Frequent Shifts in *Aspergillus flavus* Populations Associated with Maize Production in Sonora, Mexico

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ABSTRACT

Aspergillus flavus frequently contaminates maize, a critical staple for billions of people, with aflatoxins. Diversity among *A. flavus* L morphotype populations associated with maize in Sonora, Mexico was assessed and, in total, 869 isolates from 83 fields were placed into 136 vegetative compatibility groups (VCGs) using nitrate-nonutilizing mutants. VCG diversity indices did not differ in four agroecosystems (AES) but diversity significantly differed among years. Frequencies of certain VCGs changed manifold over single years in both multiple fields and multiple AES. Certain VCGs were highly frequent (>1%) in 2006 but frequencies declined repeatedly in each of the two

subsequent years. Other VCGs that had low frequencies in 2006 increased in 2007 and subsequently declined. None of the VCGs were consistently associated with any AES. Fourteen VCGs were considered dominant in at least a single year. However, frequencies often varied significantly among years. Only 9% of VCGs were detected all 3 years whereas 66% were detected in only 1 year. Results suggest that the most realistic measurements of both genetic diversity and the frequency of *A. flavus* VCGs are obtained by sampling multiple locations in multiple years. Single-season sampling in many locations should not be substituted for sampling over multiple years.

Maize is cultivated throughout Mexico regardless of elevation, soil type, rainfall, and agroecosystem (AES) (Plasencia 2004; SIACON 2010) on approximately one-third of Mexico's cultivable land (Eakin et al. 2014). The majority of the caloric daily intake for over half of Mexico's population is provided by maize (FAO 2010). However, consumption of maize can expose populations to unhealthy aflatoxin concentrations (Battilani et al. 2016; Hua et al. 2016; Plasencia 2004).

Aflatoxins are highly toxic, naturally occurring mycotoxins produced by several members of *Aspergillus* section *Flavi*, which frequently contaminate maize, cottonseed, peanut, and tree nuts in warm agricultural areas (Bandyopadhyay et al. 2016; Cotty et al. 1994). Aflatoxin B₁, the most toxic and prevalent, is classified as a group 1a carcinogen by the International Agency for Research on Cancer (IARC) and is strictly regulated across the globe through legislation (Grace et al. 2015; IARC Working Group 2002; Wu 2015). These potent toxins can induce immune system suppression, growth retardation, hepatocellular carcinoma, and death in both humans and livestock (Hernandez-Vargas et al. 2015; Probst et al. 2007; Wu 2015).

Aspergillus flavus, the most common causal agent of contamination (Amaike and Keller 2011; Cotty et al. 1994), can be subdivided in the L and S morphotypes, which differ in morphological, physiological, and genetic criteria (Cotty 1989; Ehrlich et al. 2005). The L morphotype produces abundant conidia, fewer but larger sclerotia, and variable concentrations of B aflatoxins. The S morphotype produces fewer conidia, abundant small sclerotia, and, on average, high concentrations of B aflatoxins (Cotty 1989, 1994b; Probst et al. 2007). Both morphotypes are distributed

worldwide over a great diversity of AES (Binder et al. 2007; Cotty and Mellon 2006; Jaime-Garcia and Cotty 2006; Probst et al. 2014).

Communities of aflatoxin-producing fungi are examined in order to identify the most common causal agents of contamination in a given area or cropping system (Boyd and Cotty 2001; Cardwell and Cotty 2002; Donner et al. 2009; Doster and Michailides 1994b; Ehrlich et al. 2007; Gao et al. 2007; Horn and Dörner 1999; Joffe 1969; Nesci and Etcheverry 2002; Ortega-Beltran et al. 2015; Probst et al. 2010). However, relatively few studies have examined intraspecific diversity of an aflatoxin producer across regions despite abundance of genetic diversity in relatively small areas, including individual fields (Atehnkeng et al. 2016; Bayman and Cotty 1991b; Horn et al. 1996; Mauro et al. 2013; Papa 1986; Pildain et al. 2004; Sweany et al. 2011). The state of Sonora in Mexico possesses diverse AES at elevations ranging from 0 to 2,100 m above sea level (MASL) over relatively short distances (Felger et al. 2001; INEGI 1988). Maize is cultivated in each of these AES (SIACON 2010) and *A. flavus* is associated with the maize (Ortega-Beltran et al. 2015). Knowledge of the genetic diversity within populations of aflatoxin-producing fungi resident in Sonora is needed in order to both more thoroughly determine the etiology of the state's aflatoxin contamination and optimize selection of widely distributed atoxigenic *A. flavus* genotypes for aflatoxin biological control products targeted to the examined region (Mehl et al. 2012).

Vegetative compatibility analyses (VCA) using nitrate-nonutilizing (*nit*) mutants are used to assign isolates into vegetative compatibility groups (VCGs) (Leslie 1993). The vegetative incompatibility system in the genus *Aspergillus*, as in other fungal genera such as *Fusarium*, *Neurospora*, and *Verticillium*, is regulated through several unlinked loci (Bayman and Cotty 1991b; Leslie 1993). During VCA, regions of prototrophy form between complementary mutants that are isogenic at heterokaryon incompatibility (*het*) loci controlling vegetative incompatibility. Such mutants, and the isolates from which they are derived, are said to belong to the same VCG (Bayman and Cotty 1991b; Leslie 1993; Papa 1986). In *A. flavus*, VCGs are clonal lineages and isolates within a VCG are more closely related to each other than to members of other VCGs (Bayman and Cotty 1991a; Grubisha and Cotty 2015; Mehl and Cotty 2010;

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Ortega-Beltran et al. 2016). Members of the same VCG share epidemiologic and physiologic characteristics, including aflatoxin-producing ability (Leslie 1993; Mehl and Cotty 2010, 2013).

