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# The vegetative compatibility group to which the US biocontrol agent *Aspergillus flavus* AF36 belongs is also endemic to Mexico

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## Abstract

**Aims:** To assess frequencies of the *Aspergillus flavus* atoxigenic vegetative compatibility group (VCG) YV36, to which the biocontrol agent AF36 belongs, in maize-growing regions of Mexico.

**Methods and Results:** Over 3500 *A. flavus* isolates recovered from maize agroecosystems in four states of Mexico during 2005 through 2008 were subjected to vegetative compatibility analyses based on nitrate nonutilizing mutants. Results revealed that 59 (1·6%) isolates belong to VCG YV36. All 59 isolates had the *MAT1-2* idiomorph at the mating-type locus and the single nucleotide polymorphism in the polyketide synthase gene that confers atoxigenicity. Additional degradation of the aflatoxin gene cluster was detected in three isolates. Microsatellite loci analyses revealed low levels of genetic diversity and no linkage disequilibrium within VCG YV36.

**Conclusions:** The VCG to which the biocontrol agent AF36 belongs, YV36, is also native to Mexico. The North American Free Trade Agreement should facilitate adoption of AF36 for use by Mexico in aflatoxin prevention programs.

**Significance and Impact of the Study:** An USEPA registered biocontrol agent effective at preventing aflatoxin contamination of crops in the US, is also native to Mexico. This should facilitate the path to registration of AF36 as the first biopesticide for aflatoxin mitigation of maize in Mexico. Economic and health benefits to the population of Mexico should result once aflatoxin mitigation programs based on AF36 applications are implemented.

## Introduction

Aflatoxins are highly toxic and carcinogenic mycotoxins produced by several members of *Aspergillus* section *Flavi*. These potent toxins contaminate crops posing serious health threats including growth impairment, immune system suppression, hepatocellular carcinoma, acute toxicity, and death (Cotty *et al.* 1994; CAST 2003; Probst *et al.* 2007; Liu *et al.* 2012). Aflatoxin $B_1$, the most toxic and prevalent, is classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC) (IARC 2002). Foods and feeds exceeding the stringent aflatoxin limits imposed and monitored by developed countries are rejected from premium markets, resulting in financial loss to farmers, wholesalers, and processors (Rustom 1997; Wu 2004, 2015; van Egmond *et al.* 2007). On the other hand, in emerging and developing countries, regulation of aflatoxin-contaminated commodities is poorly enforced due to lack of equipment and qualified personnel for aflatoxin quantification, and direct commodity consumption within producing villages (Plasencia 2004; Wu 2004; Shephard 2008).

In warm regions across the globe, maize, a critical staple for billions, frequently becomes contaminated with aflatoxins either prior to or after crop maturation (Bhatnagar *et al.* 1993; Bilgrami and Choudhary 1998; Plasencia 2004; Binder *et al.* 2007). Both insect activity and stress may increase maize predisposition to fungal infection and subsequent aflatoxin contamination during crop development, while high humidity, including rain, fog and irrigation during warm periods promotes contamination after crop maturity (Russell *et al.* 1976; Barry 1987; Cotty *et al.* 2008). Several cultural practices can be implemented to reduce maize susceptibility to aflatoxin contamination, although slight environmental changes may render those practices of little or no use (Lillehoj *et al.* 1984; Payne *et al.* 1986, 1989; Jones 1987; Wilson and Payne 1994; Jaime-Garcia and Cotty 2004).

Maize, which provides over half of the daily caloric intake of the majority of Mexico's population, is produced throughout Mexico primarily in regions at risk of aflatoxin contamination (INEGI 1988; Bhatnagar *et al.* 1993; Plasencia 2004; FAO 2010). However, few reports of maize aflatoxin contamination emerge in Mexico (Torreblanca *et al.* 1987; Rodríguez-Del Bosque *et al.* 1995; Rodríguez-Del Bosque 1996; Carvajal and Arroyo 1997; Guzman de Peña and Peña-Cabriales 2005; Garcia and Heredia 2006) although aflatoxin contamination events are known to occur. For example, in 2011, virtually the entire commercial maize crop in Sinaloa, the most important maize-producing state in Mexico, had to be replanted due to a rare freeze during February of that year. Replanting extended cropping until late summer, exposing the developing crop to higher temperatures which predisposed the maize to aflatoxin contamination (Bhatnagar *et al.* 1993; Wilson and Payne 1994; Guo *et al.* 2008). No written reports from this episode of contamination were made. Absence of reports in a major maize-producing state suggests that aflatoxin contamination events in other states are similarly not known to the public.

The most frequently identified causal agent of aflatoxin contamination is *Aspergillus flavus* (Klich 2007). This species is composed of numerous vegetative compatibility groups (VCGs) that behave as independent clonal lineages (Bayman and Cotty 1991, 1993). Isolates are more closely related within a VCG than between VCGs (Papa 1986; Leslie 1993). VCGs vary widely in several genetic, epidemiological, and physiological characteristics, including aflatoxin-producing ability (Bayman and Cotty 1991; Horn and Greene 1995; Mehl and Cotty 2010). Within certain VCGs, isolates may produce very high aflatoxin concentrations (>1 g $kg^{-1}$), while members of other VCGs have defects in the aflatoxin biosynthesis gene cluster and produce little or no aflatoxins (Joffe 1969; Cotty 1989). Some VCGs are called atoxigenic because they are composed entirely of individuals with no aflatoxin-producing ability (Grubisha and Cotty 2015).

