Comparison of Soil and Corn Kernel *Aspergillus flavus* Populations: Evidence for Niche Specialization

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ABSTRACT

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Aspergillus flavus is considered a generalist-opportunistic pathogen, but studies are beginning to show that *A. flavus* populations have strains specific to various hosts. The research objective was to determine whether *A. flavus* soil populations consist of solely saprophytic strains and strains which can be facultatively parasitic on corn. *A. flavus* was isolated from both corn kernels and soil within 11 Louisiana fields. Sixteen vegetative compatibility groups (VCGs) were identified among 255 soil isolates. Only 6 of the16 VCGs were identified in the 612 corn isolates and 88% of corn isolates were in two VCGs, whereas only 5% of soil isolates belonged to the same two VCGs. Isolates were characterized for aflatoxin B_1 production and sclerotial size. A random subset of the isolates (99 from corn and 91 from soil) were further characterized for simple-sequence repeat (SSR) haplotype and mating type. SSR polymorphisms revealed 26 haplotypes in the corn isolates and 78 in the soil isolates, and only 1 haplotype was shared between soil and corn isolates. Corn and soil populations were highly significantly different for all variables. Differences between corn and soil populations indicate that some soil isolates are not found in corn and some isolates have become specialized to infect corn. Further understanding of *A. flavus* virulence is important for development of resistant hybrids and for better biological control against toxigenic *A. flavus*.

Aspergillus flavus is an ascomycete fungus that infects and contaminates many economically important crops with aflatoxins. These include corn, cotton, peanut, and many tree nuts and, in Louisiana, it constantly threatens corn (20,47,51). Many strains of *A. flavus* have the ability to produce mycotoxins called aflatoxins which can be acutely toxic and carcinogenic. *A. flavus* is ubiquitous and genetically and phenotypically very diverse (20,47,51). The primary source of *A. flavus* inoculum is soil. The highest concentrations of *A. flavus* in soil are found in fields of highly susceptible crops, but *A. flavus* is also found on forest floors where there are not many known hosts, reflecting its saprophytic ability (20). In a cultivated field, the soil population of *A. flavus* increases after harvest and during hot, drought events (32) and has an aggregate or patchy spatial distribution within fields (20).

Populations of *A. flavus* isolates are characterized in many ways. One of the most common is to determine what types of toxins are produced. There are four types of aflatoxin: aflatoxin B_1 (AFB_1); aflatoxin B_2 (AFB_2), which may be produced by *A. flavus*; and aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2), produced by related *Aspergillus* spp. but not *A. flavus* (34,37,38,45). AFB_1 is the most important aflatoxin due to its greater toxicity. Another method is sclerotial size. Sclerotia are considered small if the diameter is <400 μm and large if >400 μm (1,3,14,16, 20,32,37,38,44,47). *A. flavus* strains have been characterized as belonging to cryptic species groups I or II based on five gene sequences (14,15). 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, may produce both B and G aflatoxins (14). Unfortunately, atoxigenic isolates with small sclerotia cannot be assigned to either group I or II based on sclerotial size and toxin production alone, meaning classification based on sclerotial size and toxin production does not always give insight into the genetic relatedness of strains (14). More recently, taxonomists have excluded AFG-producing ability from *A. flavus* and renamed the G-producing isolates from cryptic species II *A. minisclerotigenes* (34,45). Several DNA-fingerprinting methods have been developed to characterize isolates using simple-sequence repeat (SSR) loci and restriction fragment length polymorphisms (RFLPs) (17,18,26,30,50). Additionally, now that a sexual cycle has been identified (23), *A. flavus* isolates can be characterized into one of two mating types, *Mat1-1* and *Mat1-2* (18,36). Another classification method is based on vegetative compatibility groups (VCGs) (6,20,22,33). Isolates in the same VCG presumably have the same alleles for all compatibility loci and when paired hyphae fuse to form heterokaryons (33). Relatively few population studies use VCGs to characterize *A. flavus* isolates because determination of VCGs is labor intensive. Isolates in different VCGs differ in sclerotial size, mating type, aflatoxin production, and intraspecific aflatoxin inhibition (6,18,20,22,25,31). However, isolates in the same VCG tend to produce the same kinds of mycotoxins and have the same sclerotial size and mating type (6,18,22). It should not be surprising that strains in the same VCG would have similar characteristics because they represent a quasi-clonal lineage. Grubisha and Cotty were unable to detect migration of genes among three VCGs based on 24 SSR loci (18). However, VCG is not a barrier to mating because Horn et al. were able to produce viable sexual offspring from 11 matings between isolates of different VCGs (23). More work is necessary to determine whether or not sexual reproduction occurs in nature.