Although VCGs are useful markers of species diversity, the labor required for VCA increases with quantities of both VCGs and individuals examined. Thus, labor requirements limit use of VCA and relatively few *Aspergillus* section *Flavi* population studies report VCG diversity (Atehnkeng et al. 2016; Barros et al. 2006; Bayman and Cotty 1991b; Horn and Greene 1995; Horn et al. 1996; Houshyar-Fard et al. 2014; Mauro et al. 2013; Novas and Cabral 2002; Pildain et al. 2004; Probst et al. 2011; Sweany et al. 2011). During VCA, *nit* mutants are generated for all isolates. Mutants are classified as *cnx*, *niaD*, or *nirA*⁻ based on phenotypes on chemically defined phenotyping media (Cove 1976). Complementary *cnx* and *niaD* mutants (called a tester pair) are used to define a VCG. Formation of prototrophic growth in the zone of hyphal interaction between a *nit* mutant and the *cnx* or *niaD* mutants indicates membership in the VCG defined by that tester pair (Bayman and Cotty 1991b). When a *cnx* mutant cannot be selected, tester pairs may be composed of *niaD* and *nirA*⁻ mutants, although successful complementation may be less reliable (Bayman and Cotty 1991a; Correll et al. 1987).

The dominance of the L morphotype in *Aspergillus* communities associated with many crops (Cardwell and Cotty 2002; Cotty 1997; Doster and Michailides 1994a; Doster et al. 1996; Mauro et al. 2013), the great variability in aflatoxin-producing ability among L morphotype VCGs (Bayman and Cotty 1993; Cotty et al. 1994), and the use of L morphotype isolates as biocontrol agents for the prevention of aflatoxin contamination (Alaniz Zanon et al. 2013; Bandyopadhyay et al. 2016; Cotty et al. 2007; Mauro et al. 2015) encouraged us to investigate the dynamics of *A. flavus* L morphotype community composition in Sonora with VCA. VCA allow successes of lineages to be monitored over space and time. Assessment of aflatoxin-producing potentials of individuals within a VCG allows identification of aflatoxin-producing or atoxigenic VCGs and may facilitate design of aflatoxin management programs. Whether intrinsic characteristics of an AES influence compositions of communities of *A. flavus* L morphotype VCGs is unknown. VCG diversity within AES needs to be contrasted with diversity among AES, and VCG diversity within years needs to be contrasted with diversity among years. The current study sought to utilize VCA to evaluate AES influences on *A. flavus* community composition and to assess the extents to which both community composition and diversity vary across years. These questions were addressed by contrasting communities of the *A. flavus* L morphotype resident in soil cropped to maize in four AES of Sonora, Mexico, across 3 years. The results indicate that compositions of *A. flavus* communities associated with maize production in Sonora, Mexico, are similar among AES but fluctuate significantly across years, with dominant VCGs varying over time.

MATERIALS AND METHODS

Fungi. Genetic diversity within communities of *A. flavus* L morphotype was examined among 869 isolates recovered from 83

maize field soils collected during summer 2006 (387 isolates from 27 soil samples collected in July), summer 2007 (240 isolates from 26 soil samples collected in July), and summer 2008 (242 isolates from 30 soil samples collected in June) in Sonora, Mexico (Table 1). Seventy-three percent of the examined soils were sampled during each of the 3 years. Number of samples among and within AES differed among years due to either absence of maize production along the planned sampling route or inaccessibility as a result of flooding or security-compromised areas. Briefly, georeferenced soil samples were collected from fields previously cultivated with maize in four AES (Table 1; Fig. 1). These AES—Alamos (AL), Tesopaco (TE), Yaqui Valley (YV), and Yecora (YE)—differ in elevation, temperature, precipitation, and irrigation practice. In YV, the AES at the lowest elevation, approximately 40 crops are planted annually on more than 200,000 ha totally dependent on irrigation (Lobell et al. 2003; SIACON 2010). Crop production in the other three AES occur on fewer hectares and all planted crops are rainfall dependent (SIACON 2010; Vásquez-León and Liverman 2004; Vásquez-León et al. 2003). Maize fields were distributed across approximately 200 km at elevations ranging from 6 to 2,100 MASL. Fields >20 ha were sampled in YV, whereas small fields of <5 ha were sampled in the other AES.

The examined fungi were part of a previous study (Ortega-Beltran et al. 2015) and were obtained from soils by dilution plate technique on modified rose Bengal agar (Cotty 1994a). During that study, the recovered fungi included both morphotypes of *A. flavus*, *A. parasiticus*, *A. tamarii*, and an unknown taxon. In the current study, in order to assess both diversity within the *A. flavus* L morphotype and impacts of diversity on aflatoxin-producing potentials of *Aspergillus* communities in Sonora, *A. flavus* L morphotype isolates were subjected to VCA.

Assignment of isolates to VCG. The *nit* mutants of the 869 *A. flavus* L morphotype isolates were selected on SEL agar (Czapek-Dox broth with 25 g of KClO₃, 50 mg of rose Bengal, and 2% Bacto agar [Difco Laboratories, Detroit] per liter, pH 7.0) (Cotty 1994b) by seeding spore suspensions (approximately 1,000 spores in 15 µl) into 3-mm central wells cut into agar. Plates were incubated for up to 30 days (31°C in darkness). Spontaneous auxotrophic sectors were transferred onto MIT agar (Czapek-Dox broth with 15 g of KClO₃ and 2% Bacto agar per liter, pH 6.5) (Das et al. 2008), and incubated for 3 days (31°C in darkness) to purify mutants of residual wild-type mycelia. Mutants were then grown on 5-2 agar (5% V8 Juice [Campbell Soup Company, Camden, NJ] and 2% Bacto agar, pH 6.0) for 7 days (31°C in darkness). After incubation, *nit* mutants were stored and maintained as previously described (Probst et al. 2011).

