DISSECTION OF FERTILITY BARRIERS AMONG LINEAGES OF *GIBBERELLA ZEAE*

by

IRAZEMA FUENTES-BUENO

B.S., Instituto Tecnológico de Sonora, México, 2005

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Plant Pathology
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2012

Approved by;

Co-Major Professor
Robert L. Bowden

Approved by;

Co-Major Professor
John F. Leslie

Copyright

Abstract

Fusarium graminearum Schwabe sensu lato (teleomorph: *Gibberella zeae* (Schwein.) Petch), a homothallic ascomycete fungus, is the causal agent of Fusarium head blight (FHB) of wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), and other small grains. FHB occurs worldwide and serious outbreaks have been reported in North America, South America, Asia, and Europe. According to the phylogenetic species concept (PSC), *F. graminearum* is composed of at least 15 phylogenetic lineages known as the *Fusarium graminearum* species complex.

Although *F. graminearum* is homothallic, some members of different phylogenetic lineages are known to intercross in the laboratory. It has been suggested that *F. graminearum* sensu lato fits the biological species concept (BSC). According to the BSC, "species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups". Previous reports of intercrossing were qualitative, so the degree of reproductive isolation, if any, is not clear. Since intrinsic reproductive isolation is the key criterion to identify species by the BSC, more detailed quantitative information is needed.

Chromosome rearrangements between fungal strains may reduce fertility in sexual crosses through the production of genetically inviable recombinant progeny. As such, rearrangements can be important postzygotic reproductive barriers between species. Following methods used in *Neurospora crassa*, ascospore tetrads were analyzed for patterns of ascospore viability. Crosses were made with three lineage 7 (*F. graminearum* sensu stricto according to PSC) strains as female. Each female was a *MAT1-2* knockout mutant that rendered it obligately heterothallic. Males were several members of lineages 6 (*F. asiaticum* according to PSC) and lineage 7. Crosses with lineage 7 males formed complete asci with 8 ascospores indicating that their genomes are isosequential with the testers. Crosses with one strain from lineage 6 with two known inversions produced asci containing 8, 6, and 4 ascospores, consistent with it not being isosequential. However, three other strains of lineage 6 appeared to be isosequential with the testers. Therefore, chromosome rearrangements did not appear to be common to strains of lineage 6 and probably do not contribute significantly to reproductive isolation of lineage 6 and lineage 7.

Interlineage fertility studies with the three lineage 7 tester strains were performed to quantify interlineage fertility parameters including the total number of ascospores produced,

perithecial density, and perithecium internal development scores. All lineage 7 female testers successfully crossed to all 23 male strains from lineages 1 to 9. For total ascospore production, one female tester crossed equally well with all lineages and the other two testers showed statistically significant differences for a few lineages. For perithecial density, there was a significantly lower density with all three testers when crossed with lineage 6, but the other lineages were not statistically different from lineage 7. For perithecial development, there was large variation for every lineage. Therefore, in the crosses with reduced fertility, the reduction can be attributed to a postzygotic effect since mature perithecia and asci developed.

All of the tested lineages of the *Fusarium graminearum* species complex can produce viable progeny with *F. graminearum* lineage 7, which was the taxonomic type of the original species before it was split into phylogenetic species. There are a few examples of reduced fertility with two lineage 7 testers, the remaining tester crossed equally well with all lineages. Therefore members of lineages 1-9 all should be considered members of *Fusarium graminearum* according to the BSC. The existing female testers could be used to identify members of the *F. graminearum* clade by performing test crosses in the laboratory.

The PSC and BSC species concepts do not agree for this group of fungi. This disagreement indicates that the *F. graminearum* species complex is in the early stages of speciation. The lack of intrinsic reproductive barriers supports the hypothesis that these lineages have developed in geographic isolation. As the lineages have apparently been brought together through global trade, interlineage hybrids have been reported in the field. The discrepancy between PSC and BSC will eventually be resolved by whether the lineages fuse or remain separate in nature. Even if the lineages remain separate, this study demonstrates the potential for gene flow between lineage 7 and lineages 1 through 9.

Table of Contents

List of Figures

List of Tables

Acknowledgements

I want to express my gratitude to my advisors Dr. Robert Bowden and Dr. John Leslie for their encouragement, supervision and support through the course of this M.S. project. My committee members, Dr. Richard Todd and Dr. Barbara Valent, I appreciate the time and interest you invested in reviewing my thesis.

To the Leslie Lab staff Amy Beyer, Bruce Ramundo, QiuXia Chen, Erin Jewell, and Jasmin Hostin and to the office staff Jaime Mitchell, Diana Pavlisko, Morgan Fyffe, Jeanna Cox, and Anita Kesler for all their help and friendship.

Last but not least to Joey Carcallas-Cainong, David R. Cruz, Luisa Cruz, Ismael Badillo Vargas, Mauricio Montero Astua, Ximena Cibils, Nik Mohamed Izham Mohamed, Sandra Dunckel, and Trevor Rife who made the grad student life fun and full of adventures. One can say they are a second family I will deeply cherish.

Dedicatoria

Dedico esta tesis a mis padres, Guillermo y Sonia, a mi hermano Eduardo que siempre estuvieron a mi lado brindándome su apoyo sin importar la distancia.

Chapter 1 - Literature Review

Introduction

Gibberella zeae (Schwein.) Petch (anamorph: *Fusarium graminearum* Schwabe), a homothallic ascomycete fungus, is the causal agent of Fusarium Head Blight (FHB) of wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and other small grains. It can also infect maize (*Zea mays* L.) in which it causes *Gibberella* ear rot (69). FHB is considered one of the most destructive pathogens of cereals, resulting in billions of dollars of losses in yield and quality for the grain industry (79). FHB occurs worldwide and outbreaks have been reported in North and South America, Asia, and Europe (44).

Besides damaging production and quality of seed, the pathogen has the capability to produce mycotoxins harmful to human and animals (42, 52). The compounds produced include trichothecene mycotoxins such as deoxynivalenol (DON) and nivalenol (NIV), and estrogenic compounds, e.g. zearalenone (ZEA) (15, 42). Consumption of grain contaminated with these mycotoxins can cause vomiting, liver damage, and reproductive defects in livestock and compromises the human immune system (25, 42, 52, 76).

Control of FHB has proven difficult because cultural practices, host resistance, and chemical control are only partially effective. Since the pathogen has a wide host range and is aerially dispersed, crop rotation and tillage, though they reduce local inoculum, are inadequate (9). The Chinese wheat cultivar ‘Sumai 3’ is one of the best sources of genetic resistance to FHB, but can still suffer significant damage when disease pressure is high (7). Chemical controls, e.g. triazole fungicides, have aided in the integrated management of this disease, although they rarely achieve more than fifty percent control (49).

Epidemiology and Morphology

Source inoculum of this disease comes from infected plant debris, such as corn stalks, wheat stubble, and other host plants as mycelia or conidia (9, 29). In warm conditions, the sexual stage, *Gibberella zeae*, will develop on the infested plant debris as perithecia (69). Ascospores are forcibly ejected from the perithecia, land on susceptible wheat heads and initiate infection (9, 73). The fungus produces asexual spores (macroconidia) that are dispersed to plants by rain splash and wind (69). Fusarium Head Blight infection is favored by extended periods of high moisture or relative humidity (>90%) and moderately warm temperatures (between 15 to 30°C) (69). These conditions, when present before, during, and after flowering favor inoculum production, floret infection, and colonization of developing grains (6, 60).

Disease symptoms are visible as bleached grain heads (Figure 1.1). Infected seeds are severely damaged and have a chalky appearance. Kernels infected by *F. graminearum* may be contaminated with mycotoxins. Pink-orange mycelia and sporodochia are often noticeable at the base of the spikelets (Figure 1.1). Characteristic features of the asexual stage of this fungus are the 5-6 septate, sickle/banana-shaped macroconidia with foot-shaped basal cell and tapered apical cell (Figure 1.2) with size ranges from 25-30 × 3-4 μm (9, 39).

At the end of the season perithecia are sometimes detectable on maize or wheat stubble (60). Perithecia are black-blue fruiting bodies formed in a stroma with size ranging from 150-300 × 100-250 μm (59, 72). They have a globular shape with warty walls (72). Within a perithecium, several paraphyses (sterile hyphae) line the ostiolar canal and asci are produced from the base of the centrum (34, 59, 72). Asci are unitunicate-inoperculate (one wall and lack of operculum on the tip) and hold eight smooth, hyaline, fusiform or allantoid ascospores arranged in an overlapping uniseriate arrangement (Figure 1.2) (34, 59). Ascospores have one to four cells and their size ranges from 20-30 × 3-5 μm (9, 34, 59).

Figure 1.1 Symptoms of Fusarium Head Blight on mature wheat heads.
A) Orange mass of conidia on infected spikelets. B) Bleached spikelets are white while healthy spikelets remain green (Images by Mary Burrows, Montana State University, Bugwood.org)

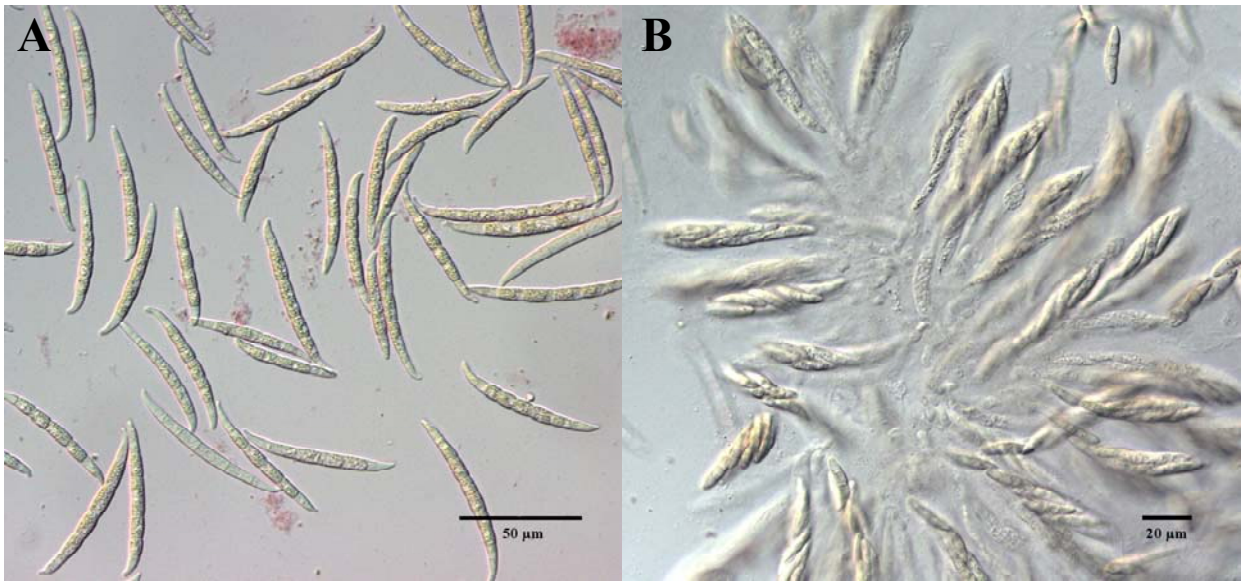

Figure 1.2 Asexual and sexual spores of *Gibberella zeae*.
A) Macroconidia, asexual spores, detached from the sporodochium. B) Rosette of asci containing hyaline ascospores, which are the sexual spores.

Describing and Naming *Gibberella zeae*

FHB was first described as “wheat scab” in 1884 although various common names have been used throughout the years including *Fusarium* blight, fusariosis, and head blight (1, 4, 23, 69). First reports in North America cited by Arthur (4) date from 1890-1898 in Ohio, Indiana, Iowa, Pennsylvania, and Nebraska. By 1917 Fusarium Head Blight was recognized across 31 states in the United States (5, 23, 66), Europe and Japan. From these first reports studies on the descriptive nature and occurrence were done.

The *Fusarium* genus was originally described by H. F. Link in 1809 where the name derives from the fusiform macroconidia; particularly based on *F. roseum* (10, 41). Schwabe later described *F. graminearum* in 1838 and linked it to the teleomorphic stage (58). For many years, proper classification, and description of *Fusarium* has been an issue among mycologists. By 1870, P.A. Saccardo had described over 1000 *Fusarium* species (55). With the publication of Wollenweber and Reinking’s monograph in 1935, the number was reduced to 65 species, 55 varieties, and 22 forms all arranged in 16 sections (80). In 1936, Thomas Petch sorted out most of the terminology of names and specimens and amended the teleomorphic stage of *F. graminearum* to *Gibberella zeae* (Schwein.) Petch based on the earliest specimen collected from Lewis David von Schweinitz in 1822 (53).

F. graminearum belongs to the section "Discolor" in the Wollenweber and Reinking taxonomy. This section is characterized by thick-walled, distinctly septate macroconidia, with the ability to produce chlamydospores, but not microconidia (10). Snyder and Hansen studied *Fusarium* variation and reduced the number of species to nine (61, 62, 63, 64). In Snyder and Hansen taxonomy, *F. roseum* was adopted for the large number of *Fusarium* species previously in sections Roseum, Arthrosporiella, Gibbosum, and Discolor. Snyder et al. (65) introduced in 1957 the cultivar concept where morphologically different strains of *F. roseum* were given cultivar names as *F. roseum* ‘Graminearum’, or if pathogenic, *F. roseum* f. sp. *cerealis* cultivar ‘Graminearum’. This system introduced a complex three or four stage nomenclature without simplification for identification purposes. Booth also reduced the number of species from the Wollenweber and Reinking taxonomy. In Booth’s monographs, the name *F. graminearum* was kept for the pathogen of FHB (10). The work done by, P.E. Nelson, T.A. Toussoun, and W.F.O.

Marasas developed a resource guide to the genus with color photographs, microphotographs, and information on how to isolate and culture *Fusarium* species (45). Leslie and Summerell (39) wrote "The *Fusarium* Laboratory Manual" which provides useful techniques to combine morphology, biological and phylogenetic species concepts for the *Fusarium* research community. This laboratory manual includes detailed chapters on techniques and methods for identifying *Fusarium* spp., taxonomy, and species descriptions of 70 *Fusarium* spp. (39). In addition to the available morphological guides; identification based on DNA sequence similarity to previously characterized species is available. FUSARIUM-ID, a publicly available database, was released in 2004 by Geiser et al. (28) and uses partial DNA sequences of the translation elongation factor 1 alpha (EF-1α) gene because of its high phylogenetic utility. This database also provides information associated with characterized isolates. The growing database contains vouchered sequences attached to publicly available cultures. Currently, the *Fusarium* Database lists a total of 76 species (33).

In 1977, two populations known as Group 1 and Group 2 were described in *F. graminearum*. Morphologically these are very difficult to differentiate but they have ecological and pathological differences (24). Group 1 causes crown rot of wheat while Group 2 causes Fusarium Head Blight. Members of Group 2 produce abundant perithecia while Group 1 does not (24). By 1999, these groups were identified as phylogenetically distinct populations, Group 1 was then renamed *F. pseudograminearum* with teleomorph *Gibberella coronicola* (2, 3).

Population Structure of the *Fusarium graminearum* Species Complex

Bowden and Leslie (12) used vegetative compatibility groups (VCGs) to show that a collection of 24 strains of *F. graminearum* from wheat in Kansas were each in different VCGs and thus were different genotypes. Although *F. graminearum* is homothallic, the high genotypic diversity suggested that sexual recombination occurs in the field. Using complementary nitrate non-utilizing (*nit*) mutants to distinguish progeny from outcrosses from those from self-crosses, Bowden and Leslie (13) showed that strains of *F. graminearum* from the USA, Nepal, Japan, and South Africa were capable of interbreeding in the laboratory. They suggested that global populations of *F. graminearum* could represent a panmictic biological species. Zeller et al. (84)

studied amplified fragment length polymorphisms (AFLPs) between strains of *F. graminearum* from small quadrats in Kansas and North Dakota. They found that genotypic diversity was very high, and that the Kansas and North Dakota populations could not be distinguished. In a larger study, Zeller et al. (85) showed that populations of *F. graminearum* in North America exhibit slight, but statistically significant, variation that is correlated with geographic distance between populations.

Phylogenetic analysis of partial DNA sequences of six nuclear genes revealed seven biogeographically structured lineages within the *F. graminearum* clade (46). Zeller et al. (87), using AFLPs, confirmed the existence of lineages in *F. graminearum* and found lineages 1, 2, 6, and 7 in populations from Uruguay and Brazil and lineage 3 from Mexico. O'Donnell et al. (47) used Genealogical Concordance Phylogenetic Species Recognition (GCPSR) analysis to formally divide the *F. graminearum* clade into 9 phylogenetic species. The name *F. graminearum* was retained for members of lineage 7, which is associated with FHB in the United States, Europe and Canada. Further GCPSR-based phylogenetic analysis of a 12 gene dataset was used to increase the number of phylogenetically distinct lineages to sixteen, of which fifteen have been formally described as species (Table 1.1) (56).

Every member of the *F. graminearum* species complex can produce type B trichothecene mycotoxins: *F. austroamericanum, F. meridionale, F. boothi, F. acaciae-mearnsii, F. asiaticum, F. graminearum, F. mesoamericanum, F. cortaderiae, F. vorosii, F. gerlachii, F. aethiopicum, F. ussurianum, F. louisianense,* and *F. nepalense* have the ability to cause Fusarium Head Blight via *in planta* assays (26, 30, 48, 56, 67, 82). Three type B trichothecene chemotypes have been identified: 3-acetyldeoxynivalenol (3ADON), 15-acetyldeoxynivalenol (15ADON), and nivalenol (NIV) (20). The distribution of the *F. graminearum* species complex chemotypes varies greatly within the lineages (Table 1.2). *F. graminearum* (lineage 7) strains in North America are commonly associated with 15ADON mycotoxins. DON production has been of interest for its contribution to disease aggressiveness (8). However, recent surveys have discovered variation of the chemotypes within isolates of the same geographic location. Schmale et al. (57) evaluated 998 isolates of *G. zeae* collected from 39 winter wheat fields in the United States (New York, Pennsylvania, Maryland, Virginia, Kentucky, and North Carolina). 92% of the isolates were 15ADON, 7% were 3ADON, and 1% were NIV chemotypes (57). Different studies show higher proportions of NIV-chemotype *F. graminearum* isolates in the United

States, a chemotype considered rare in this region (26, 67). Desjardins et al. (21) suggested that NIV-producing isolates in the United States could be the result of recent migration.

Table 1.1 Correspondence of lineage, species designation, and site of recovery for the *Fusarium graminearum* species complex.

Lineage Designation[a]	Species Designation	Recovered From	Reference
1	*F. austroamericanum*	Brazil and Venezuela	O'Donnell et al. (46) O'Donnell et al. (47)
2	*F. meridionale*	Brazil, Guatemala, South Africa, Australia, New Caledonia, Nepal, and Korea	O'Donnell et al. (46) O'Donnell et al. (47)
3	*F. boothi*	South Africa, Mexico, Guatemala, Nepal, and Korea	O'Donnell et al. (46) O'Donnell et al. (47)
4	*F. mesoamericanum*	Honduras and Pennsylvania (USA)	O'Donnell et al. (46) O'Donnell et al. (47)
5	*F. acaciae-mearnsii*	Australia and South Africa	O'Donnell et al. (46) O'Donnell et al. (47)
6	*F. asiaticum*	China, Nepal, Japan, and Korea, Brazil	O'Donnell et al. (46) O'Donnell et al. (47)
7	*F. graminearum*	Cosmopolitan (not limited to one part of the world)	O'Donnell et al. (46) O'Donnell et al. (47)
8	*F. cortaderiae*	Argentina, Brazil, Australia, New Zealand	O'Donnell et al. (47)
9	*F. brasilicum*	Brazil	O'Donnell et al. (47)
10	*F. vorosii*	Hungary and Japan	Starkey et al. (67)
11	*F. gerlachii*[c]	Minnesota, Wisconsin, and North Dakota (USA)	Starkey et al. (67)
12	*F. aethiopicum*	Ethiopia	O'Donnell et al. (48)
13	*F. ussurianum*	Eastern Russia	Yli-Mattila et al. (82)
14	*F. louisianense*	Lousiana (USA)	Sarver et al. (56)
15	*F. nepalense*	Nepal	Sarver et al. (56)

[a] Lineage designations 1 to 7 after O'Donnell et al. (46). Lineage 8-9 designated in Bowden et al. (14). Lineages 10-15 designated in this thesis.

