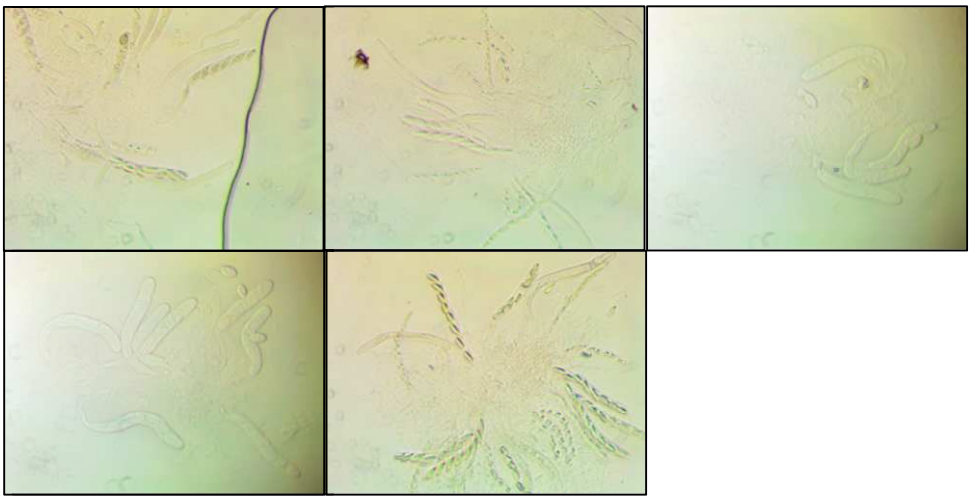

Figure 11. Dissection Results from Transformant Crosses for *N. intermedia* MSUD Screen. This figure shows rosettes dissected from 5 individual perithecia taken from cross RJP608 which contains an ectopic fragment of asm -1 localized to the Csr-1 locus

Discussion

The identification of MSUD occurring in species other than *N. crassa* could provide new evidence for the elucidation of currently unknown mechanisms within the MSUD process. In our initial crosses of *N. intermedia* strains, one of which containing an ectopic copy of Asm-1 which is a phenotypic marker used in *N. crassa* research appeared to show no signs of Asm-1 silencing. From this information we proposed two hypotheses: the first was that there was too much variation in homology between Asm-1 genes across species, and a second possible option was that there was too much background work for MSUD to produce a strong enough signal to efficiently silence Asm-1.

Following this, the Asm-1 sequence amplified from *N. intermedia* was sequenced and revealed that the sequence contains >96% homologous identities to the recorded Asm-1 sequence of *N. crassa* from the Broad Institute's genome. Theoretically, this should be sufficient homology to induce MSUD by inserting an ectopic copy. With this in mind we began to look at the idea that the level of variation across the genome between the two mating strains could sufficiently reduce the signal production for generating an effective silencing response. MSUD machinery would be spread out making aRNA signals for so many disparate sequences that the signal for Asm-1 is below the threshold for MSUD activity.

In an attempt to homogenize the genome of the mating pair the strains were repeatedly backcrossed selecting for the matA (FGSC 3416). Collected strains were saved for future analysis of MSUD and were then back crossed again. The back crossing began to fail at the F4 generation during the fifth backcross. After repeated attempts the strains successfully mated producing F4 offspring, but the mating was weak and very few perithecia were produced. From the F4 progeny two strains were successfully mating typed to matA (FGSC3416).

These strains were then crossed to the Asm-1^{ef} at the his-3. These crosses did not successfully mate. Mating with *Neurospora intermedia* is difficult because the strain has a tendency to over grow. By cutting the lid of the trays open at the sides to allow air flow, and by also opening the lids of the petri dishes every few days in a sterile environment to allow moisture content to escape the strains do not overgrow. However, mating is still not optimal in the back crossed strains. Mating tends to take longer than *Neurospora crassa* and the perithecia tend to get much larger. The larger perithecia tended to produce fewer rosettes.

The strains transformed at the Csr-1 locus were crossed to the strains isolated via back cross. The strains containing the ectopic fragment of Asm-1 did not produce any visible black spores and there was an apparent difference between the transformant crosses and the control cross. Future work for confirming this observation will need to be carried out. The single spore isolates need to be confirmed as homokaryons via PCR. The results should be reproduced using triplicate crosses of strains RJP25.7A2 and RJP24.1A. Overall, this appears to be the first evidence for MSUD

activity occurring outside of the species *Neurospora crassa*. Data obtained from the further research of MSUD within *Neurospora intermedia* could potentially reveal new information about the functioning of MSUD nuclear components, and can lead to the ability to further test the functioning of MSUD across species.