Tester pairs of VCGs were developed by pairing of an *niaD* mutant with either a *cnx* or *nirA*⁻ mutant on starch agar (SA) (36 g of dextrose, 20 g of soluble starch, 3 g of NaNO₃, and 2% Bacto agar per liter, pH 6.0) (Cotty and Taylor 2003). Complementary pairs were used to define individual VCGs. Fungal suspensions (approximately 1,000 spores in 15 µl) of the two testers and the *nit* mutant from the isolate being evaluated for membership in the VCG were seeded

TABLE 1. Four agroecosystems (AES) of Sonora, Mexico sampled from 2006 to 2008

AES	Climate	Temp (°C) ^w	Precip (mm) ^x	Elev (m) ^y	Irrigation ^z	Samples collected ^v		
						2006	2007	2008
Yaqui Valley	Very dry	24–42	200	6 to 37	IR	7 (100)	11 (70)	8 (41)
Alamos	Semi dry	28–38	700	102 to 489	RF	6 (87)	4 (52)	5 (55)
Tesopaco	Semi dry	24–38	700	338 to 648	RF	6 (87)	6 (76)	7 (62)
Yecora	Subhumid	15–31	900	1,510 to 2,118	RF	8 (113)	5 (42)	10 (84)

^v Number of samples was dependent on the availability of maize fields at the time of sampling across the AES. Numbers in parenthesis correspond to the *Aspergillus flavus* L morphotype isolates recovered and characterized into vegetative compatibility groups from each AES during each year.

^w Temperature maxima range.

^x Approximate annual precipitation.

^y Elevation of sampling locations (meters above sea level).

^z IR = irrigated land and RF = rain-fed land.

individually into one of three 3-mm wells spaced 1 cm apart in a triangular pattern on SA and incubated for 7 days (31°C in darkness). Complementary isolates were placed in the VCG defined by that tester pair. Tester pairs were generated and complementations performed until each isolate was assigned into a VCG. In a similar manner, isolates were also tested for membership in YV36, the VCG to which the biocontrol agent AF36 belongs, utilizing previously described testers ATCC 96045 and ATCC 96047 (Cotty 1994b).

Diversity indices. Simpson's diversity index (D) was calculated for *A. flavus* L morphotype VCGs in single and multiple years for both individual and combined AES by assessing proportions of the total *A. flavus* L morphotype population composed by each VCG: $D = 1 / \sum_{i=1}^S P_i^2$, where P_i is the proportion for the i th VCG and S is the total number of VCGs in the population (VCG richness). Equitability (E) was obtained by calculating the proportion of the maximum possible value of D ($D_{\max} = S$), assuming even distribution of isolates among VCGs: $E = D/S$. E assumes values between 0 and 1. The closer E gets to 1, the more equitable is the population (Begon et al. 1996). D increases with equitability for a given richness and increases with richness for a given equitability.

Shannon-Wiener diversity index (H) was calculated for single and combined years for both individual and combined AES with P_i values: $H = - \sum_{i=1}^S P_i \ln P_i$. Equitability (J) was calculated as $H/\ln S$. J assumes values between 0 and 1 (Begon et al. 1996).

A simple diversity index was calculated by dividing the number of isolates with the number of VCGs in single and multiple years for both individual and combined AES, as described by Horn and Greene (1995). Equitability for simple diversity was not calculated.

Isolate ability to produce aflatoxins. Aflatoxin-producing ability of each of the 387 *A. flavus* L morphotype isolates recovered from 2006 was assessed in a previous study (Ortega-Beltran et al. 2015).

Statistical analyses. All statistical analyses were conducted with SAS 9.2 (SAS Institute Inc., Cary, NC). VCG frequencies, diversity indices, and equitability among (same year) and within (across years) AES were summarized and analyzed with the general linear model procedure. Means of frequent VCGs were separated with Fisher's least significant difference test ($\alpha = 0.05$); means of VCG SON001 across years were analyzed with the Kruskal-Wallis test because variances were unequal. Significant differences were detected and means were separated with Dunn's multiple comparison procedure. Means of diversity indices and equitability for both Simpson's and Shannon-Wiener's methods were standardized by

dividing each value by the overall mean of the corresponding statistic. Pearson correlations of dominant VCGs between years for individual AES and the combined four AES were conducted using the CORR Procedure of SAS ($\alpha = 0.05$).

RESULTS

Association of *A. flavus* VCGs with maize soils. In a previous study, 869 *A. flavus* L morphotype isolates were recovered from 83 maize field soil samples collected from 2006 to 2008 (Ortega-Beltran et al. 2015). In the current study, those 869 isolates were assigned into 136 VCGs. The number of VCGs differed significantly in each of the 3 years. In 2007 and 2008, greater than five times more VCGs were detected than during 2006, even though more isolates were examined in 2006 (Table 2). This resulted in a simple diversity index equal to 0.03, 0.37, and 0.34 for 2006, 2007, and 2008, respectively. VCGs for which only a single member was detected ranged from 21 to 48% annually (Table 2). VCGs were grouped in five ranges based on the number of members: (i) 1, (ii) 2 to 4, (iii) 5 to 10, (iv) 11 to 20, and (v) >20 members (Table 3). The great majority of VCGs (79%) were composed of fewer than five members. More than 66% of the VCGs were found only during a single year. All VCGs composed of >20 members were detected in multiple years. In all, 87 VCGs (64%) were detected only in a single AES. Forty-four VCGs (32%) were found in more than 1 year, with 12 VCGs (9%) occurring in all 3 years (Table 3). All 12 VCGs detected in all 3 years were found in at least two AES and 8 of these VCGs (6%) were detected in all four AES (Table 3). Only 10 dominant VCGs (7%) occurred in all four AES (two of the VCGs occurring in all AES were not counted as dominant because their frequencies did not exceed 1%).

VCGs that made up >1% of the L morphotype population (14 total) across the 3 years were considered dominant (Table 4). Frequencies of the dominant VCGs fluctuated over time, with seven of them differing significantly ($P < 0.05$) among years (Table 4). For example, SON003 in 2006 was the sole VCG detected in all sampled fields during any single year when it composed more than 75% of the L morphotype population. However, SON003 was only detected in six fields across the four AES during 2007 and in two fields in only one AES in 2008, when it composed <1% of the overall population. Similarly, the second most dominant VCG in 2006, SON001, detected in 22 fields, was not detected during 2007, and only two isolates, each from a separate AES, were detected in 2008.