Table 1.2 Type B trichothecene chemotypes associated with the *Fusarium graminearum* species complex.

Species Designation	Chemotype	Reference
F. austroamericanum	3ADON[a], NIV[c], ZEA[d]	O'Donnell et al. (46)
		Ward et al. (77)
F. meridionale	NIV, ZEA	Goswami et al. (30)
		O'Donnell et al. (46)
		Ward et al. (77)
F. boothi	15ADON[b], ZEA	O'Donnell et al. (46)
		Goswami et al. (30)
		Ward et al. (77)
F. mesoamericanum	3ADON, ZEA	Goswami et al. (30)
		O'Donnell et al. (46)
		Ward et al. (77)
F. acaciae-mearnsii	3ADON, NIV	Goswami et al. (30)
		O'Donnell et al. (46)
		Ward et al. (77)
F. asiaticum	3ADON, 15ADON, NIV, ZEA	Goswami et al. (30)
		O'Donnell et al. (46)
		Suga et al. (68)
		Ward et al. (77)
F. graminearum	3ADON, 15ADON, NIV, ZEA	Goswami et al. (30)
		O'Donnell et al. (46)
		Suga et al. (68)
		Ward et al. (77)
F. cortaderiae	3ADON, NIV	Goswami et al. (30)
		Ward et al. (77)
		Ward et al. (78)
F. brasilicum	3ADON, NIV	Boutigny et al. (11)
		Ward et al. (78)
F. vorosii	15ADON	Starkey et al. (67)
F. gerlachii	NIV	Starkey et al. (67)
F. aethiopicum	15ADON	O'Donnell et al. (48)
F. ussurianum	3ADON	Yli-Mattila et al. (82)
F. louisianense	NIV	Sarver et al. (56)
F. nepalense	15ADON	Sarver et al. (56)

[a] 3-acetyldeoxynivalenol

[b] 15-acetyldeoxynivalenol

[c] Nivalenol

[d] Zearalenone

Genetic and Physical Maps of *Gibberella zeae*

There are two published genetic maps of *G. zeae*, one by Jurgenson et al., (32), and the other by Gale et al. (27). The map of Jurgenson et al. was constructed by crossing strain Z3639 (*F. graminearum* sensu stricto, or lineage 7) and R-5470 (*F. asiaticum,* which corresponds to lineage 6) isolated from Kansas and Japan, respectively. The map is based on AFLP markers and its total length is about 1286 cM. This map included nine linkage groups with 1048 polymorphic markers that mapped to 468 unique loci and detected segregation distortion on three of five linkage groups. The genetic map by Gale et al. was constructed from a cross between PH-1 (lineage 7), a strain from Michigan, and a closely related strain from Minnesota 00-676 (lineage 7). Total length of this map is 1234 cM, it has 235 loci and nine linkage groups as well. This map is based on cleaved amplified polymorphic sequence (CAPS) markers, variable number of tandem repeats (VNTR) markers and amplified fragment length polymorphisms (AFLP). While Gale's map was based on the cross of a mutant strain PH-1 and wild type strain 00-676, Jurgenson's was based on the cross between *nit* mutants of R-5470 and Kansas strain Z3639.

Lee et al. (35) aligned the physical sequence and the Jurgenson genetic map using 7 sequenced structural genes and 130 sequenced AFLP markers from all nine linkage groups (LG) of the genetic map. The total size of the Jurgenson linkage map was reduced from 1286 cM to 1140 cM. One hundred and fifteen markers were associated with nine supercontigs (SC) of the genomic sequence. Linkage groups (LG) 1, 7, 8, and 9, LG 2, LG 3, LGs 4, 5, and 6 were aligned with SC 2, and 5, SC 3, 8, and 9, SC 4 and 6, SC 1 and 7, respectively. The nine linkage groups in the previous genetic map were reduced to six linkage groups. LG1 was merged with LG 7 and LG8 (new LG1) while LG5 was merged with LG6 (new LG5). Approximately 99% of the sequence was anchored to the genetic map. This validated the high quality of the *G. zeae* genomic sequence assembly. By aligning the maps it was determined that there are four chromosomes in this fungus. Recombination between the lineage 6 and 7 genomes occurred along each of the four chromosomes. The alignment also confirmed the presence of two large heterozygous inversions in LG2:chromosome 3 and LG4:chromosome 1 of the mapping cross. Such chromosome rearrangements can be important post-zygotic reproductive barriers between species. They can affect hybrid fitness and can cause reduced gene flow through the suppression of recombination (16, 54). For example, if genes causing reproductive isolation between two

allopatric species reside within chromosomes that are rearranged between those species, the association of genes and chromosomes will persist. Heterozygosity for the rearrangements may prevent recombination between genes. Nonetheless, chromosome rearrangements following mutagenesis are not uncommon within fungal species as seen with *Neurospora* spp. (50). However there hasn't been a standardized search for them in fungal plant pathogens like *F. graminearum*.

MAT gene manipulation

Fusarium spp. have two sexual reproductive strategies: heterothallism (self-sterile) and homothallism (self-fertile). Sexual reproduction is controlled by a regulatory locus named mating type (*MAT1*). Unlike most *Fusarium* spp., *F. graminearum* is homothallic and carries both the *MAT1-1* and the *MAT1*-2 (also known as *MAT-1* and *MAT-2*, respectively) idiomorphs in a single haploid nucleus. These idiomorphs are closely linked and located on the same chromosome (36, 74, 75, 83). Δ*MAT* deletion mutants constructed by Lee et al. (36) are self-sterile. These mutants make laboratory outcrosses routine in this homothallic fungus. Δ*MAT* deletion mutants have a GFP expression cassette that replaces *MAT1-1* and *MAT1-2*. The segregation of the GFP tag allows verification that the outcross has occurred. Developing these mutants, as female-fertile tester strains, is very useful for studying sexual recombination in homothallic fungi, as well as identifying species through sexual crosses with known testers (39, 74).

Species Concepts in the *Fusarium graminearum* clade

Until relatively recently, classification and identification of *Fusarium* species relied primarily on morphological species recognition (MSR) methods. MSR looks for consistent differences in shape, size, color, or septation of macroconidia, microconidia, and ascospores as well as differences in perithecia, conidiogenous cells, chlamydospores, and sporodochia. Pigmentation and growth rate in culture were also considered important characters (39). MSR relies on type specimens to document species characteristics (71). Populations of strains that differ significantly from the type strain are candidates for recognition as new species. Because the number of morphological characters is relatively low in *Fusarium*, and characters may be under stabilizing selection, MSR typically identifies fewer species than do other methods (70).

The Biological Species Recognition method (BSR) recognizes groups of actually or potentially interbreeding populations as species (43). The key criterion for defining species in BSR is reproductive isolation, which can be tested in sexual fungal species by making crosses with known tester strains (71). This method has been used with great success in the morphologically cryptic *Gibberella fujikuroi* species complex to define Mating Groups A-I (37, 86), which constitute different reproductively isolated biological species. It has also been useful in *Neurospora* spp. (51). However, BSR cannot be applied to strains that lack the ability to form the sexual stage. Since hybrids between "good species" are not uncommon, Mayr (43) expanded the BSR concept to allow occasional production of hybrids between species as long as the hybrid zones remain limited. Mayr used the term "good species" for groups of interbreeding natural populations that are reproductively isolated from other such groups (43).

The Genealogical Concordance Phylogenetic Species Recognition method (GCPSR) relies on molecular markers such as insertions, deletions, or single nucleotide polymorphisms (SNPs) to find clusters of isolates that consistently differ at multiple loci (16, 71). As genomic sequences have been obtained and sequencing costs have declined, resequencing of particular sets of genes has become practical. Gene genealogies are constructed and when genealogies of different genes reveal similar patterns, this is considered good evidence of historical reproductive isolation between the clades (31, 71). If most gene genealogies are reciprocally monophyletic, evidence for GCPSR is considered to be strong. GCPSR can be used with strains that have

recently lost the ability to undergo sexual reproduction, but the assumptions of GCPSR are violated in strictly asexual lineages (16, 31, 71).

In *Neurospora* spp. and the *G. fujikuroi* complex, BSR and GCPSR give very similar results (22, 37, 38). However, in the *F. graminearum* clade, BSR and GCPSR apparently do not agree. Based on limited qualitative crossing data using *nit* mutants from lineages 3, 6, and 7, Bowden and Leslie (13) suggested that global populations of *G. zeae* constituted one biological species. In a preliminary report using *nit* mutants, Bowden et al. (14) reported that lineage 7 strains could cross with members from lineages 1 to 9. To date, two natural hybrids between phylogenetic species in the *F. graminearum* clade have been reported (11, 46). The Jurgenson et al. (32) lineage 7 by lineage 6 cross failed to identify polymorphisms that would merit species-level recognition (32, 35). Cumagun et al. (17) showed that progeny from the Jurgenson interlineage cross were pathologically fit since they were aggressive colonizers of wheat heads. The interlineage progeny are also capable of backcrossing to the lineage 7 parental strain (Bowden and Leslie, unpublished).

In contrast, GCPSR has been used to separate *F. graminearum* into fifteen different phylogenetic lineages (56). The rationale was that reciprocal monophyly of many gene genealogies indicated a long history of reproductive isolation between the lineages. O'Donnell et al. (47) stated that the *F. graminearum* clade was at "an advanced state of biological speciation". The second part of their justification for raising the lineages to species rank was that the lineages in the *F. graminearum* clade "…show some level of intrinsic reproductive incompatibility as evidenced by their reduced fertility in artificial crosses…" They were apparently referring to the interlineage cross of Jurgenson et al. (32) because no other data were cited.

Wu (81) emphasized that speciation is a continuous process and that BSR and GCPSR use somewhat arbitrary criteria for delimiting species boundaries. He also advocated a genic view of reproductive isolation rather than a whole genome view. He defined four stages of speciation as follows: I) populations/races with differential adaptation; reproductive isolation not apparent; II) transition between race and species with some degree of reproductive isolation; populations may fuse or diverge; III) divergent populations beyond the point of fusion but still share a portion of their genomes via gene flow; "good species"; IV) species with complete reproductive isolation. Based on current data, the *F. graminearum* clade would be at either stage II or III.

de Queiroz (18, 19) suggested that BSR and GCPSR, as well as other species concepts, could be unified by the concept of a separately evolving metapopulation lineage. In other words, a species is a series of reproductively connected subpopulations that share a common ancestor-descendant relationship. Under the Unified Species Concept (USC), any of the operational criteria (phenetic differentiation, diagnosability, reciprocal monophylly, reproductive incompatibility, ecologically distinct, etc.) could justify species recognition, but the more criteria that are met, the stronger the case for recognition.

Rationale for this Study

The existence of multiple phylogenetically structured lineages within *F. graminearum* is well established. However, whether these lineages deserve species rank has been controversial (40). Although interlineage hybrids have been produced in the laboratory and two have been found in the field, there is little qualitative and no quantitative data measuring interlineage fertility barriers in this group. This information would be useful in placing the *F. graminearum* complex at the correct stage in the Wu speciation scale. If significant fertility barriers are found, BSR will confirm recognition of the lineages as separate cryptic sibling species. This may also shed light on whether the lineages have developed intrinsic reproductive barriers that are characteristic of sympatric speciation. Alternatively, if intrinsic reproductive barriers are not found, it argues that the lineages have developed in allopatry. In that case, they might be considered incipient species or subspecies according to BSR (43).

More importantly, knowledge of reproductive barriers may help us to understand the probability of genetic introgression among lineages when they are brought together through global trade. If the members of *F. graminearum* species complex interbreed, there is potential for production of new genomes that carry novel combinations of genes for pathogenicity, host range and toxin production. Perhaps lineages will fuse when brought together. Or perhaps there are fertility barriers such as differing sex pheromones, chromosomal rearrangements, and other unknowns that can lead to reduced mate recognition, reduced fitness of progeny, inviable progeny, or suppressed recombination. The objective of this thesis is to dissect potential fertility barriers among lineages of the *F. graminearum* species complex.

References

1. Andersen, A. L. 1948. The development *Gibberella zeae* head blight of wheat. Phytopathology 38:595-611

2. Aoki, T., and K. O'Donnell. 1999. Morphological characterization of *Gibberella coronicola* sp. nov., obtained through mating experiments of *Fusarium pseudograminearum*. Mycoscience 40:443-453

3. Aoki, T., and K. O'Donnell. 1999. Morphological and molecular characterization of *Fusarium pseudograminearum* sp. nov., formerly recognized as the Group 1 population of *F. graminearum* and PCR primers for its identification. Mycologia 91:597-609

4. Arthur, J. C. 1891. Wheat scab. Purdue University Agricultural Experiment Station Bulletin 36:129-132

5. Atanasoff, D. 1920. Fusarium-blight (scab) of wheat and other cereals. Journal of Agricultural Research 20:1-32

6. Bai, G. H., and G. Shaner. 1994. Scab of wheat: Prospects for control. Plant Disease 78:760-766

7. Bai, G. H., and G. Shaner. 2004. Management and resistance in wheat and barley to *Fusarium* head blight. Annual Review of Phytopathology 42:135-161

8. Bai, G. H., A. E. Desjardins, and R. D. Plattner. 2002. Deoxynivalenol-nonproducing *Fusarium graminearum* causes initial infection, but does not cause disease spread in wheat spikes. Mycopathologia 153:91-98

9. Bockus, W., R. L. Bowden, R. Hunger, W. Morrill, T. Murray, and R. Smiley. 2010. Diseases caused by fungi and fungus-like organisms. Pages 34-36 in: Compendium of Wheat Diseases and Pests. American Phytopathological Society, St. Paul, Minnesota.

10. Booth, C. 1971. The genus *Fusarium*. Commonwealth Mycological Institute, Kew, UK.

11. Boutigny, A. L., T. J. Ward, G. J. Van Coller, B. Flett, S. C. Lamprecht, K. O'Donnell, and A. Viljoen. 2011. Analysis of the *Fusarium graminearum* species complex from wheat, barley and maize in South Africa provides evidence of species-specific differences in host preference. Fungal Genetics and Biology 48:914-920

12. Bowden, R. L., and J. F. Leslie. 1992. Nitrate-nonutilizing mutants of *Gibberella zeae* (*Fusarium graminearum*) and their use in determining vegetative compatibility. Experimental Mycology 16:308-315

13. Bowden, R. L., and J. F. Leslie. 1999. Sexual recombination in *Gibberella zeae*. Phytopathology 89:182-188

14. Bowden, R. L., Leslie, J.F., Lee, J., and Lee, Yin Won. 2006. Cross fertility of *Gibberella zeae*. Pages 54-59 in: The Global Fusarium Initiative for International Collaboration. T. Ban, J. M. Lewis, and E. E. Phipps, eds., CIMMYT, El Batan, Mexico.

15. Caldwell, R. W., J. Tuite, M. Stob, and R. Baldwin. 1970. Zearalenone production in *Fusarium* species. Applied Microbiology 20:31-34

16. Coyne, J., A., and H. A. Orr. 2004. Speciation. Sinauer Associates, Sunderland, MA, U.S.A.

17. Cumagun, C. J. R., R. L. Bowden, J. E. Jurgenson, J. F. Leslie, and T. Miedaner. 2004. Genetic mapping of pathogenicity and aggresiveness of *Gibberella zeae* (*Fusarium graminearum*) toward wheat. Phytopathology 94:520-526

18. de Queiroz, K. 2005. Ernst Mayr and the modern concept of species. Proceedings of the National Academy of Sciences of the United States of America 102(Suppl 1):6600-6607

19. de Queiroz, K. 2007. Species concepts and species delimitation. Systematic Biology 56:879-886

20. Desjardins, A. E. 2006. Fusarium mycotoxins: Chemistry, genetics and biology. APS Press, St. Paul, MN, U.S.A.

21. Desjardins, A. E., M. Busman, G. Manandhar, A. M. Jarosz, H. K. Manandhar, and R. H. Proctor. 2008. *Gibberella* Ear Rot of Maize (*Zea mays*) in Nepal: Distribution of the mycotoxins nivalenol and deoxynivalenol in naturally and experimentally infected maize. Journal of Agricultural and Food Chemistry 56:5428-5436

22. Dettman, J. R., D. J. Jacobson, E. Turner, A. Pringle, and J. W. Taylor. 2003. Reproductive isolation and phylogenetic divergence in *Neurospora*: Comparing methods of species recognition in a model eukaryote. Evolution 57:2721-2741

23. Dickson, J. G. 1942. Scab of wheat and barley and its control. USDA Farmers' Bulletin 1599:1-17

24. Francis, R. G., and L. W. Burgess. 1977. Characteristics of two populations of *Fusarium roseum* "*Graminearum*" in Eastern Australia. Transactions of the British Mycological Society 68:421-427

25. Friend, D. W., H. L. Trenholm, P. S. Fiser, K. E. Hartin, and B. K. Thompson. 1983. Effect on dam perfomance and fetal development of deoxynivalenol (vomitoxin) contaminated wheat in the diet of pregnant gilts. Canadian Journal of Animal Science 63:689-698

26. Gale, L. R., S. A. Harrison, T. J. Ward, K. O'Donnell, E. A. Milus, S. W. Gale, and H. C. Kistler. 2010. Nivalenol-type populations of *Fusarium graminearum* and *F. asiaticum* are prevalent on wheat in Southern Louisiana. Phytopathology 101:124-134

27. Gale, L. R., J. D. Bryant, S. Calvo, H. Giese, T. Katan, K. O'Donnell, H. Suga, M. Taga, T. R. Usgaard, T. J. Ward, and H. C. Kistler. 2005. Chromosome complement of the fungal plant pathogen *Fusarium graminearum* based on genetic and physical mapping and cytological observations. Genetics 171:985-1001

28. Geiser, D., M. del Mar Jiménez-Gasco, S. Kang, I. Makalowska, N. Veeraraghavan, T. Ward, N. Zhang, G. Kuldau, and K. O'Donnell. 2004. FUSARIUM-ID v. 1.0: A DNA sequence database for identifying *Fusarium*. European Journal of Plant Pathology 110:473-479

29. Goswami, R. S., and H. C. Kistler. 2004. Heading for disaster: *Fusarium graminearum* on cereal crops. Molecular Plant Pathology 5:515-525

30. Goswami, R. S., and H. C. Kistler. 2005. Pathogenicity and in planta mycotoxin accumulation among members of the *Fusarium graminearum* Species Complex on wheat and rice. Phytopathology 95:1397-1404

31. Harrington, T. C., and D. M. Rizzo. 1999. Defining species in the fungi. Pages 43-71 in: Structure and Dynamics of Fungal Populations. J. J. Worrall, ed. Kluwer Press, Dordrecht, The Netherlands.

32. Jurgenson, J. E., R. L. Bowden, K. A. Zeller, J. F. Leslie, N. J. Alexander, and R. D. Plattner. 2002. A genetic map of *Gibberella zeae* (*Fusarium graminearum*). Genetics 160:1451-1460

33. Kang, S. Fusarium-ID (http://isolate.fusariumdb.org/index.php). Access Date: January 6th 2012.

34. Kendrick, B. 1985. Kingdom Eumycota: Phylum 3: Dikaryomycota, Subphylum 1 - Ascomycotina. Pages 38-78 in: The Fifth Kingdom. Focus Publishing, R. Pullins Co., Newburyport, MA, USA.

35. Lee, J., J. E. Jurgenson, J. F. Leslie, and R. L. Bowden. 2008. Alignment of genetic and physical maps of *Gibberella zeae*. Applied and Environmental Microbiology 74:2349-2359

36. Lee, J., T. Lee, Y.-W. Lee, S.-H. Yun, and B. G. Turgeon. 2003. Shifting fungal reproductive mode by manipulation of mating type genes: Obligatory heterothallism of *Gibberella zeae*. Molecular Microbiology 50:145-152

37. Leslie, J. F. 1991. Mating populations in *Gibberela fujikuroi* (*Fusarium* section *Liseola*). Phytopathology 81:1058-1060

38. Leslie, J. F. 1995. *Gibberella fujikuroi*: available populations and variable traits. Canadian Journal of Botany 73(s1):282-291

39. Leslie, J. F., and B. A. Summerell. 2006. The *Fusarium* Laboratory Manual. Blackwell Pub., Ames, Iowa. U.S.A.

40. Leslie, J. F., and R. L. Bowden. 2008. *Fusarium graminearum*: When species concepts collide. Cereal Research Communications 36:609-615

41. Link, H. F. 1809. Observationes in ordines plantarum naturales. Dissertatio I. Magazin der Gesellschaft Naturforschenden Freunde Berlin 3:3-42

42. Marasas, W. F. O., P. E. Nelson, and T. A. Toussoun. 1984. Toxigenic *Fusarium* Species: Identity and mycotoxicology. The Pennsylvania State University Press, University Park, Pennsylvania.