A. flavus is generally thought to be an opportunistic pathogen, and it is believed that all strains are equally capable of infecting crops if the environmental conditions are conducive (20,42,

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47,51). There is conflicting evidence of specificity between different strains of *A. flavus* and infection of susceptible crops. It is commonly understood that *A. flavus* infects crops more readily than closely related *A. parasiticus*. However, it was not thought that there would be large differences among *A. flavus* strains in their abilities to infect crops (20,22). In one study, corn kernels, bean leaves, and insects became infected by all isolates of *A. flavus* when the tissues were mechanically wounded (42). *A. flavus* does not require a wound to infect a crop; therefore, this study does not show whether there are any differences in the pathogenicity of strains under natural conditions (20,29,42,47). It has been demonstrated that isolates from different crops in Argentina (44) and in Mississippi and Arkansas (1) produce different quantities of aflatoxin, indicating the possibility that there are different strains of *A. flavus* among the crops, and these different strains of *A. flavus* are better at infecting different crops. This may not actually be the case because isolates of *A. flavus* can quickly lose their ability to produce aflatoxin in serial transfers; therefore, aflatoxin production may not differentiate between strains of *A. flavus* (21). Evidence supporting specificity between peanut and different *A. flavus* VCGs in a single field showed that some VCGs only infrequently found in soil were more common in the peanut (20,22). Determining specificity for cotton and corn is more complex than for peanut because peanut plants are infected by direct contact of the peg with the soil, whereas infection of cotton and corn requires either an insect vector or airborne dissemination of conidia from the soil to infect the seed (6,20, 29,47). Vectored and airborne infections allow the source of the inoculum to come from relatively distant areas (20,29,47). In a single field in Arizona, comparisons of VCGs from the soil and cotton seed revealed that only two soil VCGs were found in the cotton seed and several VCGs were only isolated in the soil (6). Thus it appears that certain VCGs more specifically infect cotton (6,20). Results supporting this finding come from a single field in Illinois where only two RFLP genotypes (correlated with VCG) were shared between 128 corn isolates and 31 soil isolates (30,50). Unlike the studies on peanut and cotton, there were no predominant genotypes in the corn (50). There is not a clear case for specificity in corn; however, there were only two shared genotypes between the soil and corn, indicating that the soil and corn kernel populations are very different. In the present study, we have examined corn and soil *A. flavus* isolates and find, based on VCG analysis, aflatoxin production, sclerotial size, SSR haplotype, and mating type, that only certain soil isolates have become specialized to infect corn.

MATERIALS AND METHODS

Population sampling. In August 2007, soil and mature corn ears were sampled from 11 fields in seven parishes along the Red River and Mississippi River alluvial regions in Louisiana. Ten ears of corn and five soil samples were collected from each of seven fields from five parishes (Frogmore in Concordia Parish, Louisiana State University [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). The corn ears and soil samples were randomly selected from two diagonal transects originating from the middle of the outer edge of each field. Additionally, Francis Deville of Monsanto Company collected seven soil and seven corn ear samples from a field in Belcher, Caddo Parish; six corn ear and six soil samples in Chenyville, Rapides Parish; two corn ear and two soil samples in Batchelor, Point Coupee Parish; and four corn ear and four soil samples in Washington, St. Landry Parish. The corn ears were shelled and stored in paper bags at ambient temperature in the lab while the soil samples, in opened quart zip-lock bags, were allowed to dry in an exhaust hood.

Corn kernels from each shelled ear (50 ml) were surface sterilized in a 6% bleach solution, and 25 intact corn kernels were plated on *A. flavus/parasiticus* (AFPA) medium amended with hygromycin at 50 μg/ml, chlortetracycline at 1.5 μg/ml, streptomycin at 30 μg/ml, and Avermectin at 0.04 μl/ml (7,10,35). The 2,225 kernels on AFPA medium were incubated at 30°C for 5 days. AFPA is a selective and differential medium which suppresses conidiation and, when *A. flavus* or *A. parasiticus* grow, both species produce aspergillic acid that changes the color of the medium to a bright orange color (7,10,35). A plug of mycelia was aseptically removed from the orange medium below each infected kernel. The plug was then transferred to V8 medium (5% V8 juice and 2% agar, pH 5.2) containing Avermectin at 0.04 μl/ml.

Each soil sample (50 g) was suspended in 100 ml of autoclaved distilled water. A 1-ml aliquot of undiluted and two 1-ml aliquots of a 1-in-10 dilution of the soil suspension were spread on three petri dishes of amended AFPA medium. The soil dilution plates were incubated at 30°C for 5 days. For each 50-g soil sample, orange colonies were transferred onto a V8 medium plate amended with Avermectin at 0.04 μg/ml amended.

At least five orange colonies were isolated from each soil and corn ear sample to provide sufficient degrees of freedom to meet the desired level of statistical precision. Single-conidium colonies were generated by streaking conidia from V8 medium onto potato dextrose agar (PDA) and growing overnight at 30°C. A single germinating conidium was transferred onto V8 medium. The isolates were identified as *A. flavus* by the presence of smooth, olive-green conidia (28,38). A conidial suspension from the V8 medium was made for each *A. flavus* isolate in glycerol/water (1:1, vol/vol) and stored in the refrigerator or freezer.

VCGs. Nitrate nonutilizing (*nit*) mutants were generated to determine the VCG of the isolates (5,33). The *nit* mutants were characterized by the type of nitrogen source utilized and classified as either *cnx* (hypoxanthine and *nit*), *nirA* (nitrite and *nit*), or *niaD* (*nit*) mutants (5,33). All complementary *nit* mutants were plated on a starch modified Czapek-Dox medium plate 1 in. apart and incubated in ambient light at 30°C for 3 weeks (5,11,33). Plates were observed for zones of dense conidiation which indicated a successful formation of a heterokaryon between two complementary mutants in the same VCG (5,33).

