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A comparison of soil and corn kernel Aspergillus flavus populations: evidence for niche specialization

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A COMPARISON OF SOIL AND CORN KERNEL *ASPERGILLUS FLAVUS* POPULATIONS: EVIDENCE FOR NICHE SPECIALIZATION

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Dedication

I dedicate this thesis to all of my grandparents: Ruth and John Scharff, H. Paul Sweany, Donald O'Harra and Laura Francis Ray Sweany O'Harra, who through their actions or by their memories kept this city girl closely connected to agriculture and the beautiful corn fields in Illinois.

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Abstract

Aspergillus flavus is an opportunistic fungal pathogen that infects peanuts, cotton, corn and tree nuts. *Aspergillus flavus* is a major problem globally due to the production of acutely toxic and carcinogenic aflatoxins. Louisiana climatic conditions lead to annual threats of corn aflatoxin contamination. The purpose of this study was to determine the specific ability of different strains of *A. flavus* to infect corn. Five soil samples and 10 corn ears were collected from each of seven corn fields throughout Louisiana. In addition, Francis Deville of Monsanto Company collected 7, 6, 2, and 4 soil samples and corn ears from four additional fields in Louisiana. Six hundred twelve and 255 *A. flavus* colonies were isolated from the corn and soil samples, respectively. Isolates were characterized by vegetative compatibility groups (VCGs), sclerotia size, aflatoxin B1 (AFB1) production, mating type and 8 simple sequence repeat loci polymorphisms. Eighty-eight percent of corn isolates belonged to two VCGs, whereas only 5% of soil isolates belonged to the same two VCGs. Ninety-five percent of corn isolates did not produce any sclerotia, whereas 56% and 41% of soil isolates produced small and large sclerotia, respectively. The mean AFB1 production on rice for corn kernel isolates was 2314 ± 7455 ppb and 10248 ± 11430 ppb for the soil isolates. Ninety-six percent of corn isolates were in the *Mat1-2* mating type whereas only 52% of soil isolates were *Mat1-2*. SSR fingerprints revealed 26 haplotypes in the corn sample isolates and 78 in the soil sample isolates. All characteristics differed significantly between the soil and the corn kernel populations. Differences between the corn and soil populations indicate that not all soil isolates are as capable of infecting corn and that some isolates have become specialized to infect corn. Further understanding of virulence of *A. flavus* is important for the development of a better biocontrol against toxigenic *A. flavus* and possibly more resistant hybrids of corn.

Chapter 1. Introduction

Aspergillus flavus is an ascomycete fungus that infects many economically important crops including corn, cotton, peanuts and many tree nuts (16). Many strains of *A. flavus* have the ability to produce carcinogenic mycotoxins called aflatoxins which can be acutely toxic. There are four types of aflatoxins produced: aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) (24, 33, 34, 35, 36). AFB1 is the most important aflatoxin due to its greater toxicity. *Aspergillus flavus* is ubiquitous, genetically and phenotypically very diverse (16). The primary source of *A. flavus* inoculum is soil. *Aspergillus flavus* is vectored from the soil either by insects or air to infect the corn ear (16, 27, 42). Airborne conidia land on the silks, colonize the silk and mycelia grow down the silk to the kernels and establish an infection (27). Insect vectors invade the ear and eat corn kernels where conidia on the carapace or in the gut then germinate and establish an infection of the corn kernels (42). The highest concentrations of *A. flavus* in soil are found in fields of highly susceptible crops, but *A. flavus* is still found on forest floors where there are not many known hosts, reflecting its saprophytic ability (16). In a cultivated field, the soil population size of *A. flavus* increases after harvest and during hot, drought events (16, 29). *Aspergillus flavus* has an aggregate or patchy spatial distribution pattern in the soil in a cultivated field (16). Populations of *A. flavus* in northern latitudes have larger proportions of atoxigenic isolates than in southern latitudes of the United States of America (16). Until very recently, *A. flavus* was thought to be an asexual fungus. However, others postulate that the population is too diverse for a population with no genetic recombination and genetic studies reveal the possibility of recombination and two cryptic species (11, 12). *Aspergillus flavus* has a heterothallic sexual cycle with two mating type loci (19, 32).

Aspergillus flavus isolates are characterized in many ways. One of the most common is to determine what types of toxins are produced and or sclerotial size (1, 3, 13, 16, 29, 33, 34, 39). Sclerotia are considered small if the diameter is less than 400 micrometers and large if greater the 400 micrometer (1, 3, 11, 13, 16, 29, 33, 34, 39). *Aspergillus flavus* strains have been characterized as belonging to cryptic species groups I or II based on five gene sequences (11, 12). Group I consists of isolates that produce both large and small sclerotia and if toxigenic, only produce B aflatoxins, while Group II isolates produce small sclerotia and if toxigenic produce both B and G aflatoxins (11). Unfortunately, atoxigenic isolates with small sclerotia cannot be assigned to either group I or II based on sclerotia size and toxin production alone, meaning classification based on sclerotial size and toxin production does not always give any insight into the relatedness of strains (11). Now that a sexual cycle has been identified, *A. flavus* isolates can be characterized by mating type, *Mat1-1* and *Mat1-2* (19, 32). Another classification method is based on vegetative compatibility groups (VCGs) (6, 16, 18, 30). Isolates are in the same VCG, if when paired, and both isolates have the same alleles for all compatibility loci, the hyphae fuse together to form heterokaryons (30). Relatively little research has been conducted on VCGs because determination of VCGs is very labor intense. VCGs differ in sclerotial sizes, mating types and aflatoxin production and have been shown in two studies to differ in their abilities to reduce the amount of aflatoxin produced by a competing toxigenic strain (6, 14, 16, 18, 21, 26). However, isolates in the same VCG tend to produce the same kinds of mycotoxins and have the same sclerotial size and mating type. It should not be surprising that strains in the same VCG would have similar life histories because they represent a quasi-clonal lineage. There is conflicting evidence in support of sexual reproduction between VCGs in nature. Grubisha and Cotty found there is no migration of genes between three VCGs based on 24 Simple Sequence

Repeat (SSR) loci (14). But Horn et. al were able to produce viable sexual offspring from 11 matings between isolates of 9 different VCGs and isolates of 5 different VCGs *in vitro* (19). More work is necessary to determine whether or not sexual reproduction occurs in nature.

Aspergillus flavus is generally thought to be an opportunistic parasite and all isolates are equally capable of infecting crops if the environmental conditions are conducive (16, 38, 42). There is conflicting evidence of specificity between different strains of *A. flavus* and susceptible crops. It is commonly understood that *A. flavus* more readily infects crops than closely related *A. parasiticus*, but it is not thought there is any specificity between *A. flavus* strains and infection frequency of crops (16, 18). A study found there was no difference in the ability of isolates from an array of hosts to infect all the hosts. In the study corn kernels, bean leaves and insects only became infected by all strains of *A. flavus* when the tissues were mechanically wounded (38). *Aspergillus flavus* does not require a wound to infect a crop, so this study does not show if there is any differences in the pathogenicity of strains in all natural growing conditions (16, 27, 38, 42). Also the study was not conducted on fully intact plants, so we cannot know if there is no specificity with growing crops (38). It has been demonstrated that isolates from different crops in Argentina and in Mississippi and Arkansas produce different quantities of aflatoxin indicating the possibility there are different strains of *A. flavus* between the crops, and these different strains of *A. flavus* are better at infecting different crops (1, 39). This may not actually be the case because strains of *A. flavus* can quickly lose their ability to produce aflatoxin in serial plate transfers, so looking at aflatoxin production may not actually differentiate between strains of *A. flavus* (17). There is some evidence supporting specificity between peanuts and VCG (16, 18). In Georgia, a study looking at the VCGs in the soil and peanuts found some VCGs only represented in the soil isolates, and also infrequent VCGs in the soil were more common in the

peanuts (16, 18). Determining specificity for cotton and corn is more complex than peanuts because *A. flavus* has different modes of infection. Peanuts are infected by direct contact of the peg with the soil, while infection of cotton and corn require either an insect vector or airborne dissemination of conidia from the soil (6, 16, 27, 42). Vectored and airborne infections allow the source of the inoculum to come from the surrounding area (16, 27, 42). In Arizona, the VCGs in the soil were compared to the VCGs in cotton seeds (6). The results were not as straight forward as in the peanut study. Some of the VCGs found in cotton isolates were not found in the soil isolates (6). Only two of the VCGs in the cotton isolates were the same as VCGs found in the soil isolates (6). Also, some VCGs in soil were not found in cotton seeds (6). It appears that certain VCGs more specifically infect cotton, because foreign VCGs more readily infected the cotton than VCGs isolated from cotton field soil (6, 16).

The objective of this research was to determine if there is specificity in the infection of corn by different stains of *A. flavus* and to demonstrate two ecotypes of *A. flavus*, saprophytes and facultative parasites. A study was conducted to differentiate soil and corn kernel populations of *A. flavus* from one another based primarily on VCG, secondarily on mating types, aflatoxin B1 production and sclerotia production. Since the soil is the source of inoculum for infection of corn, if all strains of *A. flavus* have the same abilities to infect corn, then the probability of isolates being in the same VCG would be identical for both the soil and corn populations of *A. flavus*. But if the parasitic ability varies between strains, then it should be expected that the probability of isolates in the same VCG would differ between the soil and corn populations. The soil population of *A. flavus* would consist of both saprophytes and facultative parasites and the corn population only facultative parasites.

VCGs can be determined in two ways: complementation of *nit*-mutants and molecular fingerprinting techniques (5, 6, 14, 16, 18, 23, 28, 30). Isolates are induced to form nitrate non-utilization mutants (*nit*-mutants) on chlorate medium (5, 30). The *nit*-mutants are characterized into three different nitrogen utilization classes: *nirA, cnx* or *niaD* mutants (5, 30). Mutants of different classes are paired on starch-modified Czapek Dox medium and if a heterokaryon (characterized by dense sporulation) is formed, the two mutants are in the same VCG (5, 30). This process is very time consuming, so using molecular techniques to classify VCGs were investigated and a Restriction Fragment Length Polymorphism (RFLP) fingerprinting technique was developed (28). RFLP fingerprints are not very reproducible. Ms. Archana Jha, a student worker for Dr. Kenneth Damann, attempted unsuccessfully to use the RFLP technique to fingerprint 82 isolates representing 63 VCGs provided by Dr. Bruce Horn at the USDA-ARS National Peanut Research Laboratory in Dawson, Georgia (23) . Ms. Changwei Huang, a graduate student in Dr. Damann's lab, used simple sequence repeats (SSRs) to produce fingerprints for VCGs (22). Ms. Huang selected 8 SSR loci from a database of SSRs for *A. flavus* isolate NRRL 3357 from Dr. William Nierman of The Institute for Genomic Research (TIGR) in Rockville, Maryland. Eight primer pairs flanking eight SSRs were synthesized. The primers were used to amplify the SSRs in the 82 Geogia isolates. The amplicons were visualized and fingerprints were made for all the isolates. All VCGs produced unique fingerprints. Only two VCGs produced different fingerprints for different isolates in the same VCG. The secondary objective of this research was to determine the robustness of SSR fingerprints for determining *A. flavus* VCGs.