43. Mayr, E. 1996. What is a species, and what is not? Philosophy of Science 63:262-277

44. McMullen, M., R. Jones, and D. Gallenberg. 1997. Scab of wheat and barley: A re-emerging disease of devastating impact. Plant Disease 81:1340-1348

45. Nelson, P. E., T. A. Toussoun, and W. F. O. Marasas. 1983. *Fusarium* species: an illustrated manual for identification. Pennsylvania State University Press, University Park, Pennsylvania.

46. O'Donnell, K., H. C. Kistler, B. K. Tacke, and H. H. Casper. 2000. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. Proceedings of the National Academy of Sciences of the United States of America 97:7905-7910

47. O'Donnell, K., T. J. Ward, D. M. Geiser, H. C. Kistler, and T. Aoki. 2004. Genealogical concordance between the mating type locus and seven other nuclear genes supports

formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. Fungal Genetics and Biology 41:600-623

48. O'Donnell, K., T. J. Ward, D. Aberra, H. C. Kistler, T. Aoki, N. Orwig, M. Kimura, A. Bjørnstad, and S. S. Klemsdal. 2008. Multilocus genotyping and molecular phylogenetics resolve a novel head blight pathogen within the *Fusarium graminearum* species complex from Ethiopia. Fungal Genetics and Biology 45:1514-1522

49. Paul, P. A., P. E. Lipps, D. E. Hershman, M. P. McMullen, M. A. Draper, and L. V. Madden. 2008. Efficacy of triazole-based fungicides for *Fusarium* head blight and deoxynivalenol control in wheat: a multivariate meta-analysis. Phytopathology 98:999-1011

50. Perkins, D. D. 1997. Chromosome rearrangements in *Neurospora* and other filamentous fungi. Advances in Genetics 36:239-398

51. Perkins, D. D., B. C. Turner, and E. G. Bary. 1976. Strains of *Neurospora* collected from nature. Evolution 30:281-313

52. Pestka, J. J., and A. T. Smolinski. 2005. Deoxynivalenol: Toxicology and potential effects on humans. Journal of Toxicology and Environmental Health. Part B: Critical reviews 8:39-69

53. Petch, T. 1936. *Gibberella saubinetii* (Mont.) Sacc. Annales Mycologici 34:257-260

54. Rieseberg, L. H. 2001. Chromosomal rearrangements and speciation. Trends in Ecology and Evolution 16:351-358

55. Saccardo, P. A. 1881. Fungi Veneti novi vel critici v. mycologiae Veneti addendi. Series XII. Michelia 2:241-301

56. Sarver, B. A. J., T. J. Ward, L. R. Gale, K. Broz, H. Corby Kistler, T. Aoki, P. Nicholson, J. Carter, and K. O'Donnell. 2011. Novel *Fusarium* head blight pathogens from Nepal and Louisiana revealed by multilocus genealogical concordance. Fungal Genetics and Biology 48:1096-1107

57. Schmale, D. G., A. K. Wood-Jones, C. Cowger, G. C. Bergstrom, and C. Arellano. 2011. Trichothecene genotypes of *Gibberella zeae* from winter wheat fields in the eastern USA. Plant Pathology 60:909-917

58. Schwabe, S. H. 1839. Flora Anhaltina. Vol. 2.

59. Seifert, K. A. 1996. Notes on the typification of *Gibberella zeae*. Sydowia 48:83-89

60. Shaner, G. 2003. Epidemiology of *Fusarium* Head Blight of small grain cereals in North America. Pages 84-119 in: *Fusarium* Head Blight of Wheat and Barley. K. J. Leonard, and W. R. Bushnell, eds. APS Press, St. Paul, Minn.

61. Snyder, W. C., and H. N. Hansen. 1940. The species concept in *Fusarium*. American Journal of Botany 27:64-67

62. Snyder, W. C., and H. N. Hansen. 1941. The species concept in *Fusarium* with reference to section *Martiella*. American Journal of Botany 28:738-742

63. Snyder, W. C., and H. N. Hansen. 1945. The species concept in *Fusarium* with reference to Discolor and other sections. American Journal of Botany 32:657-666

64. Snyder, W. C., and H. N. Hansen. 1954. Variation and speciation in the genus *Fusarium*. Annals of the New York Academy of Sciences 60:16-23

65. Snyder, W. C., H. N. Hansen, and J. W. Oswald. 1957. Cultivars of the fungus *Fusarium*. Journal of the Madras University 27:185-192

66. Stack, R. W. 2003. History of *Fusarium* Head Blight with emphasis on North America. Pages 1-34 in: *Fusarium* Head Blight of Wheat and Barley. K. J. Leonard, and W. R. Bushnell, eds. APS Press, St. Paul, Minn.

67. Starkey, D. E., T. J. Ward, T. Aoki, L. R. Gale, H. C. Kistler, D. M. Geiser, H. Suga, B. Tóth, J. Varga, and K. O'Donnell. 2007. Global molecular surveillance reveals novel *Fusarium* head blight species and trichothecene toxin diversity. Fungal Genetics and Biology 44:1191-1204

68. Suga, H., G. W. Karugia, T. Ward, L. R. Gale, K. Tomimura, T. Nakajima, A. Miyasaka, S. Koizumi, K. Kageyama, and M. Hyakumachi. 2008. Molecular characterization of the *Fusarium graminearum* species complex in Japan. Phytopathology 98:159-166

69. Sutton, J. C. 1982. Epidemiology of wheat head blight and maize ear rot caused by *Fusarium graminearum*. Canadian Journal of Plant Pathology 4:195-209

70. Taylor, J. W., E. Turner, J. P. Townsend, J. R. Dettman, and D. Jacobson. 2006. Eukaryotic microbes, species recognition and the geographic limits of species: examples from the kingdom Fungi. Philosophical Transactions of the Royal Society B: Biological Sciences 361(1475):1947-1963

71. Taylor, J. W., D. J. Jacobson, S. Kroken, T. Kasuga, D. M. Geiser, D. S. Hibbett, and M. C. Fisher. 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genetics and Biology 31:21-32

72. Trail, F., and R. Common. 2000. Perithecial development by *Gibberella zeae*: A light microscopy study. Mycologia 92:130-138

73. Trail, F., H. Xu, and R. Loranger. 2002. Physiological and environmental aspects of ascospore discharge in *Gibberella zeae* (anamorph *Fusarium graminearum*). Mycologia 94:181-189

74. Turgeon, B. G. 1998. Application of mating type gene technology to problems in fungal biology. Annual Review of Phytopathology 36:115–137

75. Turgeon, B. G., O. C. Yoder, I. Kaneko, T. Arie, and S. H. Yun. 2000. Molecular organization of mating type loci in heterothallic, homothallic, and asexual *Gibberella/Fusarium* species. Fungal Genetics and Biology 31:7-20

76. Vesonder, R. F., J. J. Ellis, and W. K. Rohwedder. 1981. Swine refusal factors elaborated by *Fusarium* strains and identified as trichothecenes. Applied and Environmental Microbiology 41:323-324

77. Ward, T. J., J. P. Bielawski, H. C. Kistler, E. Sullivan, and K. O'Donnell. 2002. Ancestral polymorphism and adaptive evolution in the trichothecene mycotoxin gene cluster of phytopathogenic *Fusarium*. Proceedings of the National Academy of Sciences of the United States of America 99:9278-9283

78. Ward, T. J., R. M. Clear, A. P. Rooney, K. O'Donnell, and D. Gaba. 2008. An adaptive evolutionary shift in *Fusarium* head blight pathogen populations is driving the rapid spread of more toxigenic *Fusarium graminearum* in North America. Fungal Genetics and Biology 45:473

79. Windels, C. E. 2000. Economic and social impacts of *Fusarium* Head Blight: Changing farms and rural communities in the Northern Great Plains. Phytopathology 90:17-21

80. Wollenweber, H. W., and O. A. Reinking. 1935. Die Fusarien, ihre Beschreibung, Schadwirkung und Bekampfung, Verlag Paul Parey, Berlin.

81. Wu, C.-I. 2001. The genic view of the process of speciation. Journal of Evolutionary Biology 14:851-865

82. Yli-Mattila, T., T. Gagkaeva, T. J. Ward, T. Aoki, H. C. Kistler, and K. O'Donnell. 2009. A novel Asian clade within the *Fusarium graminearum* species complex includes a newly discovered cereal head blight pathogen from the Russian Far East. Mycologia 101:841-852

83. Yun, S.-H., T. Arie, I. Kaneko, O. C. Yoder, and B. G. Turgeon. 2000. Molecular organization of mating type loci in heterothallic, homothallic, and asexual *Gibberella/Fusarium* species. Fungal Genetics and Biology 31:7-20

84. Zeller, K. A., R. L. Bowden, and J. F. Leslie. 2003. Diversity of epidemic populations of *Gibberella zeae* from small quadrats in Kansas and North Dakota. Phytopathology 93:874-880

85. Zeller, K. A., R. L. Bowden, and J. F. Leslie. 2004. Population differentiation and recombination in wheat scab populations of *Gibberella zeae* from the United States. Molecular Ecology 13:563-571

86. Zeller, K. A., B. A. Summerell, S. Bullock, and J. F. Leslie. 2003. *Gibberella konza* (*Fusarium konzum*) sp. nov. from prairie grasses, a new species in the *Gibberella fujikuroi* species complex. Mycologia 95:943-954

87. Zeller, K. A., J. I. Vargas, G. Valdovinos-Ponce, J. F. Leslie, and R. L. Bowden. 2002. Population genetic differentiation and lineage composition among *Gibberella zeae* in North and South America. Pages 118 in: 2002 National Fusarium Head Blight Forum Proceedings. S. Canty, J. Lewis, L. Siler, and R. Ward, eds. U.S. Wheat & Barley Scab Initiative.

Chapter 2 - Methods for Detecting Chromosome Rearrangements in *Gibberella zeae*

Introduction

Gibberella zeae, teleomorph of *Fusarium graminearum* Schwabe, is the fungal pathogen of Fusarium Head Blight (FHB) of wheat and other small grains (1). *G. zeae* is a homothallic ascomycete, although high genotypic diversity is evidence that outcrossing probably occurs in field populations (2, 3, 8, 13). This species contains multiple phylogenetic lineages that share similar morphological, pathological, and toxicological traits. According to O'Donnell et al. (9) reproductive isolation of these lineages is evidenced by concordant gene genealogies, which led to the formal description of these lineages as new species (8, 9). Although the DNA marker data indicate genetic isolation, some strains from different lineages are cross fertile under laboratory conditions (3). Jurgenson et al. (4) suggested that the fertility of their lineage 6 by lineage 7 mapping cross was lower than that in some lineage 7 × 7 crosses. The possible low fertility of interlineage crosses could be due to various factors including postzygotic barriers, such as chromosome rearrangements.

Chromosome rearrangements can reduce gene flow between lineages in two ways. First, they can suppress recombination in the rearranged region, which allows chromosome segments to differentiate over time (12). Second, crossovers in the rearranged region may result in progeny with genetic duplications and/or deficiencies that render them inviable or less fit. Homothallic species, such as *G. zeae,* may be more tolerant of chromosome rearrangements than are heterothallic species if the homothallics predominantly self-fertilize. Selfed crosses of haploid organisms cannot have heterozygous rearrangements that lead to less fit or inviable progeny. Therefore, if selfing is the rule it would not be surprising to discover that rearrangements are common in *G. zeae.* Chromosome rearrangements can be detected by genetic methods such as linkage mapping, identification of regions of recombination suppression, comparative genomics, and cytological detection of pairing during meiosis. All of these methods are laborious and have a degree technical of difficulty and so an easier method is needed.

In *Neurospora crassa*, Perkins (10, 11) analyzed ejected ascospores and used the percentage of viable ascospores per ascus as an indicator of the type of rearrangement. In *Neurospora* viable ascospores are black (B) while the inviable ascospores are white (W). Crosses with 90-95% viable ascospores were considered isosequential while crosses with <90% black spores were considered candidates for having rearrangements (10).

Perkins was able to obtain unordered tetrads ejected from individual asci by collecting shot ascospores on agar slabs placed below perithecia on inverted culture plates. He identified five classes of asci: 8B:0W, 6B:2W, 4B:4W, 2B:6W, and 0B:8W. The proportions of these classes were diagnostic for different types of rearrangement. Reciprocal translocations produced equal frequencies of 8:0 and 0:8 and variable numbers of 4:4 tetrads (10). Insertional translocations were diagnosed with equal numbers of 8:0 and 4:4 with a variable number of 6:2 (10). Pericentric inversions include the centromere in the rearranged segments and are a rare class, yet they have been identified and are fully viable in *Neurospora* (10). Noncrossover and 2-strand double crossovers results in 8:0 asci (10). Other tetrad classes of pericentric inversions depend on crossing over within a long inverted segment; 6:2 and 4:4 are identified with this type of rearrangement. Paracentric inversions do not include the centromere and have not been diagnosed in *Neurospora*, possibly due to whole ascus abortion by the formation of a dicentric chromosome bridge following crossover within the pairing loop.

The objective of this study was to investigate counts of viable ascospores per ascus in *G. zeae* outcrosses as a convenient indicator of chromosomal rearrangements. Ascus pattern analyses were made by observing ascospores in rosettes of asci extruded from crushed perithecia or by observing unordered tetrads that were ejected from mature perithecia. Unlike *N. crassa*, *G. zeae* does not have colored spores and inviable ascospores never mature. Therefore the 8B:0W, 6B:2W, 4B:4W, and 2B:6W classification designed by Perkins degenerates to 8, 6, 4, and 2 viable ascospores per ascus respectively. There is no equivalent in *G. zeae* of the 0B:8W class.

Materials and Methods

Fungal strains

This study requires that all of the counted asci originate from an outcross and not from a selfed cross. In *G. zeae*, this requirement can be met by using strains that are engineered to be obligate outcrossers. Strains were constructed by Jungkwan Lee in which a portion of the mating type locus was replaced by a GFP constitutive expression cassette (6). These strains are functionally heterothallic (Figure 2.1). The *Δmat1-2* construct was transformed into three strains of *G. zeae* lineage 7, Z3639, Z3643, and H4 (11626, 11629, and 11628, respectively) that served as female tester strains. The male parents were strains Z3639 (lineage 7) and R-5470 (lineage 6). These strains are the parents of the mapping cross of Jurgenson et al. (4) where large heterozygous inversions were detected in chromosomes 1 and 3 (4, 5). Strains Z3634, 11157 (PH-1; NRRL 31084), 11784 (NRRL 5883) are additional lineage 7 strains used as controls. Additional lineage 6 strains 5048 (R-5469 wild type), 11785 (NRRL 13818), and 11786 (NRRL 26156) also were tested. Lineage 7 was chosen as the female tester because of its correspondence to *Fusarium graminearum* sensu stricto. Geographic origins of the strains used are listed in Appendix A.

Figure 2.1 Outcross between female tester 11628 (H4*Δmat1-2*) and 11784.
A) GFP expression in 4 of the 8 ascospores, B) Combination of fluorescence and differential interference contrast shows 8 mature ascospores with 4:4 segregation of GFP and confirms

sexual recombination has occurred, C) Differential interference contrast shows 8 smooth hyaline fusiform ascospores.

Crossing technique

Carrot agar (CA) plates were used to induce the sexual stage as described by Leslie and Summerell (7). Plates were 60 mm in diameter with an area of 28.3 cm^2.Female testers were cultured on carrot agar plates for 7 days at 24˚C in the dark prior to fertilization. Male strains were cultured on Potato Dextrose Agar (PDA) (DIFCO Laboratories Michigan, USA.) slants for 7 days. Conidia were harvested the day of fertilization. To fertilize female testers, a suspension of 1 ml of 2.5% Tween 60 carrying at least 5×10^5 conidia from the male strains was dispensed onto the CA plates and worked into the mycelia with a glass rod until the solution was absorbed (Figure 2.2). Self-fertilizations were made by substituting sterilized 2.5% Tween 60 solution for the spore suspension. Following fertilization, crosses were incubated at 24˚C with a 12 hr photoperiod. Perithecia were observable after seven days. There were two replicate plates per cross.

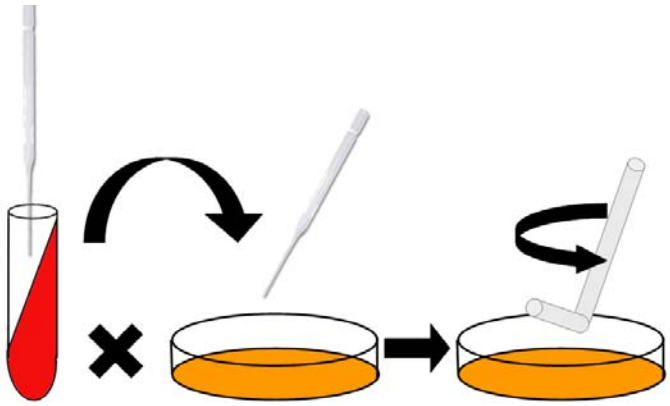

Figure 2.2 Crossing technique to produce perithecia.
Spore suspension scraped from Potato Dextrose Agar slants after 7 days of incubation at 24˚C with a 12 hr photoperiod.

Ejected tetrad method

From the outcross plate, a slice of CA with mature perithecia was placed inverted on the lid of a 3% water agar (WA) plate. Diameter of disposable Petri plate was 100 mm .There was at least 1 mm between the perithecia and the surface of the WA to avoid hyphal growth on the plate. Ascospores were ejected on the water agar. The lid was rotated every 20-30 seconds to avoid overlapping of ejected ascospore clumps (Figure 2.3). Only isolated clumps of ascospores were counted and placed in the 8, 6, 4, and 2 classes. Counting was continued until 100 tetrads were recorded.

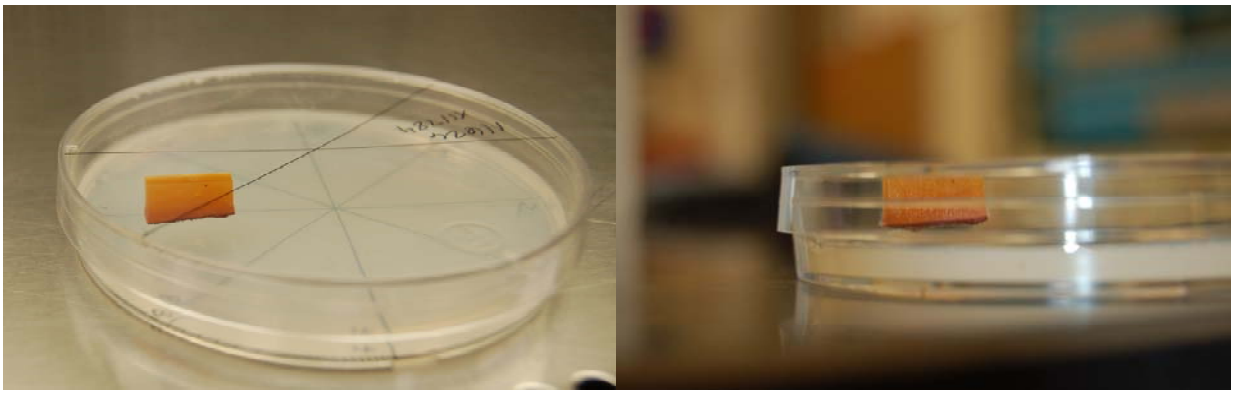

Figure 2.3 Ejected tetrad method.
Slice of carrot agar carrying mature perithecia from an outcross wass inverted. Ascospores were shot and landed on 3% water agar plate.

Rosette method

The rosette method was used to corroborate the ejected tetrad method. Several perithecia from a single cross were removed with a scalpel from the CA plate onto a glass slide with several drops of water to wash off the excess media, hyphae, and any macroconidia attached to the surface. If needed, perithecia were transferred to a new glass slide with water. A scalpel (blade no. 22) and tweezers (Dumostar No. 5 non-magnetic and non-corrosive) were used to dissect perithecia without disrupting the rosette or the asci within it. Once the rosettes were extruded, the perithecial wall was removed from the glass slide. All rosettes were viewed at 40× using Nomarski or Differential Interference Contrast (DIC) and epifluorescence imaging was

performed with a Zeiss Axioplan 2 IE MOT microscope. Fluorescence was observed with a 100 Watt FluoArc or an X-Cite®120 (EXFO Life Sciences) mercury lamp source. Filter set used was GFP (excitation 480 ± 10 nm, emission 510 ± 10 nm, filter set 41020, Chroma Tech. Corp., Rockingham, VT). Asci were considered mature when the ascospore wall and septa were delineated. Asci were scored as being in class 2, 4, 6, or 8 (Figure 2.4). Observations were difficult since asci frequently were overlapping. Only unambiguous asci were counted.