Phenotypic traits: aflatoxin and sclerotial size. Each *A. flavus* isolate's conidial suspension (20 μl) was inoculated on autoclaved rice (5 ml of rice and 5 ml of H_2O in a 20-ml scintillation vial) for

TABLE 1. Eight simple-sequence repeat (SSR) loci forward and reverse primers (26)

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

[z] First three digits indicate length of the amplified fragment based on the genome sequence of the *Aspergillus flavus* isolate (for example, NRRL 3357 = 347), central letters and numbers indicate composition of SSR locus (for example, ACT70 indicates a locus consisting of 70 ACT repeats), F means forward, and R means reverse.

AFB_1 production (39). The conidial suspension concentrations were not standardized because Wicklow et al. (48) found that aflatoxin production was more a function of the amount of substrate rather than inoculum size. After 5 days at 30°C in ambient light, the vial was filled with chloroform and soaked overnight to extract the aflatoxin from the rice and fungus (39). The chloroform extract was then filtered through a Whatman number 1 100-mm filter paper funnel into a 100-ml glass beaker. The chloroform was allowed to evaporate under an exhaust hood and the aflatoxin was resuspended in 0.5 ml of a methanol/water (80:20, vol/vol) mixture (39). The extract was diluted with 0.5 ml of acetonitrile and filtered through a cleanup column packed with 200 mg of basic aluminum oxide into an autosampler vial (40).

The aflatoxin was quantified with reversed-phase high-performance liquid chromatography (HPLC) 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 (27). A post-column derivatization step was conducted by exposure to UV light in a Photochemical Reactor for Enhanced Detection (Aura Industries Inc., New York) (27). The mobile phase was HPLC grade methanol/HPLC grade acetonitrile/distilled water (22.5:22.5:55, vol/vol) at 1 ml/min. The stationary phase was an Acclaim 120 C18, 3-µm, 120-Å, 4.6-by-150-mm column (Dionex Corporation). AFB_1 was detected at 9.2 min. The G toxins were not detected because the rice substrate created background peaks in the chromatograms, which eluted at the same time as G toxins would if they were present.

All of the isolates were grown on 4-ml PDA slants in 15-by-100-mm test tubes in an ambient light incubator at 30°C for 1 month. Diameters of at least 10 sclerotia were determined to be large or small on a compound light microscope with the aid of an ocular micrometer. Sclerotia >400 µm were classified as large and sclerotia <400 µm were classified as small (1,3,14,16,20,32, 37,38,44,47). Also, some isolates produced sclerotia of both sizes. If only a few sclerotia were of a different size, the isolate was considered the majority class; however, if sclerotia of both size classes were observed with the same frequency, the isolates were put into a fourth class of both large and small sclerotia.

Genotypic traits: SSR haplotypes and mating type loci. SSR fingerprints and mating types were obtained from a subsample of nine random number-selected corn isolates and nine random number-selected soil isolates from each of 11 fields, except only two isolates from the soil samples from Beggs, St. Landry Parish and eight isolates from the soil samples from Frogmore, Concordia Parish were selected. In total, 99 of 612 corn isolates and 91 of 255 soil isolates were fingerprinted and haplotypes determined.

Each of the 190 isolates was grown on PDA in a standard 100-by-15-mm petri dish. All the conidia were then scraped into 600 µl of Nuclei Lysis solution (Promega Corp., Madison, WI) in a 1.5-ml microcentrifuge tube, 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 Tris-EDTA buffer, pH 8.

Eight SSR loci were selected by C. Huang (25,26) using sequence data of *A. flavus* isolate NRRL 3357, provided by W. Nierman of the J. Craig Venter Institute in Rockville, MD. 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 1). Amplifications were done in a Cetus DNA Thermal cycler (Perkin-Elmer Inc., Massachusetts) using PuReTaq Ready-To-Go polymerase chain reaction (PCR) beads (GE Healthcare, Buckinghamshire, UK). The PCR reactions were done according to the manufacturer's guidelines with a final concentration of 0.24 µM for both the forward and reverse primers for a particular SSR locus and 10 ng of DNA. The PCR program consisted of: an initial 210-s denaturation step at 95°C; 35 cycles of 15 s of denaturation at 95°C, a 20-s annealing step at the temperature specified in Table 1, and a 30-s extension step at 72°C; and final extension at 72°C for 120 s. The samples were held at 4°C.

The mating types were determined in a multiplex PCR reaction (36). A concentration of 0.5 µM of both the *Mat1-1* and *Mat1-2* forward and reverse primers and 10 ng of DNA were added to 0.5-ml tubes of PuReTaq Ready-To-Go PCR beads (GE Healthcare) and mixed to the manufacturer's guidelines (36). The PCR amplifications were conducted in a Cetus DNA Thermal cycler (Perkin-Elmer Inc.) with an initial 5-min denaturation step at 95°C and 40 cycles of 30 s at 95°C, 60 s at 54°C, and 45 s at 72°C.