VCGs SON004, SON007, SON008, SON050, and SON122 were detected in low frequencies in either one or two AES during 2006 but were more common during 2007 and/or 2008. VCG SON034 was ninefold more common in 2008 than in 2007 ($P < 0.05$) but was not detected in 2006. VCG SON024 was the sole dominant VCG detected in only a single year (2008); however, SON024 frequencies were not significantly ($P = 0.2855$) different across years. Frequencies of dominant VCGs during 1 year were not correlated with frequencies in other years when examining individual AES or the

Fig. 1. Approximate location of collection sites of fields cropped to maize in four agroecosystems of Sonora, Alamos (1), Tesopaco (2), Yaqui Valley (3), and Yecora (4), from which populations of *Aspergillus* section *Flavi* were sampled from 2006 to 2008.

TABLE 2. *Aspergillus flavus* L morphotype isolates and vegetative compatibility groups (VCGs) detected in four agroecosystems (AES) of Sonora, Mexico

Variable	Year		
	2006	2007	2008
Isolates ^a	387 (0.95)	240 (0.38)	242 (0.47)
Total VCGs ^b	15 (1)	91 (46)	84 (41)
VCGs with only 1 member ^c	4	44	18

^a *A. flavus* L morphotype isolates collected in the four AES. Numbers in parenthesis correspond to proportions of the L morphotype within the *Aspergillus* section *Flavi* population during the indicated year.

^b Number of *A. flavus* L morphotype VCGs detected in each year across the four AES. Italicized numbers in parenthesis correspond to VCGs found solely during that year.

^c VCGs detected once during the indicated year.

combined four AES (Table 5). Representative examples of variation in incidences of VCGs among AES and across years are given in Figure 2. In all cases, relatively high VCG frequencies were detected only during a single year and, sometimes, only in a single AES.

VCG YV36, to which the atoxigenic biocontrol agent *Aspergillus flavus* AF36 belongs (Cotty et al. 2007; Ehrlich and Cotty 2004), was detected all 3 years in each AES, with no significant differences in

frequencies across years. AF36 has not been applied to maize or any other crop in the examined AES. Indeed, there is no registration for AF36 use in Mexico. The detected YV36 isolates from Sonora belong to a fairly common YV36 population endemic to Mexico (Ortega-Beltran et al. 2016).

Aflatoxin-producing ability. In a previous study, 95% of the *A. flavus* L morphotype isolates from 2006 produced aflatoxin

TABLE 3. Detection of *Aspergillus flavus* L morphotype vegetative compatibility groups (VCGs) in four agroecosystems (AES) of Sonora, Mexico

FG ^x	Single year ^w					Two years ^w					3 years ^w					Total VCGs ^y
	VCG	AES				VCG	AES				VCG	AES				
		1	2	3	4		1	2	3	4		1	2	3	4	
1 (rare)	52	52	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	52
2–4	34	27	7	0	0	20	6	11	3	0	1	0	1	0	0	55
5–10	3	2	0	1	0	8	0	3	5	0	4	0	1	2	1	15
11–19	1	0	1	0	0	4	0	2	2	0	1	0	0	0	1	6
>20 (common)	0	0	0	0	0	2	0	0	0	2	6	0	0	0	6	8
Total VCGs ^z	90	...	...	...	...	34	...	...	...	...	12	...	...	...	...	136

^w Number of VCGs detected during either a single year, 2 years, or 3 years. Number of VCGs per classification and the number of AES in which VCGs were detected is indicated. For example, for VCGs composed of 2 to 4 members and detected during a single year (34 total), 27 VCGs were detected in a single AES while 7 VCGs were detected in two AES. NA indicates not applicable, for examples, VCGs with 1 member, can only be detected in 1 AES, and only in 1 year. Bold numbers indicate where VCGs were detected.

^x Frequency group (FG). Isolates were assigned to VCGs through complementation of nitrate nonutilizing mutants. Five ranges were used to classify VCGs: VCGs with (i) 1, (ii) 2 to 4, (iii) 5 to 10, (iv) 10 to 20, and (v) >20 members.

^y Number of VCGs within each FG summed across the 3 years.

^z Sum of VCGs detected during 1, 2, or 3 years.

TABLE 4. Frequencies (percentages) of vegetative compatibility groups (VCGs) that composed more than 1% of the entire *Aspergillus flavus* L morphotype population in four agroecosystems (AES) of Sonora, Mexico over a 3-year period^w

VCG ^x	2006						2007						2008						Across years ^y
	AES					Loc	AES					Loc	AES					Loc	
	YV	AL	TE	YE	Mean		YV	AL	TE	YE	Mean		YV	AL	TE	YE	Mean		
SON001	2.2	4.4	2.3	3.9	3.3 b	22	0	0	0	0	0 e	0	0.4	0	0	0.4	0.3 b	2	A, B, AB*
SON003	17.8	17.1	16.5	24.0	19.5 a	27	1.7	0.8	0.4	0.4	1.4 a-e	6	0	0	1.7	0	0.7 b	2	A, B, B***
SON004	0.5	0	0	0.5	0.3 c	2	1.3	0.4	0.8	0.4	1.2 b-e	6	0.4	0.4	2.9	0.4	1.7 ab	7	A, A, A
SON006	0.8	0.5	1.6	0.3	0.8 c	8	0	0	0.4	0	0.2 de	1	0	0	0	0.4	0.2 b	1	A, B, B*
SON007	1.3	0	0	0	0.3 c	1	1.7	0.4	3.3	0.4	2.4 a-c	8	0.4	0	0	0.4	0.3 b	2	A, A, A
SON008	0.3	0	0.8	0	0.3 c	2	2.9	0.4	2.1	1.7	3.0 ab	11	0.4	0	0.8	0	0.5 b	3	B, A, B*
SON024	0	0	0	0	0 c	0	0	0	0	0	0 e	0	0	0	5.4	0.8	2.6 ab	3	A, A, A
SON026	0	0	0	0	0 c	0	1.7	0	0.8	0	1.0 c-e	6	0	6.6	0	3.7	4.3 ab	6	A, A, A
SON027	0	0	0	0	0 c	0	1.3	0	0.8	0.4	1.0 c-e	6	0	0	4.1	0.8	2.0 ab	4	A, A, A
SON034	0	0	0	0	0 c	0	0	0.8	0	0	0.3 de	2	0.8	2.5	2.1	1.7	2.9 ab	8	B, B, A*
SON040	0	0	0	0	0 c	0	0.8	0	2.1	1.7	1.9 a-d	6	0.8	0	0.4	0	0.5 b	2	B, A, AB
SON050	0	0	0	0	0 c	0	0.8	1.7	0.8	0.4	1.6 a-e	6	0	0	0.8	0	0.3 b	1	B, A, B*
SON122	0.3	0	0	0	0.1 c	0	0.8	3.8	1.7	1.3	3.1 a	11	1.2	0.4	0	1.2	1.2 ab	7	B, A, AB*
YV36	0.3	0.5	0.3	0.3	0.3 c	5	0.4	0	1.3	0	0.7 c-e	3	0	0	0	5.0	1.2 ab	1	A, A, A
P value ^z	...	...	...	...	<0.0001	...	...	...	...	...	0.0065	...	...	...	...	...	0.4544	...	...