Chapter 2. Materials and Methods

2.1 Sample Collection

In August 2007, to determine whether soil and corn *Aspergillus flavus* populations are different, soil and mature corn ears were sampled from 11 fields in seven parishes along the Red River and Mississippi River alluvial ecoregions in Louisiana. Ten ears of corn and five soil samples were collected from each of seven fields from five parishes (Frogmore in Concordia parish, LSU AgCenter Macon Ridge Research Station and Crowville in Franklin parish, Torbert in Point Coupee parish, Beggs in St. Landry parish, and LSU AgCenter Northeast Research Station and St. Joseph in Tensas parish) as seen in Figure 2.1. Additionally, Francis Deville of Monsanto Co. collected 7 soil and 7 corn ear samples from a field in Belcher, Caddo parish, 6 corn ear and 6 soil samples in Chenyville, Rapides parish, 2 corn ear and 2 soil samples in Batchelor, Point Coupee parish, and 4 corn ear and 4 soil samples in Washington, St. Landry parish.

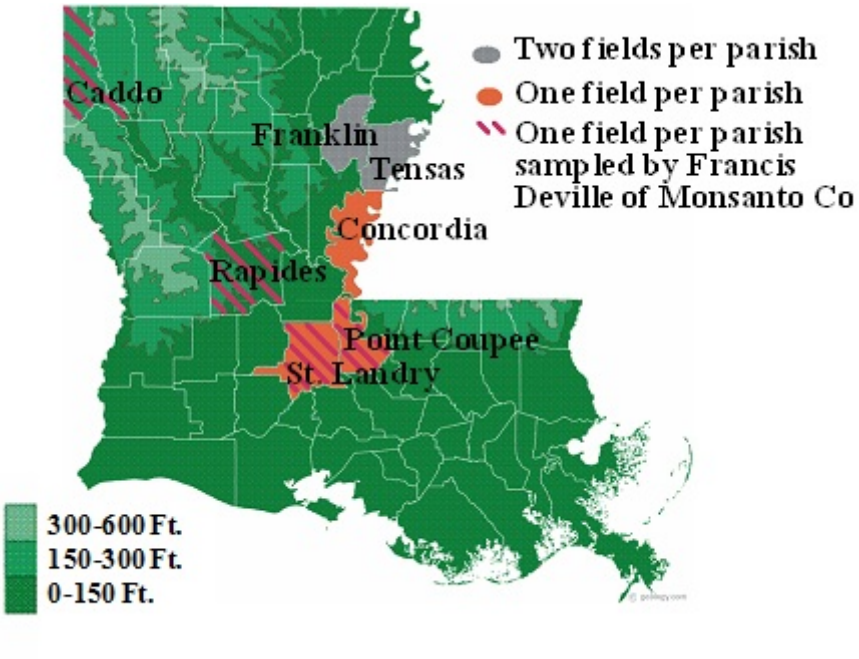

Figure 2.1 Map of corn fields where corn and soil samples were collected.

2.2 Fungal Isolation and Identification

Twenty-five corn kernels from each ear were surface sterilized in a 6% bleach solution and were plated on a *Aspergillus flavus/parasiticus* medium(AFPA) amended with 50 µg/ml hygromycin, 1.5 µg/ml chlorotetracycline, 30 µg/ml streptomycin and, 0.04 µl/ml Avermectin (7, 9, 31). AFPA is a selective and differential medium which suppresses conidiation and when *Aspergillus flavus* or *A. parasiticus* grow, both species produce aspergillic acid that then changes the color of the medium to a bright orange color (7, 9, 31). The 2225 kernels on AFPA medium were incubated at 30°C for 5 days. A plug of mycelium was aseptically removed from the orange medium below each infected kernel. The plug was then transferred to V8 medium containing 0.04 µl/ml of Avermectin.

Fifty grams of each soil sample was suspended in 100 ml of autoclaved distilled water. A 1-ml aliquot of undiluted and two, 1-ml aliquots of a one in ten dilution of the soil suspension were spread on three petri dishes with amended AFPA medium. The soil dilution plates were incubated at 30°C for 5 days. For each soil sample, all orange colonies were transferred onto 0.04 µg/ml Avermectin amended V8 medium from the petri dish with the smallest number of colonies, with a minimum of five colonies. If there were fewer than five colonies on one petri dish, colonies were isolated from multiple plates to obtain five isolates.

Single-conidium colonies were isolated from all the soil and corn kernel isolates by streaking conidia on potato dextrose agar (PDA) and growing over night at 30°C. A single germinating conidium was isolated and plated on V8 medium. The isolates were identified as *Aspergillus flavus* by the presence of smooth, olive-green conidia (25, 34). There were a few isolates that looked similar to *A. flavus*; but the conidia of one type was orange and the other was gray. An isolate with each these characteristics was sent to Dr. Maren Klich at the USDA,

Southern Regional Research Center in New Orleans to be identified. She identified them as *A. alliaceus* and *A. fumigatus*. For colonies confirmed to be *A. flavus*, a conidial suspension was made in 50 parts glycerol: 50 parts water and stored in the refrigerator or freezer.

2.3 Aflatoxin Quantification

All *A. flavus* isolates were grown on rice to quantify the aflatoxin B1 production ability. Five ml of rice were soaked overnight in 5 ml of distilled water in a 20 ml scintillation vial (35). The rice was then autoclaved once for 20 minutes at 20 psi and 121°C. Twenty µl of each isolate spore suspension was added to the vials of rice and the rice was agitated with the pipette tip. The caps were loosely fitted on the vials and incubated for five days at 30°C (35). After five days, the vial was filled with chloroform and soaked overnight to extract the aflatoxin from the rice and fungus. The chloroform extract was then filtered through a Whatman no. 1 100 mm filter paper funnel into a 100 ml glass beaker. The chloroform was allowed to evaporate and the aflatoxin was resuspended in 0.5 ml of a 80 methanol: 20 water mixture. The extract was diluted with 0.5 ml acetonitrile and filtered thru a cleanup column, packed with 200 mg basic Aluminum oxide into an auto-sampler vial (36).

The aflatoxin was then quantified with reversed-phase high performance liquid chromatography using a Summit HPLC System (Dionex Corporation, California) with a P580 pump, ASI-100 automated sample injector, RF2000 fluorescence detector, and Chromeleon software version 6.20 (24). A post-column derivatization step was conducted by exposing the extract to a UV light in a PHRED (Aura Industries Inc., New York) (24). The mobile phase was 22.5 HPLC grade Methanol: 22.5 HPLC grade Acetonitrile: 55 distilled water mixture at 1ml/min. The stationary phase was an Acclaim 120 C18, 3µm, 120Å 4.6X150mm long column (Dionex, California). Aflatoxin B1 was detected at 9.2 minutes. The G toxins were hard to

detect because in the chromatograms the rice substrate created lots of background noise peaks, some of which eluted at the same time as the G toxins peaks.

2.4 Sclerotia Measurement

All of the isolates were grown on 4 ml PDA slants in 15mm X 100 mm test tubes in an ambient light incubator at 30°C for one month. Sclerotia diameters were measured on a Ortholux II compound light microscope (Leitz, Wetzlar, Germany) with the aid of an ocular micrometer. For each isolate the diameters of at least ten sclerotia were measured. Sclerotia larger than 400 μm were classified as large and sclerotia smaller than 400 μm were classified as small (1, 3, 11, 13, 16, 29, 33, 34, 39). Some isolates did not produce sclerotia which was noted. Also some isolates produced sclerotia of both sizes, if only one or two sclerotia was a different size, they were considered the majority size, but if around 50% of the sclerotia were the different size it was noted there was an equal amount of the different sizes.

2.5 VCG Identification

In order to determine the VCG of the isolates, nitrate non-utilization (*nit*) mutants were produced by growing the isolates on chlorate amended Czapek dox medium plates (5, 30). The isolates were incubated at 30°C for at least two weeks or until a *nit*-mutant was produced. The mutants were distinguished by production of fine hyaline mycelia, with no conidial production, while the wild types produce copious conidia. The growing tip of the mutant was transferred to Czapek dox medium (CD) to confirm that a *nit*-mutant was produced (30). The *nit*-mutants produce very few conidia on CD, resulting in nearly hyaline growth (30).

The *nit*-mutants were characterized by the type of nitrogen the mutant could utilize. The sodium nitrate in CD was replaced with either hypoxanthine, ammonium tartrate or sodium nitrite (30). If the mutant did not produce conidia on the hypoxanthine medium then it was

considered a *cnx* mutant. If no conidia were produced on the sodium nitrite medium then it was considered a *nirA* mutant. Finally, if the mutant produced conidia on all three media then it was a *niaD* mutant (30). When two different mutant types are paired on a starch modified CD plate and the hypha fuse and produce a zone of dense conidiation the mutants have formed a heterokaryon and are in the same VCG (30).

In order to determine the VCG groups within this study, the *cnx* and *nirA* mutants of different isolates were paired on starch medium plates. These were chosen for two reasons: *cnx* mutants have been reported to be the best at complementing other *nit*-mutants, and both the *cnx* mutants and the *nirA* mutants are the least likely mutants to be produced (5, 18, 30). The pairings were incubated at 30°C for three weeks. Pairs of mutants which produced dense zones of conidia were placed in the same VCG. There were multiple large VCG groups found; one *cnx* and one *nirA* mutant was selected from each group and these were paired with the remaining *niaD* mutants. Additionally all *cnx* and *nirA* mutants that did not fall into a VCG were also paired with the remaining *niaD* mutants and grown for three weeks. Unfortunately, not all the isolates could be assigned to a VCG. In order to discover if these are singletons or members of a larger VCG, additional types of *nit*-mutants need to be produced for these isolates. Due to time constraints, this was not done, however these isolates are not in the VCGs assigned in the earlier steps.

2.6 Simple Sequence Repeat Fingerprints and Mating Type Loci

SSR fingerprints were obtained for nine randomly selected corn isolates and nine randomly selected soil isolates from all eleven fields. Unfortunately, there were only two isolates from the soil samples from Beggs, St. Landry parish and one of the isolates from the soil in Frogmore, Concordia parish was *A. fumigatus*. In total, 99 out of 612 corn isolates and 91 out of

255 soil isolates were fingerprinted. The fingerprints were obtained primarily to differentiate the soil and corn kernel *A. flavus* populations. Secondarily, fingerprints were used to determine the robustness of using SSR fingerprinting to determine VCG. Additional SSR fingerprints were obtained from 28 isolates, representing cryptic species 1 and 2, provided by Dr. David Geiser at Pennsylvania State University in State College, PA (11, 12).

2.6.1 DNA Extraction

Genomic DNA was extracted from 190 isolates from the corn kernel and soil isolates. Each isolate was grown on a standard 100X15 mm petri dish with potato dextrose agar. All the conidia were then scraped into a 1.5 ml micro-centrifuge tube with 600µl Nuclei Lysis solution (Promega, Wisconsin), ground with a micropestle, and DNA was extracted following the Promega protocol (2). The concentration of DNA was measured with a ND-1000 spectrophotometer (Nanodrop, Delaware) and then all the extracts were diluted to 10 µg/ml with pH 8 TE buffer.

2.6.2 Amplification

Eight simple sequence repeat loci were selected by Changwei Huang using sequence data of *A. flavus* isolate, NRRL 3357, provided by William Nierman at the Institute for Genomic Research in Rockville, Maryland (22). These consisted of three (TTC)n repeats, one (AC)n repeat, one (ACT)n repeat, two (TTA)n repeats, and one (TTTC)n repeat. Forward and reverse primers flanking these loci were used to amplify the SSRs (Table 2.1). Amplifications were done in a Cetus DNA Thermal cycler (PerkinElmer Inc., Massachusetts) using PuReTaq Ready-To-Go PCR beads (GE Healthcare, Buckinghamshire, United Kingdom). The PCR reactions were done according to manufacture's guidelines with a final concentration of 0.24 µM for both the forward and reverse primers for a particular locus and 10 ng of DNA. The PCR program

consisted of: an initial 210 sec. denaturation step at 95°C, 35 cycles of a 15s denaturation at 95°C, a 20 s annealing step at the temperature specified in Table 2.1, and 30 s extension step at 72°C, and final extension at 72°C for 120s, the samples were held at 4°C.

