Distribution of active ingredients of a commercial aflatoxin biocontrol product in naturally occurring fungal communities across Kenya

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Summary

Human populations in Kenya are repeatedly exposed to dangerous aflatoxin levels through consumption of contaminated crops. Biocontrol with atoxigenic Aspergillus flavus is an effective method for preventing aflatoxin in crops. Although four atoxigenic A. flavus isolates (C6E, E63I, R7H and R7K) recovered from maize produced in Kenya are registered as active ingredients for a biocontrol product (Aflasafe KE01) directed at preventing contamination, natural distributions of these four genotypes prior to initiation of commercial use have not been reported. Distributions of the active ingredients of KE01 based on haplotypes at 17 SSR loci are reported. Incidences of the active ingredients and closely related haplotypes were determined in soil collected from 629 maize fields in consecutive long and short rains seasons of 2012. The four KE01 haplotypes were among the top ten most frequent. Haplotype H-1467 of active ingredient R7K was the most frequent and widespread haplotype in both seasons and was detected in the most soils (3.8%). The four KE01 haplotypes each belonged to large clonal groups containing 27–46 unique haplotypes distributed across multiple areas and in 21% of soils. Each of the KE01 haplotypes belonged to a distinct vegetative compatibility group (VCG), and all A. flavus with haplotypes matching a KE01 active ingredient belonged to the same VCG as the matching active ingredient as did all A. flavus haplotypes differing at only one SSR locus. Persistence of the KE01 active ingredients in Kenyan agroecosystems is demonstrated by detection of identical SSR haplotypes six years after initial isolation. The data provide baselines for assessing long-term influences of biocontrol applications in highly vulnerable production areas of Kenya.

Introduction

Several species in Aspergillus section Flavi produce aflatoxins (highly toxic, carcinogenic fungal metabolites) during infection of host crops. Aflatoxin contaminates 25% of world food crops, and 4.5 billion people in developing countries, especially in Africa and Asia, are chronically exposed to aflatoxins through consumption of staple crops (Williams et al., 2004). Maize is the primary staple in Kenya, a major ‘hotspot’ for aflatoxin (Mutegi et al., 2018) with exposure frequently documented as aflatoxin B1-lysine adduct in serum of children (Gong et al., 2012) and aflatoxin M1 in mother’s milk (Mutegi et al., 2018). Indeed, Kenya repeatedly experienced epidemics of lethal aflatoxicosis since 1981 (Liu and Wu, 2010; WHO, 2011) and nearly 317 cases of acute aflatoxicosis with over 125 deaths occurred in 2004 in the Coastal and Eastern provinces of Kenya (Azziz-Baumgartner et al., 2005). In a 2010, human serum aflatoxin levels in Kenyans were among the highest ever recorded (Obura, 2013) and a cross-sectional serosurvey documented a widespread urgent need to implement interventions to minimize aflatoxin exposure (Yard et al., 2013).

The Centers for Disease Control and Prevention (CDC) and the Kenya Ministry of Public Health and Sani-
tation focused on efforts to reduce aflatoxin exposure (Mutegi et al., 2018). Biocontrol of aflatoxin-producing fungi is a promising method for reducing crop contamination (Probst et al., 2011). Atoxigenic strains of A. flavus are used to alter natural fungal communities with non-
aflatoxin-producing (atoxigenic) A. flavus so that aflatoxin producers are less common and the average aflatoxin-
producing potentials of fungal communities are reduced (Cotty et al., 2008; Bandyopadhyay et al., 2016). This change in the fungal community results in greatly reduced aflatoxin concentrations in crops. Atoxigenic strain-based biocontrol has been successfully used in the United States for over two decades in a variety of crops, such as cotton (Cotty, 1994a,b; Cotty et al., 2007), groundnut (Dornier et al., 1998), pistachio (Doster et al., 2014) and maize (Dornier et al., 1999). Influences of atoxigenic biocontrol products extend beyond the season of treatment as a result of survival of the atoxigenic active ingredients in soils (Cotty et al., 2007; Bandyopadhyay et al., 2016). Recently, biocontrol technology has been adapted to reduce aflatoxin in maize and groundnut in Kenya and several other African nations through a partnership between the International Institute of Tropical Agriculture (IITA), the USDA-Agricultural Research Service (USDA-ARS) and several National Agricultural Research Services including the Kenya Agricultural and Livestock Research Organization (KALRO) (Bandyopadhyay and Cotty, 2013; Bandyopadhyay et al., 2016).