^w Percentage of the total population (869 isolates) composed of each VCG in each AES: YV = Yaqui Valley, AL = Alamos, TE = Tesopaco, and YE = Yecora. Mean = average VCG frequency across the four AES. Values correspond to the average incidence during the 3 years. VCG means with the same lowercase letters are not significantly different by Fisher's protected least significant difference (LSD) test ($\alpha = 0.05$). Loc = number of locations in which each VCG was detected.

^x Code used to identify VCGs in Sonora. The listed VCGs composed more than 1% of the *A. flavus* L morphotype population during the 3 years of the study. Frequencies were determined by assigning 869 isolates recovered across 3 years in four AES to VCGs with analyses based on nitrate-nonutilizing mutants.

^y Separation of means among years based on Fisher's protected LSD test ($\alpha = 0.05$), except for SON001, where Kruskal-Wallis test and Dunn's multiple comparison ($\alpha = 0.05$) were used due to unequal variances. First, second, and third uppercase letters correspond to VCG frequencies during 2006, 2007, and 2008, respectively. A single asterisk indicates $P < 0.05$. Triple asterisks indicate $P < 0.0001$.

^z P value of mean separation of frequencies of VCGs in single years based on Fisher's protected LSD test ($\alpha = 0.05$).

TABLE 5. Pearson correlation coefficients for the comparison of frequencies of dominant vegetative compatibility groups between years for individual and combined agroecosystems (AES)

AES ^z	Period		
	2006–2007	2006–2008	2007–2008
AL	0.0054 ($P = 0.9853$)	–0.1458 ($P = 0.6189$)	–0.0885 ($P = 0.7634$)
TE	–0.2361 ($P = 0.4164$)	0.0074 ($P = 0.9797$)	–0.4297 ($P = 0.1252$)
YE	–0.0786 ($P = 0.7894$)	–0.2185 ($P = 0.4528$)	–0.4009 ($P = 0.1554$)
YV	0.2253 ($P = 0.4386$)	–0.2234 ($P = 0.4425$)	–0.0573 ($P = 0.8455$)
All four AES	–0.0244 ($P = 0.934$)	–0.2078 ($P = 0.4758$)	–0.3216 ($P = 0.262$)

^z AL = Alamos, TE = Tesopaco, YE = Yecora, and YV = Yaqui Valley.

B₁ (range = 45 to 24,000 ng/g of mycelium weight) in liquid fermentation (Ortega-Beltran et al. 2015). In the current study, aflatoxin-producing potential was associated with VCG grouping. Seventeen isolates did not produce detectable amounts of aflatoxins and these were recovered from all AES, with five each in YV and TE, four in YE, and three in AL. The atoxigenic isolates belonged to eight VCGs (data not shown). Only two of these VCGs contained exclusively atoxigenic members, one of them being YV36 (Ortega-Beltran et al. 2016). Six of the dominant VCGs (Table 4) contained at least one atoxigenic isolate (data not shown), including one isolate belonging to SON003, the most dominant VCG during 2006.

Diversity indices. In each year, VCG diversities did not differ ($P < 0.05$) among AES but diversity within each AES and the combined AES differed significantly between years. Diversity results were similar with each of the three diversity indices. Therefore, only values for Simpson's diversity index are presented in Table 6. Equitability results were also similar for Simpson's and Shannon-Wiener and only the results from the former are presented. Less ($P < 0.001$) diversity was detected in the examined populations during 2006 than in either 2007 or 2008 (Table 6). Simpson's diversity indices were greater in both 2007 and 2008 than in 2006 both within each AES and for the combined four AES (Table 7). When analyzing VCG diversity indices in all possible combinations of 2 years, similar diversity was detected both within each AES and for the combined four AES (Table 7). However, diversity was higher when the data from all 3 years were combined than it was for any 2 years combined. The greatest diversity was detected when only a single year or a single AES was examined (Table 7).