Management to reduce aflatoxin accumulation may be directed at the contamination process *per se* or the aflatoxin-producing fungi themselves (Cotty *et al.* 2008). An effective, simple, and environmentally sound method to reduce aflatoxin accumulation in crops is use of atoxigenic genotypes of *A. flavus* as biocontrol agents. Atoxigenics both influence aflatoxin biosynthesis during crop infection and displace aflatoxin producers when applied at appropriate crop growth stages (Cotty *et al.* 1994; Dorner *et al.* 1999; Dorner 2004; Cotty 2006). Through displacement of aflatoxin producers, the aflatoxin-producing potential of fungal communities associated with crops is reduced resulting in less crop contamination (Cotty *et al.* 2008). Atoxigenic isolates are usually dispersed with a nutrient (i.e. wheat, sorghum, or barley grain) on which the atoxigenic isolate produces conidia for dispersal to the target crop (Cotty and Mellon 2006). The private food source provides both reproductive and dispersal advantage over aflatoxin-producers residing within the soil matrix (Cotty *et al.* 2008). A central feature of biocontrol with atoxigenic isolates is that application influences extend over multiple years and benefit multiple crops with area-wide, long-term, and additive effects (Mehl *et al.* 2012).

In the US, there are two atoxigenic genotypes registered with USEPA for prevention of contamination (Cotty 2006; Dorner and Lamb 2006; Cotty *et al.* 2008; Dorner 2009). One genotype, *Aspergillus flavus* AF36, is produced and distributed by the farmer-governed and financed Arizona Cotton Research and Protection Council (Cotty *et al.* 2007). AF36, originally isolated in Arizona (Cotty 1989), was the first genotype registered with the USEPA for aflatoxin control; it continues to be used on maize, cottonseed, figs, and pistachio (Cotty 2006; Cotty *et al.* 2007; Doster *et al.* 2014; T.J. Michailides, personal communication). Registration of a biopesticide may take several years and requires environmental and health risk assessment. During the registration period use of the biopesticide by farmers is limited (Cotty and Mellon 2006; Cotty *et al.* 2008).

Members of the VCG to which AF36 belongs, YV36, bear a single nucleotide polymorphism (SNP) that causes an early stop codon in the polyketide synthase (*aflC*) gene (Grubisha and Cotty 2015). The enzyme coded by this gene is essential for aflatoxin biosynthesis, and thus the *aflC* SNP confers atoxigenicity to YV36 and several other atoxigenic VCGs (Ehrlich and Cotty 2004). However, SNPs, insertions, and deletions in the aflatoxin biosynthesis gene cluster all may cause atoxigenicity within *A. flavus* (Chang *et al.* 2005; Donner *et al.* 2014).

Maize production in Mexico occurs in regions where aflatoxin management is needed to reduce human expo-

sure to aflatoxins. Biocontrol products with atoxigenic *A. flavus* active ingredients are effective management tools. In this study, frequencies of VCG YV36 within aflatoxin-producing fungal communities associated with maize were examined between 2005 and 2008 in four states of Mexico. Presence of the *aflC* SNP within each member of YV36 from Mexico was assessed. In addition, genetic variability among YV36 isolates from Mexico was characterized with microsatellite markers (Grubisha and Cotty 2009), a PCR assay for mating-type locus idiomorphs (Ramirez-Prado *et al.* 2008), and a PCR assay to monitor indels in the aflatoxin biosynthesis gene cluster and adjacent regions (Callicott and Cotty 2015) to determine extents to which members of YV36 from Mexico are divergent from the biocontrol agent AF36.

The primary objective of this study was to determine whether VCG YV36 is endemic to Mexico. Natural distribution in target areas is a common sense requirement for biocontrol agents. Endemic, locally adapted atoxigenic genotypes are preferred as biocontrol agents because introduced agents have potential to cause unanticipated negative effects on the ecology and/or cropping system (Cotty 2006; Probst *et al.* 2011). In addition, endemic atoxigenic isolates are already successful in the target environment demonstrating ability to both effectively compete for resources provided by cropping systems and resist seasonal fluctuations (Probst *et al.* 2011; Mehl *et al.* 2012). Biopesticides directed at reducing aflatoxin contamination and utilizing endemic, well adapted, atoxigenic genotypes of *A. flavus* as active ingredients are registered for use in the US, Nigeria and Kenya, and are under development in several other nations in Africa, the Americas, and Europe (Cotty 2006; Atehnkeng *et al.* 2014; Chulze *et al.* 2015; Mauro *et al.* 2015). In the current report members of VCG YV36, the VCG to which the registered biopesticide AF36 belongs, are shown to be widely distributed in several maize-producing regions of Mexico. This supports utilization of YV36 isolates, including AF36, as active ingredients for biopesticides directed at reducing aflatoxins in maize produced in Mexico. Such products could contribute to reduction of human exposure to aflatoxins.

## Materials and methods

### Isolation of *Aspergillus flavus*

Maize and soils from maize fields were collected in the states of Sonora, Sinaloa, Nayarit, and Tamaulipas (Fig. 1), from 2005 through 2008 in order to isolate aflatoxin-producing and closely related fungi. In total, 3581 *A. flavus* isolates were recovered. Maize field soil samples (~150 g each) from Nayarit (2007), Sonora (2006 to 2008), and Tamaulipas (2005) were composed of multiple subsamples (40–50) from three random locations of each field to a depth of 2 cm (Bayman and Cotty 1991; Cotty 1997). Commercial maize from Sonora (2006 and 2009)


**Figure 1** Locations (●) from which fungi belonging to *Aspergillus flavus* VCG YV36 used in this study were collected. Includes the location from which the original AF36 isolate was collected in Arizona.

and Tamaulipas (2005), and maize land races (MLRs) accessions from Sinaloa (2006), Sonora (2007 and 2008), and Nayarit (2004 and 2006), were imported into the US under an APHIS *Permit to Move Live Plant Pests and Noxious Weeds* and maintained at the USDA-ARS aflatoxin research laboratory in the School of Plant Sciences, University of Arizona, Tucson. *Aspergillus flavus* isolates were obtained by dilution plate technique using modified rose Bengal agar (Cotty 1994a) and maintained as previously described (Probst *et al.* 2007; Ortega-Beltran *et al.* 2015).