The amplified DNA from the PCR reactions and an Ultraclean 20-bp ladder (MoBio Laboratories Inc., California) were separated with a subcell agarose gel electrophoresis system (model 192; Bio-Rad Laboratories, Hercules, CA) with a 3% GenePure Sieve GQA Agarose gel (ISC BioExpress, Utah) made with 0.5× Tris-borate-EDTA. The DNA was stained with 3-EZ-vision DNA dye as loading buffer (Amresco Inc., Ohio), which fluoresced when exposed to UV light, and a digital image was captured 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). The *Mat1-1* mating type diagnostic amplicon is 395 bp and *Mat1-2* is 273 bp (36). The SSR bands were assigned to different size classes 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 classes for each isolate were combined and considered a haplotype or fingerprint.

Analysis. Descriptive statistics and hypothesis testing were evaluated using SAS for Windows (version 9.2; SAS Institute, Cary, NC). Multicategory logit generalized linear models were created to compare soil and corn kernel populations with VCGs, mating type loci, sclerotia groups, and AFB_1 groups (13). In these models, the fields were pooled for each linear model. In all models, soil and corn source served as the explanatory variable and the VCG, mating type loci, sclerotia, and AFB_1 group membership were the response variables. Only the nine most abundant VCGs were used in the VCG linear model. Inclusion of additional VCGs resulted in unacceptably high overdispersion, as measured by χ^2 or degrees of freedom; therefore, only those nine VCGs were analyzed. Although sclerotia were measured, all isolates were classified as either having none, small (<400 µm), or large sclerotia (>400 µm) (1,3,14,16,20,32,37,38,44). Continuous AFB_1 data were converted to categories (zero, low, medium, and high) because of nonhomogenous variances between soil and corn sample groups. The models were interpreted with an α of 0.05, and goodness-of-fit (i.e., whether one type of explanatory variable was a better description of the data compared with competing alternatives) was assessed with the Akaike information criterion (AIC), with lower AIC values suggesting better fit and more explanatory ability. Each unique combination of SSR alleles found among the isolates was assigned a new haplotype. Unbiased haplotype diversities were calculated for both the soil and corn kernel isolates in each field based on the proportion of different haplotypes in a field (19,46). A value of one meant the field was completely diverse (no two isolates alike) and zero meant there was no diversity in the field (all isolates identical). The formula for unbiased haplotype diversity was $H_e = [n/(n-1)] \times (1 - \Sigma p_i^2)$, where i was the ith haplotype and n was the number of corn

kernels or soil isolates within a field (46). Analysis of molecular variance was performed using the differences in SSR bands for all isolates to determine whether the soil and corn kernel populations were different using Arlequin (version 3.11; Computational and Molecular Population Genetics Lab, University of Berne, Switzerland) (19,40,46).

RESULTS

Population sampling. In total, 867 *A. flavus* colonies were isolated: 612 isolates from corn kernels from 89 corn ears and 255 isolates from 54 soil samples. The frequency of *A. flavus* isolation varied between fields and between soil and corn samples. The mean number of *A. flavus* colonies isolated from a field was 23 ± 4 (standard error [SE]) colonies in the soil and 56 ± 12 (SE) in the corn kernels. The mean number of colonies in a soil sample was 6 ± 1 (SE) and 7 ± 1 (SE) in a corn ear sample.

VCGs. In all, 16 different VCGs were identified out of 594 corn *nit* mutants and 235 soil *nit* mutants. A total of 18 corn isolates and 20 soil isolates failed to produce *nit* mutants after successive tries. In total, 32 of 594 corn *nit* mutants and 129 of 235 soil *nit* mutants did not form heterokaryons with any *nit* mutants in the 16 VCGs and, thus, were distinct from isolates within the 16 VCGs. It is unknown whether these isolates are in singleton VCGs or in multiple different VCGs because they were not tested against each other due to the fact that only one *nit* mutant was produced per isolate. Multiple VCGs were found in both single soil and single corn ear samples. VCGs 1 and 4 were the only VCGs to be found in all 11 fields and accounted for 88% of corn kernel isolates (Table 2). Eight VCGs consisted of isolates with consistent sclerotial size. From VCG 10 and 13, 5 of 12 and 2 of 3 isolates, respectively, produced an equal proportion of small and large sclerotia. Isolates in 10 VCGs all produced AFB_1 in the same AFB_1 quantity category.

Soil isolates were represented in all VCGs whereas corn isolates were only found in six VCGs (Table 2). The proportion of soil isolates and corn isolates in the same VCG varied for each VCG (Fig. 1). The probability of recovering isolates in VCGs 1, 2, 3, 4, 5, 7, 8, 9, and 10 varied significantly between the corn and soil populations (χ^2 = 553.41, df = 8, P value < 0.0001, AIC = 1,895).

Phenotypic traits: aflatoxin and sclerotial size. All *A. flavus* isolates produced a mean value of AFB_1 at 4,658 ± 9,526 (SE) ppb. The mean aflatoxin AFB_1 for corn kernel isolates was 2,314 ± 7,455 (SE) ppb and 10,248 ± 11,430 (SE) ppb for the soil isolates. There was a higher proportion of soil isolates with high levels of AFB_1 and there was a higher proportion of the corn isolates with medium, low, and no toxin (Fig. 2). The probability of isolates in the different aflatoxin production groups differed significantly between the corn kernel and soil population (χ^2 = 334.79, df = 3, P value < 0.0001, AIC = 2,376).