DISCUSSION

In Mexico, maize is cultivated regardless of elevation, soil type, rainfall, and AES (INEGI 1988; Plasencia 2004; SIACON 2010). The state of Sonora, located in northwest Mexico, has AES representative of Mexico's maize-producing environments over relatively short distances (Ortega-Beltran et al. 2015). Thus, Sonora is an excellent location to study influences of AES on population

dynamics of aflatoxin-producing fungi associated with maize. The current study examined genetic variability exclusively within the *A. flavus* L morphotype resident in maize field soils of four AES in Sonora (Table 1). We prioritized the analysis on the L morphotype because (i) this morphotype dominates aflatoxin-producing fungal communities associated with many crops, including maize (Cardwell and Cotty 2002; Cotty 1997; Donner et al. 2009; Doster and Michailides 1994a; Horn and Dörner 1999; Mauro et al. 2013; Ortega-Beltran et al. 2015); (ii) aflatoxin-producing ability varies greatly among L morphotype VCGs (Bayman and Cotty 1993; Cotty et al. 1994); and (iii) atoxigenicity in L morphotype VCGs is a trait exploited to prevent crop aflatoxin contamination (Alaniz Zanon et al. 2013; Atehnkeng et al. 2014; Bandyopadhyay et al. 2016; Cotty et al. 2007; Dörner 2009; Mauro et al. 2015; Mehl et al. 2012). Genetic diversity was assessed within the *A. flavus* L morphotype by assigning all 869 isolates recovered during the course of 3 years into VCGs by employing *nit* mutants on VCA (Bayman and Cotty 1991a). This allowed comparison with previous, less-extensive efforts to detect shifts in aflatoxin-producing fungal genotypes (Barros et al. 2006; Bayman and Cotty 1991b, 1993; Horn and Greene 1995; Mauro et al. 2013; Novas and Cabral 2002; Pildain et al. 2004; Sweany et al. 2011) and studies examining genetic diversity among atoxigenic genotypes (Atehnkeng et al. 2016; Horn and Dörner 1999; Probst et al. 2011).

VCA were conducted immediately after *nit* mutant recovery by testing for membership in VCG YV36. Interest in investigating frequencies of this VCG stems from use of AF36 as the active ingredient in the first biopesticide registered by the U.S. Environmental Protection Agency for use in mitigation of aflatoxin contamination. AF36 continues to be used in cotton, maize, and pistachio (Cotty et al. 2007; Doster et al. 2014). YV36 frequencies span over large portions of the United States, including the Sonoran Desert (Cotty 1989, 1997; Grubisha and Cotty 2015). Sonora and Arizona share over 350 miles of border and large portions of these states belong to the Sonoran Desert (Ricketts et al. 1999), a region in which aflatoxin-producing fungi are ubiquitous in both agricultural and noncultivated areas (Boyd and Cotty 2001; Cotty 1989).

Fig. 2. Variation in frequencies of common *Aspergillus flavus* L morphotype vegetative compatibility groups in the examined agroecosystems across 3 years. YV = Yaqui Valley, AL = Alamos, TE = Tesopaco, and YE = Yecora.

Detection of YV36 in maize field soils of Sonora was expected. VCG YV36 was widely distributed in Sonora (Ortega-Beltran et al. 2016) (Table 4). Approximately 1% of the L morphotype isolates were assigned to YV36. The current study is the first to address frequencies of additional VCGs in Sonora.

The L morphotype composed a greater proportion of the *A. flavus* population during 2006 than in either 2007 or 2008 (Ortega-Beltran et al. 2015) (Table 2). Over 35% more isolates were recovered during 2006 compared with the other years and, thus, greater VCG diversity was expected during 2006 because genetic diversity in microorganism populations tends to increase with the number of examined individuals (Grünwald et al. 2003). However, this was not the case, and fivefold more VCGs were detected in 2007 and 2008 than in 2006 (Table 2). The number of VCGs detected during 2006

(15 total) was apparently atypical. Studies examining diversity within aflatoxin-producing species generally report large numbers of VCGs even when examining relatively few individuals (Barros et al. 2006; Bayman and Cotty 1991b; Horn and Greene 1995; Mauro et al. 2013; Novas and Cabral 2002). Surprisingly, most VCGs detected in 2006 became either infrequent or absent in 2007 or 2008 (Tables 3 and 4). VCGs dominating communities in 2007 or 2008 were found at low frequencies in other years (limit of detection = 7% within fields). This suggests that dominance of VCGs is transient (Fig. 2). This transience is detected as statistically significant variation in VCG proportions among years (Table 4).

Genotypes of *A. flavus* vary in sporulation during competition on crops, with incidence of certain genotypes increasing during crop production (Mehl and Cotty 2010). Results of the current study may reflect increased sporulation by highly frequent VCGs on unknown substrates and rapid dispersal of conidia over relatively large distances. Long-distance movement of fungi through atmospheric pathways occurs regularly (Isard et al. 2005). However, founder effects likely also played an important role in dictating VCG success prior to each sampling period. The current results suggest that success on one crop during one period does not guarantee subsequent dominance. Competition among genotypes is influenced by host (Mehl and Cotty 2010, 2013) and substrate but other biotic or abiotic factors may also influence compositions of aflatoxin-producing fungal communities. These influences, along with impacts of founder effects, may explain the annual shifts in *A. flavus* population composition observed in the current work. Certainly there are more VCGs in the region than we detected and the purpose of the current work was not to detect all VCGs in an area. Indeed, no study has been designed that could achieve this because there are likely thousands of VCGs (Bayman and Cotty 1991b; Mehl and Cotty 2010). However, the current design was sufficient to detect the most frequent VCGs in each of the sampled AES in each of the 3 years. These differences were sufficiently consistent that the preplanned statistical comparisons detected significant differences in frequencies of the major VCGs across years. This is a much more robust sampling, with more isolates better distributed in a planned manner and more sampled fields than any *A. flavus* VCG study previously undertaken.

In most cases, AES had no influence on frequencies of dominant VCGs, with these VCGs occurring at similar frequencies across AES (Tables 3 and 4). When the study was designed, it was anticipated that adaptive differences among VCGs would result in detection of AES-specific VCGs because *A. flavus* populations vary over geography (Jaime-Garcia and Cotty 2006) and are influenced by host species (Mehl and Cotty 2013) and crop rotation (Jaime-Garcia and Cotty 2010). However, we detected that several VCGs increased in frequency in multiple AES in a single year (Tables 3 and 4) regardless of differences among AES in elevation, soil pH, climate, precipitation, and cropping system (Table 1).