### Assignment of isolates to VCG YV36

Vegetative compatibility analyses (VCA) have been used to identify AF36 during the pesticide registration process (USEPA 2003), for isolate tracking during efficacy trials (Cotty 1994b), and for quality control tests during manufacture (Cotty *et al.* 2007). Indeed, in these cases, membership in VCG YV36 is accepted as a proxy for the AF36 genotype. The same testing procedures were used in this study to determine membership of isolates from Mexico in VCG YV36. The 3581 *A. flavus* isolates originating from Mexico were subjected to VCA using nitrate nonutilizing auxotrophs on complementation agar (Cotty and Taylor 2003) following protocols previously described (Bayman and Cotty 1991; Cotty 1994b; Grubisha and Cotty 2010).

### Mating-type idiomorph characterization

*Aspergillus flavus* isolates contain one of two idiomorphs, *MAT1-1* or *MAT1-2*, at the mating-type locus (Ramirez-Prado *et al.* 2008). Frequencies of mating-type idiomorphs among YV36 isolates from the current study were characterized by multiplex-PCR amplification of segments of *MAT1-1* and *MAT1-2* using primers M1F, M1R, M2F, and M2R (Ramirez-Prado *et al.* 2008). Isolates with the *MAT1-1* idiomorph yield a PCR-product of 390 bp, while isolates with the *MAT1-2* idiomorph yield a PCR-product of 270 bp. PCR-products were visualized on agarose gels. Minor modifications to the original multiplex-PCR conditions were made, as described previously (Grubisha and Cotty 2010, 2015).

### Validation of the SNP associated with atoxigenicity in YV36

Members of VCG YV36 contain a single nucleotide polymorphism (SNP) in the polyketide synthase (*aflC*) gene that introduces an early stop codon and results in loss of aflatoxin-producing ability (Ehrlich and Cotty 2004). The G to A polymorphism is located at nucleotide 591 of *aflC*. A multiplex-PCR assay was used to detect this SNP. The assay utilizes two primer pairs (pair 1: SNP36Ta CCTTGGTCTACCATTGTTAGGGG and SNP36Ca AGA ATTGCAGGCCAGGTGGA; pair 2: SNP36Tb GCTGAA GGCTCCTCTTGCTG and SNP36Cb GCTGGGGATCCA GAACTCA), with each pair having a primer binding site incorporating the SNP but on opposite strands: SNP36Ta matches the *aflC* sequence of toxigenic isolates and points towards the telomere, while SNP36Cb matches the *aflC* sequence of YV36 and points towards the centromere. In both cases, the SNP is at the -2 position of the primer, and both primers also incorporate an intentional mismatch at -4 position to destabilize binding by the 'wrong' primer (Kwok *et al.* 1990). Oligonucleotide primers were designed with Geneious Pro 4.8.2 (Biomatters Ltd, Auckland, New Zealand, available from http://www.geneious.com/) and Primer 3 (Rozen and Skaletsky 2000) to amplify either a 920 bp amplicon (SNP present in AF36; amplified with primer pair 2) or a 1225 bp amplicon (SNP present in isolates that produce large quantities of aflatoxins; amplified with primer pair 1). PCR reactions were conducted in a 20 $\mu$l volume using Accupower® Hoststart (Bioneer, Alameda, CA) PCR Pre Mix tubes with 6 ng genomic DNA, $2{\cdot}5e^{-7}$ mol $l^{-1}$ of each primer, and 0·0015 mol $l^{-1}$ $MgCl_2$. PCR amplification conditions started with a denaturing step (94°C, 5 min) followed by 35 cycles of amplification (94°C, 30 s; 61·3°C, 30 s; 72°C, 30 s), and finalized with an elongation step (72°C, 5 min). Amplicons were separated on 1% agarose gels and visually inspected for size and quantity of amplicons per isolate.

### Detection of aflatoxin biosynthesis genes

Mechanisms of atoxigenicity in *A. flavus* may include partial or complete deletions in the aflatoxin biosynthesis gene cluster (Chang *et al.* 2005; Donner *et al.* 2010). Therefore, known indels influencing genes involved in biosynthesis of either aflatoxins or cyclopiazonic acid are routinely monitored with the CAPs multiplex-PCR assay (Callicott and Cotty 2015). All YV36 isolates examined in the current study were subjected to the CAPs assay in order to determine if the entire aflatoxin cluster was retained by all isolates. In total, 32 markers are monitored with the CAPs assay.

### Additional deterioration in *aflC*

During the CAPs assay, three YV36 isolates did not amplify marker AC11 located in *aflC* downstream from the SNP associated with YV36 atoxigenicity (Ehrlich and Cotty 2004). In order to examine this apparent deletion, the amplicon obtained with primers SNP36Tc