REFERENCES

Alexander, W.G., N.B. Raju, H. Xiao, T.M. Hammond, T.D. Perdue, R.L. Metzenberg, P.J. Pukkila, and P.K.T. Shiu. 2008. DCL-1 colocalizes with other components of the MSUD machinery and is required for silencing. Fungal Genet. Biol. 45: 719-727.

Bacon, C.W., D.M. Hinton, J.K. Porter, A.E. Glenn, & G. Kuldau. 2004. Fusaric acid, a *Fusarium verticillioides* metabolite, antagonistic to the endophytic biocontrol bacterium Bacillus mojavensis. Can J Botany 82: 878–885.

Brown, D.B., R. A. E. Butchko, M. Busman, and R.H. Proctor. 2007. The *Fusarium verticillioides* FUM Gene Cluster Encodes a Zn(II)2Cys6 Protein That Affects FUM Gene Expression and Fumonisin Production. Eukaryotic Cell. 6(7) 1210-1218.

Carroll, A. M., J. A. Sweigard, and B. Valent, 1994. Improved vectors for selecting resistance to hygromycin. Fungal Genet. Newsl. 41: 22.

CFSAN. Draft guidance for industry: Fumonisin levels in human foods and animal feeds. USFDA Center for Food Safety and Applied Nutrition and the Center for Veterinary Medicine. 2000 http://www.vm.cfsan.fda.gov/dms/fumonbg1.html (verified April 6, 2002)

Choi, H.-W., Kim, J.-M., Hong, S. K., Kim, W. G., Chun, S.-C., & Yu, S.-H. (2009). Mating Types and Optimum Culture Conditions for Sexual State Formation of *Fusarium fujikuroi* Isolates. *Mycobiology*, *37*(4), 247–250. doi:10.4489/MYCO.2009.37.4.247

Desjardins, A. E., R. D. Plattner, D. D. Shackelford, J. F. Leslie, and P. E. Nelson. 1992. Heritability of fumonisin B, production in Gibberella fujikuroi mating population A. Appl. Environ. Microbiol. 58:2799-2805

Dohlman, E. 2011. Mycotoxin hazardsand regulations: impacts on food and animal feed crop trades. USDA Econ. Res. Arch. 828: 97-108.

Fazekas, B., E., Bajmocy, R., Glavits, A., Fenyvesi, and J., Tenyi (1998) Fumonisin B1 contaminatino of maize and experimental acute fumonisin toxicosis in pigs. J. Vet. Met. B. 45:171-181

Gelderblom, W. C. A., K. Jaskiewicz, W.F.O. Marasas, P.G., Thiel, R.M. Horak, R. Vleggaar, and N.P.J. Kriek. 1988. Fumonisins- Novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliformine*.

Hammond, T. M., W. G. Spollen, L. M. Decker, S. M. Blake, G. K. Springer et al., 2013a Identification of small RNAs associated with meiotic silencing by unpaired DNA. Genetics 194: 279-284.

Hammond, T. M., H. Xiao, E. C. Boone, L. M. Decker, S. A. Lee, et al., 2013b Novel proteins required for meiotic silencing by unpaired DNA and siRNA generation in Neurospora crassa. Genetics 194: 91–100.

Hammond, T. M., Xiao, H., Boone, E. C., Perdue, T. D., Pukkila, P. J., & Shiu, P. K. T. (2011). SAD-3, a Putative Helicase Required for Meiotic Silencing by Unpaired DNA, Interacts with Other Components of the Silencing Machinery. *G3: Genes|Genomes|Genetics*, *1*(5), 369–376. doi:10.1534/g3.111.000570

Hammond, T.M., D.G. Rehard, H. Xiao, and P.K.T. Shiu. 2012. Molecular dissection of Neurospora killer meiotic drive elements. PNAS: 109:30 12093-12098.

Howard, P.C. et al. 2001. Fumonisin B1 carcinogenicity in a two year fedding study using F344 rats and B6C3F1 mice. Environ. Health Perspect. 109 (S2): 277-282.

Jaskiewicz K, Marasas WFO, van der Walt FE. 1987. Oesophageal and other main cancer patterns in four districts of Transkei, 1981-1984. S Afr Med J 72:27–30

Keunsub, L. , J.J. Pan, & G. May. 2009. Endophytic *Fusarium verticillioides* reduces disease severity caused by Ustilago maydis on maize. FEMS Microbiol Lett 299: 31–37

Kusano, A., C. Staber, H.Y.Chan and B. Ganetzky. 2003. Closing the (Ran)GAP on segregation distortion in Drosophila. BioEssays 25:108–115.