All isolates were classified as either having none, small (<400 µm), or large sclerotia (>400 µm) (1,3,14,16,20,32,37, 38,44). The majority (95%) of corn kernel isolates produced no sclerotia, whereas the majority (97%) of soil isolates produced sclerotia (56% small and 41% large sclerotia). The probability of isolates producing the same sclerotial size varied significantly between the corn kernel and soil populations (χ^2 = 1,094.02, df = 2, P < 0.0001, AIC = 1,454).

Genotypic traits: SSR haplotypes and mating type loci. In all, 103 different haplotypes were found within the 190 isolate subsample of the corn kernel and soil isolate population. In total, 26 haplotypes were found in the corn kernels, 78 haplotypes were in the soil samples, and only 1 haplotype in VCG1 was shared between soil and corn kernel isolates (Supplemental Table 1). Multiple haplotypes were found in the fields in both the soil and corn kernel samples and only 10 haplotypes were found in more than one field (Table 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 (Rst = 0.6033, df = 1, P value < 0.0001). Multiple isolates for 13 of the 16 VCGs were characterized by SSR fingerprints; of those, only VCG 10 had a single haplotype (Table 2). Two VCGs had one SSR haplotype that was shared with an isolate that was determined to not be in the same VCG by *nit* complementation (Table 2).

Of the 99-isolate corn-kernel subsample, 96% 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*). The probability of corn and soil isolates in the two mating types differed significantly (χ^2 = 110.44, df = 1, P < 0.0001). Each VCG is only represented by one mating type, with

TABLE 2. Characteristics of *Aspergillus flavus* isolates in 16 vegetative compatibility groups (VCGs)

		Number of isolates in			Sclerotia (%)[u]			Toxin (%)[v]				Number of SSR haplotypes[w]		
VCG	Count	Soil	Corn	Fields	Zero	Small	Large	Zero	Low	Medium	High	Finger	Unique	Shared
1	487	4	483	11	99.8	0.2	0	5.4	89.5	5.2	0.0	70	6	13 A, 7 B, 31 C[x], 7 D, 6 E
2	29	29	0	2	0.0	0.0	100.0	0.0	0.0	0.0	100.0	7	4	3 F
3	6	6	0	4	0.0	100.0	0.0	0.0	0.0	0.0	100.0	3	3	0
4	61	5	56	11	98.4	1.6	0.0	0.0	0.0	3.3	96.7	14	6	4 G, 4 H
5	11	10	1	3	0.0	100.0	0.0	0.0	0.0	0.0	100.0	5	3	2 I[y]
6	5	5	0	2	0.0	100.0	0.0	20.0	80.0	0.0	0.0	2	2	0
7	12	2	10	2	0.0	75.0	25.0	0.0	100.0	0.0	0.0	3	3	0
8	14	14	0	4	0.0	92.9	7.1	0.0	14.3	7.1	78.6	6	4	2 J
9	16	14	2	4	12.5	12.5	75.0	0.0	0.0	0.0	100.0	8	4	4 K[x]
10	12	2	10	2	50.0[z]	41.7[z]	41.7[z]	83.3	16.7	0.0	0.0	5	0	5 L
11	2	2	0	2	0.0	0.0	100.0	0.0	0.0	0.0	100.0	1	1	0
12	4	4	0	3	0.0	100.0	0.0	0.0	25.0	0.0	75.0	2	2	0
13	3	3	0	1	0.0	66.7[z]	100.0[z]	0.0	100.0	0.0	0.0	0	…	…
14	2	2	0	1	0.0	50.0	50.0	0.0	100.0	0.0	0.0	1	1	0
15	3	3	0	2	0.0	100.0	0.0	0.0	0.0	0.0	100.0	2	2	0
16	2	2	0	1	0.0	0.0	100.0	0.0	0.0	0.0	100.0	2	1	1 M[y]

[u] Small sclerotia diameters are <400 µm and large sclerotia diameters are >400 µm.

[v] Low aflatoxin B_1 (AFB_1) = AFB_1 >0 and ≤20 ppb, medium = AFB_1 >20 and ≤300 ppb, and high = AFB_1 >300 ppb.

[w] Finger = fingerprinted. Letters within Shared column represent a unique simple-sequence repeat (SSR) haplotype found for multiple isolates within this study, numbers are the number of isolates with the SSR haplotype found within the VCG.

[x] SSR haplotype was shared with two additional isolates that were not characterized by VCG due to lack of a nitrate nonutilizing (*nit*) mutant.

[y] SSR haplotype was shared with one isolate that was determined not to be in any of the 16 VCGs due to that lack of *nit* mutant complementation with any of the VCGs.

[z] Total percentage of isolates in VCG producing sclerotia is >100 because some isolates produced both large and small sclerotia.

the exception of VCG1, with only 1 of the 71 isolates in the *Mat1-1* mating type (Fig. 3).

DISCUSSION

The objective of this research was to determine whether certain strains of *A. flavus* in the soil were more specialized to infect corn than others. Such a result would imply that there were two ecotypes of *A. flavus*: one primarily saprophytic and the other facultatively parasitic. Because soil is the source of inoculum for infection of corn, it should have contained isolates of both ecotypes. The results showed that different VCGs predominated on the corn and in the soil, providing evidence for two *A. flavus* ecotypes with different abilities to occupy these two niches. Isolates within VCGs varied in their production of aflatoxin and sclerotia. The soil and corn *A. flavus* populations differed in the sclerotial sizes, AFB_1 production, mating types, and eight SSR locus haplotypes. Even though these variables differentiated the soil and corn kernel *A. flavus* populations, these metrics were not as good as VCGs for comparing strains of *A. flavus*.