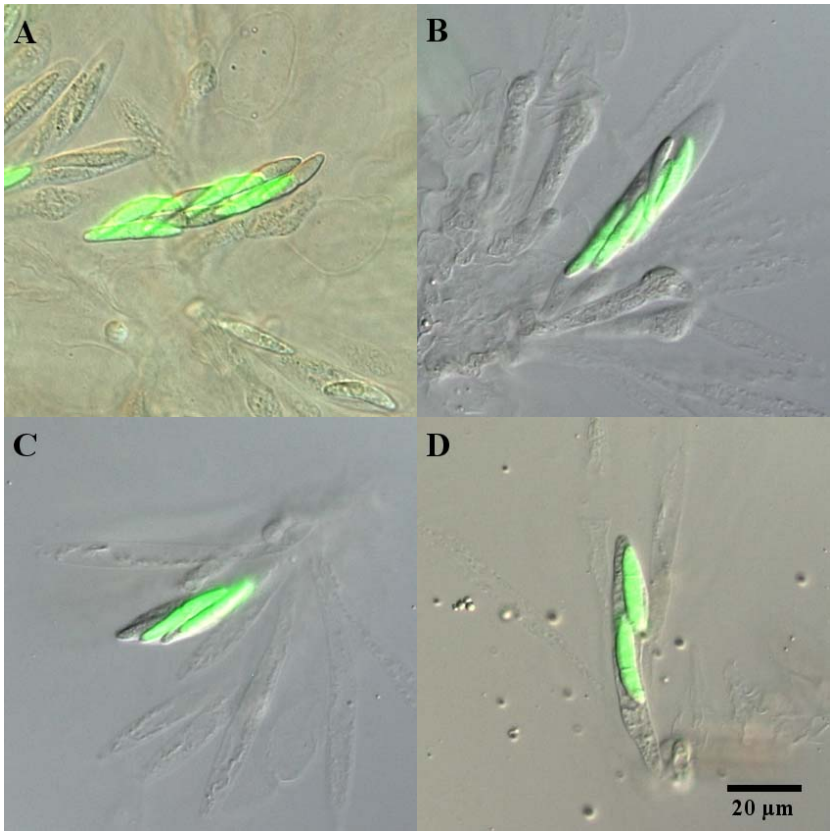

Figure 2.4 Four classes of tetrads in outcrosses of *Gibberella zeae*.
The rosette method showing ascospore classes of A) eight, B) six, C) four, D) two ascospores within an ascus.

Results

Selfed crosses served as negative controls for the detection of rearrangements because selfed crosses of haploid organisms are isosequential (i.e., no rearrangements) by definition. Selfed crosses predominantly produced the normal complement of eight ascospores per ascus whether using the ejected tetrad or rosette method. For both methods, a few asci contained either 6 or 4 ascospores, but these did not exceed 5% of the total for any culture (Figure 2.5). Since both methods gave similar results, data from the two methods were combined.

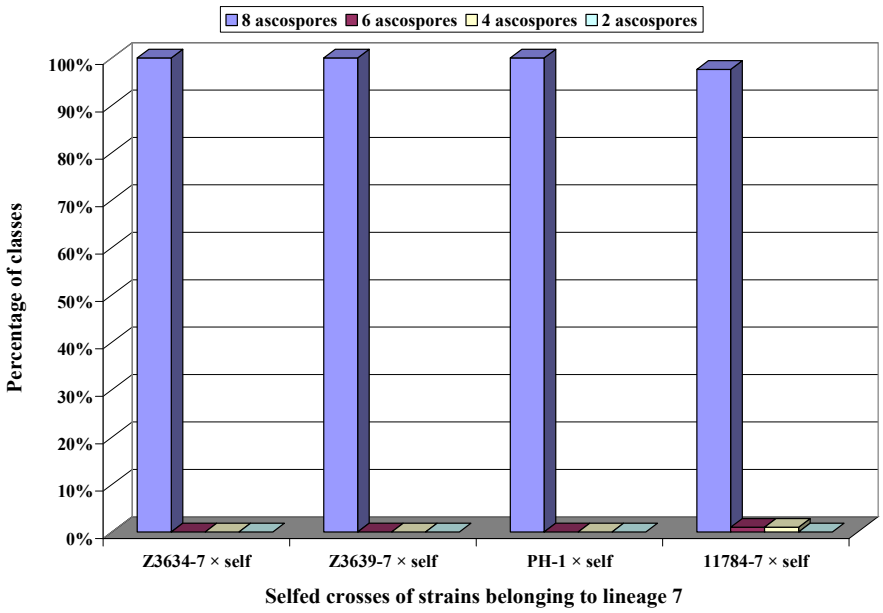

Figure 2.5 Selfed crosses that do not carry chromosome rearrangements. Results of rosette and ejected tetrad analysis from isosequential selfed crosses were combined bringing a total of ~200 tetrads analyzed. They serve as negative controls for detection of rearrangements. The X-axis lists the isolates and the Y-axis shows the percentage of the tetrad classes identified for each cross.

The parents of the mapping cross of Jurgenson et al. (4) differ by two known large inversions, which were confirmed by the alignment of the genetic and physical maps by Lee et al. (5). A cross between these parents served as the positive control for the method. In crosses with the lineage 7 female tester strains, strain Z3639 (lineage 7) consistently produced eight

ascospores per ascus, indicating that it is isosequential with the testers. In contrast, strain R-5470 (lineage 6) consistently produced 20% or more asci containing 6, 4, or 2 ascospores, indicating that it is not isosequential with the three testers (Figure 2.6).

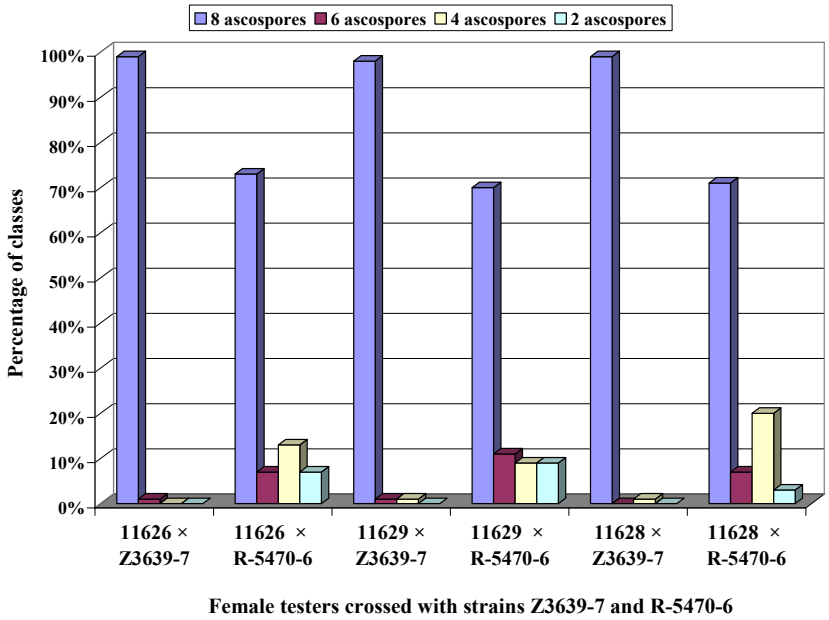

Figure 2.6 Crosses with mapping parents that carry known chromosome rearrangements. Combined results of rosette and ejected tetrad analysis (~200 tetrads analyzed) from three female testers crossed to mapping population parents. The X-axis lists the crosses and the Y-axis shows the percentage of the tetrad classes identified for each cross.

To test if the rearrangements in R-5470 are characteristic of lineage 6, additional crosses with lineage 6 males were conducted. The data show that there is variation between members of lineage 6 when crossed to the lineage 7 female tester (Figure 2.7). The mapping parent, R-5470, had the lowest number of normal asci with 8 ascospores. The other three strains had 90-95% normal asci.

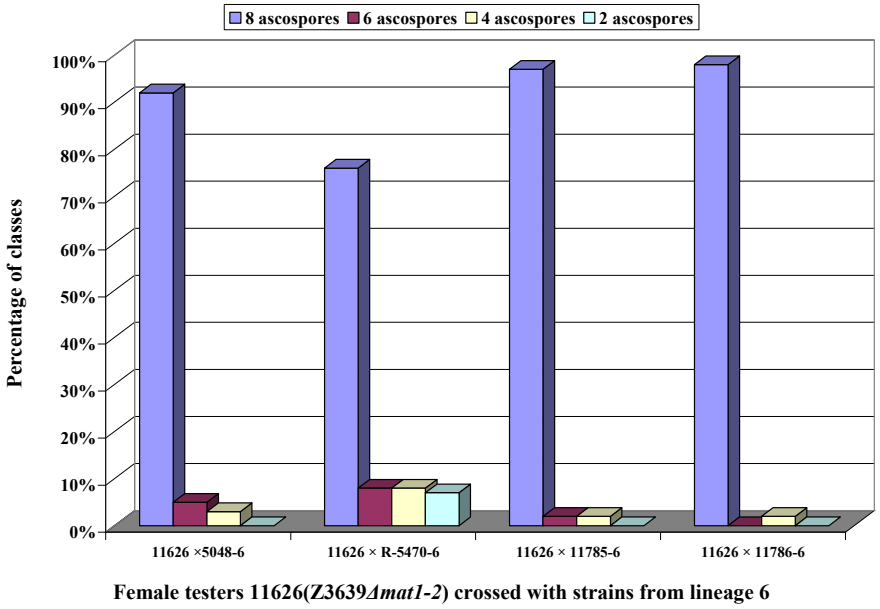

Figure 2.7 Crosses with additional strains of lineage 6.

Female tester 11626 (*Z3639Δmat1-2*) crossed to lineage 6 strain R-5470 had the lowest percentage of the 8 class tetrad. Whereas crossed to strains 5048, 11785, and 11786 produced over 90% of the 8 class tetrad. X-axis lists the crosses and the Y-axis shows the percentage of the tetrad class identified for each cross. Results of both methods were combined bringing a total of ~200 tetrads.

Discussion

In this study, we adapted to *Gibberella zeae* the tetrad analysis methods that Perkins (10, 11) developed to detect chromosome rearrangements in *Neurospora crassa*. One of the keys to success with this method was the *MAT* knockout female tester strains that allowed us to examine only heterozygous tetrads. Without these obligate heterothallic strains, tetrad analysis could not be efficiently applied in *G. zeae*. Since inviable ascospores in *G. zeae* fail to mature, the Perkins method was modified to place tetrads into four classes of 8, 6, 4, or 2 viable ascospores instead of Perkin's five classes of 8B:0W, 6B:2W, 4B:4W, 2B:6W, and 0B:8W. All four modified tetrad classes were observed in crosses with known chromosome rearrangements (Figure 2.6), so these classes appear to be valid in *G. zeae.* In addition to observing ejected ascospores tetrads on WA, we also examined rosettes of intact asci that contained mature ascospores prior to their ejection. Both methods gave similar results for the test crosses.

There was lower ascospore viability in crosses that contained known chromosome rearrangements, which differentiated them from known isosequential crosses (Figures 2.6, 2.7). In isosequential *Neurospora* crosses, 90-95% of the tetrads are "8B:0W" (10). In *G. zeae*, isosequential crosses had >95% of asci in the "8" category. Crosses with known rearrangements had approximately 70% of asci in the "8" category. Is 70% normal asci consistent with the two known inversions in the mapping cross? Inviability due to duplications and deficiencies in crosses that are heterozygous for inversions is caused by odd numbers of crossover events in an inverted segment. The frequency of those recombination events are proportional to the length of the inverted segment but also depends on rates of recombination suppression. Therefore, 70% normal asci is consistent with expectations. Perkins (10) stated that single exchanges and 3-strand doubles would result in 6B:2W tetrads, while 4-strand double exchanges would result in 4B:4W tetrads. Perkins figures for crosses with pericentric inversions showed that 6B:2W were several times more common that 4B:4W tetrads. Our results did not show that the "6" class was more frequent than the "4" class. The reason for the discrepancy is unknown.

The lineage 6 parent of the mapping cross was clearly shown to be the one harboring rearrangements relative to three lineage 7 female strains (Figure 2.7). It had previously been ambiguous which parent was rearranged in relation to lineage 7 reference strains (5). We also

demonstrated that the three female tester strains are isosequential with Z3639. Among four lineage 6 strains used as males with the Z3639 female tester strain, only the mapping parent (R-5470) produced 75% normal asci whereas the other males produced >90% normal asci (Fig. 2.8). This suggests that one or both of the chromosome rearrangements in R-5470 may not be shared by the population of lineage 6, and therefore may not be a significant barrier to fertility between the lineages.

Although these detection methods provided a method to screen strains for rearrangements relative to standard testers, performing such an analysis was time consuming. With the rosette method, it was not always clear whether the asci sampled had reached maturity and therefore could be analyzed. The preparation method for rosettes also was technically difficult. Frequently, overlapping structures prevents accurate counts of ascospores. Several pictures of rosettes showed broken rosettes and/or asci, made it a difficult task to identify the number of replicates needed. For the ejected tetrads, there was concern that asci with 6 or fewer ascospores might not be ejected efficiently. Perkins (10) also was concerned about the ballistic disadvantages of abnormal asci possibly introducing bias into counts.

References

1. Bockus, W., R. L. Bowden, R. Hunger, W. Morrill, T. Murray, and R. Smiley. 2010. Diseases caused by fungi and fungus-like organisms. Pages 34-36 in: Compendium of Wheat Diseases and Pests. American Phytopathological Society, St. Paul, Minnesota.

2. Boutigny, A. L., T. J. Ward, G. J. Van Coller, B. Flett, S. C. Lamprecht, K. O'Donnell, and A. Viljoen. 2011. Analysis of the *Fusarium graminearum* species complex from wheat, barley and maize in South Africa provides evidence of species-specific differences in host preference. Fungal Genetics and Biology 48:914-920

3. Bowden, R. L., and J. F. Leslie. 1999. Sexual recombination in *Gibberella zeae*. Phytopathology 89:182-188

4. Jurgenson, J. E., R. L. Bowden, K. A. Zeller, J. F. Leslie, N. J. Alexander, and R. D. Plattner. 2002. A genetic map of *Gibberella zeae* (*Fusarium graminearum*). Genetics 160:1451-1460

5. Lee, J., J. E. Jurgenson, J. F. Leslie, and R. L. Bowden. 2008. Alignment of genetic and physical maps of *Gibberella zeae*. Applied and Environmental Microbiology 74:2349-2359

6. Lee, J., T. Lee, Y.-W. Lee, S.-H. Yun, and B. G. Turgeon. 2003. Shifting fungal reproductive mode by manipulation of mating type genes: Obligatory heterothallism of *Gibberella zeae*. Molecular Microbiology 50:145-152

7. Leslie, J. F., and B. A. Summerell. 2006. The *Fusarium* Laboratory Manual. Blackwell Pub., Ames, Iowa. U.S.A.

8. O'Donnell, K., H. C. Kistler, B. K. Tacke, and H. H. Casper. 2000. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of

Fusarium graminearum, the fungus causing wheat scab. Proceedings of the National Academy of Sciences of the United States of America 97:7905-7910

9. O'Donnell, K., T. J. Ward, D. M. Geiser, H. C. Kistler, and T. Aoki. 2004. Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. Fungal Genetics and Biology 41:600-623

10. Perkins, D. D. 1974. The manifestation of chromosome rearrangements in unordered asci of *Neurospora*. Genetics 77:459-489

11. Perkins, D. D. 1997. Chromosome rearrangements in *Neurospora* and other filamentous fungi. Advances in Genetics 36:239-398

12. Rieseberg, L. H. 2001. Chromosomal rearrangements and speciation. Trends in Ecology and Evolution 16:351-358

13. Zeller, K. A., R. L. Bowden, and J. F. Leslie. 2003. Diversity of epidemic populations of *Gibberella zeae* from small quadrats in Kansas and North Dakota. Phytopathology 93:874-880

Chapter 3 - Time Course Studies I and II of Perithecia and Asci Maturity

Introduction

Gibberella zeae (Schwein.) Petch (anamorph: *Fusarium graminearum* Schwabe), a homothallic filamentous fungus, can produce both sexual spores (ascospores) and asexual spores (macroconidia) for inoculum dispersal. *Gibberella* belongs in the Hypocreaceae family which is characterized by forming dark blue or black perithecia. Perithecia can be easily detected in the field, particularly on corn and wheat debris (10, 15).

The perithecium is the sexual fruiting body of *G. zeae* (Figure 3.1). In the interior of a perithecium is found the hymenium (specialized hypha for ascus production and also intermixed with paraphyses), asci (definitive character of the ascomycetes, specialized cells formed after karyogamy and that form ascospores endogeneously), periphyses (short sterile hyphae lining the wall of the ostiolar canal of a perithecium) and paraphyses (sterile hypha-like structures located in the hymenium of the Ascomycota) (16). Karyogamy takes place within the ascus and after two meiotic and one mitotic divisions, eight ascospores are delimited. These ascospores are forcibly discharged from the perithecium once the ascus that contains them reaches maturity (3, 13, 16).

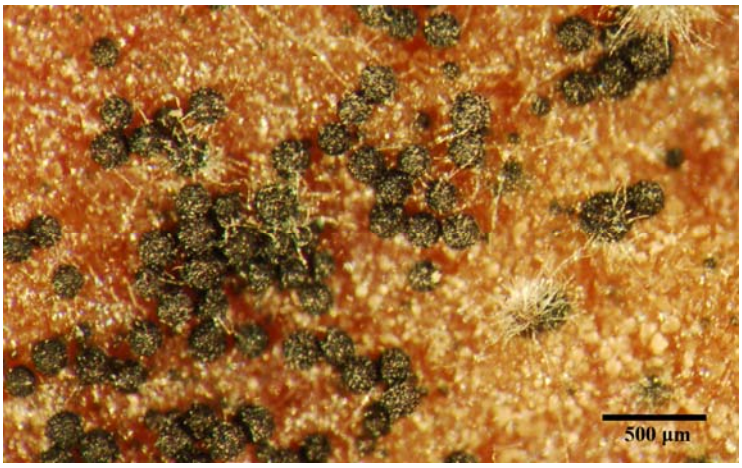

Figure 3.1 Perithecia, the sexual fruiting body of *Gibberella zeae*.
Macrophotograph of perithecia from a Z3639 selfed.

Development of perithecia has been thoroughly studied due to the important role of ascospores in the epidemiology of the disease. Such studies include optimum substrate and environmental factors, as well as details of formation (12, 14, 15). Tschanz et al. (14) determined that perithecia are produced within 14 days after induction. The number increases with rising temperatures from 15 up to 28.5˚C. Trail et al. (12) observed and described how perithecial initials are visible within 24 hrs of induction. The hymenium, paraphyses, and periphyses are formed soon thereafter. Seven days after induction, ascospores are discharged from mature functional perithecia.

The previous study (Chapter 2 – Methods for Detecting Chromosome Rearrangements in *Gibberella zeae*) suggested that differences in time to reach maturity could be a source of error in tetrad analysis. Timing differences could also affect counts of the number of perithecia and ascospores produced. Two time course studies were performed with several diverse crosses to test if timing differences are a significant problem and to determine optimal times for sampling. A new method, spore settling towers, was used to collect and count ejected ascospores. A second issue raised by the previous study was the difficulty in visually identifying the different classes of asci. Staining and fluorescent tagging are widely used to highlight structures in biological tissues (2, 5, 6, 7, 8, 9, 11). DAPI nuclear stain was tested for the ability to make observations of rosettes easier. A third issue that arose was that certain cultures produced relatively few macroconidia on potato dextrose agar (PDA) slants. Macroconidia are used as spermatia, so large numbers are required. Mung bean broth (MBB) is commonly used to produce large quantities of macroconidial inoculum of *F. graminearum* for pathogenicity testing (1). Therefore, the suitability of MBB for producing macroconidia for crossing was tested.

Objectives

1. Determine optimal timing for counting perithecia and the number of ascospores using several diverse intralineage and interlineage crosses.
2. Determine the utility of DAPI stain for observing and distinguishing the diverse classes of asci described in Chapter 2.
3. Evaluate MBB for production of macroconidia by several strains from different lineages.