Table 2.1 SSR loci primers and annealing temperatures (22)

Primer	Annealing temperature	Sequence 5'-3'
347-ACT70-F*	51°C	CAAGGTTGGCTAATCGGCA
347-ACT70-R	51°C	TAACAGGCGGTAGCAGAGCA
327-TAA41-F	51°C	TGCCTAAAGCTCCTTCCTCC
327-TAA41-R	51°C	CGGCTGTGTCGGCTATTA
277-TTC32-F	50°C	CAACCCAGGAGTTCTGATGC
277-TTC32-R	50°C	TGCTATCTGCCTTGGAGACG
250-TTC23-F	50°C	GTGGTTCCTGTTTTGCATGG
250-TTC23-R	50°C	CTTTCTTGCCTTAGGCAGTCT
205-TTTC17-F	52°C	CTCTTCTTCGCCGGTCTTGT
205-TTTC17-R	52°C	GCAGTGAGGCCCTTTTCTTG
146-TTC18-F	51°C	GCGACCAGGATAAGCTCAAAG
146-TTC18-R	51°C	ACACGGTGCGAGAGACTTCA
177-TAA18-F	53°C	AGGAGAGGGAACCCAAGTCA
177-TAA18-R	53°C	CATTAAACGGTGCAGGATGGC
123-AC27-F	52°C	ACCCACCTTACCCACACCAAC
123-AC27-R	52°C	CAACCCTGCCAATCTTCCTC

Table 2.1 *347 is the length of the amplified fragment from NRRL 3357, F means forward, ACT70 means the SSR locus for NRRL 3357 is composed of 70 ACT repeats. This formula follows for the remaining primers with R meaning reverse.

The mating types were determined in a multiplex PCR reaction (31). A concentration of 0.5 μM of both the *Mat1-1* and *Mat1-2* forward and reverse primers and 10 ng of gDNA were

added to 0.5 ml tubes of PuReTaq Ready-To-Go PCR beads (GE Healthcare, United Kingdom) and mixed to manufacturer's guidelines. The PCR amplifications were conducted in a Cetus DNA Thermal cycler (PerkinElmer Inc., Massachusetts) with an initial 5 min. denaturation step at 95°C and 40 cycles of 30 s at 95°C, 60s at 54°C and 45s at 72°C.

2.6.3 SSR Band Size and Mating Type Determination

The amplified DNA from the PCR reactions were separated with a Sub-cell Model 192 (Bio-Rad Laboratories, California) agarose gel electrophoresis system. Two 51 sample, 0.75mm thick combs were placed at 1.5 cm and 13cm in a 24.5 X 25.5 X 0.5 cm^3, 3% GenePure Sieve GQA Agarose (ISC BioExpress, Utah) gel made with 0.5 X TBE. Ten µl of DNA for each sample was added to 2 µl 3-EZ- vision DNA dye as loading buffer (Amresco Inc., Ohio) and 5 µl of Ultraclean 20 bp ladder (MoBio Laboratories Inc., California) was added to 2 µl loading buffer and loaded in the gel. Three ladders were run for each row of samples, two at the end positions and one in the middle. Gels were run initially at 150 volts for 15 minutes and then 120 volts for 2.5 hours using a PS250/2.5 amp transphor/electrophoresis DC power supply (HSI Hoefer, California). The dyed DNA bands fluoresced when exposed to UV light and an image was captured by a digital camera in the Gel logic 1500 Imaging system (Carestream Health Inc., New York). Photos of the gels were edited with Kodak Molecular Imaging Software Version 4.5 (Kodak, New York). The sizes of the SSR and mating type bands were determined using BioNumerics version 3.0 (Applied Maths BVBA, Ghent, Belgium). *Mat1-1* mating type diagnostic amplicon is 395 bp and *Mat1-2* is 273 bp. The bands from the 190 isolates and the 28 isolates from Dr. Geiser were added to an already established BioNumerics database from Changwei Huang's work that had 63 VCGs from 82 isolates provided by Dr. Bruce Horn from the USDA, National Peanut Lab in Dawson, GA (11, 12, 22). The bands were assigned to

different size classes or alleles using BioNumerics software and were placed in different groups using the tolerance settings of 0.50% optimization and 1.00% position tolerance. Additionally, the band assignments were double checked by eye and new band classes were assigned as needed. The eight SSR alleles for each isolate were combined and considered a haplotype or fingerprint.

2.7 Analysis

Descriptive statistics, and hypothesis testing were calculated using SAS for Windows version 9.2 (SAS, North Carolina). Multicategory logit generalized linear models were created to compare soil and corn kernel populations with VCG groups, sclerotia groups and aflatoxin B1 groups. Each unique combination of SSR alleles found among the isolates was assigned into a new haplotype. Unbiased haplotype diversities were calculated for both the soil and corn kernel isolates in each field based on the proportion a different haplotypes in a field (36). A value of one means the field is completely diverse and zero means there is no diversity in the field. The formula for unbiased haplotype diversity is $H_e = \{n/(n-1)\}*(1-\Sigma p_i^2)$ where I is the ith haplotype and n is the number of corn kernel or soil isolates within a field (40). Analysis of molecular variance was performed using the differences in SSR bands for all isolates to determine if the soil and corn kernel populations were different using Arlequin version 3.11 (Computational and Molecular Population Genetics Lab, University of Berne, Switzerland) (15, 37, 40). To investigate the robustness of the SSR fingerprinting technique and see if VCGs cluster, Bionumerics software calculated a multidimensional scaling model which calculates the spatial distribution of isolates based on similarities between all isolates. Also Bionumerics software was used to determine if SSR haplotypes were good predictors of VCGs using the Jackknife method of group violation measurement. In the analysis, for every VCG, all isolates were removed from

a VCG, one at a time and then assigned to the VCG it belonged to based on the new average similarities in the truncated group.

Chapter 3. Results

3.1 *A. flavus* Isolation

A total of 867 *A. flavus* colonies were isolated: 612 isolates from corn kernels from 70 corn ears and 255 isolates from 54 soil samples. The frequency of *A. flavus* isolation varied between fields and between soil and corn samples (Figure 3.1).

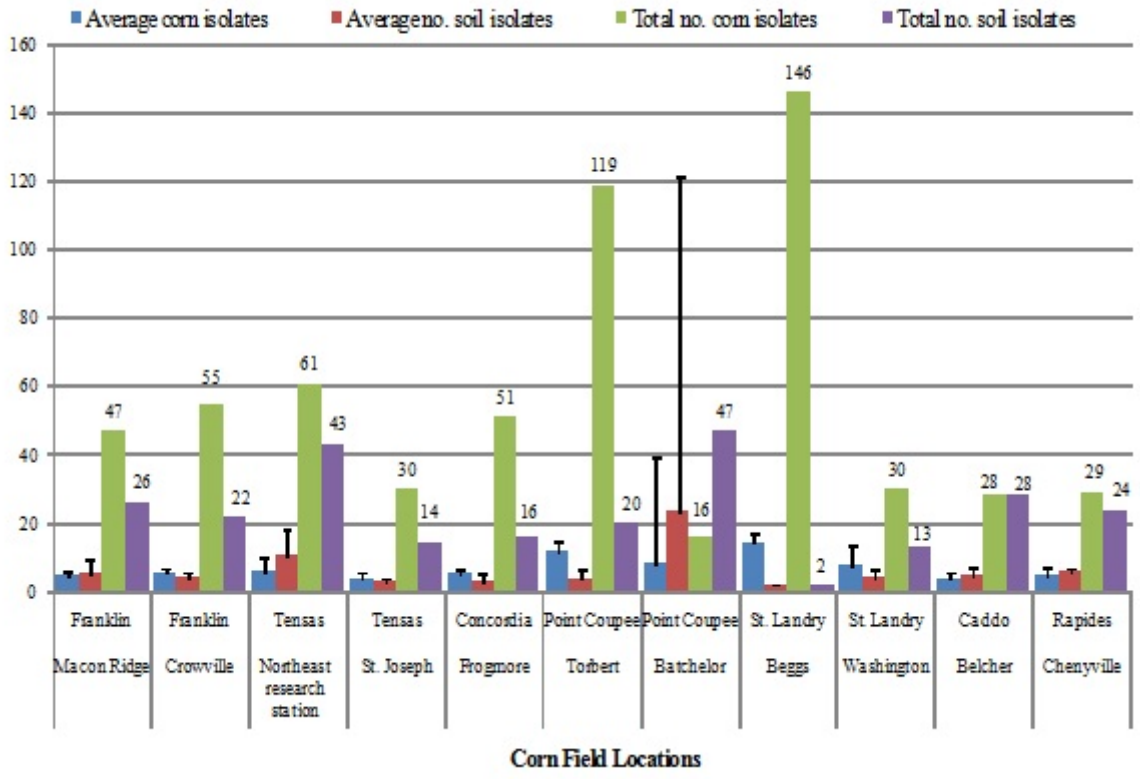

Figure 3.1 Isolation of *Aspergillus flavus* from corn kernels and soil.
Average isolates per corn ear or soil sample within a field with 95% upper confidence limit error bars and total *A. flavus* isolates from corn ear and soil samples from corn fields in Louisiana.

3.2 VCG Groups

Sixteen different vegetative compatibility groups (VCG) were identified. *Nit*-mutants were not obtained from sixteen out of 612 corn isolates and sixteen out of 255 soil isolates.

Additionally, determination of heterokaryon formation between 2 corn isolates and 4 soil isolates with the 16 determined VCGs was not complete due to either lack of growth of the *nit*-mutant or contamination of the *nit*-mutant. For hypothesis testing, it was assumed there was no heterokaryon formation between the six isolates and the VCGs that were inconclusive. Thirty-two out of 594 corn *nit*-mutants and 129 out of 235 soil *nit*-mutants did not form heterokaryons with any *nit*-mutants in the 16 VCG groups. It is unknown whether these isolates are in singleton VCG groups or in multiple different VCGs because they were not tested against each other.

Nine VCGs consisted of isolates with consistent size sclerotia (Table 3.1). VCG 10 and 13 had 5 out of 12 and 2 out of 3 isolates respectively which produced an equal proportion of small and large sclerotia. Isolates in 10 VCGs all produced aflatoxin B1 in the same aflatoxin B1 quantity category (Zero, low (AFB1 $\leq$ 20 ppb), medium (20<AFB1 $\leq$300 ppb), and high (AFB1>300ppb). Different VCGs were found in both single soil and in a single corn ear samples. VCG 1 and 4 were the only VCGs to be found in all 11 fields and accounted for 88% of corn kernel isolates.

Soil isolates were represented in all VCGs whereas corn isolates were only found in six VCGs (Table 3.1). The proportion of soil isolates and corn isolates in the same VCG group varied for each VCG (Figure 3.2).

A multicategory logit generalized linear model was constructed to determine if the probability of isolates in the 16 VCGs differed between the soil and corn kernel *A. flavus* population. Initially a full model was created that accounted for the fields as blocks. The model was not able to converge because the negative Hessian values. The fields were pooled because VCG1 and VCG4 were found in all fields and coefficient of variation (see Table 3.1) for VCG1 and VCG4 were small. Since VCG1 and VCG4 accounted for 88% of the corn isolates it was

Table 3.1 Characteristics of *A. flavus* isolates in 16 vegetative compatibility groups. Coefficient of variation = 100 x standard deviation/mean proportion of isolates in VCG in a sample. Proportions are the number of isolates in category X within VCGX divided by total isolates in VCGX. Small sclerotia are less that 400 µm and large sclerotia are larger than 400 µm. *Proportion of isolates in VCG producing sclerotia is more than one because some isolates produced both large and small sclerotia. Low toxin is AFB1 ≤ 20 ppb. Medium toxin is 20<AFB1 ≤300 ppb. High toxin is AFB1>300ppb.