Our results were similar to recent findings on cotton and peanut. These indicate that VCGs varied between soil *A. flavus* isolates and cotton and peanut *A. flavus* isolates and demonstrated that isolates of different VCGs have different specific abilities to infect peanut and cotton (6,22). Like peanut and cotton, the VCG assemblages varied significantly between soil and corn populations in this study. The soil contained all of the six identified VCGs from the corn (15% soil isolates and 93% corn isolates) as well as additional identified (27% soil isolates) and unidentified VCGs (51% soil isolates); this suggests a greater diversity of VCGs in the soil than in the corn (Table 2; Fig. 1). Consequently, it appears that only some VCGs are specialized to inhabit the corn niche. The four most abundant VCGs (VCGs 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 that these isolates are potential threats to contaminate the crops with aflatoxin (32,37). Our results suggest that the most abundant VCGs confined to soil (VCGs 2, 5, 8, 9, and undetermined VCGs) may not infect corn and, therefore, do not threaten to contaminate the corn with aflatoxin. Here, the abundance of a soil VCG was not predictive of the *A. flavus* strains that would be a threat to the crop. The majority of corn kernel isolates consisted of two VCGs (81% VCG1 and 9% VCG4) which were found at only 1.7 and 2.1%, respectively, in the soil. These two VCGs were isolated from corn kernels in all 11 fields whereas they were only isolated from soil samples in 3 fields: 1 field with VCG1, 1 with both VCG1 and -4, and 1 with VCG4. The fact that these two VCGs were so abundant in corn compared with soil may indicate that they were better adapted to live in the corn niche than other VCGs found in the soil and may suggest that the source of inoculum for corn is outside the boundaries of the field.

Previous work has demonstrated that VCGs consist of isolates with the same sclerotia and aflatoxin production phenotypic characteristics as well as the same mating type (6,18,20,22, 23,30,31,47). Our results confirmed that most isolates within a VCG also had the same mating type locus. However, 1 isolate in VCG1 had a different mating type than the other 70 isolates. This anomalous isolate could be the result of the reassortment of genes between isolates from two different VCGs. The occurrence of mating is supported by the fact that the SSR haplotype is very different than any of the other SSR haplotypes within this VCG. This may be the first evidence of the sexual cycle of *A. flavus* occurring in nature. Isolates within the same VCGs in this study produced similar quantities of AFB_1. However, VCGs 1, 4, 7, 8, 9, 10, 13, and 14 were composed of isolates which produced different sclerotial size classes. In VCGs 10 and 13, 5 of 12 and 2 of 3 isolates, respectively, produced an equal proportion of small and large sclerotia. This indicates that classifying isolates as

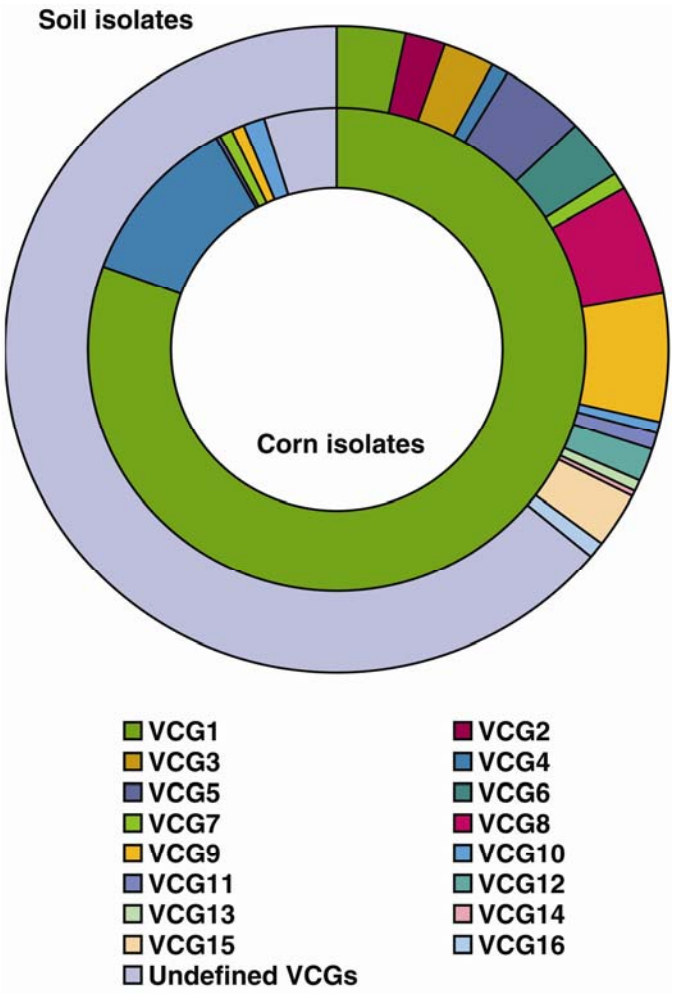

Fig. 1. Different vegetative compatibility group (VCG) assemblages between soil and corn kernel *Aspergillus flavus* populations. Circle slices are the mean proportion of corn or soil *A. flavus* isolates in all samples.