TABLE 6. Variability in *Aspergillus flavus* L morphotype vegetative compatibility groups (VCG) diversity among four agroecosystems (AES) of Sonora, Mexico

AES ^x	Simpson's diversity ^w			Mean ^y
	2006	2007	2008	
YV				
<i>D</i>	2.1 (0.15)	24.8 (1.88)	29.5 (2.24)	1.42 a
<i>E</i>	0.15 (0.30)	0.65 (1.36)	0.87 (1.81)	1.16 x
AL				
<i>D</i>	1.6 (0.12)	16.7 (1.27)	7.4 (0.56)	0.65 a
<i>E</i>	0.41 (0.85)	0.58 (1.20)	0.39 (0.81)	0.95 x
TE				
<i>D</i>	1.8 (0.14)	25.8 (1.95)	15.3 (1.15)	1.08 a
<i>E</i>	0.22 (0.47)	0.63 (1.31)	0.48 (0.99)	0.92 x
YE				
<i>D</i>	1.4 (0.11)	18.8 (1.42)	12.9 (0.98)	0.84 a
<i>E</i>	0.24 (0.50)	0.75 (1.56)	0.42 (0.87)	0.98 x
Mean <i>D</i> ^z	0.13 B	1.63 A	1.24 A	...
Mean <i>E</i> ^z	0.53 C	1.36 D	1.12 D	...

^w Simpson's diversity index (*D*) and equitability (*E*) (Simpson 1949) were calculated each year for VCGs detected in each AES. Untransformed *D* and *E* values are indicated for their corresponding year in regular font (i.e., *D* in YV during 2006 is 2.1). Numbers in parenthesis and in italics were obtained by dividing the untransformed value of *D* by the overall mean of *D*, taking into account each AES during the 3 years, and the same criterion was used to calculate values of *E* in italics. This was done in order to homogenize *D* and *E* variances among AES and within years.

^x YV = Yaqui Valley, AL = Alamos, TE = Tesopaco, and YE = Yecora.

^y Means of the transformed *D* and *E* values among AES were separated using Fisher's protected least significant difference (LSD) test ($\alpha = 0.05$). Transformed *D* means with the same lowercase letter (a) are not significantly different. Transformed *E* means with the same lowercase letter (x) are not significantly different.

^z Means of the transformed *D* and *E* values across years were separated using Fisher's protected LSD test ($\alpha = 0.05$). Transformed *D* means with the same uppercase letter (A, B) are not significantly different. Transformed *E* means with the same uppercase letter (C, D) are not significantly different.

TABLE 7. Variability in Simpson's diversity and equitability indices for vegetative compatibility groups (VCGs) of *Aspergillus flavus* L morphotype populations among four agroecosystems (AES) for individual and combined years

Period ^z	Simpson's diversity and equitability of VCGs ^y									
	YV		AL		TE		YE		All AES	
	<i>D</i>	<i>E</i>	<i>D</i>	<i>E</i>	<i>D</i>	<i>E</i>	<i>D</i>	<i>E</i>	<i>D</i>	<i>E</i>
2006	2.1	0.15	1.6	0.41	1.8	0.22	1.4	0.24	1.7	0.11
2007	24.8	0.65	16.7	0.58	25.8	0.63	18.8	0.75	37.0	0.41
2008	29.5	0.87	7.4	0.39	15.3	0.48	12.9	0.42	29.6	0.35
2006 and 2007	3.4	0.07	3.3	0.10	3.1	0.07	4.6	0.16	3.9	0.04
2006 and 2008	4.1	0.10	2.9	0.13	2.9	0.05	3.7	0.11	3.7	0.04
2007 and 2008	3.2	0.05	2.6	0.06	3.0	0.05	2.9	0.06	3.7	0.03
All 3 years	7.4	0.11	6.8	0.15	10.6	0.17	4.9	0.10	7.0	0.05

^y Simpson's diversity index (*D*) and equitability (*E*) (Simpson 1949) were calculated for each AES and the four AES combined during the specified period. YV = Yaqui Valley, AL = Alamos, TE = Tesopaco, and YE = Yecora.

^z Period during which VCGs were analyzed.

Frequencies of dominant VCGs were not correlated between years (Table 5). This was observed in each individual AES as well as in the combined four AES. Increased frequencies of a dominant VCG may have resulted from a founder event followed by rapid reproduction and dispersal rather than from superior adaptation to specific ecological pressures (Isard et al. 2005). In the short term (i.e., over the course of a season), optimal adaptation to an AES may not be a critical factor for rapid genotype increases. Adaptation may be more important for long-term (i.e., multiple years) residence of a genotype in a target AES. Greater persistence in soils (Jaime-Garcia and Cotty 2006, 2010) and organic matter (Jaime-Garcia and Cotty 2004) provides greater opportunity to participate in founder effects during subsequent crop production.

In 2006, VCG SON003 dominated every maize field soil (Table 4), with similar frequencies across AES ($P = 0.5572$, data not shown). More than 75% of the L morphotype population of 2006 was composed of members of SON003. Other important VCGs during 2006 included SON001 and YV36, with 12 and 1% of the population, respectively (Table 4). High incidences of these three VCGs masked detection of other VCGs and, as a result, fewer VCGs were detected during 2006 in comparison with both 2007 and 2008. Similar high frequencies of an *Aspergillus* VCG over a large area have not been previously reported. The sole report of a VCG dominating aflatoxin-producing fungal communities in an unusual fashion was a VCG composing 55% of the population associated with maize substrates collected from 11 fields in Mississippi (Sweany et al. 2011). Dominant VCGs in other reports have frequencies of <25% and, commonly, <10% (Barros et al. 2006; Bayman and Cotty 1991b; Horn and Dörner 1999; Horn and Greene 1995; Houshyar-Fard et al. 2014; Mauro et al. 2013; Novas and Cabral 2002; Pildain et al. 2004; Probst et al. 2011). However, unlike the current study, all previous studies of VCG diversity among aflatoxin-producing fungal communities assigned only a portion of the examined isolates into VCGs. In any given study, unsuccessful assignment of all recovered isolates into their corresponding VCG may skew frequencies. For example, if isolates belonging to SON003 were not placed into a VCG, SON001 would have been the most common VCG detected in 2006.