Klittich, Carla JR, and John F. Leslie. 1988. Klittich, C. J. R., and J. F. Leslie, 1988 Nitrate reduction mutants of *Fusarium moniliforme* (*Gibberella fujikuroi*). Genetics 118:417–423.

Klittich, Carla JR, and John F. Leslie. 1992. Identification of a second mating population within the Fusarium moniliforme anamorph of Gibberella fujikuroi. *Mycologia*: 541-547.

Koch, A., Kumar, A., Weber, L., Keller, H., Imani, J., Kogel, K.H. 2013 Host-induced gene silencing of cytochrome P450 lanosterol C14α-demethylase–encoding genes confers strong resistance to Fusarium species. PNAS 110:48: 19,324-19,329

Kriek NPJ, T.S. Kellerma, W.F.O. Marasas. 1981. A comparative study of the toxicity of *Fusarium verticillioides* (= F. moniliforme) to horses, primates, pigs, sheep and rats. Onderstepoort J Vet Res48:129–131.

Leslie, J. F. 1996. Introductory biology of Fusarium moniliforme ,p.153 – 164. In L. S. Jackson, J. W. De Vries, and L. B. Bullerman (ed.), Fumonisins in food. Plenum Press, New York, N.Y.

Lee, D. W., R. J. Pratt, M. Mclaughlin, and R. Aramayo, 2003. An Argonaute-like protein is required for meiotic silencing. Genetics 164: 821–828.

Lyon, M.F. 2003. Transmission ration distortion in mice. Ann rev genetics: 37 393-508.

Makaula NA, Marasas WFO, Venter FS, Badenhorst CJ, Bradshaw D, Swanevelder S. 1996. Oesophageal and other cancer patterns in four selected districts of Transkei, southern Africa: 1985-1990. Afr J Health Sci 3:11–15.

Marliére, C.A., R.C.J. Pimenta , A.C. Cunha. 2009. Fumonisin as a risk factor to esophageal cancer: a review. Applied Cancer Research. ;29(3):102-105.

Marasas, W.F.O. 2001. Discovery and occurrence of the fumonisins: a historical perspective. Environ. Health Perspect.109(S2),239–243.

Marasas, W.F.O. et al. 2004. Fumonisins Disrupt Sphingolipid Metabolism, Folate Transport, and Neural Tube Development in Embryo Culture and In Vivo: A Potential Risk Factor for Human Neural Tube Defects among Populations Consuming Fumonisin-Contaminated Maize1. J. Nutri. 134(4): 711-716.

Nelson, P. E. 1992. Taxonomy and biology of Fusarium moniliforme . Myco- pathologia 117: 29 – 36.

Oren, L., Ezrati, S., Cohen, D., and Sharon, A. 2003. Early events in the *Fusarium verticillioides*-maize interaction characterized by using green fluorescent protein-expressing transgenic isolate. Appl And Environ Micro. 69: 1695-1701.

Pietri, A., M. Zanetti, and T. Bertuzzi. 2009. Distribution of aflatoxins and fumonisins in dry-milled maize fractions. Food Additives & Contaminants: Part A 26:3 DOI: 10.1080/02652030802441513

Proctor, R.H., Brown, D.W., Plattner, R.D., and Desjardins, A.E. Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis.* Fungal Gen. and Bio. 38: 237-249 (2003).

Samarajeewa, D.A., P. A. Sauls, K.J.Sharp, Z.J. Smith, H. Xiao, K.M. Groskreutz, T. L. Malone, E. C. Boone, K.A. Edwards, P.K.T. Shiu, E.D. Larson, and T.M. Hammond. Efficient detection of unpaired DNA requires a member of the Rad54-like 1 family of homologous recombination proteins. Genetics.

Shiu, P. K. T., N. B. Raju, D. Zickler, and R. L. Metzenberg, 2001. Meiotic silencing by unpaired DNA. Cell 107: 905–916.

Shiu, P. K. T., Zickler, D., Raju, N. B., Ruprich-Robert, G., & Metzenberg, R. L. (2006). SAD-2 is required for meiotic silencing by unpaired DNA and perinuclear localization of SAD-1 RNA-directed RNA polymerase. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(7), 2243–2248. doi:10.1073/pnas.0508896103

Stevens, R. B. 1974. Mycology Guidebook. University of Washington Press, Seattle, WA. 703 pp

Turner, B.C. 2001 Geographic distribution of Neurospora *Spore killer* strains and strains resistant to killing. Fungal Gen and Bio: 32, 93-1074.