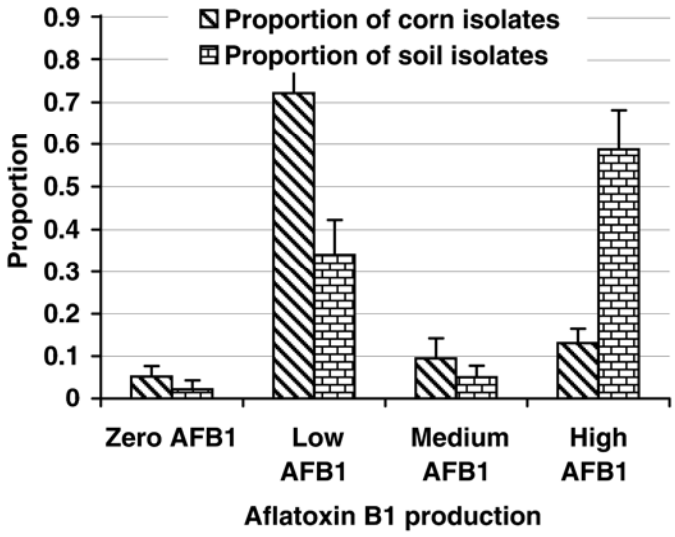

Fig. 2. Difference in the aflatoxigenicity of corn and soil isolates of *Aspergillus flavus*. Bars represent the mean proportion of soil or corn *A. flavus* isolates with aflatoxin B_1 (AFB_1) production category X in each sample. Error bars are upper 95% confidence limit. Low AFB_1 = AFB_1 >0 and ≤20ppb AFB_1, Medium = AFB_1 >20 ppb and ≤300 ppb, and High = AFB_1 >300 ppb.

producing small or large sclerotia does not account for all the phenotypic variability within strains of *A. flavus*.

Sclerotia and toxin production are not ideal characteristics to show the differences between populations. Geiser showed that large and small sclerotia are not phylogenetically related characteristics by comparing three genes of 28 different *A. flavus* isolates (14). Therefore, small or large sclerotial size does not show if isolates within a population are related, making it hard to compare populations of *A. flavus* using this criterion. The sclerotial production and size may be affected by growing conditions. It has been found that some isolates of *A. flavus* will produce sclerotia more readily in the dark; accordingly, our sclerotial results are for growth on PDA in ambient light at 30°C (8,49). Regardless, only 5% of corn isolates produced sclerotia, whereas 97% of soil isolates produced sclerotia under these conditions. Sclerotia are important for survival of *A. flavus* in the harsh soil environment. The fact that the majority of corn isolates failed to produce sclerotia indicates that these isolates may not be as specialized to inhabit soil but, indeed, have become specialized to inhabit the corn niche. Also, the genes for aflatoxin production are located in the subtelomeric region of chromosome III, leading to potentially high mutation rates and large variability in aflatoxin synthesis (8,21,51). Aflatoxin synthesis has been shown to be quickly lost after serial transfers on PDA; therefore, 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 (21). It is noteworthy that the majority of corn isolates produce only small quantities of AFB_1, indicating that the production of aflatoxin is not essential for infection of corn, whereas the majority of soil isolates produced large amounts of aflatoxin, indicating that aflatoxin production might be important for survival in the soil niche. AIC values are used to compare the goodness of fit of general linear models; the smallest AIC is best. Accordingly, the general linear model with sclerotia size classes had the lowest AIC value of 1,454 which meant that, statistically speaking, the difference in sclerotia sizes best explained the differences between soil and corn kernel populations, not toxin production or VCG. 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 isolates produced low levels of aflatoxin in the corn (Table 2). VCG1 accounted for 81% and VCG4 for 9% of corn *nit* mutants and both accounted for only 4% of the soil *nit* mutants. In summary, the difference in VCGs better differentiated the soil and corn kernel isolates and, thus, the corn and soil *A. flavus* populations.

Eight SSR loci and mating type loci were also used to compare the soil and corn kernel populations (Table 3) (26). Similar to a RFLP study conducted in a corn field in Illinois, the SSR loci haplotypes were very different between the soil and corn kernel populations (50). Only one haplotype was shared between the soil and corn kernel isolates in this study. This was a haplotype for VCG1 and was represented by 1 soil isolate and 12 corn kernel isolates. In the Illinois study (50), no genotypes predominated in the corn kernel or soil isolates but, among the Louisiana isolates, there were only 26 different haplotypes out of 99 corn kernel isolates and 78 different haplotypes out of 91 soil isolates, and the most common haplotype was found 35 times in the corn. However, much like in the Illinois corn field, the corn isolate haplotypic diversity was smaller than soil isolates in every field (Table 3) (50). The fact that only one haplotype was shared between the corn and soil isolates and the soil isolates were more diverse supports the VCG results that isolates in the corn have become specialized to infect the corn. The distribution of *Mat1-1* and *Mat1-2* loci was different between the soil and corn kernel