Forces driving SON003 to dominate across diverse AES (Table 1) in 1 year and then decline in subsequent years have not been identified. Incidences of SON003 across the examined AES resembled incidences of atoxigenic *A. flavus* strains used as active ingredients in biopesticides directed at preventing aflatoxin contamination (Atehnkeng et al. 2014; Bandyopadhyay et al. 2016; Cotty et al. 2007). During the treatment year, the active ingredient VCG dominates the *A. flavus* community on the treated crop. In subsequent years, the atoxigenic VCG declines in frequency (Cotty and Mellon 2006). In the current study, increases and decreases in frequency were also detected for other VCGs, although at a lower magnitude than that of SON003 (Fig. 2; Table 4). Eight of the most frequent VCGs had >20 members and were all found in all four AES; six of those VCGs were found in all 3 years (Table 3). High frequencies of VCGs during a given year were associated with lower frequencies during previous or subsequent years (Fig. 2). Occurrence of VCGs at high frequencies in single or multiple AES may be dictated by founder effects. This was previously observed in a cotton field in the Yuma Valley, AZ, where high frequencies of VCGs during single years were attributed to founder effects (Bayman and Cotty 1991b).

Cropping systems in YV differ greatly from those in the other AES (Ortega-Beltrán et al. 2015) but such differences had little or no effect on VCG distribution (Table 4). This observation supports the notion that atoxigenic biocontrol products may be useful across multiple AES. Whether biotic or abiotic factors trigger the temporary dominance and proliferation of a VCG across contrasting AES is unknown. Mechanisms that might allow individual founder effects to influence *A. flavus* communities over areas as large as those observed in the current work have not been described.

A. flavus may vary in virulence, aflatoxin-producing potential, and other potentially adaptive traits (Cotty 1989; Cotty et al. 1994), and adaptive differences among VCGs may be sufficient to drive small shifts in VCG composition (Mehl and Cotty 2013; Mehl et al. 2012). However, increased frequencies of an individual VCG across several AES during a single year followed by rapid posterior decline in subsequent years cannot be explained by known adaptive differences among *A. flavus* VCGs.

VCG diversity varies with cropping system and location (Barros et al. 2006; Bayman and Cotty 1991b; Horn and Dörner 1999; Horn and Greene 1995; Horn et al. 1996; Mauro et al. 2013; Novas and Cabral 2002; Pildain et al. 2004; Probst et al. 2011; Sweany et al. 2011). However, VCG diversity is frequently examined in similar locations and/or during single years. In the current study, we examined VCG diversity across largely contrasting AES over multiple years and VCG diversity indices significantly ($P < 0.05$) differed among years (Table 6). This suggests that examining genetic diversity during a single year in either single or multiple AES has considerable limitations in estimating genetic diversity and identifying highly frequent VCGs for use as active ingredients in biopesticide formulations. Similar levels of VCG diversity were detected when examining any combination of 2 years in either a single AES or the combined four AES (Table 7). Studies examining VCG diversity should include samples collected over at least 2 years and, for more realistic estimations of VCG diversity, additional years (Table 7).

Labor requirements and other costs limit extensive examination of aflatoxin-producing genotypes in any given area. Tester pair combinations including a *cnx* mutant are preferred because pairings of a *cnx* mutant with *niaD* mutants of the same VCG result in reliable complementation (Bayman and Cotty 1991b; Cove 1976). On average, *niaD* and a *nirA*⁻ pairings result in weaker reactions and occasional failure to detect membership in a VCG (Bayman and Cotty 1991a; Correll et al. 1987; Pildain et al. 2004). In the current study, *cnx* mutant yields were low (<3% of the recovered mutants; data not shown). Low *cnx* frequencies increase the required labor for developing tester pairs. In all, 91% of the developed tester pairs were composed of a *cnx* and a *niaD* mutant, while the remainder was a *nirA*⁻/*niaD* combination. Before including a *nirA*⁻ in a tester pair, more than 100 mutants of that isolate were generated, stabilized, and phenotyped without finding a *cnx* mutant. Development of culture media promoting increased *cnx* frequencies is needed in order to facilitate and encourage studies examining VCG diversity in aflatoxin-producing fungi. However, regardless of the difficulty in finding reliable tester pairs, this is the first report on *A. flavus* in which each isolate was assigned to a VCG. Self-incompatible isolates were not detected as in other studies on both aflatoxin-producing fungi and other species (Barros et al. 2006; Brooker et al. 1991; Correll et al. 1987, 1988, 1989; Horn and Dörner 1999; Horn and Greene 1995; Hyakumachi and Ui 1987; Mauro et al. 2013; Novas and Cabral 2002; Pildain et al. 2004).

Aflatoxin management strategies have not been developed and disseminated in Mexico even though this country has vast tropical and subtropical regions in which production of maize is at high risk of aflatoxin contamination (Cotty and Jaime-Garcia 2007; Cotty et al. 1994; Ortega-Beltrán et al. 2015; Plasencia 2004; Rodríguez-Del-Bosque 1996). However, maize germplasm and atoxigenic fungi with the potential to decrease exposure of Mexico's population to aflatoxins are known and could be used to implement aflatoxin management (Ortega-Beltrán et al. 2014; Ortega-Beltrán et al. 2016). Atoxigenic VCGs detected in the current study represent genetic resources endemic to Mexico that should be considered for utilization in biocontrol programs for limiting aflatoxin contamination of maize (Cotty 2006; Mehl et al. 2012; Probst et al. 2011).

Knowledge of causes of the observed shifts in compositions of aflatoxin-producing fungal populations may lead to methods for predicting aflatoxin outbreaks (Cotty and Jaime-Garcia 2007) and allow development of practices that promote proliferation of beneficial VCGs (i.e., atoxigenic). Shifts in compositions of populations of

aflatoxin-producing fungi should occur relatively frequently in both highly mechanized and rural agricultural areas with or without the use of atoxigenic biocontrol agents.

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