Materials and Methods

Time Course Study I

Strains from lineages 6 and 7 were crossed with female tester 11626. These crosses were used to determine the optimum day to observe and record perithecia production. Nuclear staining with DAPI also was evaluated in this experiment.

Fungal strains

Female strain tester 11626 (Z3639*Δmat1-2*) was used as the female parent. Strains R-5470-6 (FGSC 8631/NRRL 13819), 11786-6 (NRRL 26156), 11784-7 (NRRL 5883), 11157-7 (PH-1/ FGSC 9057/ NRRL 31084), Z3639-7 (FGSC 8630/NRRL 29169), and Z3643-7 were used as male parents. Selfed crosses of 11626 and Z3639-7 served as negative and positive controls respectively. Hosts and isolation sites are listed in Appendix A.

Crossing technique

Female tester 11626 was cultured on carrot agar (CA) plates in the dark 7 days prior fertilization as described by Leslie and Summerell (4). Male strains were grown on PDA slants (DIFCO Laboratories Michigan, USA.) for 7 days. The sexual stage was induced as described in Chapter 2 (Figure 2.2). The crosses were divided into three groups and each group had two replicates per cross. Each group of crosses was repeated five times.

Quantification of perithecia

Fertilized plates were photographed daily with a Nikon D50 professional camera attached to a Nikon SMZ1000 dissecting scope. Percentage of coverage of the CA plate by perithecia was estimated visually from the photographs and recorded fifteen days after fertilization.

DAPI staining

Staining was done with 4′, 6-diamidino-2-phenylindole (DAPI) with a final concentration of 1.5 µg/mL (Invitrogen, Carlsbad, CA) and a fixation solution of 4% paraformaldehyde: Approximately 10 µL of 1× phosphate buffered saline (PBS) was placed on one end of the glass slide and 10 µL of the fixation solution (4% paraformaldehyde) on the other. With a pair of tweezers perithecia were picked from the CA plate and placed on the PBS end to wash off the macroconidia and maintain a constant pH. Washed perithecia were transferred to the other end of the glass slide and dissected to extrude the rosettes into the fixation solution. Rosettes were incubated at room temperature for 5 to 10 minutes. A wash with polymerization buffer (PME) followed and 2 µL of DAPI were added to the specimen. Slides were left at room temperature for 5 min before finally adding 20 µl of 1× PBS and placing the cover slide on top. Recipes for buffers are listed in Appendix C.

Extruded rosettes were observed using differential interference contrast (DIC). Epifluorescence imaging was performed with a Zeiss Axioplan 2 IE MOT microscope. Fluorescence was observed with a 100 Watt FluoArc or an X-Cite®120 (EXFO Life Sciences) mercury lamp source. Filter sets used were GFP excitation 480 ± 10 nm, emission 510 ± 10 nm, and DAPI excitation 359 ± 10 nm, emission 461 ± 10 nm, (Chroma Tech. Corp., Rockingham, VT). Images were acquired with an Axiocam HRc camera and Axiovision® software version 4.6. Microscopy components were obtained from Carl Zeiss (Oberkochen, Germany).

Time Course Study II

The second experiment included strains from lineages 1, 2, 6, and 7 crossed with the three female testers. Perithecial production was compared for macroconidial spermatia produced on either PDA or MBB. The number of ascospores produced was sampled using spore settling towers.

Fungal strains

The three female tester strains 11626 (Z3639*Δmat1-2*), 11629 (Z3643 *Δmat1-2*), and 11628 (H4 *Δmat1-2*), were used. Male parents strains were 11796-1 (NRRL 28718), 11793-2

(NRRL 28436), R-5470-6 (FGSC 8631/NRRL 13819), 11785-6 (NRRL 13818), and Z3639-7 (FGSC 8630/NRRL 29169) were used. Host and isolation site are listed in Appendix A.

Production of macroconidial spermatia

Male strains were cultured on PDA slants at 20-24˚C with a 12 hr photoperiod for 7 days. Macroconidia were scraped from PDA slants and collected in approximately 5 mL of sterile 2.5% Tween 60.

MBB was prepared from 40 g of mung beans steeped for 7 min in 1L of freshly boiled dH_2O (modified from (1)). With a ceramic funnel and sterilized cheesecloth, about 75 mL of the broth was filtered into a 125 mL Erlenmeyer flask. The flasks were autoclaved for 20 minutes at 121 ˚C. Prior to broth preparation the male strains were cultured on PDA plates and kept at 24˚C for 4 to 5 days. Once the broth had been autoclaved and cooled, two to three mycelial plugs were transferred to the MBB flasks and incubated on a table top shaker at 200 rpm for 4 days at 20-24˚C (Figure 3.2).

After spores were collected from PDA or MBB, macroconidia were filtered to remove mycelial fragments. The filtered macroconidia were concentrated and transferred into a microcentrifuge tube and centrifuged at 20,000 ×g for 5 min at 18 ˚C. The pellet of spores was re-suspended in 1 mL of 2.5% Tween 60, and the suspension was adjusted to 1×10^6 spores/mL.

Crossing technique

Female testers 11626 (*Z3639Δmat1-2*), 11629 (*Z3643 Δmat1-2*), and 11628 (H4 *Δmat1-2*) were cultured on CA as previously described. Crosses were fertilized with macroconidia harvested from MBB or PDA. One mL of macroconidial suspension was deposited on top of the female culture. Aerial mycelia were knocked down and the spore suspension was worked into the mycelia with a sterilized bent glass rod. Crosses were divided by female tester into three batches with two replicates of each cross per batch. Each batch was repeated three times. Z3639 (selfed) served as a positive control and each of the female testers (selfed) served as a negative control. Self-fertilizations were made by knocking down the aerial mycelium with sterilized 2.5% Tween 60 lacking male conidia. The plates were incubated at 24˚C with a 12 hr photoperiod. Perithecia were visible within seven days.

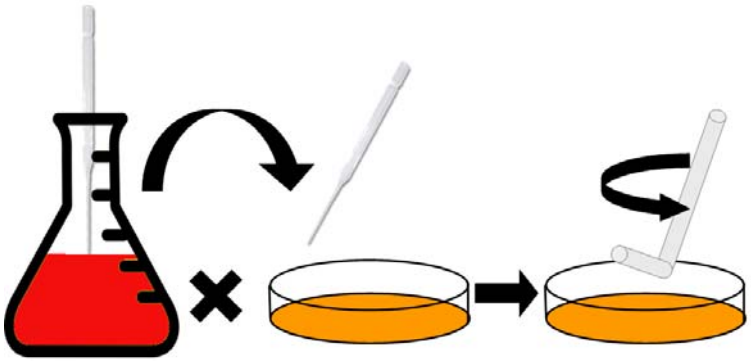

Figure 3.2 Crossing technique with mung bean broth.
Male parents were grown and macroconidia were harvested, filtered, and centrifuged from MBB. The spore suspension was filtered and centrifuged, the resulting pellet was mixed with 2.5% Tween 60 to obtain a concentration of × 10^6 macroconidia per mL. One mL of macroconidial suspension was deposited on top of the female culture. Aerial mycelia were knocked down and the spore suspension was worked into the mycelia with a sterilized bent glass rod.

Quantification of perithecia

Fertilized plates were photographed day 7 after fertilization with a Nikon D50 professional camera attached to a Nikon SMZ1000 dissecting scope. Percentage of coverage of the CA plate by perithecia was estimated visually from the photographs and recorded. The day of maximum coverage was read directly from the data and no statistical analyses were performed.

Quantification of ascospores using settling towers

On days 7, 9, 11, 13, and 15 after fertilization, an overall count of total ascospores ejected was obtained by inverting the cross plates for 16 h overnight on a 40 cm height settling tower made of 10 cm diameter PVC pipe (Figure 3.3). Ejected ascospores were deposited on water agar plates at the base of the towers. Ascospore counts were made in five pre-selected fields of view per plate and then averaged. Ascospore counts collected from the settling towers were highly variable. To improve homogeneity of variance, the counts were log(X+1) transformed.

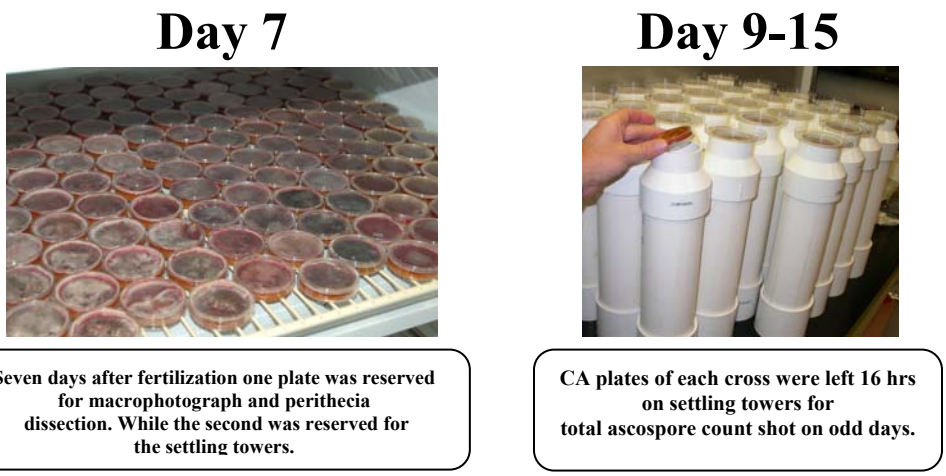

Figure 3.3 Time Course Study II

Seven days after fertilization perithecia density was recorded with macrophotography. Total count of ascospores shot were collected overnight from the settling towers on days 9-15.

Results

Time Course Study I

Development of perithecia over time

The outcrosses between female strain tester 11626 (Z3639*Δmat1-2*) and strains R-5470-6, 11786-6, 11784-7, 11157-7, Z3639-7, Z3643-7 and selfed cross of Z3639-7 were assessed for percentage of visible fruiting bodies, or perithecia, across the carrot agar (CA) plates. At 7 days after fertilization, up to 40% of CA plate surface had visible perithecia for crosses with strains R5470-6, Z3643-7, PH-1, Z3639-7, and Z3634-7. Crosses with strains 11784-7 and 11786-6 showed up to 40% in coverage on day 9 after fertilization. Selfed control Z3639-7 displayed, on day 3 after fertilization, 40% coverage and by day 9 up to 80% of the CA plate was covered with fruiting bodies (Figure 3.4).

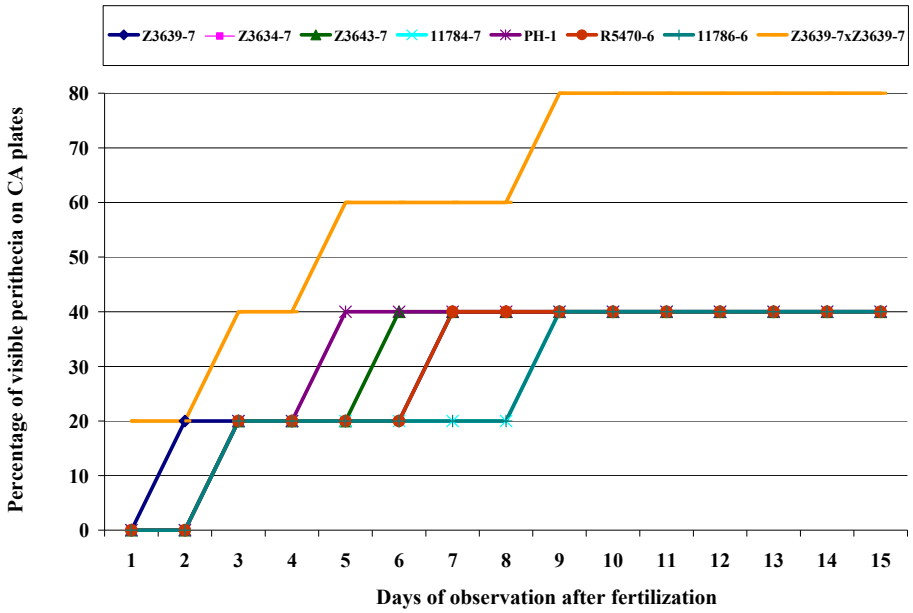

Figure 3.4 Percentage of coverage by perithecia produced from the crosses of female tester 11626 by strains 11157-7 (PH-1), Z3643-7, Z3639-7, Z3634-7, 11784-7, 11786-6 and R-5470-6.

The X-axis corresponds to day of observation and Y-axis to percentage of plate covered by perithecia. Lineages designation of the strains used as male parents are followed by " - ". Selfed cross of Z3639 was used as a reference.

DAPI staining

DAPI staining hindered rather than helped the identification and classification of the different ascus types (Figure 3.5). Although nuclei were successfully stained, DAPI increased the time needed to prepare the specimens and made it difficult to distinguish ascospores that covered others due to their lateral arrangement. The buffers used in this experiment also proved to be incompatible with the GFP signal needed to confirm sexual recombination has occurred.

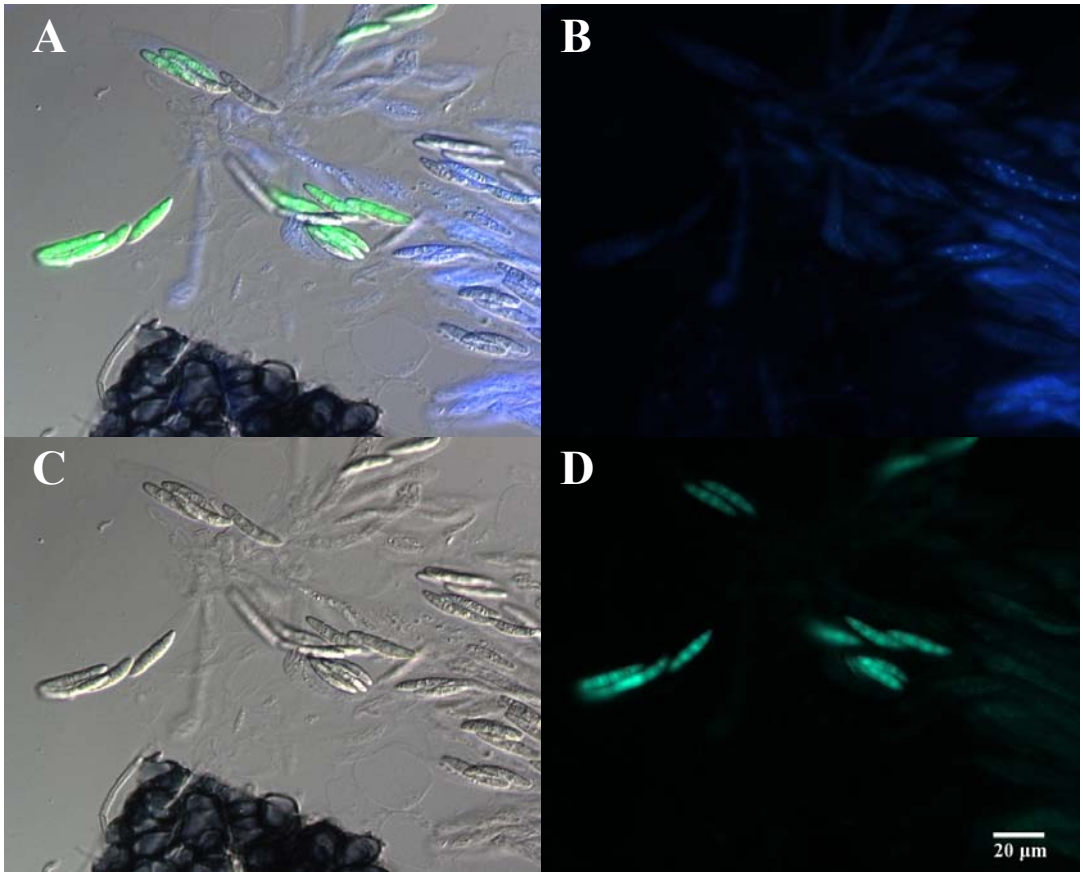

Figure 3.5 DAPI staining of *Gibberella*.

Cross of female tester 11628 with male strain R-5470-6 with DAPI+dH_2O. A) composite photo with DIC+GFP+DAPI signals, B) DAPI view, C) DIC view, and D) GFP view.

Time Course Study II

Performance of mung bean broth

On water agar, macroconidia harvested from MBB and PDA both showed 100% germination (data not shown). The results in Table 3.1 show a slight increase in perithecia distribution on average, but it was not statistically significant (paired *t*-test at α=0.05). Nevertheless, suspensions from MBB were faster and more convenient to prepare than suspensions from PDA. *F. graminearum* strains cultured on PDA slants require 7 to 10 days of incubation for macroconidia production (4). Strains that were grown on MBB had an incubation period of 4 days (1).

Table 3.1 Percentage of perithecia coverage on carrot agar plates for MBB and PDA.

	FEMALE							
	11626 (Z3639Δ*mat1-2*) ×		**11628 (H4Δ*mat1-2*) ×**		**11629 (Z3643Δ*mat1-2*) ×**		**Z3639-7 ×**	
MALE	**MBB**	**PDA**	**MBB**	**PDA**	**MBB**	**PDA**	**MBB**	**PDA**
Z3639-7	20	10	15	10	30	5	-[a]	-
Z3634-7	15	15	5	5	10	5	-	-
Z3643-7	5	5	5	10	10	1	-	-
R-5470-6	10	10	20	10	5	5	-	-
11785-6	10	15	20	20	1	1	-	-
11793-2	10	5	30	65	5	1	-	-
11796-1	5	10	50	25	1	1	-	-
11626	0	0	0	0	0	0	-	-
11628	0	0	0	0	0	0	-	-
11629	0	0	0	0	0	0	-	-
Z3639-7	-	-	-	-	-	-	80	80

[a] Cross was not done

Production of ascospores over time

Strain 11626 (derived from Z3639-7) produced the highest number of ascospores when crossed to Z3639-7, which was essentially a self-cross. However, it produced no ascospores with isolate 11793-2. Strains 11628 (derived from isolate H4) and 11629 (derived from Z3643-7) produced high numbers of ascospores with all males from all the lineages. The highest numbers of ascospores shot from perithecia were on days 9 to 13 after fertilization for all of the crosses except 11626 x Z3639-7 in which day 7 was equally high (Figure 3.6).

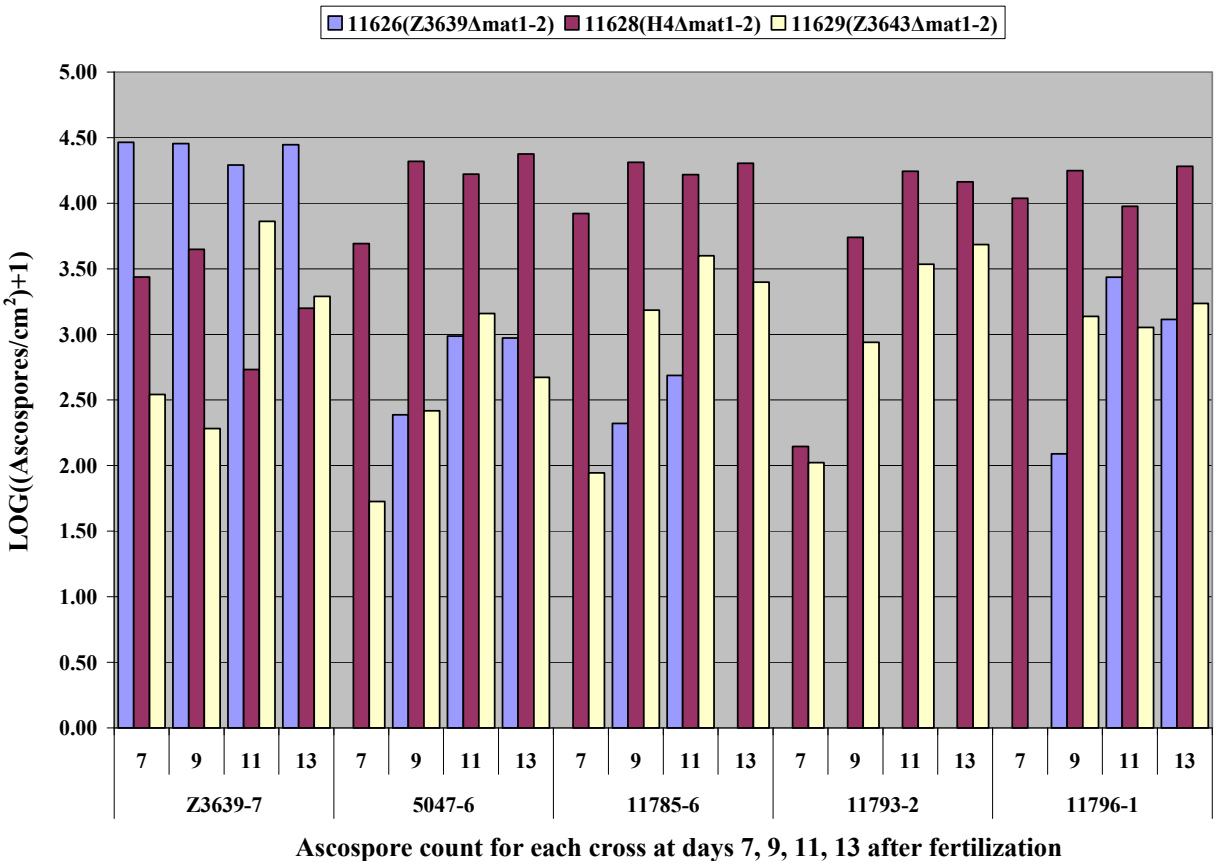

Figure 3.6 Ascospores per cm^2 collected from settling towers.
Log transformation of ascospores per cm^2 counted from WA plates at base of settling towers at 7, 9, 11, and 13 days after fertilization.