VCG	Count	CV	# soil	# corn	# fields	prop. no sclerotia	prop. small sclerotia	prop. large sclerotia	prop. no toxin	prop. low toxin	prop. medium toxin	prop. high toxin
1	487	79	4	483	11	0.998	0.002	0	0.054	0.895	0.052	0
2	29	931	29	0	2	0	0	1.00	0	0	0	1.00
3	6	707	6	0	4	0	1.00	0	0	0	0	1.00
4	61	196	5	56	11	0.984	0.016	0	0	0	0.033	0.967
5	11	740	10	1	3	0	1.00	0	0	0	0	1.00
6	5	850	5	0	2	0	1.00	0	0.200	0.800	0	0
7	12	862	2	10	2	0	0.750	0.250	0	1.00	0	0
8	14	479	14	0	4	0	0.929	0.071	0	0.143	0.071	0.786
9	16	464	14	2	4	0.125	0.125	0.750	0	0	0	1.00
10	12	727	2	10	2	0.500*	0.417*	0.417*	0.833	0.167	0	0
11	2	894	2	0	2	0	0	1.00	0	0	0	1.00
12	4	681	4	0	3	0	1.00	0	0	0.250	0	0.750
13	3	1150	3	0	1	0	0.667*	1.00*	0	1.00	0	0
14	2	1150	2	0	1	0	0.500	0.500	0	1.00	0	0
15	3	946	3	0	2	0	1.00	0	0	0	0	1.00
16	2	1150	2	0	1	0	0	1.00	0	0	0	1.00

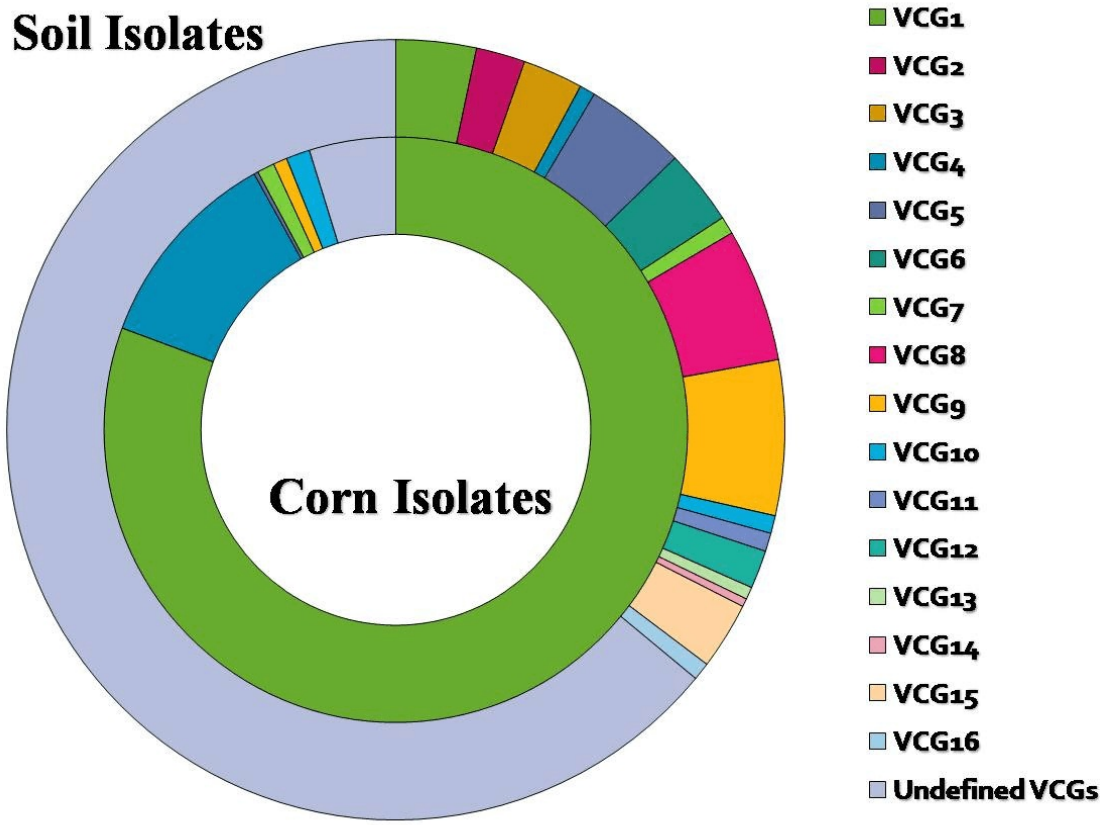

Figure 3.2 Difference in mean proportion of soil and corn kernel isolates in vegetative compatibility groups.

determined the corn population was highly uniform. A second model was created with the pooled fields, this model also failed to converge. This was due to having too many zeros and negative Hessian values. The model was run, removing the least abundant VCG, until the model finally converged when only the top nine VCGs were used in the model (see Table 3.2). The probability of isolates in VCG 1, 2, 3, 4, 5, 7, 8, 9 and 10 varied significantly between the corn and soil populations ($X2=553.41$, d.f. = 8, p-value <0.0001). The same method was used to develop the model for the subsample of 190 isolates for the SSR fingerprint study. The fields

were pooled and the eight most abundant VCGs were used. The probability of isolates in VCGs 1, 2, 3, 4, 5, 8, 9 and 10 differed significantly between the soil and corn population as well (X2=125.25, d.f. = 7, p-value <0.0001).

Table 3.2 Test statistics to differentiate between the soil and corn kernel populations. Test statics to differentiate the corn kernel and soil populations calculated with the proportion of isolates in different VCGs, aflatoxin production groups (zero, low, medium and high), and sclerotia types (none, small and large) and the SSR haplotype diversity in different fields. AIC values are measures of goodness of fit of the model, the smallest AIC is the best model. AIC for haplotype diversity cannot be compared to others because the test statistic is different. ǂ Only VCGs 1, 2, 3, 4, 5, 7, 8, 9, 10 used in model. ɳ Only VCGs 1, 2, 3, 4, 5, 8, 9, 10 used in model. * Not all isolates in the model, only 190 randomly chosen isolates for the SSR study.

Variable	Test statistic	Degrees of Freedom	p-value	AIC
VCGs ǂ	$X^2 = 553.41$	8	<0.0001	1895.4957
AflatoxinB1	$X^2 = 334.79$	3	<0.0001	2376.3063
Sclerotia	$X^2 = 1094.02$	2	<0.0001	1454.2896
Haplotype diversity *	Rst= 0.6033	1	<0.0001	n.a.
VCGs *ɳ	$X^2 = 125.25$	7	<0.0001	522.1333
AflatoxinB1 *	$X^2 = 82.17$	3	<0.0001	595.5433
Sclerotia *	$X^2 = 248.81$	2	<0.0001	456.6368
Mating type *	$X^2 = 110.44$	1	<0.0001	327.1213

3.3 Aflatoxin B1 Production

All isolates of *A. flavus* produced a mean value of 4658 ppb aflatoxin B1 (AFB1) with a standard error of 9526. The mean aflatoxin AFB1 for corn kernel isolates was 2314 ± 7455 ppb and 10248 ± 11430 ppb for the soil isolates. The corn kernel isolates appeared to produce less aflatoxin B1 than the soil isolates, but the AFB1 data did not meet all the assumptions of an ANOVA. Therefore, the aflatoxin values were categorized into four different classes, zero AFB1, low AFB1 greater than zero and less than or equal to 20 ppb AFB1, medium AFB1 greater than

20 ppb and less than or equal to 300 ppb AFB1, and high AFB1 greater than 300 ppb. There was a higher proportion of soil isolates with high levels of AFB1 and there was a higher proportion of the corn isolates with medium, low and no toxin (Figure 3.3). The fields were pooled together and a multicategory logit generalized linear model was made of the proportion of isolates in the different toxin categories and in the soil and corn kernel population. The probability of isolates in the different aflatoxin production groups differed significantly between the corn kernel and soil population (Table 3.2, $X^2 = 334.79$, d.f. = 3, and p-value<0.0001) The probability of isolates in the different aflatoxin production groups also differed significantly between the corn kernel and soil subpopulation for the SSR fingerprint study ($X^2 = 82.17$, d.f. = 3, p-value<0.0001).

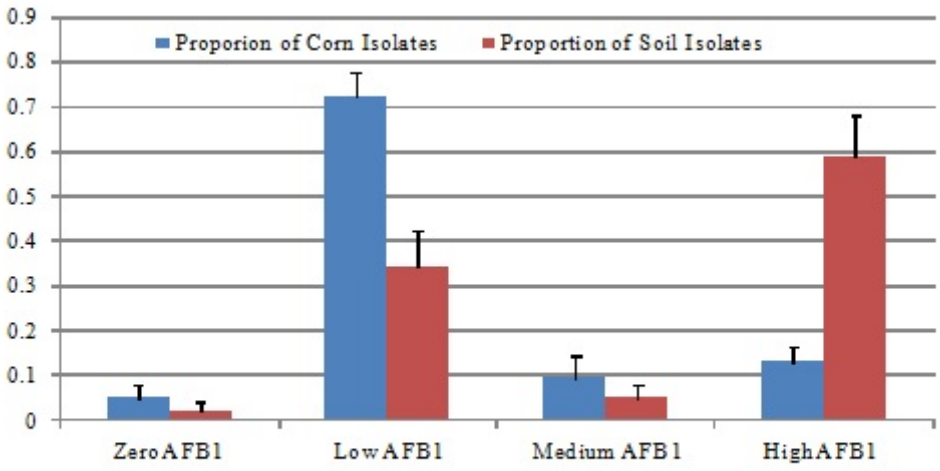

Figure 3.3 Difference in the proportion of soil and corn kernel isolates in different aflatoxin B1 categories. Mean proportion of corn kernel and soil isolates from different samples. The error bars are upper 95% confidence limit. Low AFB1 is AFB1 greater than 0 and less than or equal to 20ppb AFB1, Medium AFB1 is AFB1 greater than 20 ppb and less than or equal to 300 ppb, and High AFB1 is AFB1 greater than 300ppb.

3.4 Sclerotia

All isolates were classified as either having none, small (less than 400 micrometers) or large sclerotia (greater than 400 micrometers) (1, 3, 11, 13, 16, 29, 33, 34, 39). The majority (95%) of corn kernel isolates produced no sclerotia (Figure 3.4) whereas the majority (97%) of soil isolates produced sclerotia (56% small and 41% large sclerotia). The fields were pooled and probability of isolates producing the same size sclerotia varied significantly between the corn kernel and soil populations (Table 3.2, $X^2 = 1094.02$, d.f. = 2, p-vaule<0.0001). The probability of isolates producing the same size sclerotia also varied significantly between the soil and corn kernel population in the subsample used to evaluate the SSR fingerprinting technique ($X^2 = 248.81$, d.f. = 2, p-value<0.0001).