(CCTTGGTCTACCATTGTTTGAGG) and SNP36Ca (from the above *aflC* SNP assay) was sequenced. These primers were used in all four combinations of forward and reverse primers to amplify from the three unusual isolates as well as other members of YV36. PCR reactions were conducted as above using relaxed conditions including a denaturing step (94°C, 5 min) followed by 38 cycles of amplification (94°C, 20 s; 54°C, 30 s; 72°C, 90 s), and an elongation step (72°C, 5 min). Amplicons were separated on 1% agarose and visually inspected for size and singularity. PCR-products were purified from excess primers and unincorporated nucleotides using 1 $\mu$l of ExoSAP-IT (USB Corporation, Cleveland) in 18 $\mu$l of PCR-product (1 h at 37°C followed by 15 min at 85°C). Once purified, PCR-products were sequenced in each direction by the University of Arizona Genetics Core with a 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA). Bidirectional sequences were assembled, visually inspected and aligned with the MUSCLE algorithm (Edgar 2004) with standard options of Geneious Pro 4.8.2. A BLAST search (www.ncbi.nlm.nih.gov) was performed to identify nucleotide sequence nonhomologous to *aflC* in the three YV36 isolates that did not amplify AC11 (Fig. 2). Amplicon portions not homologous to *aflC* were 100% identical to a portion of the chromosome arm opposite the arm bearing *aflC*. Two additional primer pairs were designed to study this putative translocation event. Primers SNP36Ta (the first primer from the above *aflC* SNP assay) and pksAac2R (ACTGAT AAAGCTGACTGGGAGAA) amplify a portion of *aflC*. Primers oryreg2F (TCCGGGGACAGCGGGTAGTT) and oryreg4R (TGCCCAGGTACAACAGCTCACT) amplify a region on scaffold SC023 of *Aspergillus oryzae* RIB40 containing the region homologous to the insert detected in *aflC* (Fig. 2). PCR amplification conditions for both primer pairs included a denaturing step (94°C, 5 min) followed by 30 cycles of amplification (94°C, 20 s; 58°C, 30 s; 72°C, 90 s), and an elongation step (72°C, 5 min). Amplicon purification and sequencing was conducted as above. Bidirectional sequences were assembled, visually inspected, and aligned as above.

### Microsatellite genotyping

YV36 isolates were genotyped using 24 microsatellite markers for *A. flavus* (Grubisha and Cotty 2009). DNA isolation, microsatellite multiplex-PCR locus combinations, and microsatellite genotyping were conducted as previously described (Grubisha and Cotty 2009, 2010). Over 20% of isolates were subjected to at least three independent PCR and genotyping assays for all loci for verification of results.

### Population genetic analyses

YV36 isolates were placed into nine *a priori* populations based on origin and year of isolation (Table 1). Haplotypes and allele frequencies were assessed with GENODIVE 2.0b11 (Meirmans and Van Tienderen 2004). Genetic structure within YV36 was assessed with Bayesian clustering program STRUCTURE 2.2.3 (Pritchard *et al.* 2000) to define genetic groups within YV36. Haplotypes were assigned to $K$ populations using the admixture model and default parameters, and Markov Chain Monte Carlo (MCMC) simulations were run for $K = 1$–9 (the nine *a priori* populations). Simulations (10 total) were run with a burn-in length of 100 000 MCMC generations followed by 1 million MCMC iterations for each $K$. The optimal number of populations was obtained using Structure Harvester (Earl and VonHoldt 2012) which calculates the rate of change in the log probability of data between successive runs of $K$ (Evanno *et al.* 2005). Linkage disequilibrium for each pair of polymorphic loci of the 60 YV36 isolates was estimated using MULTILOCUS 1.3b (Agapow and Burt 2001). The most variable locus, AF48, was excluded from the analysis. Linkage disequilibrium was estimated using the index of association ($\bar{r}_d$) standardized for the included loci. Significance was based on 1000

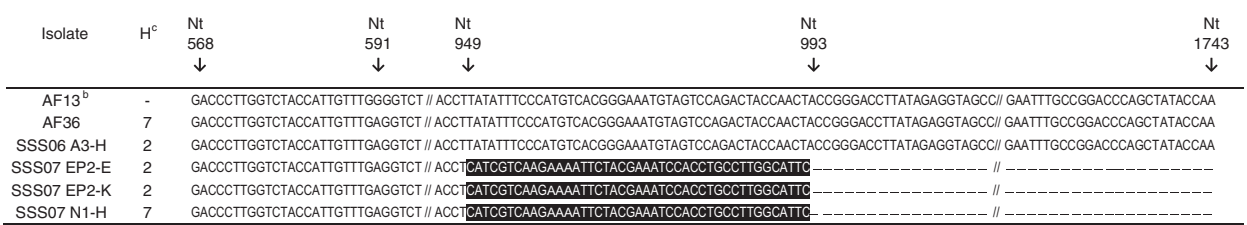


**Figure 2** Comparison of several portions of the *aflC* gene among AF36, four *Aspergillus flavus* isolates endemic to Mexico and belonging to VCG YV36, and AF13, a highly toxigenic *A. flavus* isolate included to illustrate absence of the *aflC* SNP that confers atoxigenicity at nucleotide 591. A 43 bp insert (highlighted in black) was detected in isolates SSS07 EP2-E, SSS07 EP2-K, and SSS07 N1-H. H: haplotype; Nt: nucleotide; SSS06: isolated from Sonora soil samples collected in 2006; SSS07: isolated from Sonora soil samples collected in 2007.

**Table 1** *Aspergillus flavus* VCG YV36 isolates listed by year of collection, substrate, location of isolation, number of isolates, and population. The isolate collected in 1987 from Arizona cotton field soil is AF36

| Year of collection | Substrate | Location | Isolates | Population |
|---|---|---|---|---|
| 1987 | Cotton field soil | Arizona, US | 1 | AZ87 |
| 2004 | Maize land race | Nayarit, Mexico | 2 | NAY04 |
| 2005 | Maize land race | Nayarit, Mexico | 16 | NAY05 |
| 2005 | Maize land race | Sinaloa, Mexico | 6 | SIN05 |
| 2005 | Commercial maize | Tamaulipas, Mexico | 3 | TAM05 |
| 2005 | Maize field soil | Tamaulipas, Mexico | 1 | TAM05 |
| 2006 | Maize land race | Nayarit, Mexico | 3 | NAY06 |
| 2006 | Maize field soil | Sonora, Mexico | 5 | SON06 |
| 2007 | Maize land race | Sonora, Mexico | 5 | SON07 |
| 2007 | Maize field soil | Sonora, Mexico | 4 | SON07 |
| 2008 | Maize land race | Sonora, Mexico | 2 | SON08 |
| 2008 | Maize field soil | Sonora, Mexico | 12 | SON08 |

Populations were constructed prior to genetic analyses based on origin and the year of collection. AZ, Arizona; NAY, Nayarit; SIN, Sinaloa; TAM, Tamaulipas; SON, Sonora.

randomizations and compared the observed value of $\bar{r}_d$ to that expected under the null hypothesis of random mating (Agapow and Burt 2001).