Discussion

The primary objective of both time course studies was to optimize sampling times with respect to perithecium and ascus maturity. Time Course Study I showed that perithecia reached maximum coverage of the plate by day 9 after fertilization for crosses involving both lineages 6 and 7. Time Course Study II used spore settling towers to show that the number of ascospores ejected from CA plates was already high by day 7 for most crosses, but then showed a broad plateau from days 9 to 13. The results were similar for all three female testers and five different males comprising a total of four lineages. Therefore, it was concluded that sampling of perithecia

or asci should be on days 9-13 following fertilization regardless of the *F. graminearum* parental strains involved.

The second objective of this work was to test the DAPI stain for improving the observation of asci extruded from perithecia. DAPI staining successfully stained the ascospore nuclei of *F. graminearum* in rosettes of asci. However, it did not provide enough power of identification or classification of asci. Ascospores in the ascus overlap and some spores cover up their sister spores. When using the DAPI stain, the covered ascospores could not be detected with greater accuracy. Since the method was laborious and did not improve the quality of observations, DAPI is not recommended for observing asci of *F. graminearum*.

The third objective was to test the performance of mung bean broth for production of macroconidia to be used as spermatia. Modifying the crossing technique by changing the medium to MBB was an improvement. It reduced the culture time for the male strains from 7 to 4 days, and was quicker and easier to prepare. It allowed harvesting a larger number of macroconidia per strain for an equivalent amount of labor. Subsequent experiments will use MBB in order to more easily standardize the spore suspension to 1×10^6 spores/mL.

An interesting observation in these two time course studies was that all males, regardless of lineage, produced ascospore progeny with at least two of the lineage 7 female tester strains. Individual pairings of lineage 7 females with males from other lineages often had fertility comparable to lineage 7 × lineage 7 crosses. However, some crosses also produced relatively few progeny, indicating the possible existence of pre- or post-zygotic fertility barriers.

The methods developed in this chapter, including macrophotographs for counting perithecia, microphotographs of rosettes using fluorescence and differential interference contrast microscopy for assessing ascus development, and settling towers for counting ejected ascospores, have all been shown to be useful. In the next chapter, an experiment will be conducted using these methods with a larger set of crosses, including all lineages available, and a larger number of replications.

References

1. Bai, G. H., and G. Shaner. 1996. Variation in *Fusarium graminearum* and cultivar resistance to wheat scab. Plant Disease 80(9):975-979

2. Freitag, M., P. C. Hickey, N. B. Raju, E. U. Selker, and N. D. Read. 2004. GFP as a tool to analyze the organization, dynamics and function of nuclei and microtubules in *Neurospora crassa*. Fungal Genetics and Biology 41:897-910

3. Kendrick, B. 1985. Kingdom Eumycota: Phylum 3: Dikaryomycota, Subphylum 1 - Ascomycotina. Pages 38-78 in: The Fifth Kingdom. Focus Publishing, R. Pullins Co., Newburyport, MA, USA.

4. Leslie, J. F., and B. A. Summerell. 2006. The *Fusarium* Laboratory Manual. Blackwell Pub., Ames, Iowa. U.S.A.

5. McClintock, B. 1945. Neurospora. I. Preliminary observations of the chromosomes of *Neurospora crassa*. American Journal of Botany 32:671-678

6. Moore, E. 1933. The use of phenosafranin for staining fungi on culture media or in host tissue. Science 77:23-24

7. Prigione, V., and V. Filipello Marchisio. 2004. Methods to maximise the staining of fungal propagules with fluorescent dyes. Journal of Microbiological Methods 59:371-379

8. Shoemaker, R. A. 1964. Staining asci and annellophores. Stain Technology 39:119-121

9. Son, H., K. Min, J. Lee, N. B. Raju, and Y.-W. Lee. 2010. Meiotic silencing in the homothallic fungus *Gibberella zeae*. Fungal Biology 115:1290-1302

10. Sutton, J. C. 1982. Epidemiology of wheat head blight and maize ear rot caused by *Fusarium graminearum*. Canadian Journal of Plant Pathology 4:195-209

11. Taga, M., and M. Murata. 1994. Visualization of mitotic chromosomes in filamentous fungi by fluorescence staining and fluorescence *in situ* hybridization. Chromosoma 103:408-413

12. Trail, F., and R. Common. 2000. Perithecial development by *Gibberella zeae*: A light microscopy study. Mycologia 92:130-138

13. Trail, F., H. Xu, and R. Loranger. 2002. Physiological and environmental aspects of ascospore discharge in *Gibberella zeae* (anamorph *Fusarium graminearum*). Mycologia 94:181-189

14. Tschanz, A. T., R. K. Horst, and P. E. Nelson. 1975. A substrate for uniform production of perithecia in *Gibberella zeae*. Mycologia 67:1101-1108

15. Tschanz, A. T., R. K. Horst, and P. E. Nelson. 1976. The effect of environment on sexual reproduction of *Gibberella zeae*. Mycologia 68:327-340

16. Ulloa, M., and R. T. Hanlin. 2001. Illustrated Dictionary of Mycology. APS Press, St. Paul, MN. U.S.A.

Chapter 4 - Patterns of Cross Fertility in the *Fusarium graminearum* clade

Introduction

Recognition of species limits is typically done by a combination of morphological species recognition (MSR), biological species recognition (BSR), and phylogenetic species recognition (PSR) methods. Recognizing species with just one recognition method, usually the PSR, has increased recently (33). PSR recognizes phylogenetic species using genealogical concordance of multiple independent loci. This process has become very popular since obtaining large amounts of DNA sequence has become easier over the years with constant upgrading of technology (10, 33).

In many cases, the different recognition methods agree on species limits (5). *Gibberella fujikuroi* constitutes a species complex in which BSR and PSR usually identify the same group of species (18, 19, 32). *Neurospora* spp., a model fungal organism, has been the subject of studies describing biochemical genetics, cell biology, genome organization, cytogenetics, natural populations, species recognition, and sexual reproduction (1, 26, 28, 29). This model organism has also been analyzed by MSR, BSR, and PSR methods. Genealogical concordance of four unlinked nuclear loci was used to recognize phylogenetic species in *Neurospora* spp. (7). Comprehensive BSR was applied by performing crossing experiments and delineating biological species based on patterns of reproductive success (8). PSR and BSR placed individuals in the same species groups (7, 8), and four cryptic species were recognized within what was thought originally to be *N. crassa* and *N. intermedia*.

Initial phylogenetic studies of the *Fusarium graminearum* clade, also known as the *Fusarium graminearum* species complex, showed that it consisted of seven biogeographically structured lineages based on the genealogies of six nuclear genes: β-tubulin, elongation factor α-1, reductase, phosphate permease, ammonia ligase, and 3-o-acetyltransferase by O'Donnell et al. (23). This study concluded that the lineage divergence in this complex was due to geographic and host isolation. However, in this study a hybrid between lineages 2 and lineage 6 was

detected. The authors suggested this hybrid did not have a fitness advantage over strains from the other lineages (23). Hybrids may be rare in nature and could have reduced fitness, or they may survive and provide advantageous combinations of characters to the population of which they are part. O'Donnell et al. subsequently expanded the number of phylogenetic species to nine (24), with a total of fifteen now identified (23, 24, 25, 30, 31, 36).

Bowden and Leslie (3) showed that strains of *F. graminearum* could intercross under laboratory conditions utilizing *nit* mutants of strains of lineages 3, 6, and 7 isolated from the USA, Japan and Nepal. They expanded this type of analysis to include representative members the of *F. graminearum* species complex with lineages 1 to 9 in a diallel cross (4). Members of lineage 7 produced ascospores with all lineages. Members of every lineage managed to cross with members of at least two different lineages (4). BSR sets species limits based on reproductive isolation of groups of individuals. Since the lineages are not reproductively isolated in laboratory crosses, the species complex appears to constitute only one biological species.

It appears, therefore, that PSR and BSR disagree in the *F. graminearum* clade. O'Donnell et al. (24) claimed that the lineages comprising the *F. graminearum* clade showed a clear history of reproductive isolation, and some level of intrinsic reproductive incompatibility. Yet they did not perform reproductive tests. The data collected by Bowden and Leslie (3, 4) indicates that sexual recombination occurs among members of different lineages. However the currently available data are both limited and qualitative, and insufficient to conclude that the members of the *F. graminearum* species complex lack fertility barriers between them. We need more definitive quantitative information to determine which components of fertility are reduced, if any, in crosses between lineages.

The following experiments were done with three female tester strains which are *ΔMAT1-2* mutants. The mating type gene in these strains is replaced with a GFP constitutive expression cassette (16). This mutation renders these strains heterothallic, and ensures that progeny resulting from crosses with these strains are the result of an outcross. These female tester strains all belong to lineage 7, which contains the original type of *Fusarium graminearum* Schwabe. By using these three female testers the scope of the study is limited to a manageable number of crosses that can be tested in detail.

Objective

To determine and evaluate the components of fertility: perithecia density, number and quality of asci, and total number of ascospores produced in sexual crosses between female testers strains from lineage 7 and representative members of lineages 1 through 9.

Fertility Studies

Fertility studies were done on two separate occasions, the first by Ms. Amy Beyer in fall 2004, and the second by me during fall 2010. The experiments were labeled "FSA" and "FSI" respectively.

Materials and Methods

Fungal strains

Three female tester strains 11626 (*Z3639Δmat1-2*), 11629 (*Z3643Δmat1-2*), and 11628 (*H4Δmat1-2*) were used as female parents. As male parents, 23 strains from lineages 1 to 9 were used. Cultures and the identifications of those from lineages 1-9 were provided by Dr. Kerry O'Donnell (USDA NCAUR, Peoria, IL, USA). All cultures were purified by single spore-isolation with a micromanipulator (20). For prolonged storage, a mix of mycelia fragments and spores were kept at – 80°C in 15% glycerol. For short-term storage, cultures were maintained on complete medium slants at 4°C. Host and isolation site, are given in Appendix A as well as their corresponding Kansas State University (KSU – Manhattan, KS, USA), Fusarium Research Center (FRC – Penn State University, University Park, PA, USA), Fungal Genetics Stock Center (FGSC – University of Missouri, Kansas City, Missouri, USA), and ARS Culture Collection (NRRL – USDA NCAUR, Peoria, IL, USA) collection numbers. Throughout the chapter, strains will be referenced by their KSU collection number followed by their lineage designation.

Crossing technique

FSA

To induce the sexual stage, female testers 11626, 11628, and 11629 were cultured on carrot agar 7 days prior to fertilization at 24˚C with a 12 hr photoperiod. Male donors were grown on either Potato Dextrose Agar (PDA) or Spezieller Nährstoffarmer Agar (SNA) slants for 7 days prior to crossing. Macroconidia were used as the fertilizing agent. These spores were

collected by rinsing the slants with a sterile 2.5% Tween 60 solution and then standardized to 5×10^5 macroconidia/mL. One mL of suspension was added per plate and aerial mycelium was knocked down while spreading the suspension with a bent glass rod (20). All plates were incubated at 24˚C with a 12 hr photoperiod. There were two replicate plates per cross and the experiment was repeated three times.

FSI

Female testers 11626, 11628, and 11629 were cultured as previously described in Chapter 3. Crosses were fertilized with macroconidia harvested from Mung Bean Broth (MBB) as described in Chapter 3 of this thesis. There were two replicate plates per cross and the experiment was repeated seven times.

Settling towers for total ascospore counts

Overall counts of total ascospores were produced by inverting plates on a 40-cm spore settling tower made of 10-cm diameter PVC pipe as described in Chapter 3.

FSA

Ten days after fertilization, fertility was measured by counting ascospores ejected overnight (14-16 hrs) on 2% water agar plates. Five pre-selected fields of view were examined for each plate, using a Nikon SMZ1000 dissecting scope at 10×. The average number of ascospores deposited per cm^2 was recorded for each plate.

FSI

On days 9, 10, and 11 after fertilization, the plates were left overnight on the settling towers. As in FSA, ascospores per cm^2 were counted with a Nikon SMZ1000 dissecting scope.

Perithecia density with macrophotographs

FSA

No macrophotographs were taken in this experiment.

FSI

Fertilized plates were photographed with a Nikon D50 professional camera attached to a Nikon SMZ1000 dissecting scope. Perithecia were counted in five pre-selected areas or fields of view on the carrot agar (CA) plates eight days after fertilization. Counts were made by two different individuals and averaged.

Microphotographs and perithecial development scores

FSA

Microphotographs and the rosette method were not used for in this experiment.

FSI

Several perithecia from each cross were removed with a scalpel from the CA plate onto a glass slide with several drops of water to wash the excess of media, hyphae, and any macroconidia attached to the surface. If needed, perithecia were transferred to a new glass slide with water and/or 0.25% gelatin suspension. With the use of both a scalpel (blade no. 22) and tweezers (Dumostar No. 5 non-magnetic and non-corrosive), perithecia were dissected. Once the rosettes were excised, the perithecium wall was removed from the glass slide. An additional drop of the gelatin solution was added and then a glass cover slip was placed on top. Ascospores were considered mature when ascospore walls and septa were delineated. Microscopy was done on days 9, 10, and 11 after fertilization by using differential interference contrast (DIC) and fluorescence imaging as described in Chapter 3 of this thesis. A rating scale for reproductive development of perithecia was modified from Dettman et al. (8) to accommodate our system since *F. graminearum* does not mature non-viable spores (Table 4.1).

Table 4.1 Modified rating scale for perithecial development.

Rating	Category Description
0	Sterile, no perithecia produced
1	Barren perithecia, no asci developed
2	Perithecia and asci developed but not ascospores
3	<1% of ascospores developed to maturity
4	1-15% of ascospores developed to maturity
5	15-50% of ascospores developed to maturity
6	>50% of ascospores developed to maturity

Statistical analysis

Ascospore and perithecia counts were standardized to numbers per cm^2 and log-transformed to reduce variance heterogeneity. The data were analyzed as a randomized complete block design with individual runs (ten for ascospore counts, seven for perithecia counts), each consisting of a single replicate with two repeated observations, treated as random (block) effects. The treatment structure included the fixed effects of female, lineage, male nested within lineage, and all interactions. Analyses were conducted using Proc Mixed in SAS (SAS Institute, Cary, N.C.), and effect means were separated using Bonferroni's adjustment for multiple comparisons.

Results

Ascospore total counts

All lineage 7 female testers successfully crossed with the 23 male strains and produced ascospores. We detected significant difference in female fertility of the testers strains at the $\alpha = 0.05$ level (Table 4.2). The results were separated into three graphs (Fig. 4.1) because there was a significant interaction between female tester and the lineage to which the male belonged. For female tester 11626 (Z3639*Δmat1-2*), males from lineage 7 produced the highest count of ascospores. These results were significantly different from those for lineages 2, 4, 5, 6 and 9, but not lineages 1, 3, and 8 (Figure 4.1A). Female tester 11628 (H4Δ*mat1-2*) produced high numbers of ascospores with strains from all lineages without any significant differences between them (Figure 4.1B). For female tester 11629 (Z3643*Δmat1-2*), males from lineage 7 produced the highest count of ascospores. These results were significantly different from those for lineages 2, 3, 5, 6, but not lineages 1, 4, 8, and 9 (Figure 4.1C).

Table 4.2 Female main effects for total ascospore production.

Female tester	Log(Ascospores/cm^2)
11626 (Z3639*Δmat1-2*)	1.46b[1]
11628 (H4*Δmat1-2*)	2.14a
11629 (Z3643*Δmat1-2*)	1.48b

[1] Same letters indicate that they were not significantly different from each other (α=0.05).

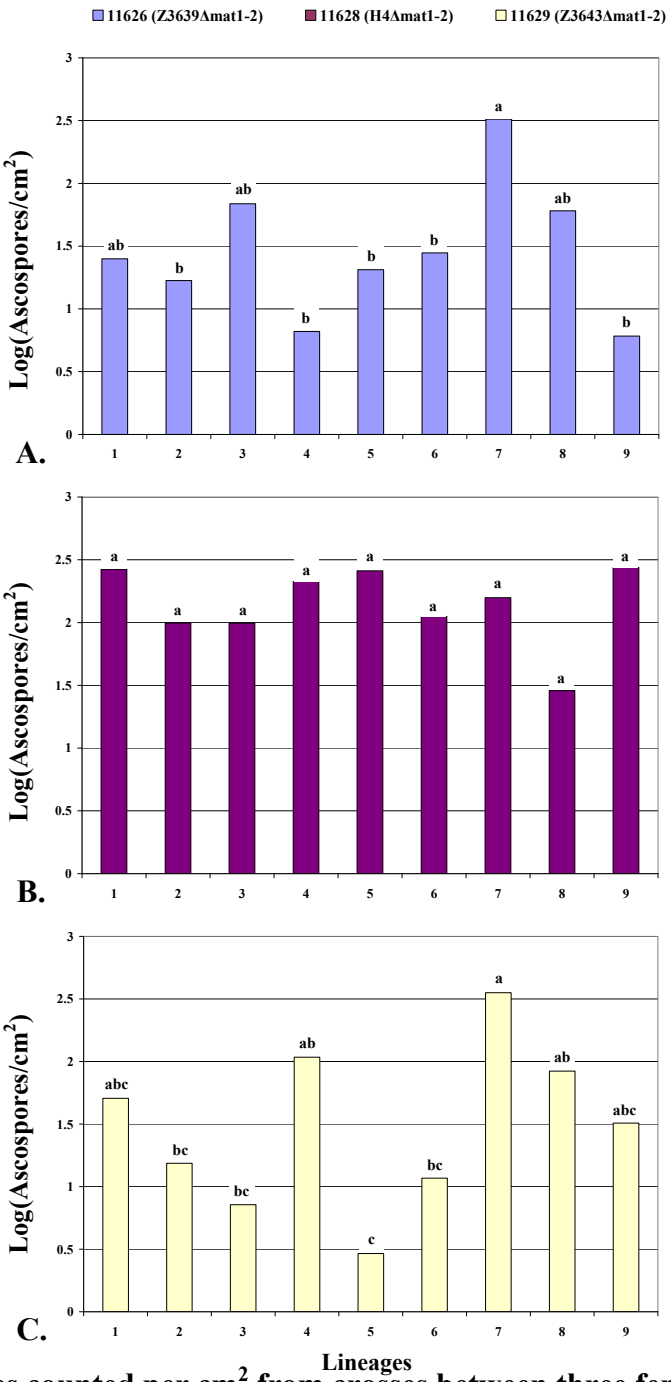

Figure 4.1 Ascospores counted per cm^2 from crosses between three female testers and strains from lineages 1 to 9.

A) 11626 (Z3639*Δmat1-2*), B) 11628 (H4*Δmat1-2*), C) 11629(Z3643*Δmat1-2*). The X-axis lists male strains combined by lineage. Counts for male strains were combined by lineage. The Y-axis refers to log transformation of ascospores per cm^2 counted. Same letters indicate they were not significantly different from each other (*α*=0.05).

Perithecia density

A significant difference in the tester strains was detected in the density of perithecia produced among the lineages (Table 4.3). The lineage of the male, but not the female strain by lineage interaction, was significant. Therefore the female tester strains were combined into a single bar graph (Fig. 4.2). All of the crosses produced perithecia. Lineage means were between 1.2 and 1.6 units of log-transformed perithecia per cm^2. Except for the males from lineage 6, males from the other seven lineages did not significantly differ from lineage 7 males in the density of perithecia produced in crosses with the female testers.