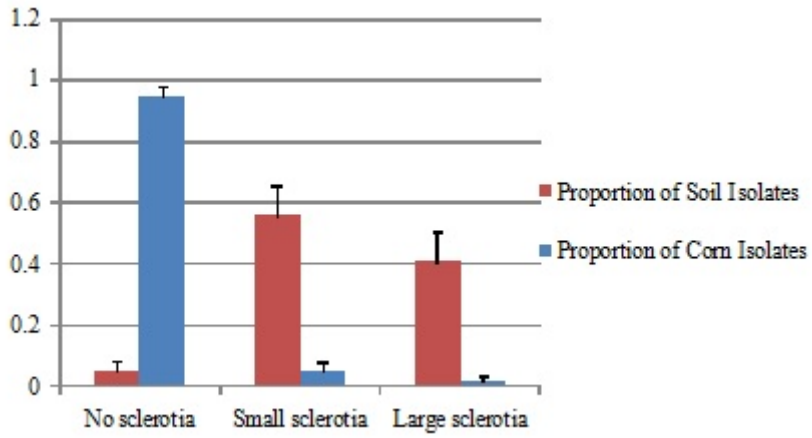

Figure 3.4 Differences in sclerotia production by corn kernel and soil *A. flavus* isolates. Mean proportion of corn kernel and soil isolates with different sclerotia sizes in samples. The error bars are 95% upper confidence limits.

3.5 Mating Types

Ninety-six percent of the 99 isolate, corn-kernel subsample was *Mat1-2* mating type whereas the 91 isolate soil subsample was more evenly distributed between the two mating types (48 % *Mat1-1* and 52% *Mat1-2*) (Figure 3.5). The probability of corn and soil isolates in the

two mating types differed significantly (Table 3.2, X^2= 110.44, d.f. = 1,p-value<0.0001). Each VCG is only represented by one mating type with the exception of VCG1 with only one of the 71 isolates in the *Mat1-1* mating type. Distribution of mating types is less skewed in the sclerotia and aflatoxin B1 production groups.

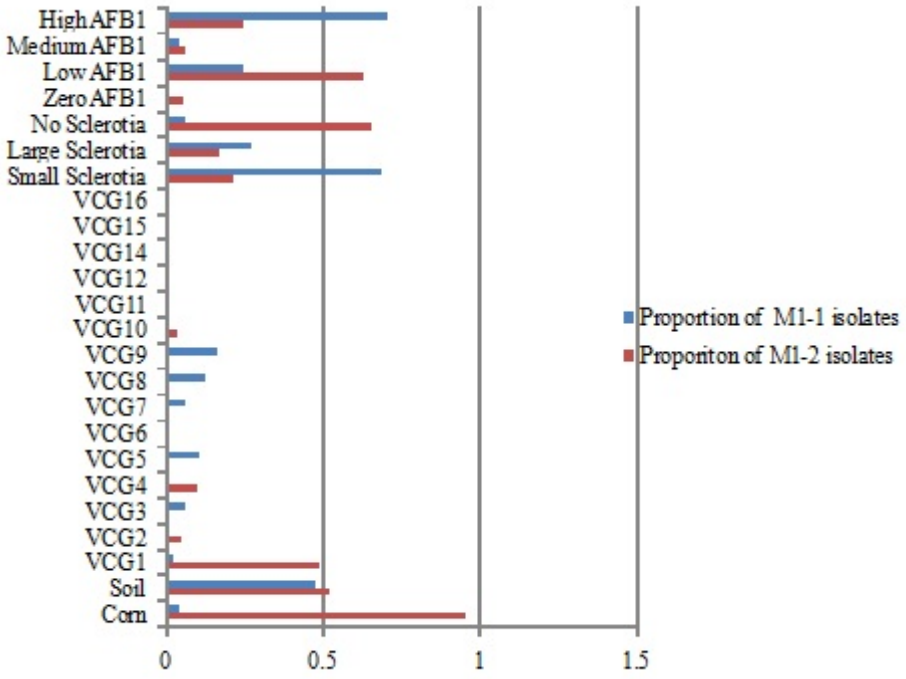

Figure 3.5 Proportion of mating types in soil and corn isolates, VCG, sclerotia, and aflatoxin groups.

3.6 SSR Fingerprints

One hundred and two different haplotypes were found within the 190 isolate subsample of the corn kernel and soil isolate population. Twenty-six haplotypes were found in the corn kernels and 78 haplotypes were in the soil samples and only one haplotype in VCG1 was shared between soil and corn kernel isolates. Multiple haplotypes were found in the fields in both the soil and corn kernel samples (Table 3.3). Within a field, the haplotypic diversities were higher for the soil samples than the corn samples. The mean differences in SSR loci varied significantly between the soil and corn kernel populations (Table 3.2, Rst= 0.6033, d.f.=1, p-value<0.0001).

Table 3.3 Difference in haplotype diversities in soil and corn populations in corn fields.
* Haplotype diversity calculated $H_e = \{n/(n-1)\}*(1-\Sigma p_i^2)$ where I is the ith haplotype and n is the number of isolates from either the soil or corn kernels within a field.

Field	# soil isolates	# of soil haplotype	soil haplotype* diversity	# corn isolates	# of corn haplotype	corn haplotype diversity
Batchelor	9	7	0.9	9	3	0.7
Beggs	2	2	1	9	3	0.7
Belcher	9	6	0.9	9	3	0.4
Chenyville	9	8	1	9	2	0.6
Crowville	9	8	1	9	5	0.8
Frogmore	8	7	0.9	9	3	0.4
Macon Ridge Research Station	9	8	1	9	4	0.8
St. Joseph	9	8	1	9	3	0.7
Northeast Research Station	9	9	1	9	8	1
Torbert	9	9	1	9	4	0.6
Washington	9	9	1	9	4	0.6

Each VCG with more than one isolate had more than one haplotype (Table 3.4). The different mating types, toxin and sclerotia groups were all represented by multiple haplotypes. For each VCG the number of polymorphisms in each SSR locus may vary. Out of the 102 only 1 haplotype was shared by two VCGs. This haplotype only consisted of two isolates, one was in VCG16 and the other was not in VCG 16 , but the actual VCG was undetermined. When the 28 isolates representing cryptic species I and II were added into the database, all isolates were represented by 28 different haplotypes. Isolate 14-1 shared a haplotype with six isolates from the soil and corn kernels. Four of the isolates were in VCG4 and two were of unknown VCG because a *nit*-mutant was not obtained.

Table 3.4 Number of genotypes, haplotypic diversities and SSR polymorphisms in each VCG, Sclerotia type and Toxin group. Haplotypic diversity calculated $H_e = \{n/(n-1)\}*(1-\Sigma p_i^2)$ where I is the ith haplotype within a category.

				Number of polymorphisms in SSR locus							
Category	N	# haplotype	haplotype diversity	SSR 123	SSR 146	SSR 177	SSR 205	SSR 250	SSR 277	SSR 327	SSR 347
VCG1	71	12	0.76	6	2	2	3	2	2	3	4
VCG2	7	5	0.9	2	1	2	2	3	2	2	2
VCG3	3	3	1.0	2	1	1	1	1	1	2	2
VCG4	14	8	0.87	4	2	1	2	2	1	3	1
VCG5	5	4	0.9	3	2	1	1	2	2	2	2
VCG6	2	2	1.0	2	1	1	1	1	1	2	2
VCG7	3	3	1.0	2	1	1	1	2	2	1	1
VCG8	6	5	0.9	2	2	2	3	3	2	3	3
VCG9	8	5	0.8	3	2	1	2	2	2	2	3
VCG10	5	1	0	1	1	1	1	1	1	1	1
VCG11	1	1	n.a.	1	1	1	1	1	1	1	1
VCG12	2	2	1.0	2	1	1	1	2	2	2	2
VCG13	0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
VCG14	1	1	n.a.	1	1	1	1	1	1	1	1
VCG15	2	2	1	1	1	1	2	1	2	2	1
VCG16	2	2	1	2	1	2	1	1	1	1	1
Zero AFB1	8	3	0.64	4	3	2	3	1	2	3	3
Low AFB1	102	41	0.88	15	3	4	4	5	4	5	11
Medium AFB1	11	10	0.98	7	4	4	5	3	2	6	6
High AFB1	69	51	0.98	15	9	6	11	10	7	12	15
No sclerotia	96	24	0.85	11	7	3	5	5	5	8	8
Small sclerotia	64	55	0.99	14	7	6	7	9	6	13	18
Large sclerotia	37	30	0.99	12	6	5	9	7	7	10	13
Mat1-1	48	41	0.99	14	6	6	11	9	6	12	13
Mat1-2	142	62	0.93	19	7	6	11	8	7	10	14

Multidimensional scaling (MDS) analysis arranged isolates in 3-dimensional space based on the similarity matrix using all 8 SSR loci. Similarity coefficients range from 1 to zero, with zero being the most similar and one the most different. VCG 1, the green dots, had multiple clusters and in two VCG 1 clusters they were closely associated with VCG 4, the yellow dots (Figure 3.6). Also VCG 7(orange), 9 (olive-green) and 10 (white) each had only one cluster which were separate from one another. VCG 1, 2, 3, 5, 6, and 8 all consisted of at least one isolate that was very different from the others and not located in the same space.

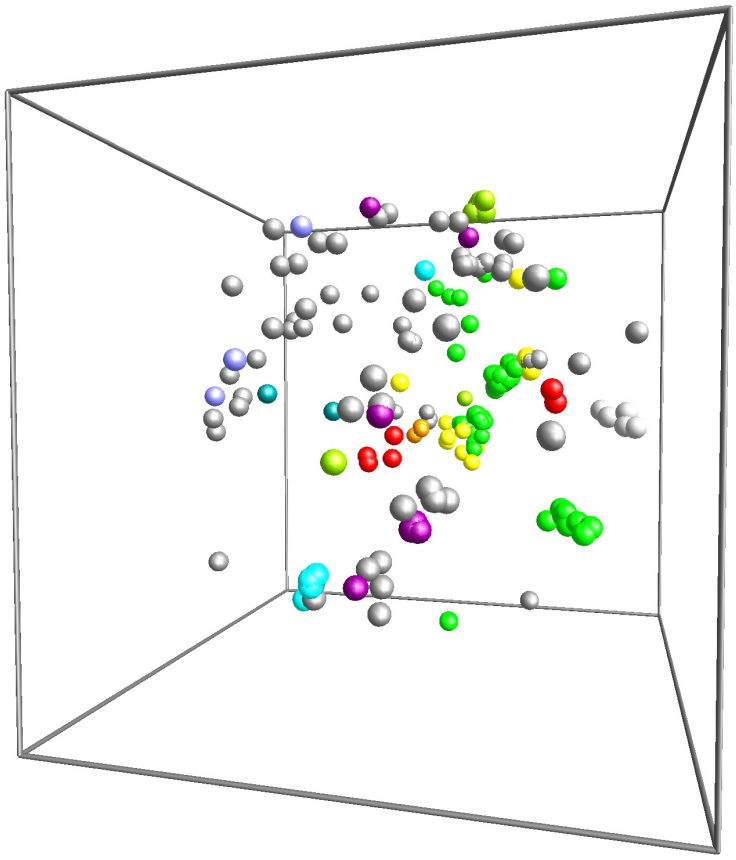

Figure 3.6 Multidimensional space model of VCG groups based on similarities of all eight SSR loci between only corn and soil isolates. Light green circles = VCG1, red = VCG2, blue = VCG3, yellow = VCG4, light blue = VCG5, blue-green = VCG6, orange = VCG7, purple = VCG8, olive-green = VC9, white = VCG10, gray = VCG 11, 12, 14, 15, 16 and unknown VCG.