## Results

### Assignment of *Aspergillus flavus* isolates into VCG YV36

*Aspergillus flavus* isolates belonging to VCG YV36 were detected in each of the four states sampled in Mexico (Fig. 1). Members of YV36 were not detected in maize soils from Nayarit in 2007, nor in commercial maize kernel samples from Sonora in 2006 and 2009. In the rest of the examined substrates, YV36 ranged from 0·2% (1 isolate from commercial maize from Tamaulipas in 2005) to 9·8% (16 isolates from MLRs from Nayarit in 2005). Overall, YV36 composed 1·6% (59 isolates) of the examined *A. flavus* isolates (3581 total). The 59 isolates belonging to YV36 originating from Mexico, along with AF36, are listed in Table 1 by origin and year of isolation. The 59 YV36 isolates from Mexico and the biocontrol isolate AF36, grown from the mother culture, were further subjected to genetic analyses.

### Mating-type idiomorph characterization in VCG YV36

All 59 YV36 isolates endemic to Mexico contain the *MAT1-2* idiomorph at the mating-type locus, as do all examined YV36 isolates from the US (Grubisha and Cotty 2015). Each isolate amplified only a single PCR amplicon of approximately 270 bp (*data not shown*) which is typical of the *MAT1-2* idiomorph (Ramirez-Prado *et al.* 2008). No isolates displayed the 395 bp amplicon associated with the *MAT1-1* idiomorph, and no isolates containing both idiomorphs were detected.

### Polyketide synthase (*aflC*) gene SNP associated with atoxigenicity in YV36

Each of the 59 YV36 isolates endemic to Mexico along with AF36 produced a 920 bp amplicon associated with the *aflC* SNP that confers atoxigenicity to AF36 (Ehrlich and Cotty 2004). Aflatoxin producers belonging to other VCGs produced the 1225 bp amplicon typical of wild-type, functional *aflC* gene (Ehrlich and Cotty 2004). Negative PCR controls produced no amplicon.

### Indels in aflatoxin biosynthesis genes

Fifty-six YV36 isolates from Mexico along with AF36 amplified each of the 32 markers of the CAPs multiplex-PCR assay (Callicott and Cotty 2015). However, three YV36 isolates collected from Sonora maize field soil in 2007 amplified each marker except marker AC11 (536 bp) which is located 44 nucleotides downstream from the SNP associated with atoxigenicity.

### Additional deterioration in *aflC*

Absence of amplification of the AC11 marker in three isolates led to investigate an *aflC* region 35 bp downstream from the *aflC* SNP using primers SNP36Tc and SNP36Ca. Isolates, including AF36, which displayed marker AC11 in the CAPs assay, produced a 1120 bp amplicon from PCR with primers SNP36Tc and SNP36Ca. The three isolates that failed to amplify marker AC11 produced a 369 bp amplicon. There was 100% sequence identity between the amplicon and the published *aflC* sequence from AF36 (Ehrlich *et al.* 2005) from the start of nucleotide 626 of *aflC* to nucleotide 949 (88% of the amplicon; Fig. 2). The 43 additional nucleotides were identical to a segment from the arm of chromosome 3 opposite from *aflC* (on scaffold SC023 in the *A. oryzae* genome). Primer pairs 3 and 4 (Figs. 3 and 4) were designed to amplify across the translocation. Primers 3a and 3b (from *aflC*) produced a 1116 bp amplicon in AF36 but not in the three isolates with the translocation (Fig. 3). Likewise, primers 4a and 4b (from scaffold SC023 of the *A. oryzae* genome) produced a 578 bp amplicon in AF36 but not in the three isolates with the translocation (Fig. 3). On the other hand, primers 3a and 4b (which are located on opposite arms of the *A. oryzae* chromosome 3) produced a 670 bp amplicon in the three

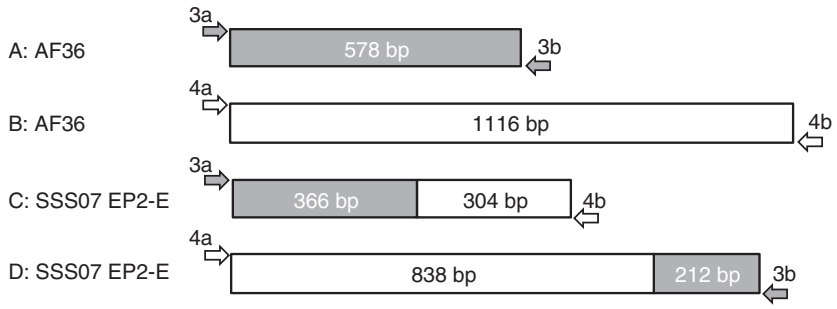


**Figure 3** Evidence for a translocation between the long and short arms of chromosome 3 detected in three YV36 isolates, represented by SSS07 EP2-E. Amplicons A and B are the expected products from amplification of AF36 using primers SNP36Ta (3a) and pksAac2R (3b) and primers oryreg2F (4a) and oryreg4R (4b), respectively. Three isolates with a translocation in *aflC* produce amplicons C and D after amplification with primers 3a and 4b, and primers 4a and 3b, respectively. Sequence analyses revealed that amplicon C is composed of a segment homologous to 366 bp from *aflC* and another segment of 304 bp homologous to part of scaffold SC023 from the *A. oryzae* RIB40 genome; amplicon D is composed of 838 bp homologous to part of scaffold SC023 and a 212 bp segment homologous to part of *aflC*. (■ Sequence homologous to *aflC*; □ sequence homologous to *A. oryzae* scaffold SC023).