Table 4.3 Female main effects for perithecia production.

Female tester	Log(Pertihecia/cm^2)
11626 (Z3639*Δmat1-2*)	1.43a[1]
11628 (H4*Δmat1-2*)	1.48a
11629 (Z3643*Δmat1-2*)	1.18b

[1] Same letters indicate they were not significantly different from each other (α=0.05).

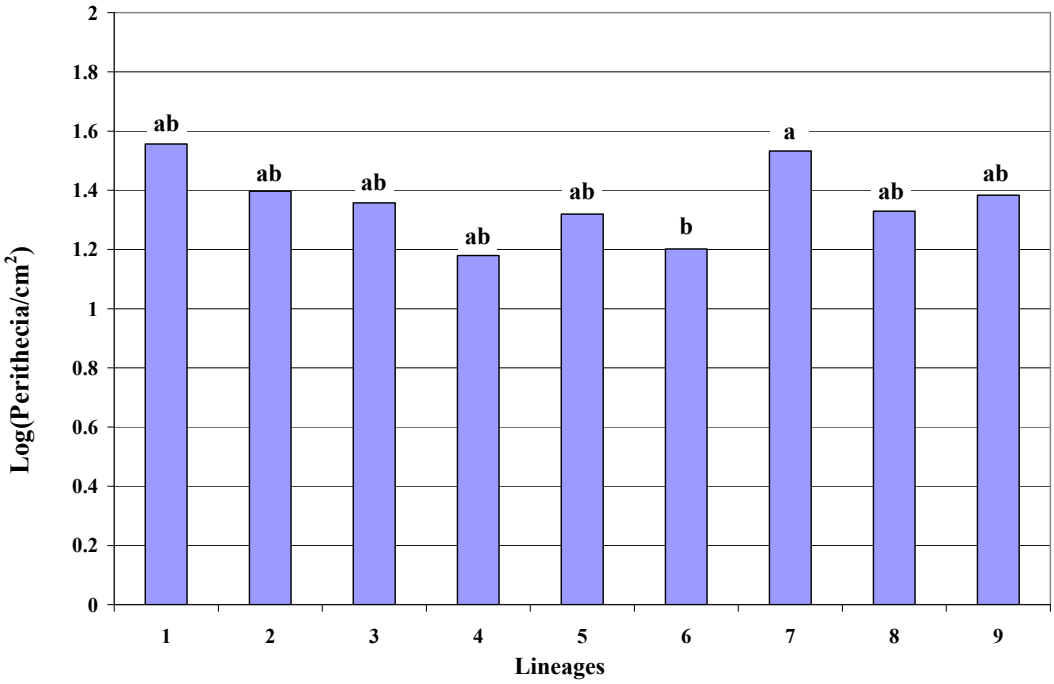

Figure 4.2 Perithecia density in crosses with three lineage 7 female testers.
The X-axis lists lineages of males and the Y-axis shows log transformation of perithecia per cm^2. Same letters indicate they were not significantly different from each other (α=0.05). The power of different statistical comparisons varied depending on the number of male available to represent a lineage.

Perithecial development scores with microphotographs

A female effect was not detected therefore female testers were combined and the SAS UNIVARIATE procedure was used (SAS Institute, Cary, N.C.) to calculate basic statistical measures. Histograms for each lineage were constructed based on the number of the ratings (Figure 4.3). As in the results for perithecia distribution, no significant difference between the female testers was detected at the 0.05 level. Therefore the results were pooled by female tester strain. This experiment showed the quality of a particular outcross evaluated from within. All lineages had ratings of at least 3 or 4, <1% of ascospores developed and 1-15% of ascospores developed respectively. Lineage 7 again, had higher ratings (5 and 6 or 15-50% and >50% of ascospores developed) than the other lineages.

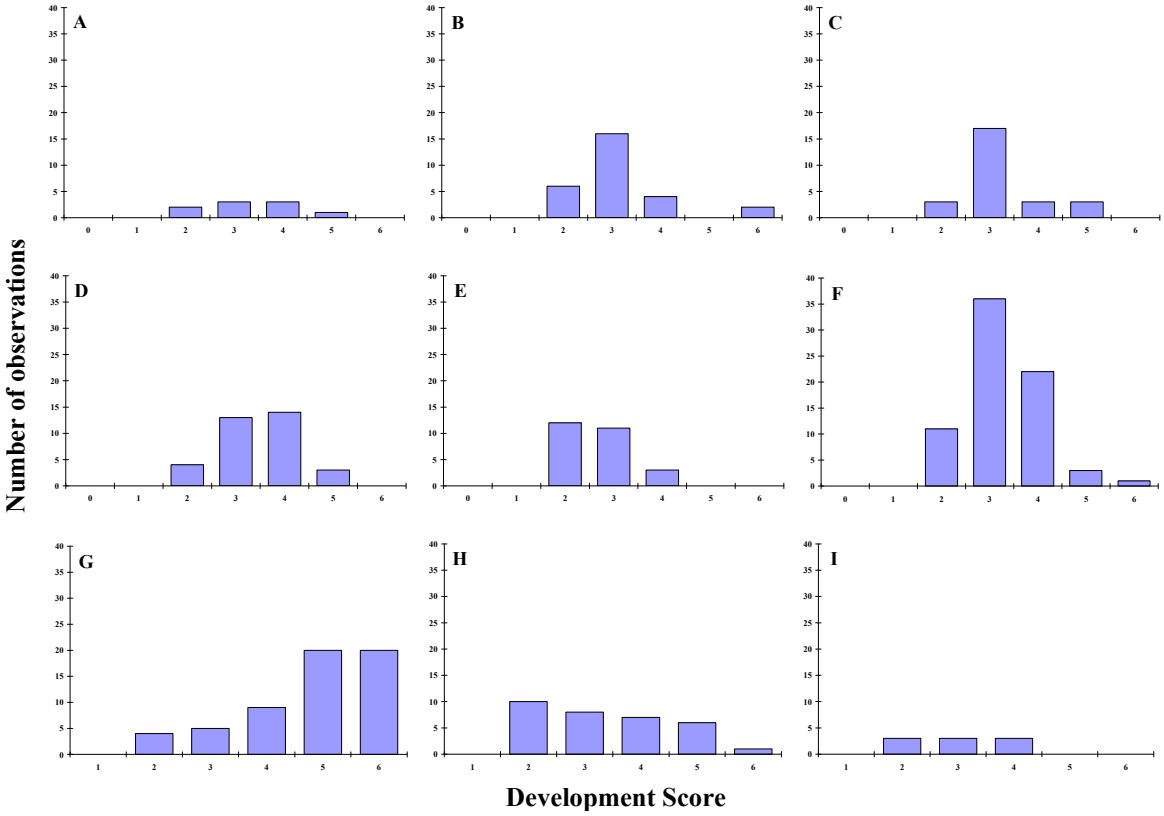

Figure 4.3 Perithecial development score histograms for each lineage as the male. A) Lineage 1, B) Lineage 2, C) Lineage 3, D) Lineage 4, E) Lineage 5, F) Lineage 6, G) Lineage 7, H) Lineage 8, and I) Lineage 9. Rating categories are listed on the x-axis and frequency of each category on the y-axis. Data was combined for female testers.

Discussion

All three lineage 7 female testers successfully crossed to all 23 male strains from lineages 1 to 9. Heterothallic crosses were confirmed by the segregation of the GFP marker in individual perithecia. The results collected from this experiment were consistent with the qualitative results obtained by Bowden et al. (3, 4) from interlineage crosses with *nit*-mutants. Since none of the lineages were reproductively isolated from the lineage 7 testers in laboratory crosses, we conclude that all of the lineages are members of the same species according to the Biological Species Concept (BSC) as originally proposed by Mayr (21), "Species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups."

One of the criticisms of Mayr's original biological species definition is that complete reproductive isolation is an overly conservative criterion for setting species limits (5). Even Mayr recognized that hybrids between "seemingly good sympatric species" are not uncommon (22). According to Coyne and Orr's (5) modified version of the BSC, "distinct species are characterized by substantial, but not necessarily complete reproductive isolation." If intralineage crosses in the *Fusarium graminearum* species complex typically have high reproductive success while interlineage crosses show low reproductive success, then the modified BSC would recognize the existence of multiple biological species in this group even though reproductive barriers are incomplete. Therefore, it is important to quantify the degree of cross fertility in the species complex to test if substantial reproductive barriers exist.

The settling tower method for counting ejected ascospores was an easy and objective method to quantify cross fertility in the *F. graminearum* species complex. In *Neurospora*, Dettman et al. (8) used the number of ejected black viable ascospores versus white inviable ascospores to quantify reproductive success. Based on a comprehensive set of crosses with 73 individuals identified as *N. crassa* or *N. intermedia*, they considered crosses that produced >50% black ascospores to be members of the same biological species. Unlike *Neurospora* spp., *F. graminearum* does not eject mature inviable ascospores; essentially all ejected ascospores can germinate on water agar. Therefore, we used total viable counts rather than percent viability as the metric. Like the *Neurospora* method, counting ejected ascospores in *F. graminearum* is

simple, fast, and unambiguous. The advantage is that it is a more comprehensive way to estimate the fertility of crosses because the quantity of viable ascospores rather than just the percent viability of ascospores is measured. The disadvantage is that there is no established threshold for identifying members of the same biological species. Consequently, we used analysis of variance and multiple comparison tests to detect significant differences in fertility.

Patterns of cross fertility tended to be strain-specific rather than lineage-specific. Using log-transformed counts of ejected ascospores, tester 11628 crossed equally well to males from every lineage with no significant differences between them (Fig. 4.1). In contrast, female testers 11626 and 11629 both produced the highest number of ascospores in intralineage crosses, but the means for three to four other lineages did not differ significantly from lineage 7. Males from the remaining lineages produced significantly fewer ascospores than lineage 7 males, but only lineages 2, 5, and 6 were significantly lower for both females. Decreases in fertility in some cases were 10- or even 100-fold, which is clearly substantial. Despite the significant reproductive barriers in certain combinations, there were no consistent quantitative reproductive barriers between lineages 1 to 9 and the lineage 7 testers. Therefore, the modified BSC of Coyne and Orr (5), in which limited hybridization is allowed between species, would still identify this group as one biological species.

These results contradict the claim of O'Donnell et al. (24) that the lineages in the *F. graminearum* clade "...show some level of intrinsic reproductive incompatibility as evidenced by their reduced fertility in artificial crosses...". They cited only the work of Jurgenson et al. (12) involving a lineage 6 by 7 mapping cross. However, that cross is unusual for several reasons. In Chapter 2, we conducted tetrad analyses that showed reduced fertility for the mapping cross, but other lineage 6 by 7 crosses showed negligible effects on tetrad fertility. The mapping cross has two large inversions, one of which may be unique to the strain R-5470 parent. R-5470 also carries a pleiotropic mutation that affects female fertility (14, 15). Although fertility of the mapping cross was indeed low, the mapping cross is not representative of interlineage crosses in the *F. graminearum* clade.

The high variation in ascospore production from different crosses between members of the *F. graminearum* species complex was similar to the pattern for *N. intermedia*, which also consists of a single biological species (8). From a total of 438 intraspecific cross attempts, most had >50% black ascospores and received the highest fertility rating of "6" (8). However, 201

(46%) did not meet the biological species criterion of >50% black ascospores and 124 of those (28% of total) were sterile. Even reciprocal crosses often gave conflicting results. In those cases, Dettman et al. (8) used only the highest score to reflect the maximum potential of the pairing. Their rationale was that a sexual function, such as female fertility, is much easier to lose than it is to gain *de novo*. Thus, the variation in reproductive success for strains of *F. graminearum* is not unusual for members of a biological species in the ascomycete fungi.

Fertility barriers can be divided into prezygotic and postzygotic types (5, 34). In *F. graminearum*, full-sized mature perithecia develop only after fertilization (14). Accordingly, perithecia density is a measure of the amount of successful fertilizations and an indicator of prezygotic fertility barriers. All of the outcrosses produced mature perithecia with only slight differences in distribution or coverage of the carrot agar plate. (Fig. 4.2) There was no significant interaction between the lineage of the males and the individual female tester strains, but female 11629 produced significantly fewer perithecia than the others. Only males from lineage 6 produced a significantly lower density of perithecia than lineage 7 males. This could reflect a slight prezygotic barrier in lineage 6×7 crosses. However, if prezygotic barriers were important in the species complex, we would have expected significantly fewer perithecia in more of the crosses. Therefore, the cause of low fertility in certain pairings appears to be primarily postzygotic.

Perithecial development scores are semi-quantitative estimates of the percent viability of pre-ejection ascospores and were intended to be an indicator of intrinsic postzygotic barriers. Due to small sample sizes for individual strains, data were aggregated by lineage for presentation (Fig. 4.3). Scores for all nine lineages were at least “4”, three lineages had maximum scores of “5”, and four lineages had maximum scores of “6”. Scores for lineage 7 were most heavily weighted towards ratings of “5” or “6”. Most lineages included scores that spanned four or five rating classes, and even lineage 7 had perithecia ratings that ranged from “2” to “6”. The large variation in scores occurred within individual pairings and is clearly due to experimental variation. Although the data suggest postzygotic fertility difficulties in some cases, the results are not conclusive. Different methods may be needed to study intrinsic postzygotic fertility barriers in this group.

An important implication of this study is that any trait that is polymorphic between any strains or lineages in the species complex can be genetically analyzed because all tested strains,

regardless of lineage, successfully crossed as males to the lineage 7 females. For example, female tester 11628 (derived from strain H4) consistently crossed with different lineages with no observable statistical differences. Such behavior may be attributable to genetic differences related to geographic or host origin. Strain 11628 is from corn in South Korea, while 11626 and 11629 are both from wheat in Kansas. It should be possible to genetically map the loci in 11628 that confer high fertility in wide crosses as was done with pathogenicity and aggressiveness loci using the Jurgenson mapping cross (6).

When O'Donnell et al. (24) elevated the lineages of *F. graminearum* to species rank using PSR they used DNA sequences from multiple loci to show that the *F. graminearum* clade is composed of multiple reciprocally monophyletic lineages. Reciprocal monophyly indicates that the lineages have a history of reproductive isolation from each other. Reciprocal monophyly is actually a conservative criterion for recognizing phylogenetic isolation. According to Hudson and Coyne (11) "it would take more than 1 million years after speciation before species would be delimited if 15 loci were sampled in species with an effective population size (N_e) of 100,000, assuming one generation per year. In larger populations, the number of years that must pass before the species would be recognized increases proportionally". Based on their analyses, O'Donnell et al. (24) claimed that the *Fg* clade has reached "an advanced state of biological speciation."

In contrast, our data on the high degree of cross fertility in the *F. graminearum* clade indicates that this species complex is still in the early stages of speciation. Wu (35) described four stages of speciation: I) populations/races with differential adaptation; reproductive isolation not apparent, II) transition between race and species with some degree of reproductive isolation; populations may fuse or diverge; III) divergent populations beyond the point of fusion but still share a portion of their genomes via gene flow; good species, and IV) species with complete reproductive isolation. Global trade has apparently brought the lineages back together in various locations and natural hybrids between lineages have been detected twice in the field (2, 23). Our study has shown that intrinsic reproductive isolation is minimal, at least with lineage 7. Lee et al. (17) showed that lineage 6 (*F. asiaticum*) may have a host preference for rice. Boutigny et al. (2) suggested that lineage 3 (*F. boothii*) may have a host preference for maize. These characteristics could indicate that the *F. graminearum* clade is at Stage I or II. Although we did not find significant intrinsic reproductive isolation, there could be significant extrinsic reproductive

isolation whereby hybrids have poor fitness. That would be consistent with Stage III. Our data rule out Stage IV.

Reduced hybrid ecological fitness is a type of postzygotic barrier that is difficult to identify or to predict. Numerous species pairs form stable hybrid zones presumably because the hybrids are unfit and do not spread into the parental ranges (5). Interspecific hybrids are not uncommon amongst some closely related *Phytophthora* species (13). These hybrids have been created in the laboratory, and most importantly detected in the field (13). Natural hybrid variant, *P. alni* subsp. *alni* appears to be more aggressive towards the host than either of its parents (9). In the *F. graminearum* clade, Cumagun et al. (6) found that those progeny from a lineage 6×7 cross that produced deoxynivalenol were more aggressive than those that produced nivalenol. Therefore the members of species complex could interbreed to produce a new genome that carries novel combinations of genes for pathogenicity, host range and toxin production.

The disagreement between PSR and BSR will ultimately be resolved by whether the lineages fuse or remain separate in the field. *Fusarium graminearum* is cosmopolitan, found primarily on wheat, maize and barley, but also known from other annual and perennial plants (20). In the midst of an increase in global trade and with >30 countries producing wheat, hybridization between *F. graminearum* species complex members of different lineages will continue to occur. Even if hybrid formation presents no threat to the integrity of the species, there is still the possibility of heightened and more widely distributed variability within the species via introgression (27). As mentioned in the literature review chapter of this thesis every member of the species complex can produce type B trichothecene mycotoxins, and members of lineages 1 to 9 are each associated with at least two chemotypes (Chapter 1 Table 1.2). Therefore it is important to realize that gene exchange could occur between the members of the *F. graminearum* species complex whenever they meet in nature, regardless of species names that we apply.

References

1. Barry, E. G. 1967. Chromosome aberrations in *Neurospora*, and the correlation of chromosomes and linkage groups. Genetics 55:21-32

2. Boutigny, A. L., T. J. Ward, G. J. Van Coller, B. Flett, S. C. Lamprecht, K. O'Donnell, and A. Viljoen. 2011. Analysis of the *Fusarium graminearum* species complex from wheat, barley and maize in South Africa provides evidence of species-specific differences in host preference. Fungal Genetics and Biology 48:914-920

3. Bowden, R. L., and J. F. Leslie. 1999. Sexual recombination in *Gibberella zeae*. Phytopathology 89:182-188

4. Bowden, R. L., Leslie, J.F., Lee, J., and Lee, Yin Won. 2006. Cross fertility of *Gibberella zeae*. Pages 54-59 in: The Global Fusarium Initiative for International Collaboration. T. Ban, J. M. Lewis, and E. E. Phipps, eds., CIMMYT, El Batan, Mexico.

5. Coyne, J., A., and H. A. Orr. 2004. Speciation. Sinauer Associates, Sunderland, MA, U.S.A.

6. Cumagun, C. J. R., R. L. Bowden, J. E. Jurgenson, J. F. Leslie, and T. Miedaner. 2004. Genetic mapping of pathogenicity and aggresiveness of *Gibberella zeae* (*Fusarium graminearum*) toward wheat. Phytopathology 94:520-526

7. Dettman, J. R., D. J. Jacobson, and J. W. Taylor. 2003. A multilocus genealogical approach to phylogenetic species recognition in the model eukaryote *Neurospora*. Evolution 57:2703-2720

8. Dettman, J. R., D. J. Jacobson, E. Turner, A. Pringle, and J. W. Taylor. 2003. Reproductive isolation and phylogenetic divergence in *Neurospora*: Comparing methods of species recognition in a model eukaryote. Evolution 57:2721-2741

9. Érsek, T., and Z. Nagy. 2008. Species hybrids in the genus *Phytophthora* with emphasis on the alder pathogen *Phytophthora alni*: a review. European Journal of Plant Pathology 122:31-39

10. Hausdorf, B. 2011. Progress toward a general species concept. Evolution 65:923-931

11. Hudson, R. R., J. A. Coyne, and J. Huelsenbeck. 2002. Mathematical consequences of the genealogical species concept. Evolution 56:1557-1565

12. Jurgenson, J. E., R. L. Bowden, K. A. Zeller, J. F. Leslie, N. J. Alexander, and R. D. Plattner. 2002. A genetic map of *Gibberella zeae* (*Fusarium graminearum*). Genetics 160:1451-1460

13. Kroon, L. P. N. M., H. Brouwer, A. W. A. M. de Cock, and F. Govers. 2011. The Genus *Phytophthora* Anno 2012. Phytopathology 102:348-364

14. Lee, J., J. F. Leslie, and R. L. Bowden. 2008. Expression and function of sex pheromones and receptors in the homothallic ascomycete *Gibberella zeae*. Eukaryotic Cell 7:1211-1221

15. Lee, J., J. E. Jurgenson, J. F. Leslie, and R. L. Bowden. 2008. Alignment of genetic and physical maps of *Gibberella zeae*. Applied and Environmental Microbiology 74:2349-2359

16. Lee, J., T. Lee, Y.-W. Lee, S.-H. Yun, and B. G. Turgeon. 2003. Shifting fungal reproductive mode by manipulation of mating type genes: Obligatory heterothallism of *Gibberella zeae*. Molecular Microbiology 50:145-152

17. Lee, J., I.-Y. Chang, H. Kim, S.-H. Yun, J. F. Leslie, and Y.-W. Lee. 2009. Genetic diversity and fitness of *Fusarium graminearum* populations from rice in Korea. Applied and Environmental Microbiology 75:3289-3295

18. Leslie, J. F. 1991. Mating populations in *Gibberela fujikuroi* (*Fusarium* section *Liseola*). Phytopathology 81:1058-1060

19. Leslie, J. F. 1995. *Gibberella fujikuroi*: available populations and variable traits. Canadian Journal of Botany 73(s1):282-291

20. Leslie, J. F., and B. A. Summerell. 2006. The *Fusarium* Laboratory Manual. Blackwell Pub., Ames, Iowa. U.S.A.

21. Mayr, E. 1942. Systematics and the origin of species from the viewpoint of a zoologist, *Systematics and the origin of species from the viewpoint of a zoologist.* Columbia University Press, New York, U.S.A.