U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, Center for Veterinary Medicine, Guidance for Industry: Fumonisin Levels in Human Foods and Animal Feeds, June 6, 2000; revised November 9, 2001

Vogel, H. J., 1956 A convenient growth medium for Neurospora (Medium N). Microb. Genet. Bull. 13: 42–43.

Westergaard M., and H. K. Mitchell, 1947 Neurospora V. A synthetic medium favoring sexual reproduction. Am. J. Bot. 34: 573–577.

Wilson BJ. 1971 Recently discovered metabolites with unusual toxic manifestations. In: Mycotoxins in Human Health (Purchase JFH, ed). London:Macmillan. 223–229

Xiao, H., Alexander, W. G., Hammond, T. M., Boone, E. C., Perdue, T. D., Pukkila, P. J., & Shiu, P. K. T. (2010). QIP, a Protein That Converts Duplex siRNA Into Single Strands, Is Required for Meiotic Silencing by Unpaired DNA. *Genetics*, *186*(1), 119–126. doi:10.1534/genetics.110.118273

Xu, J.R. and J.F. Leslie. 1996 A genetic map of Gibberella fujikuroi mating population a (Fusarium moniliforme). Genetics 143: 175-189.

Yu J.-H. H., Hamari Z., Han K.-H. H., Seo J.-A. A., Reyes-Domínguez Y., Scazzocchio C., 2004 Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. Fungal Genet. Biol. 41: 973–981.

Zain, M.E. 2011 Impacts of mycotoxins on humans and animals. J. Saudi Chem. Soc. 15, 129-14

APPENDIX A

SUPPLEMENTARY TABLE 1. RFLP RAW DATA

Strain	RFLP6b	RFLP1b	RFLP2b	RFLP3	RFLP4b	RFLP5b
60	7	7	7	7	7	3
61	7	7	7	7	3	3
62	7	7	7	7	7	7
63	7	7	7	7	7	7
64	7	7	7	7	7	7
65	7	7	7	7	7	7
66	7	7	7	7	7	7
67	7	7	7	7	7	3
68	7	7	7	7	7	7
69	7	7	7	7	7	3
70	7	7	7	7	7	3
71	7	7	7	7	3	3
72	7	7	7	7	7	7
73	3	7	7	7	7	7
74	7	7	7	7	7	7
75	7	7	7	7	3	3
76	7	7	7	7	7	3
77	7	7	7	7	7	3
78	7	7	7	7	7	7
79	7	7	7	7	7	3
80	7	7	7	7	3	3
81	7	7	7	7	7	7
82	7	7	7	7	7	3
83	7	7	7	7	7	7
84	7	7	7	7	7	3
85	7	7	7	7	7	7
86	7	7	7	7	7	7
87	7	7	7	7	3	3
88	7	7	7	7		3
89	7	7	7	7	7	3

7	7603 Genotype
3	3125 Genotype
	No Data
	Strains of Interest

(Table Continues)

Strain	RFLP6b	RFLP1b	RFLP2b	RFLP3	RFLP4b	RFLP5b
90	7	7	7	7	7	3
91	7	7	7	7	7	7
92	7	7	7	7	7	3
93	7	7	7	7	7	3
94	3	7	7	7	7	3
95	7	7	7	7	7	7
96	7	7	7	7	7	7
97	3	7	7	7	7	7
98	7	7		7	7	7
99	7	7	7	7	7	7
100	7	7	7	7	7	3
101	7	7	7	7	3	3
102	7	7	7		7	7
103	7	7	7	7	7	7
104	3	7	7	7	7	3
105	3	7	7	7	7	3
106	3	7	7	7	3	3
107	7	7	7	3	7	7
108	7	7	7	7	3	7
109	7	7	7	7	7	7
110	3	7	7	7	3	3
111	3	3	3	3	3	7
112	7	7	7	7	7	7
113	7	7	7	7	7	7
114	7	7	7	7	7	7
115		7	7	7	7	7
116	7	7	7	7	3	3
117	7	7	7	7	7	7
118	3	3	3	7	7	7
119	7	7	7	7	3	3

Note. Supplementary Table 1. This table shows the raw RFLP data collected from N=60 strains of *Fusarium verticillioides.*