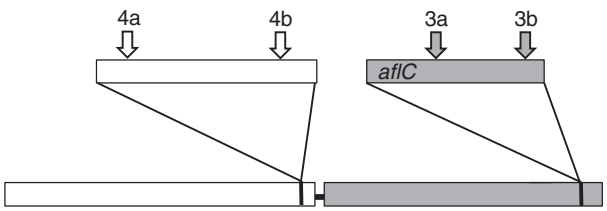


**Figure 4** Schematic representation of chromosome 3 of *Aspergillus oryzae* RIB40 and *Aspergillus flavus* AF36 indicating locations of primers used to assess the translocation between opposite arms of chromosome 3 detected in three YV36 isolates from Sonora, Mexico. With AF36 amplicons formed with primers 3a (SNP36Ta) and 3b (pksAac2R) on the short arm and primers 4a (oryreg4F) and 4b (oryreg2R) on the long arm. No amplicons were generated with these primer pairs from isolates bearing the translocation (Fig. 3).

isolates with the translocation but not in AF36 (Fig. 3). Similarly, primers 4a and 3b produced a 1050 bp amplicon in the three isolates with the translocation but not in AF36 (Fig. 3).

### Microsatellite genotyping

Fungi belonging to VCG YV36 from Mexico were further characterized into multilocus, haploid microsatellite genotypes (haplotypes). Single electropherogram peaks for 23 microsatellite loci were obtained for all isolates. Locus AF26 was not reliably amplified and was excluded from analyses. Eleven loci (AF17, AF22, AF25, AF27, AF31, AF33, AF53, AF54, AF55, AF63, and AF66) were monomorphic across isolates. Locus AF48 was removed from analyses because it was hypervariable with 21 alleles across the 60 isolates. The number of alleles in the remainder of the loci (11 total) was low and ranged from 2 to 7 (mean = 3·2). These 11 polymorphic loci were used for further analyses.

### Genotypic diversity

Twenty-one haplotypes were detected among the 60 isolates of VCG YV36 based on 11 informative microsatellite loci (Table 2). Eight haplotypes were detected at least twice. The most common haplotype of YV36 (haplotype 1, 19 isolates), was detected in all four states sampled in Mexico and in each of the 4 years. The second most common haplotype (haplotype 2, 9 isolates) was detected only in Sonora from 2006 through 2008. The haplotype to which AF36 belongs, haplotype 7, was detected only once in Mexico, in Sonora (SON07). Thirteen haplotypes were represented by a single isolate.

### Population structure

Assessment of genetic composition was conducted using STRUCTURE (Pritchard *et al.* 2000) by grouping isolates into inferred populations. In the Structure algorithm, initial conditions assumed nine genetic groups based on origin and year of isolation. Structure resolved a single genetic group. Linkage disequilibrium analyses indicate that the examined YV36 isolates form a single population with all microsatellite loci in equilibrium ($\bar{r}_d$ = 0·029, $P$ = 0·087).

## Discussion

Humans consume over 80 000 metric tons of maize daily in Mexico with maize providing over half the caloric intake of the population, primarily in the form of tortillas (Plasencia 2004; FAO 2010). Vast agricultural regions are dedicated to maize production, mostly in climates conducive to aflatoxin contamination (Levy and van Wijn-

**Table 2** Frequencies of haplotypes of VCG YV36 detected in four states of Mexico. The biocontrol agent *Aspergillus flavus* AF36 is included (haplotype 7, isolated from a cotton field soil in Arizona during 1987)

| Haplotype* | *n*† | Origin‡ | Substrate§ | Years of isolation |
|---|---|---|---|---|
| 1 | 19 | NAY, SIN, SON, TAM | CM, MLR, MFS | 2005, 2006, 2007 and 2008 |
| 2 | 9 | SON | MFS | 2006, 2007 and 2008 |
| 3 | 5 | SON | MFS | 2008 |
| 4 | 4 | NAY | MLR | 2005 |
| 5 | 3 | NAY | MLR | 2004 and 2005 |
| 6 | 3 | SIN | MLR | 2005 |
| 7 | 2 | AZ, SON | CFS, MFS | 1987 and 2007 |
| 8 | 2 | NAY | MLR | 2006 |
| 9 | 1 | NAY | MLR | 2004 |
| 10 | 1 | NAY | MLR | 2005 |
| 11 | 1 | NAY | MLR | 2005 |
| 12 | 1 | NAY | MLR | 2005 |
| 13 | 1 | NAY | MLR | 2006 |
| 14 | 1 | SON | MFS | 2007 |
| 15 | 1 | SON | MFS | 2007 |
| 16 | 1 | SON | MLR | 2007 |
| 17 | 1 | SON | MFS | 2008 |
| 18 | 1 | SON | MLR | 2008 |
| 19 | 1 | SON | MLR | 2008 |
| 20 | 1 | TAM | CM | 2005 |
| 21 | 1 | TAM | MFS | 2005 |

*Haplotypes were detected with GENODIVE ver. 2.0b11 (Meirmans and Van Tienderen 2004) using 22 microsatellite loci (Grubisha and Cotty 2009). Locus AF26 was not reliably amplified and was excluded from analyses. Locus AF48 was hypervariable with 21 alleles across the 60 isolates and was excluded from analyses. Eleven loci (AF17, AF22, AF25, AF27, AF31, AF33, AF53, AF54, AF55, AF63, and AF66) were monomorphic across isolates. Number of alleles in the included polymorphic loci (11 total) ranged from 2 to 7 (mean = 3·18).