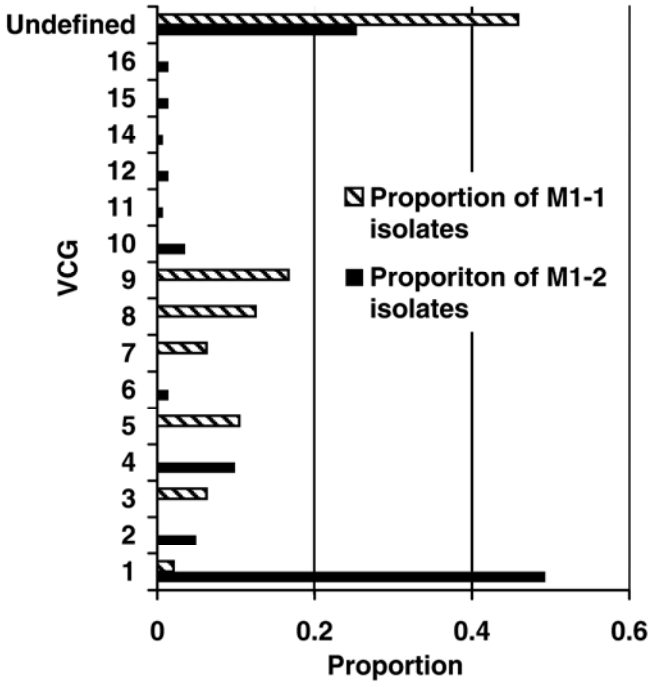

Fig. 3. *Mat*1-1 and *Mat*1-2 mating type locus distribution among *Aspergillus flavus* vegetative compatibility groups (VCGs). Bars represent the proportion of *Mat1-1* or *Mat1-2 A. flavus* isolates in each VCG.

TABLE 3. Corn and soil *Aspergillus flavus* simple-sequence repeat (SSR) haplotype diversity (HD)

	Soil				Corn			
	Number of				Number of			
Field	Isolates	Haplotypes	Shared[y]	HD[z]	Isolates	Haplotypes	Shared[y]	HD[z]
Batchelor	9	7	1 P	0.9	9	3	2 C	0.7
Beggs	2	2	0	1	9	3	1 B, 4 C, 4 E	0.7
Belcher	9	6	0	0.9	9	3	7 C, 1 E	0.4
Chenyville	9	8	1 P	1	9	2	4 C, 5 G	0.6
Crowville	9	8	2 K, 1 R	1	9	5	3 C, 3 D, 1 H	0.8
Frogmore	8	7	0	0.9	9	3	7 A, 1 C	0.4
Macon Ridge Research Station	9	8	2 H	1	9	4	4 D, 3 H	0.8
St. Joseph	9	8	0	1	9	3	4 A, 4 C	0.7
Northeast Research Station	9	9	1 R	1	9	8	1 C	1
Torbert	9	9	0	1	9	4	1 A, 6 B, 1 C, 1 E	0.6
Washington	9	9	1 A	1	9	4	6 C	0.6

[y] Haplotypes shared between fields. Letters represent unique SSR haplotypes (corresponding to letters in Table 2).

[z] HD calculated as $H_e = [n/(n-1)] \times (1 - \Sigma p_i^2)$, where p_i is the proportion of corn kernel or soil *A. flavus* isolates within field in the *i*th haplotype and *n* is the number of isolates from either the soil or corn kernels within a field.

isolates of *A. flavus*. The two *Mat* loci were evenly distributed among soil isolates whereas 96% of the corn isolates were *Mat1-2*. The even distribution of *Mat* loci is consistent with the soil being the site of sexual reproduction (36). The predominance of *Mat1-2* among the corn isolates may suggest that it confers a selective advantage to colonize corn. 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 *Mat1-2* mating type and these accounted for 87% of the corn kernel subsample isolates and only 3% of the soil subsample isolates.

The eight SSR loci were poor predictors of VCGs (43) in contrast to the results of Grubisha and Cotty (18). 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 polymerase slippage, leading 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 population differentiation and migration and genetic drift of alleles (4). Stepwise mutation rates of single nucleotide differences are much better understood and have been incorporated into population genetics models; therefore, better genetic markers would consist of genes with single nucleotide differences (4).

More work needs to be done to understand why certain VCGs are more specialized to infect corn. The pathogenicity factors associated with certain VCGs could play a role in the differences in ability to colonize the plant. Oxylipin-generating dioxygenase mutants have been shown to affect the pathogenicity of *A. flavus* (24). 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 (9,12,24,51).

The fact that some isolates of *A. flavus* have a greater propensity to infect corn has several implications for aflatoxin contamination management. Currently, there are two avenues being explored to alleviate aflatoxin contamination: first is the use of a nontoxigenic *A. flavus* biocontrol and second is breeding resistant cultivars of corn (9,18,25,31,51). In order to develop a successful biocontrol, the nontoxigenic biological control needs to be highly specific to infect corn in order to stack the deck in favor of biological control. Also, when screening for resistance to *A. flavus*, isolates should be chosen based on their demonstrated ability to infect corn under natural growing conditions. Our results indicate that there are differences between strains of *A. flavus* in their abilities to infect corn under natural growing conditions, and it should be assumed that there are genetic differences among strains and that these genetic differences affect pathogenicity.

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