Jack-knife group separation analysis takes one isolate within a VCG based on *nit*-complementation out at a time and recalculates the average similarities of all the VCGs based on the 8 SSR loci then determines which VCG the isolate should be reassigned to based on how similar it is to the new groups. The result of the group separation analysis showed that when an isolate was removed from *nit*-complementation VCGs 2, 3, 6, 7, 10, 11, 14, 15, 16 it reassigned into the same VCG based on SSR loci one hundred percent of the time (Table 3.5). The remaining VCGs had isolates that were reassigned to different VCGs; only VCG 5 and 9 had the majority of its isolates reassigned to the proper VCG. Even though it appeared that VCG1 and VCG4 to overlap in the MDS (Figure 3.6), isolates of both VCG1 and VCG4 were not reassigned to each others' group, instead the isolates from both were reassigned to VCG2, VCG7 and VCG14.

Table 3.5 VCG group separation matrix based on 8 SSR loci. Row headings are VCGs based on *nit*-complementation and column headings are VCGs based on 8 SSR similarities. The values in each column are the percent of isolates in the VCGX that are assigned to the VCGY after Jackknife analysis of the average similarities within VCGX.

	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[14]	[15]	[16]
[1]	23.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
[2]	4.2	100	0.0	28.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
[3]	0.0	0.0	100	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
[4]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
[5]	0.0	0.0	0.0	0.0	80.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
[6]	0.0	0.0	0.0	0.0	0.0	100	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
[7]	46.5	0.0	0.0	57.1	0.0	0.0	100	0.0	12.5	0.0	0.0	0.0	0.0	0.0	0.0
[8]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
[9]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	75.0	0.0	0.0	0.0	0.0	0.0	0.0
[10]	18.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100	0.0	0.0	0.0	0.0	0.0
[11]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100	0.0	0.0	0.0	0.0
[12]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	50.0	0.0	0.0	0.0
[14]	5.6	0.0	0.0	14.3	20.0	0.0	0.0	28.6	0.0	0.0	0.0	50.0	100	0.0	0.0
[15]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100	0.0
[16]	1.4	0.0	0.0	0.0	0.0	0.0	0.0	71.4	12.5	0.0	0.0	0.0	0.0	0.0	100

Chapter 4. Discussion

The objective of this research was to determine if there are two ecotypes of *A. flavus*: saprophytes and facultative parasites. Soil is the source of inoculum for infection of corn, therefore soil should have both *A. flavus* saprophytes and facultative parasites and corn should only have *A. flavus* facultative parasites. The probability of finding the same VCGs in the soil and corn varied between the soil and corn *A. flavus* populations giving evidence for two ecotypes and niche specialization. Isolates within VCGs varied in their production of aflatoxin and sclerotia. The soil and corn *A. flavus* populations differed in the sclerotia sizes, aflatoxin B1 production, mating types and 8 SSR loci haplotypes. Even though these variables differentiated the soil and corn kernel *A. flavus* populations these metrics are not as good as VCGs for comparing strains of *A. flavus*. The secondary objective of this research was to determine if the 8 SSR loci selected by Changwei Huang correctly determine VCGs (22). Unfortunately, the 8 SSR loci haplotypes did not predict VCG.

Two studies, one on peanuts and the other on cotton, indicate that VCGs varied between soil *A. flavus* isolates and cotton and peanut *A. flavus* isolates, demonstrating VCGs have different specific abilities to infect peanuts and cotton (6, 18). Like peanuts and cotton, the VCG assemblages varied significantly between soil and corn populations. Sixteen VCGs were identified for 235 soil *nit*-mutants and six of these VCGs were found in 594 corn kernel *nit*-mutants. Since the soil *A. flavus* population consisted of all sixteen VCGs it indicated the soil is composed of both facultative parasites as well as saprophytes, whereas the corn population only consisted of the facultative parasite subset of VCGs. Only two VCGs had similar between the corn and soil; VCGs 7 and 10 were both 0.85% of soil *nit*-mutants and 2.5% of corn *nit*-mutants. Otherwise the VCGs were different between the soil and corn population. The four most

abundant VCGs (VCG 2, 5, 8 and 9) in the soil were isolated 29, 10, 14 and 14 times respectively and not as readily isolated from the corn kernels (0, 1, 0 and 2 isolates). Previous studies characterize *A. flavus* isolates in the soil and indicate these isolates are potential threats to contaminate the crops (29, 33). But the fact that abundant VCGs in the soil are not as frequently found in the crop shows that soil isolates will not necessarily be capable of infecting corn and are not predictive of which *A. flavus* strains will be a threat to crops. The majority of corn kernel *nit*-mutants consisted of two VCGs; 81% of corn kernel *nit*-mutants were VCG1 and 9% of corn kernel *nit*-mutants were VCG4. These two VCGs were much less abundant in the soil; 1.7% of soil *nit*-mutants were VCG1 and 2.1% of soil *nit*-mutants were VCG4. The two VCGs were isolated from corn kernels in all eleven fields whereas they were only isolated from soil samples in 3 fields: one field with VCG1, one with both VCG1 and 4, and one with VCG4. The fact that these two VCGs were so abundant in corn indicated they are better adapted to live in the corn niche than other VCGs. The data from VCGs indicates there are two ecotypes present in the soil and only facultative parasites in the corn, and some VCGs have become highly specialized to inhabit the corn niche.

Previous work has demonstrated that VCG groups consist of isolates with the same sclerotia and aflatoxin production phenotypic characteristics as well as the same mating type (6, 14, 16, 18, 19, 26, 28, 42). All isolates within the same VCG also had the same mating type locus. There was one isolate in VCG1 that had a different mating type than the remaining 70 isolates; this was probably due to a contaminate because this was the only isolate to produce a sclerotium and the SSR haplotype was very different from the others. Isolates within the VCGs in this study produced similar quantities of aflatoxin B1. But isolates within VCGs 1, 4, 7, 8, 9, 10, 13 and 14 produced different sizes of sclerotia. In previous work sclerotia sizes are

considered to be large (greater than 400 micrometers) or small (less that 400 micrometers) (1, 3, 11, 13, 16, 29, 33, 38, 42). VCGs 10 and 13 had 5 out of 12 and 2 out of 3 isolates, respectively, which produced an equal proportion of small and large sclerotia. This indicates that classifying isolates as producing small or large sclerotia does not account for all the phenotypic variability between strains of *A. flavus*.

Previous *A. flavus* population studies characterize isolates by the type and amount of aflatoxin production and the production of large or small sclerotia (1, 3, 13, 16, 29, 33, 39, 42). Populations of *A. flavus* differed between regions and substrates in abilities to produce aflatoxin and sclerotia (3, 13, 16, 33, 39, 42). One study showed corn *A. flavus* population produced statistically different amount of aflatoxin than the combined aflatoxin production of peanut, rice and soil isolates (1). The study also showed the corn population was no different in sclerotial production than soil and peanut isolates. The study did not have enough isolates to determine if the soil and corn populations were different based on toxin production and showed the populations produced similar size sclerotial. Sclerotia and toxin production are not ideal characteristics to show the differences between populations. Geiser showed large and small sclerotia are not phylogenetically related characteristics by comparing 3 genes of 28 different *A. flavus* isolates (11). Therefore small or large sclerotia sizes do not show if isolates within a population are related, making it hard to compare populations of *A. flavus*. Also, the genes for aflatoxin production are located in the sub-telomeric region of chromosome III leading to high mutation rates and large variability in aflatoxin synthesis (8, 17, 41). Aflatoxin synthesis has been shown to be quickly lost after serial transfers on PDA, so the aflatoxin quantification of isolates may not represent what the isolate produced in the field, additionally closely related isolates may have very different aflatoxin synthesis abilities (16). In spite of these concerns, the

soil and corn kernel populations varied significantly in the aflatoxin B1 production and sclerotial production. Based on the smallest AIC values (Table 3.2), the best model for predicting the difference between the soil and corn populations is the sclerotia size rather than VCG or aflatoxin production. In spite of this, a closer examination of these differences reveals that the difference in sclerotia and aflatoxin production between the two populations was a result of the absence of sclerotia in VCG1 and VCG4 and 90% of VCG1 and 80% of VCG4 isolates produced low levels of aflatoxin in the corn. These two VCGs accounted for 90% of the corn *nit*-mutants and accounted for only 4% of the soil *nit*-mutants.

Eight SSR loci and mating type loci were also used to compare the soil and corn kernel populations (22). The SSR loci haplotypes were very different between the soil and corn kernel populations. Only one haplotype was shared between the soil and corn kernel isolates. This was a haplotype for VCG1 and was represented by one soil isolate and 12 corn kernel isolates. There were 26 different haplotypes out of 99 corn kernel isolates and 78 different haplotypes out of 91 soil isolates. Corn isolates haplotypic diversity was smaller than soil isolates in the in every field. The fact that only one haplotype was shared between the corn and soil isolates and the soil isolates were more diverse suggests that the soil and corn kernel populations are different and that isolates in the corn have become specialized to infect the corn. The distribution of mating type 1-1 and mating type 1-2 loci was different between the soil and corn kernel isolates of *A. flavus*. Much like the aflatoxin and sclerotia production the difference in mating types was directly related to the predominance of VCG1, 4 and 10 in the corn kernel population. All the corn kernel isolates in VCG1, 4 and 10 were 1-2 mating type and these accounted for 87% of the corn kernel sub-sample isolates and only 3% of the soil sub-sample isolates.

The 8 SSR loci are poor predictors of VCGs. Many isolates from VCG1 and VCG4, the most common VCGs, clustered together in the multidimensional scaling analysis based on the 8 SSR similarity matrix (Figure 3.6). Also 47% of VCG1 and 57% of VCG4 isolates, were reassigned into VCG7 instead of being reassigned into VCG1 or VCG4 in the jackknife analysis based on the 8 SSR loci similarity matrix. Additionally isolates from VCG1 were only reassigned to VCG1 24% of the time and isolates from VCG4 were never reassigned to VCG4. This indicated the SSR haplotypes within a VCG were diverse and very similar to haplotypes of isolates from other VCGs making it impossible to predict VCG based on SSR haplotypes. This presents the need to identify better genetic markers to compare *A. flavus* populations. SSR loci, even if good predictors of VCGs, are not good genetic markers due to their high mutation rates (4). The mutation rates are high in SSR loci because of DNA replicase slippage, this slippage will lead to either the addition of repeats or the truncation of multiple repeats during DNA replication in mitosis. Currently there are no good population genetic models that account for the strange mutation rates of SSRs (4). This makes it hard to understand gene flow within a population and to be able to investigate, populations differentiation, migration and genetic drift of alleles (4). Step-wise mutation rates of single nucleotide differences are much better understood and have been incorporated into population genetics models, so better genetic markers would consist of genes with single nucleotide differences (4).

The corn kernel populations and soil *A. flavus* populations are very different in the composition of VCGs, SSR haplotypes, sclerotial and aflatoxin B1 production. The best metric to compare the populations is VCG. This due to the fact the isolates within a VCG are genetically related because they must share the same het loci allele (6, 16, 18, 19, 28, 30). The use of small and large sclerotia is not preferable for three reasons: 1. small and large does not

account for all phenotypic variability among isolates, 2. small and large sclerotia trait does not hold up phylogenetically and 3. in this study the difference in sclerotia between the corn and soil populations was due to predominance of two VCGs. Aflatoxin production is not a good trait because it is a highly mutable trait that can be very different between closely related isolates, and in this study the difference was due to the predominance of two VCGs in the corn kernels. The mating type loci are not good parameters to compare populations because they do not account for enough difference between isolates as is seen by the fact the difference in the two populations was due to the predominance of VCG1 and 4 in the corn kernel isolates which both had the same mating type locus. Finally, the 8 SSR haplotypes are not good genetic markers to differentiate the populations because the mutation rates are too high and they cannot distinguish between the different VCGs. The difference between VCGs in the soil and corn population indicate there are two ecotypes of *A. flavus*: saprophytes (present in the soil) and facultative parasites (present in the soil and corn). The fact that different VCGs are more common in the corn than other VCGs also indicates that some VCGs are more adept at inhabiting the corn niche. This is supported by recent studies. Two studies have demonstrated that different VCGs have differing abilities to inhibit the toxin production of other toxigenic *A. flavus* isolates (21, 26). Also it has been demonstrated using real time PCR that during the intraspecific aflatoxin inhibition between different VCGs some VCGs actually grow more than others in corn kernels (26).