†Number of isolates in the haplotype. Eight haplotypes contain at least two isolates. Thirteen haplotypes are composed of a single isolate.

‡States in which the haplotypes were detected. AZ: Arizona; NAY: Nayarit; SIN: Sinaloa; SON: Sonora; TAM: Tamaulipas.

§Substrates from which haplotypes were isolated. CM: commercial maize; CFS: cotton field soil; MFS: maize field soil; MLR: maize land race.

bergen 1992; Cotty *et al.* 1994). Documented episodes of aflatoxin contamination of maize occurred in Tamaulipas during the 1990s (Rodríguez-Del Bosque *et al.* 1995; Carvajal and Arroyo 1997). In other states, reports are not common, but the risk of contamination is considered high. For example, aflatoxin-producing fungi are closely associated with maize in Sonora regardless of elevation, soil type, and agroecosystem (Ortega-Beltran *et al.* 2015). Indeed, aflatoxin-producing fungi are associated with maize in all regions of Mexico examined to date (Torreblanca *et al.* 1987; Rodríguez-Del Bosque 1996; Plasencia 2004; Guzman de Peña and Peña-Cabriales 2005; Valencia-Botin 2011).

Aflatoxins are typically not quantified by wholesalers, formal markets, food processing industries, and in informal markets in Mexico. Therefore, maize is often consumed without knowledge of aflatoxin content (Torreblanca *et al.* 1987; Resnik *et al.* 1995; CAST 2003; Plasencia 2004; Garcia and Heredia 2006). Efficient methods to prevent contamination are necessary to avoid human exposure. Practical, efficient aflatoxin preventive methods may have lower cost than combined costs of maize sampling and aflatoxin quantification for regulatory purposes (Wu *et al.* 2008).

The biocontrol agent *Aspergillus flavus* AF36 belongs to VCG YV36 (Cotty and Mellon 2006; Cotty *et al.* 2007, 2008), which is widely distributed across the US from California to Georgia (Ehrlich and Cotty 2004). Wide distribution in the US led us to investigate frequencies of YV36 across Mexico. Thousands of *A. flavus* isolates from maize-growing regions in four states of Mexico (Nayarit, Sinaloa, Sonora, and Tamaulipas) were subjected to VCA to determine incidences of YV36. Fifty-nine isolates (1·6% of the population) were found to belong to YV36 (Table 1); YV36 was found in each sampled state over varying soil types, elevations, and cropping systems. Our results indicate that YV36 is a VCG endemic to Mexico that is well adapted to maize produced in both highly mechanized and traditional agricultural systems. All YV36 members endemic to Mexico possess the *MAT1-2* idiomorph at the mating-type locus, the SNP in the *aflC* gene conferring atoxigenicity, and low levels of genetic diversity based on microsatellite analyses. These same genetic characteristics exist throughout YV36 populations spanning the US (Grubisha and Cotty 2015). The current results support prior suggestions that atoxigenicity is a stable trait across YV36 populations. The observed stability adds additional confidence in the use of AF36 as a biopesticide for aflatoxin prevention.

Genetic variability within YV36 collected from Mexico was examined using microsatellite markers distributed across the eight *A. flavus* chromosomes (Grubisha and Cotty 2009). Although members of a VCG descend from a common ancestor, genetic variation within a VCG may exist. Our results revealed 21 haplotypes among the 59 YV36 isolates from Mexico (Table 2). A single haplotype (haplotype 1) dominated the YV36 population with presence in all four states sampled. The second most common haplotype, haplotype 2, was detected in several maize soil samples from Sonora collected over a three year period (2006 through 2008; Table 2). These results suggest that haplotypes 1 and 2 may be rapidly expanding their range in a clonal fashion faster than both drift in microsatellite sequence and gene flow within the

VCG. However, regardless of the high number of haplotypes detected within the YV36 population endemic to Mexico, STRUCTURE analysis revealed that a single YV36 population exists across Mexico. Linkage disequilibrium analyses revealed microsatellite loci within this VCG are in linkage equilibrium ($\bar{r}_d = 0{\cdot}029$, $P = 0{\cdot}087$) which indicates free genetic exchange among members of YV36. Equilibrium provides additional support that YV36 isolates from Mexico belong to a single genetic population that also contains AF36. The results agree with previous observations based on genetic clustering revealed by STRUCTURE and linkage disequilibria among the microsatellite loci when additional VCGs are included that there is strong evidence for clonality (Tibayrenc and Ayala 2012; Grubisha and Cotty 2015). Thus, VCG YV36 is naturally distributed in a single population that extends from Arizona in the US through several states in Mexico. The existing variability among YV36 isolates collected in Mexico suggests that YV36 has long been endemic to Mexico. If presence of YV36 in Mexico were the result of a recent dispersal to Mexico as a result of applications of the biopesticide AF36 in Arizona, California, and Texas (Cotty *et al.* 2007), the AF36 haplotype would have been the dominant haplotype in Mexico. However, only a single YV36 isolate with the AF36 haplotype was detected in Mexico in the current study (Table 2).

Examination of genetic variability within YV36 populations from the US revealed that the *aflC* SNP, which confers atoxigenicity to AF36 and the VCG as a whole (Ehrlich and Cotty 2004), is present in isolates recovered from fields where AF36 has been applied as well from isolates originating from both noncultivated areas and agricultural areas that have never been treated with AF36 (Grubisha and Cotty 2015). In the current study, all YV36 isolates endemic to Mexico were found to uniformly harbour the *aflC* SNP. This indicates that the *aflC* SNP is maintained in members of YV36 regardless of origin, year of isolation, or the environmental pressures to which YV36 members have been subjected.