22. Mayr, E. 1996. What is a species, and what is not? Philosophy of Science 63:262-277

23. O'Donnell, K., H. C. Kistler, B. K. Tacke, and H. H. Casper. 2000. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. Proceedings of the National Academy of Sciences of the United States of America 97:7905-7910

24. O'Donnell, K., T. J. Ward, D. M. Geiser, H. C. Kistler, and T. Aoki. 2004. Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. Fungal Genetics and Biology 41:600-623

25. O'Donnell, K., T. Ward, D. Aberra, T. Aoki, and H. Kistler. 2008. A novel species within the *Fusarium graminearum* complex from Ethiopia detected by a multilocus genotyping assay and molecular phylogenetics. J. Plant Pathol. 90:S3.21

26. Perkins, D. D. 1974. The manifestation of chromosome rearrangements in unordered asci of *Neurospora*. Genetics 77:459-489

27. Perkins, D. D. 1994. How should the infertility of interspecies crosses be designated? Mycologia 86:758-761

28. Perkins, D. D. 1997. Chromosome rearrangements in *Neurospora* and other filamentous fungi. Advances in Genetics 36:239-398

29. Raju, N. B. 1978. Meiotic nuclear behavior and ascospore formation in five homothallic species of *Neurospora*. Canadian Journal of Botany 56:754-763

30. Sarver, B. A. J., T. J. Ward, L. R. Gale, K. Broz, H. Corby Kistler, T. Aoki, P. Nicholson, J. Carter, and K. O'Donnell. 2011. Novel *Fusarium* head blight pathogens from Nepal and Louisiana revealed by multilocus genealogical concordance. Fungal Genetics and Biology 48:1096-1107

31. Starkey, D. E., T. J. Ward, T. Aoki, L. R. Gale, H. C. Kistler, D. M. Geiser, H. Suga, B. Tóth, J. Varga, and K. O'Donnell. 2007. Global molecular surveillance reveals novel *Fusarium* head blight species and trichothecene toxin diversity. Fungal Genetics and Biology 44:1191-1204

32. Steenkamp, E. T., B. D. Wingfield, T. A. Coutinho, K. A. Zeller, M. J. Wingfield, W. F. O. Marasas, and J. F. Leslie. 2000. PCR-based identification of *MAT-1* and *MAT-2* in the *Gibberella fujikuroi* species complex. Applied and Environmental Microbiology 66:4378-4382

33. Taylor, J. W., D. J. Jacobson, S. Kroken, T. Kasuga, D. M. Geiser, D. S. Hibbett, and M. C. Fisher. 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genetics and Biology 31:21-32

34. Turner, E., D. J. Jacobson, and J. W. Taylor. 2010. Reinforced postmating reproductive isolation barriers in *Neurospora*, an Ascomycete microfungus. Journal of Evolutionary Biology 23:1642-1656

35. Wu, C.-I. 2001. The genic view of the process of speciation. Journal of Evolutionary Biology 14:851-865

36. Yli-Mattila, T., T. Gagkaeva, T. J. Ward, T. Aoki, H. C. Kistler, and K. O'Donnell. 2009. A novel Asian clade within the *Fusarium graminearum* species complex includes a newly discovered cereal head blight pathogen from the Russian Far East. Mycologia 101:841-852

Chapter 5 - Final Thoughts

The different definitions for a species influence whether a group of morphologically identical strains deserve species rank or if an already established species needs to be separated into different species. Such is the case with *Fusarium graminearum*. If phylogenetic species recognition (PSR) is used, then *F. graminearum* should been separated into fifteen different phylogenetic lineages, also known as the *Fusarium graminearum* species complex (12). Using the Coyne and Orr (2) quantitative version of biological species recognition (BSR), the experiments I have conducted provide evidence that the members of lineages 1-9 of the *F. graminearum* should be considered a single species. Thus, the PSR and BSR approaches to delimiting species do not give the same conclusion.

Ideally all methods should be considered, as robust species descriptions usually rely on more than just one recognition method. Phylogenetic species concepts may fail if hybridization between members of the different groups occur frequently enough (8). Biological species concepts cannot be applied if the strains involved cannot cross sexually with one another (8). In my opinion, *F. graminearum* is in the early stages of speciation as it has met one of the criteria for species recognition, i.e., genealogical concordance.

Whether the lineages in the *F. graminearum* clade deserve species rank is ultimately a subjective decision. de Queiroz (4) proposed that meeting any of several recognition criterion could justify separate species status. The known reciprocally monophyletic gene genealogies within *F. graminearum* would suffice for de Queiroz to elevate the various lineages to species status. Mayr would probably consider the lineages to be subspecies. Two characteristics emphasize the close relationship of all strains in *F. graminearum*. First, members of all nine lineages are cross-fertile with members of lineage 7. The observed levels of cross fertility within *F. graminearum,* if the strains belong to different species, are quite high. Second, DNA sequencing shows that the species are very similar to each other. The percent sequence similarity between members of lineage 7 and the other lineages ranges from 99.1% to 99.9% (15). Additional cross fertility experiments and progeny fertility assays with more members of the *F. graminearum* species complex would be helpful. This study included one or a few members from each of lineages 1 to 9. Currently, 15 phylogenetic species have been recognized, and

members of lineages 10-15 need to be tested in a similar manner. Testing a larger number of strains from lineages 1-9 would increase confidence in the generalization from work with a few strains to conclusions regarding all of the members of the lineage.

Several methodological advances were essential for the success of my thesis project. The three heterothallic female tester strains (9) used in this study allowed us to collect progeny from only outcrossed perithecia. The segregation of the GFP tag enabled verification that an outcross had occurred. The use of mung bean broth for production of large numbers of macroconidia to be used as spermatia removed previous roadblocks standardized crosses and reduced the time to prepare male inocula from 7 to 4 days.

In the Ascomycota, ascospore production is a good indicator of reproductive success, since the sexual cycle is relatively easy to follow once ascospores are produced (6, 13, 14). The use of settling towers for counting ejected ascospores was a straightforward and objective method to quantify cross fertility in the *F. graminearum* species complex. Finally, measuring perithecial density appears to be a good measure of prezygotic barriers between strains in this group because perithecial density depends on the number of successful fertilizations.

Methodological challenges remain partially unsolved. In Chapter 2 of this thesis, I attempted to identify postzygotic barriers by detecting chromosome rearrangements. A question raised was whether the ejected tetrad counts were biased by poor ballistic performance of tetrads with two or four ascospores. Examining rosettes from excised asci also was not satisfactory. Ascospores in the ascus overlap and some spores cover up one or more of their sister spores, which makes it difficult to observe all of the spores. DAPI staining did not provide enough contrast to distinguish the diverse classes of asci. Performing these analyses was both laborious and time consuming, while also raising questions about possible errors in the interpretation of the data. Perithecial development scoring suffered from similar problems.

Although biogeographic survey data and my outcrossing data support allopatric origins for the lineages, global movement of agricultural commodities has apparently brought the lineages back together in some places. Multiple lineages now occur in the same area, and sometimes on the same hosts, in Nepal (5), South Korea (10), South America (11, 18), Japan (16), and South Africa (1). Therefore, we expect hybrids to be increasingly frequent in those regions with mixed populations of lineages.

Despite the reported frequent sympatric occurrence of lineages, only two natural hybrids between lineages have been reported in the literature (1, 11). One hybrid from Nepal was found to carry alleles from lineages 2 and 6 (11). The sample of twenty-seven *Fg* clade strains was not randomly selected, so the 3.7% hybrid frequency is probably not representative of natural populations. One hybrid from South Africa was found between lineages 3 and 7 (1). The overall frequency of reported hybrids was 1 in 560 (0.18%) in a set of samples from South African wheat, barley, and maize (1).

One possible reason for the low frequency of reported hybrids is that detection methods have been inadequate. The multilocus genotyping (MLGT) assay developed by Ward et al. (17) was used by Boutigny et al. (1). The MLGT uses six genes on two chromosomes. Two genes (*Tri12* and *Tri13*) are tightly linked on scaffold 2 in the middle of chromosome 2. A third locus, reductase, is also on scaffold 2. *MAT1* and *EF1α* are linked on scaffold 5 of chromosome 2. The sixth locus, *Tri101*, is on chromosome 4. Therefore, the middle of chromosome 2, and one locus on chromosome 4 are represented, but the rest of the genome is not sampled in this assay. More powerful methods of detecting hybrids are needed.

Genotyping-by-sequencing (GBS) is an inexpensive, simple, and quick method that could be used for detecting hybrids. GBS uses next generation DNA sequencing technologies to generate tens of thousands of genome-wide markers (7). The existing genomic sequence of *F. graminearum* could be used to order the markers or to develop new linkage maps, if needed. The GBS markers can be used to characterize reference isolates of the lineages, and then can be used to classify unknowns. Due to the density of GBS markers, even small introgressed segments should be easily detected in hybrid strains.

Another possible reason for the low frequency of reported hybrids is that there are important fertility barriers that remain unrecognized. Our data do not support the existence of intrinsic prezygotic or postzygotic barriers between lineages. However, it is possible that there exist significant extrinsic postzygotic barriers such as reduced hybrid fitness. Cumagun et al. showed that progeny from a cross with lineage 6 and lineage 7 parent may be highly aggressive on wheat (3). However, there may be other components of fitness, e.g. competitive saprophytic ability on the host crop debris, effects on developmental phenology, or effects on male or female fertility, that also are important determinants of long-term viability.

As a result of this work, all of these phenotypes are now open to genetic analysis. Future studies can easily create biparental mapping populations between strains with contrasting phenotypes. Genetic maps can quickly be generated using GBS markers. Quantitative trait locus (QTL) analysis can be used to identify loci that affect the trait of interest. Identifying the genetic basis of such traits as host preference may eventually be useful for developing novel forms of genetic resistance in the host.

References

1. Boutigny, A. L., T. J. Ward, G. J. Van Coller, B. Flett, S. C. Lamprecht, K. O'Donnell, and A. Viljoen. 2011. Analysis of the *Fusarium graminearum* species complex from wheat, barley and maize in South Africa provides evidence of species-specific differences in host preference. Fungal Genetics and Biology 48:914-920

2. Coyne, J., A., and H. A. Orr. 2004. Speciation. Sinauer Associates, Sunderland, MA, U.S.A.

3. Cumagun, C. J. R., R. L. Bowden, J. E. Jurgenson, J. F. Leslie, and T. Miedaner. 2004. Genetic mapping of pathogenicity and aggresiveness of *Gibberella zeae* (*Fusarium graminearum*) toward wheat. Phytopathology 94:520-526

4. de Queiroz, K. 2005. A unified concept of species and its consequences for the future of taxonomy. Proceedings of the California Academy of Sciences 56:196-215

5. Desjardins, A. E., and R. H. Proctor. 2011. Genetic diversity and trichothecene chemotypes of the *Fusarium graminearum* clade isolated from maize in Nepal and identification of a putative new lineage. Fungal Biology 115:38-48

6. Dettman, J. R., D. J. Jacobson, E. Turner, A. Pringle, and J. W. Taylor. 2003. Reproductive isolation and phylogenetic divergence in *Neurospora*: Comparing methods of species recognition in a model eukaryote. Evolution 57:2721-2741

7. Elshire, R. J., J. C. Glaubitz, Q. Sun, J. A. Poland, K. Kawamoto, E. S. Buckler, and S. E. Mitchell. 2011. A Robust, Simple Genotyping-by-Sequencing (GBS) Approach for High Diversity Species. PLoS ONE 6:1-10

8. Hausdorf, B. 2011. Progress toward a general species concept. Evolution 65:923-931

9. Lee, J., T. Lee, Y.-W. Lee, S.-H. Yun, and B. G. Turgeon. 2003. Shifting fungal reproductive mode by manipulation of mating type genes: Obligatory heterothallism of *Gibberella zeae*. Molecular Microbiology 50:145-152

10. Lee, J., I.-Y. Chang, H. Kim, S.-H. Yun, J. F. Leslie, and Y.-W. Lee. 2009. Genetic diversity and fitness of *Fusarium graminearum* populations from rice in Korea. Applied and Environmental Microbiology 75:3289-3295

11. O'Donnell, K., H. C. Kistler, B. K. Tacke, and H. H. Casper. 2000. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. Proceedings of the National Academy of Sciences of the United States of America 97:7905-7910

12. O'Donnell, K., T. J. Ward, D. M. Geiser, H. C. Kistler, and T. Aoki. 2004. Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. Fungal Genetics and Biology 41:600-623

13. Perkins, D. D. 1994. How should the infertility of interspecies crosses be designated? Mycologia 86:758-761

14. Perkins, D. D., B. C. Turner, and E. G. Bary. 1976. Strains of *Neurospora* collected from nature. Evolution 30:281-313

15. Sarver, B. A. J., T. J. Ward, L. R. Gale, K. Broz, H. Corby Kistler, T. Aoki, P. Nicholson, J. Carter, and K. O'Donnell. 2011. Novel *Fusarium* head blight pathogens from Nepal and Louisiana revealed by multilocus genealogical concordance. Fungal Genetics and Biology 48:1096-1107

16. Suga, H., G. W. Karugia, T. Ward, L. R. Gale, K. Tomimura, T. Nakajima, A. Miyasaka, S. Koizumi, K. Kageyama, and M. Hyakumachi. 2008. Molecular characterization of the *Fusarium graminearum* species complex in Japan. Phytopathology 98:159-166

17. Ward, T. J., R. M. Clear, A. P. Rooney, K. O'Donnell, and D. Gaba. 2008. An adaptive evolutionary shift in *Fusarium* head blight pathogen populations is driving the rapid spread of more toxigenic *Fusarium graminearum* in North America. Fungal Genetics and Biology 45:473

18. Zeller, K. A., J. I. Vargas, G. Valdovinos-Ponce, J. F. Leslie, and R. L. Bowden. 2002. Population genetic differentiation and lineage composition among *Gibberella zeae* in North and South America. Pages 118 in: 2002 National Fusarium Head Blight Forum Proceedings. S. Canty, J. Lewis, L. Siler, and R. Ward, eds. U.S. Wheat & Barley Scab Initiative.

APPENDIX A – *Fusarium graminearum* species complex strains

Species designation for *Fusarium graminearum* lineages with different collection numbers, host and isolation site used for this study. Lineage designation corresponds to digit after " - "in KSU collection number column.

Taxon (Lineage)	KSU[a]	FRC[b]	FGSC[c]	NRRL[d]	Species	Host/substrate	Origin
F. graminearum (1)	11796-1			28718	*F. austroamericanum*	Corn (*Zea mays*)	Brazil
F. graminearum (2)	11793-2			28436	*F. meridionale*	Orange twig (*Citrus sinensis*)	New Caledonia
F. graminearum (2)	11795-2			28723	*F. meridionale*	Corn (*Zea mays*)	Nepal
F. graminearum (3)	11257-3				*F. boothii*	Corn (*Zea mays*)	Korea
F. graminearum (3)	11791-3			29020	*F. boothii*	Corn (*Zea mays*)	South Africa
F. graminearum (4)	11789-4			25797	*F. mesoamericanum*	Banana (*Musa acuminata*)	Honduras
F. graminearum (4)	11790-4			29148	*F. mesoamericanum*	Grape ivy (*Vitis vinefera*)	Pennsylvania, USA
F. graminearum (5)	11787-5			26752	*F. acaciae-mearnsii*	*Acacia mearnsii*	South Africa
F. graminearum (5)	11788-5			26754	*F. acaciae-mearnsii*	*Acacia mearnsii*	South Africa
F. graminearum (6)	5048-6	R-5469			*F. asiaticum*	Barley (*Hordeum vulgare*)	Japan
F. graminearum (6)	5047-6	R-5470	8631	13819	*F. asiaticum*	Barley (*Hordeum vulgare*)	Japan
F. graminearum (6)	11785-6			13818	*F. asiaticum*	Barley (*Hordeum vulgare*)	Japan
F. graminearum (6)	11786-6			26156	*F. asiaticum*	Wheat (*Triticum aestivum*)	China
F. graminearum (7)	11157-7		9075	31084	*F. graminearum*	Corn (*Zea mays*)	Michigan,USA
F. graminearum (7)	11784-7			5883	*F. graminearum*	Corn (*Zea mays*)	Ohio, USA
F. graminearum (7)	Z3634-7				*F. graminearum*	Wheat (*Triticum aestivum*)	Kansas, USA
F. graminearum (7)	Z3639-7		8630	29169	*F. graminearum*	Wheat (*Triticum aestivum*)	Kansas, USA
F. graminearum (7)	Z3643-7				*F. graminearum*	Wheat (*Triticum aestivum*)	Kansas, USA
F. graminearum (8)	14954-8			31205	*F. cortaderiae*	Pampa grass (*Cortaderia selloana*)	New Zealand
F. graminearum (8)	14953-8			31185	*F. cortaderiae*	Pampa grass (*Cortaderia selloana*)	New Zealand
F. graminearum (8)	14952-8			29306	*F. cortaderiae*	Pampa grass (*Cortaderia selloana*)	New Zealand
F. graminearum (8)	14951-8			29297	*F. cortaderiae*	Pampa grass (*Cortaderia selloana*)	New Zealand
F. graminearum (9)	14947-9			31238	*F. brasilicum*	Oat (*Avena sativa L.*)	Brazil

[a] Kansas State University, Manhattan, KS, USA

[b] Fusarium Research Center, Penn State University, University Park, PA, USA

[c] Fungal Genetics Stock Center, University of Missouri, Kansas City, Missouri, USA

[d] ARS Culture Collection, USDA NCAUR, Peoria, IL, USA

APPENDIX B – *Gibberella zeae* female testers

Species designation for female testers used in the project. Collection numbers, host and isolation site are described.

Taxon (Lineage)	KSU[a]	Host/substrate	Origin
F. graminearum (7)	11626 (Z3639*Δmat1-2*)	Wheat (*Triticum aestivum*)	Kansas, USA
F. graminearum (7)	11628 (H4*Δmat1-2*)	Corn (*Zea mays*)	Kanwon, Korea
F. graminearum (7)	11629 (Z3643*Δmat1-2*)	Wheat (*Triticum aestivum*)	Kansas, USA

[a] Kansas State University, Manhattan, KS, USA

APPENDIX C – Buffers used for staining

1× PBS (Phosphate Buffered Saline)

Dissolve the following in 800ml distilled H_2O:

- 8g of 0.14M NaCl (Sodium chloride)
- 0.2g of 0.0027M KCl (Potassium chloride)
- 1.44g of 0.010M Na_2HPO_4 (Disodium hydrogen phosphate)
- 0.24g of 0.002M KH_2PO_4 (Potassium phosphate monobasic)
- Adjust pH to 7.4
- Adjust volume to 1L with additional distilled H_2O
- Sterilize by autoclaving

PME (Polymerization Buffer)

- 50 mL 0.5M PIPES (1,4-Piperazinebis ethanesulfonic acid)
- 10 mL 0.5M EGTA (Ethylene glycol tetraacetic acid)
- 2.5 mL 1M $MgSO_4$ (Magnesium sulfate anhydrous)
- Adjust pH to 6.7
- Complete to 500 mL with distilled H_2O

Fixation Solution of 4% EM quality paraformaldehyde

Dissolve the following in fume hood:

- 0.6 g of paraformaldehyde
- 15 mL PME
- Dissolve at 68˚C by stirring (~ 45 min)
- Cool to room temperature before use