More work needs to be done to understand why different VCGs were better at infecting the corn. Possibly a dose dependent field study can be conducted. In this study corn plants would be exposed to the same concentrations of a VCG widely found in the soil and not found in the corn and another VCG that was widely found in the corn and see if there are difference in the amount of corn kernels infected by the different VCGs. Also better genetic markers need to

be identified to compare the populations. Now that *A. flavus* genome has been sequenced maybe better genetic markers could be putative *het* genes and pathogenicity genes (8, 10, 41). Oxylipin-generating dioxygenase mutants have been shown to affect the pathogenicity of *A. flavus* (20). Perhaps sequencing oxylipin-generating dioxygenases, other pathogenicity genes, or het genes would differentiate the isolates widely found in the corn and ones found in the soil (8, 10, 20, 41). The first documented cases of aflatoxin poisonings were in the 1960s. It would be interesting to find if there were differences in any of these genes, and how long ago the genes diverged. Possibly *A. flavus* did not infect crops before this time or perhaps aflatoxin poisoning has been a long unrecognized problem.

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Appendix A. A Field View of *A. flavus* VCG Assemblages

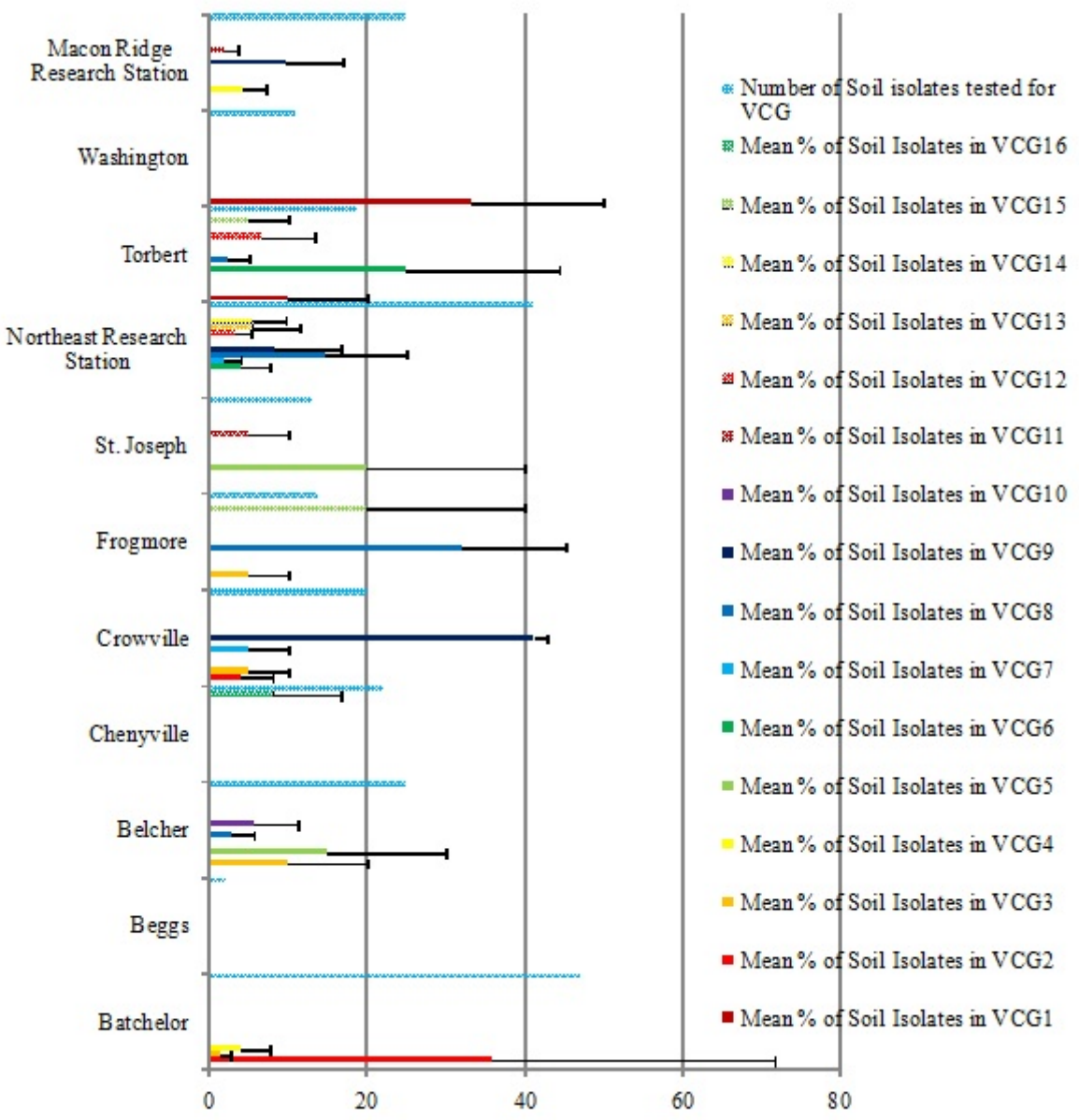

Figure A.1 Different VCG assemblages in soil *A. flavus* isolates collected for different corn fields. Large standard error bars around the mean percentage of isolates in a VCG in each field revealed that VCG assemblages were very different between the soil samples within a field.

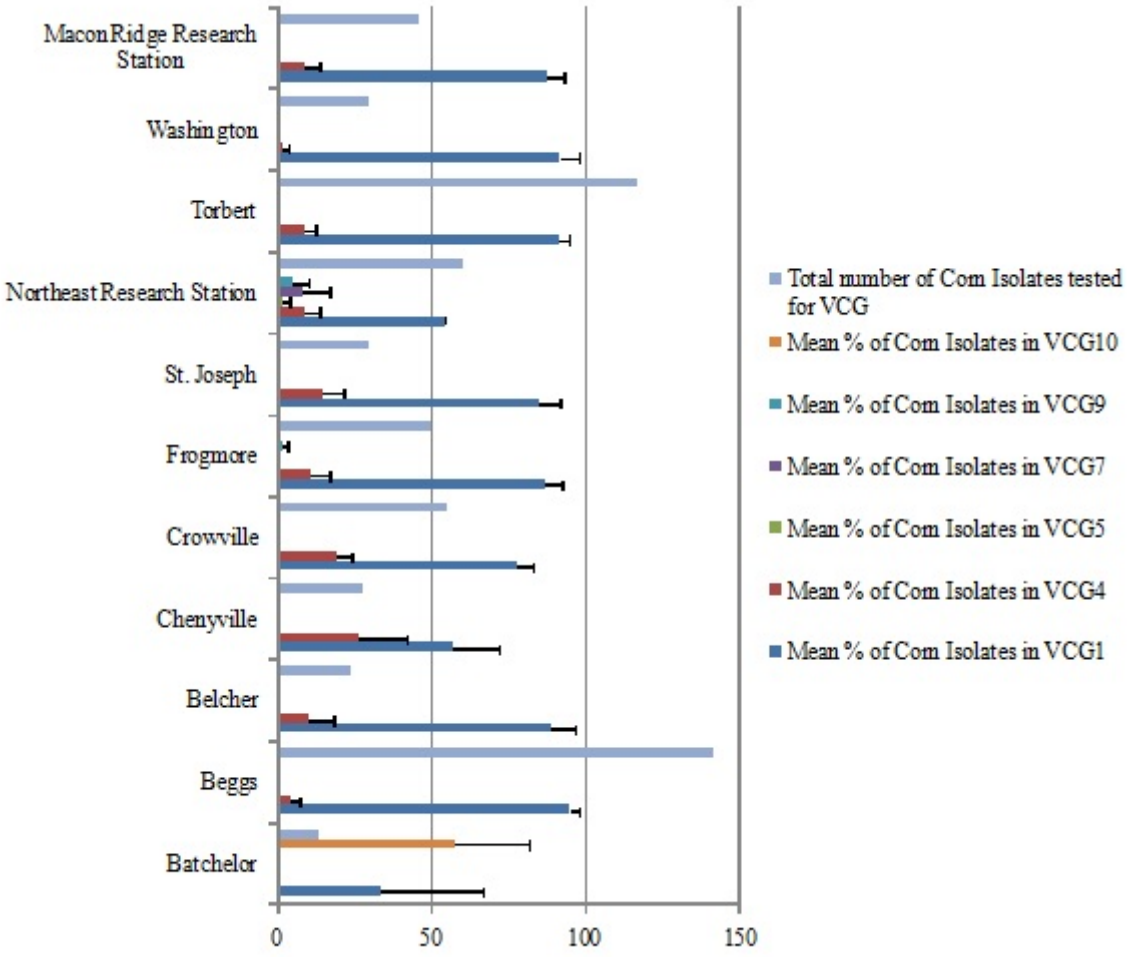

Figure A.2 Similar VCG assemblages in corn kernel *A. flavus* isolates from eleven different corn fields in Louisiana. Small standard error bars about mean percentage of isolates in VCG1 in most fields revealed that there was an similar distribution of isolates in VCG1 in corn ears samples for most fields. VCG 2, 3, 6, 8, 11, 12, 13, 14, 15 and 16 were not found in any of the corn kernel isolates but were found in the soil isolates.