While applying a PCR-based screening tool developed to detect deletions in the aflatoxin gene cluster (Callicott and Cotty 2015), deterioration of *aflC*, beyond the SNP at nucleotide 591, was detected in three YV36 isolates collected from Sonora maize field soils sampled in 2007. The predominant genetic defect in the three isolates is a translocation between the two arms of chromosome 3 with the breakpoint in the middle of *aflC* (Figs. 2 and 3). Primers targeting portions of *aflC* did not work for the three isolates with the translocation, but were positive for other YV36 isolates (Fig. 3). However, primer pair 3a and 4b, and primer pair 4a and 3b bridge between *aflC* and a region of the chromosomal arm opposite the arm normally bearing the aflatoxin gene cluster (Fig. 4). Only the three isolates that underwent the translocation yielded an amplicon when conducting PCR with primers from opposite arms of the chromosome (Fig. 3). Two of these isolates belong to haplotype 2 and originate from the same location; the other, from a location eight km away, belongs to haplotype 7, the same haplotype as AF36. Haplotypes 2 and 7 are identical at all microsatellite loci except AF10 (*data not shown*). The *aflC* gene in YV36 is not functional as a result of the premature stop codon caused by the SNP at nucleotide 591. Thus, the translocation is evidence of additional deterioration in *aflC*. Deterioration of *aflC* apparently has no influence on success of AF36 in the environment. Other mutations in the AF36 aflatoxin gene cluster are also known (Ehrlich and Cotty 2004) and are expected to increase as VCG YV36 continues to evolve.

In fungi, the parasexual cycle may occur through heterokaryon formation, nuclear fusion, and mitotic recombination (Pontecorvo 1956). In *A. flavus*, the parasexual cycle has been documented only under laboratory conditions (Papa 1973). Recently, Grubisha and Cotty (2015) pointed out that data on populations of YV36 in the US are consistent with this phenomenon occurring in natural populations of *A. flavus*. The translocation event appears to have occurred during mitosis because meiotic recombination in the YV36 population from Mexico is not supported by the microsatellite analyses. As mentioned previously, microsatellite loci of all isolates included in this study were in equilibrium. If the translocation observed in these three isolates occurred during meiosis, greater allelic diversity would have been detected by our microsatellite analyses (Grubisha and Cotty 2015). In *Aspergillus nidulans*, parasexuality occurs during environmental challenges leading to novel chromosomal rearrangements that may result in superior adaptation to the subjected pressures (Schoustra *et al.* 2007). The translocation observed in the current study may be an example of such a rearrangement. Whole genome screening strategies directed at identifying translocations need to be developed in order to clarify extents to which translocation events occur in *A. flavus* populations.

Mexico needs practical aflatoxin management (Rodríguez-Del Bosque *et al.* 1995; Carvajal and Arroyo 1997; Plasencia 2004; Ortega-Beltran *et al.* 2015). In the state of Sinaloa, Mexico's largest maize producer with over 22% of the national production from just 6·2% of the plantings (SIACON 2010), all the maize had to be replanted due to a rare freeze in 2011. Replanting extended cropping until late summer, exposing the developing crop to high temperature and greater aflatoxin risk. However, there were no practical aflatoxin preventive measures available. Aflatoxin levels exceeded 500 ppb in some Sinaloa maize that year and most the maize

exceeded the allowable aflatoxin levels (20 ppb) for human consumption (J. Ureta and P.J. Cotty, unpublished data). Availability of effective aflatoxin biocontrol would have allowed the Sinaloa farmers to mitigate this anticipated contamination. Farmers with traditional cultivation systems should be sensitized to the risk of contamination (Ortega-Beltran *et al.* 2015) and be provided with inexpensive biocontrol products in a manner similar to that underway in several African nations (Bandyopadhyay *et al.* 2013; Schmidt 2013). This will provide both health and animal productivity benefits to rural Mexico.

Results from this study indicate that members of YV36, the VCG to which the biocontrol agent *Aspergillus flavus* AF36 belongs, are distributed across vast regions of Mexico where maize is produced in both commercial and traditional agriculture. Wide distribution of atoxigenic genotypes within a target area is one criterion for utilization as biocontrol agents (Probst *et al.* 2011). Utilization of these native atoxigenic genetic resources within Mexico could reduce human exposure to aflatoxins in areas with high risk of contamination regardless of production scale. Endemic atoxigenic genotypes well adapted to target regions are preferred as biocontrol agents for the prevention of aflatoxin contamination because greater adaptation of endemic organisms to target regions is thought to facilitate efficacy (Mehl *et al.* 2012). Use of endemic organisms also avoids risks to ecosystems posed by nonendemic organisms (Probst *et al.* 2011). AF36 has been rigorously evaluated for risks to the environment and to both humans and animals during the registration process with the USEPA (USEPA 2003; Cotty and Mellon 2006; Cotty *et al.* 2008). Over 20 years ago, the three countries of North America (i.e. Canada, Mexico and the US) signed the North American Free Trade Agreement (NAFTA) (NAFTA 1993). This agreement includes the facilitation of exchange of agricultural technologies. In addition, the Commission for Environmental Cooperation of NAFTA mandates increased cooperation between the parties to better conserve, protect, and enhance the environment, and to promote economically efficient and effective environmental measures. Use of AF36 in maize fields within Mexico would contribute to mandate fulfilment. Geopolitical borders should not be an impediment to restrict the use of AF36 as a biocontrol agent in Mexico as VCG YV36 is endemic to both the US and Mexico. Thus, both the evidence provided in this study that AF36, an USEPA registered biocontrol agent for use in the US, is endemic to Mexico, and the environmental, economic, and commercial mandates of NAFTA should be used to facilitate rapid registration of this agent within Mexico and provide farmers within the country with their first practical tool for preventing pre- and postharvest aflatoxin contamination of maize.

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## Conflict of Interest

No conflict of interest declared.

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