Appendix B. A Detailed Look at *A. flavus* SSR Loci Fingerprints

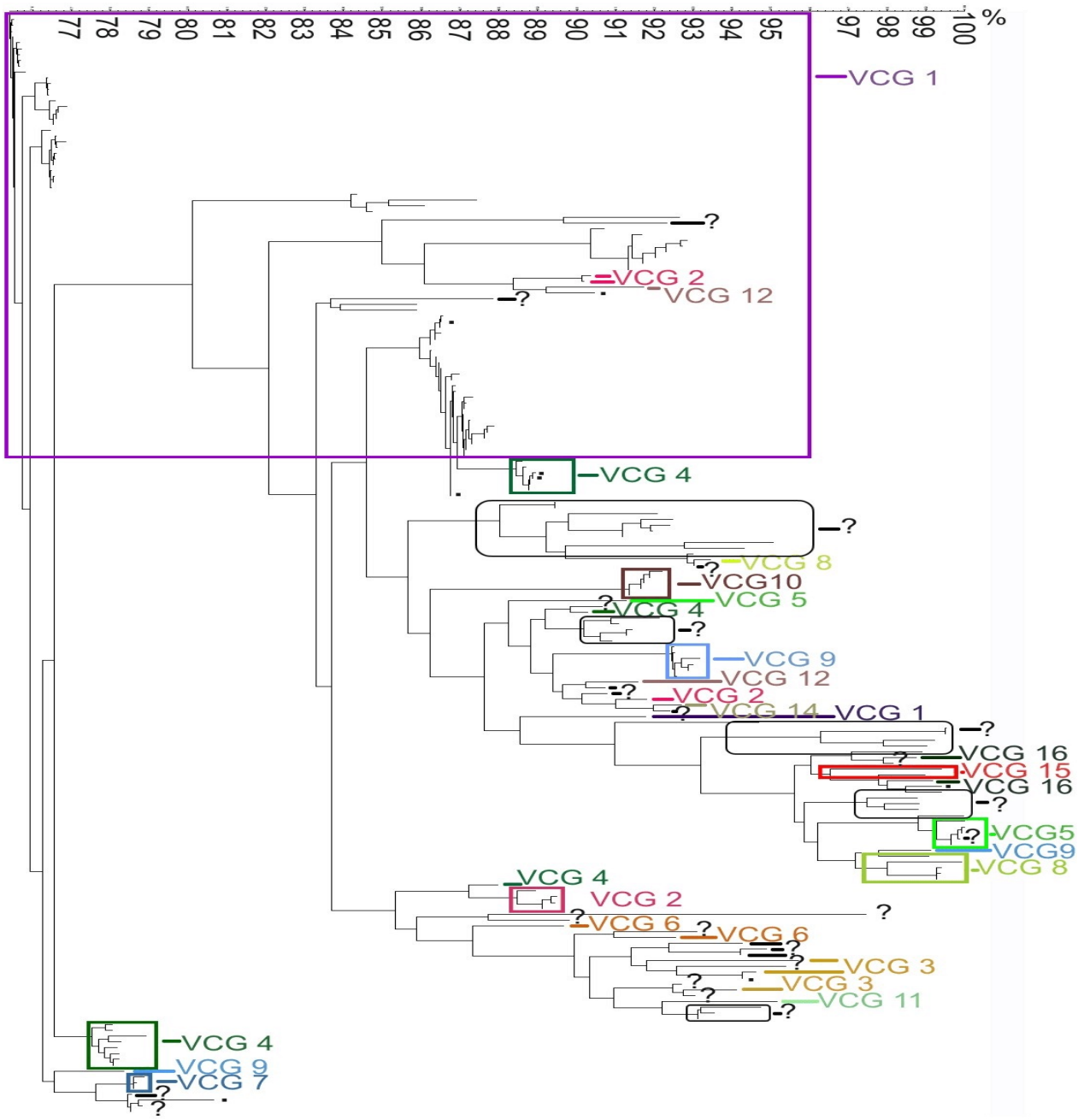

Figure B.1 Neighbor Joining tree of corn and soil isolates constructed based on SSR fingerprint haplotypes. Isolates are identified by their VCG. ? are isolates that did not fuse with VCG 1- 16 and VCG has not be defined and . are isolates where no nit-mutant was obtained, so VCG information is unknown. The ruler measures the percent similarity between isolates based on SSR fingerprints.

Table B.1 One-hundred two SSR haplotypes for 190 *A. flavus* isolates from soil and corn ear samples.

			SSRhaplotypes are the molecular weights for each SSR locus							
# Isolates	Source	VCG	SSR 123	SSR 146	SSR 177	SSR 205	SSR 250	SSR 277	SSR 327	SSR 347
1	Corn	1	118	125	156	179	175	213	287	173
13	12 Corn 1 Soil	1	253	125	156	179	175	213	287	420
1	Corn	1	253	125	156	188	175	213	287	420
1	Soil	1	263	125	156	179	167	213	287	343
1	Corn	1	263	125	156	179	167	213	287	420
1	Corn	1	236	125	156	179	175	213	253	420
7	Corn	1	263	125	156	179	175	213	287	388
35	Corn	31 are VCG1, 4 Not VCG1-16, Undetermined	263	125	156	179	175	213	287	420
7	Corn	1	263	125	156	188	175	213	287	420
6	Corn	1	174	125	156	179	175	213	287	420
1	Corn	1	134	125	156	179	175	213	287	388
1	Soil	2	97	113	150	134	175	213	180	388
3	Soil	2	97	113	156	134	175	213	180	388
1	Soil	2	106	113	156	134	167	213	180	388
1	Soil	2	106	113	156	134	175	213	180	388
1	Soil	2	106	113	156	142	184	229	183	627
1	Soil	3	126	136	156	134	201	213	183	203
1	Soil	3	130	136	156	134	201	213	180	217
1	Soil	3	130	136	156	134	212	213	183	203
1	Soil	4	97	161	156	179	193	213	287	388
1	Corn	4	126	161	156	179	201	213	301	388
6	Corn	4 in VCG4, 2 Unknowns	130	161	156	179	201	213	287	388
1	Corn	4	136	161	156	179	201	213	287	388

(Table B.1 cont)

# Isolates	Source	VCG	SSR 123	SSR 146	SSR 177	SSR 205	SSR 250	SSR 277	SSR 327	SSR 347
1	Corn	4	130	167	156	179	201	213	287	388
1	Corn	4	130	167	156	179	201	213	301	388
4	Corn	4	130	167	156	198	201	213	287	388
1	Corn	4	130	167	156	198	201	213	357	388
1	Soil	5	106	108	144	255	167	197	183	466
1	Soil	5	97	146	144	255	167	197	324	146
1	Soil	5	101	146	144	255	167	213	324	146
3	Soil	2 in VCG5, 1 Not 1-16, Undetermined	101	146	144	255	175	213	324	146
1	Soil	6	97	108	136	142	175	213	227	217
1	Soil	6	97	108	136	142	175	213	216	203
1	Corn	7	106	108	136	134	175	197	216	217
1	Corn	7	112	108	136	134	175	197	216	217
1	Corn	7	112	108	136	134	175	213	216	217
1	Soil	8	126	108	136	134	175	197	253	242
2	Soil	8	92	113	156	142	201	213	180	149
1	Soil	8	92	113	156	142	201	213	180	157
1	Soil	8	92	113	156	142	212	213	183	157
1	Soil	8	92	113	156	149	201	213	183	149
1	Soil	9	97	113	156	134	187	213	216	149
1	Corn	9	154	117	156	179	187	225	199	296
1	Soil	9	164	117	156	179	184	225	199	279
1	Soil	9	164	117	156	179	187	225	199	269
4	Soil	9	164	117	156	179	187	239	199	269
5	Corn	10	118	113	156	213	175	213	357	173
1	Soil	11	88	113	156	179	167	213	357	217
1	Soil	12	136	104	144	169	167	258	287	466
1	Soil	12	143	104	136	169	156	248	271	343

(Table B.1 cont)

# Isolates	Source	VCG	SSR 123	SSR 146	SSR 177	SSR 205	SSR 250	SSR 277	SSR 327	SSR 347
1	Soil	14	92	104	136	134	175	197	238	686
1	Soil	15	118	117	156	134	175	213	180	149
1	Soil	15	118	117	156	142	175	225	183	149
2	Soil	1 is VCG16, 1 Not 1-16, Undetermined	154	108	156	179	201	239	253	149
1	Soil	16	164	108	150	179	201	23	253	149
2	Corn	Not 1-16, Undetermined	118	104	136	134	175	197	238	279
1	Corn	Not 1-16, Undetermined	130	104	136	142	175	197	227	279
2	Corn	Not 1-16, Undetermined	126	108	136	134	175	197	216	263
1	Soil	Not 1-16, Undetermined	92	104	144	156	167	213	389	183
1	Soil	Not 1-16, Undetermined	97	104	136	134	167	197	227	343
2	Soil	Not 1-16, Undetermined	106	104	136	134	175	197	216	242
1	Soil	Not 1-16, Undetermined	106	104	136	134	175	197	238	242
2	Soil	Not 1-16, Undetermined	112	104	130	134	175	197	216	388
1	Soil	Not 1-16, Undetermined	112	104	136	134	175	197	216	388
1	Soil	Not 1-16, Undetermined	112	104	136	134	175	197	238	343
1	Soil	Not 1-16, Undetermined	112	104	136	142	175	213	253	203
1	Soil	Not 1-16, Undetermined	112	104	136	179	167	197	324	163
1	Soil	Not 1-16, Undetermined	118	104	136	134	175	197	216	686
1	Soil	Not 1-16, Undetermined	130	104	136	134	175	197	216	232
1	Soil	Not 1-16, Undetermined	130	104	136	134	175	213	238	466
1	Soil	Not 1-16, Undetermined	136	104	136	134	137	197	238	343
1	Soil	Not 1-16, Undetermined	148	104	136	134	175	197	216	217
1	Soil	Not 1-16, Undetermined	148	104	136	142	184	213	238	466
1	Soil	Not 1-16, Undetermined	92	108	136	134	167	197	216	203
1	Soil	Not 1-16, Undetermined	92	108	136	134	175	197	216	203
2	Soil	Not 1-16, Undetermined	97	108	136	134	175	197	238	217
1	Soil	Not 1-16, Undetermined	97	108	150	179	212	213	199	149

(Table B.1 cont)

# Isolates	Source	VCG	SSR 123	SSR 146	SSR 177	SSR 205	SSR 250	SSR 277	SSR 327	SSR 347
1	Soil	Not 1-16, Undetermined	106	108	136	134	175	197	216	242
1	Soil	Not 1-16, Undetermined	106	108	136	134	175	213	216	217
1	Soil	Not 1-16, Undetermined	106	108	171	134	175	197	183	149
1	Soil	Not 1-16, Undetermined	112	108	136	134	175	197	216	242
1	Soil	Not 1-16, Undetermined	112	108	163	188	167	239	324	149
1	Soil	Not 1-16, Undetermined	118	108	136	42	184	213	216	203
1	Soil	Not 1-16, Undetermined	118	108	136	142	184	213	227	203
1	Soil	Not 1-16, Undetermined	118	108	156	179	201	272	389	163
1	Soil	Not 1-16, Undetermined	143	108	136	134	175	197	216	203
1	Soil	Not 1-16, Undetermined	148	108	136	142	175	213	238	263
1	Soil	Not 1-16, Undetermined	182	108	156	179	175	213	277	149
1	Soil	Not 1-16, Undetermined	88	113	156	198	201	258	324	420
1	Soil	Not 1-16, Undetermined	106	113	156	134	184	213	183	420
1	Soil	Not 1-16, Undetermined	118	113	156	179	229	272	271	360
1	Soil	Not 1-16, Undetermined	130	113	163	255	184	239	301	149
1	Soil	Not 1-16, Undetermined	130	113	171	255	184	258	287	149
1	Soil	Not 1-16, Undetermined	118	117	156	134	175	213	183	149
1	Soil	Not 1-16, Undetermined	118	125	156	179	239	248	253	388
2	Soil	Not 1-16, Undetermined	130	136	136	213	161	197	216	163
1	Soil	Not 1-16, Undetermined	130	161	156	188	201	213	301	388
1	Corn	Unknown	118	104	136	142	175	197	227	279
1	Soil	Unknown	85	104	144	324	175	197	389	242
1	Soil	Unknown	112	104	136	134	175	197	238	343
1	Soil	Unknown	106	125	136	179	161	197	357	149
1	Soil	Unknown	126	136	163	134	201	213	183	203

Vita

Rebecca Sweany was born in New Orleans in March 1980. She was raised in New Orleans by parents from Illinois and Michigan and she attended Orleans public schools. Her vacations were spent in the Illinois countryside with her grandparents. She moved far away from home to LSU in Baton Rouge to study for her bachelor's degree in 1998 and has not left yet. She graduated in 2003 with a Bachelor of Science with a wildlife and fisheries conservation major and a minor in biology and chemistry. She worked for Dr. Kenneth Damann in the Department of Plant Pathology and Crop Physiology for 4 years as az research associate and chose to study with him for a master's degree in 2007. While at LSU she met her husband and married Dr. Michael Kaller in 2005.