Aspergillus flavus is commonly divided into two strains or morphotypes, the L-strain morphotype and the S-strain morphotype, which differ in not only morphology but also physiology, genetics and consistency of aflatoxin production (Cotty, 1989). Almost all members of the S-strain morphotype produce high levels of aflatoxins (Cotty, 1989); however, atoxigenic strains of the L-strain morphotype are frequent in crops and soils (Cotty, 1990, 199a,b, 1997). Many of these atoxigenic strains are effective as biocontrol agents for aflatoxin management (Mehl et al., 2012; Bandyopadhyay and Cotty, 2013; Atehnkeng et al., 2014; Ehrlich, 2014; Bandyopadhyay et al., 2016; Agbetiameh et al., 2019; Moral et al., 2020).

Atoxigenic A. flavus-based aflatoxin biocontrol products directed at various regions in Africa are named Aflasafe™ with a country-specific suffix. Each Aflasafe product contains atoxigenic strain active ingredients endemic to the target nation. Aflasafe KE01™ (hitherto called KE01) is registered with the Pest Control Products Board (PCPB) of Kenya as a Kenya-specific aflatoxin biocontrol product. This product contains four distinct atoxigenic A. flavus L-morphotype active ingredients (C6E, E63l, R7H and R7K; Bandyopadhyay and Cotty, 2013; Bandyopadhyay et al., 2016; Moral et al., 2020). These active ingredients were isolated from maize produced in Kenya from 2004 to 2006 with the intent of selecting effective fungi well adapted to maize agroecosystems in Kenya (Probst et al., 2011). However, natural distributions of these selected genotypes in Kenyan agroecosystems beyond the grain from which they were isolated are unknown. Data on distribution of KE01 active ingredients prior to initiation of commercial treatments are necessary to quantify extents of long-term, cumulative benefits in treated regions of Kenya.

Natural populations of A. flavus are composed of hundreds of vegetative compatibility groups (VCGs) (Bayman and Cotty, 1991; Cotty et al., 1994). Vegetative compatibility groups are defined by a heterokaryon incompatibility system that allows anastomosis and gene flow among closely related genotypes but restricts anastomosis among dissimilar individuals (Grubisha and Cotty, 2010, 2015). Aspergillus flavus VCGs evolve as clonal lineages (Papa, 1986; Leslie, 1993; Ehrlich et al., 2007; Grubisha and Cotty, 2010). Vegetative compatibility analyses (VCA) are used to track and characterize A. flavus active ingredients (Bayman and Cotty, 1993; Cotty, 1994a,b; Atehnkeng et al., 2014; Agbetiameh et al., 2020; Senghor et al., 2020). However, VCA are laborious culture-based techniques, and each VCG must be assayed independently with the workload multiplying by the number examined. Vegetative compatibility analyses also require maintenance of living, functional tester mutants for each VCG (Bayman and Cotty, 1993; Horn et al., 1996; Probst et al., 2011), and these mutants must be shared among laboratories wishing to identify or monitor active ingredients. In this regard, DNA technologies can be advantageous (Shenge et al., 2019).

Simple sequence repeats (SSR) are effective at differentiating genotypes of A. flavus (Grubisha and Cotty, 2015; Picot et al., 2018; Senghor et al., 2020). Fungal VCGs frequently contain multiple closely related SSR haplotypes (Berbegal et al., 2010; Chang et al., 2014). SSR markers are valuable tools for genotyping A. flavus from agricultural (Tran-Dinh et al., 2009; Grubisha and Cotty, 2010, 2015; Sweeney et al., 2011; Ortega-Beltran et al., 2020) and clinical settings (Hadrich et al., 2010; Dehghan et al., 2014).

In the present study, 17-SSR loci distributed across all eight chromosomes of A. flavus were used to determine distributions of the four active ingredients of KE01 in soils under maize cultivation in seven counties across Kenya. An objective of this study was to determine relationships of the active ingredient SSR haplotypes to VCA used to monitor the active ingredients in Kenya. In addition, this work sought to characterize natural frequencies and distributions across fungal communities in Kenyan agroecosystems with high incidences of aflatoxin contamination prior to initiation of aflatoxin management programmes based on the biocontrol product. During the process, genetic lineages containing each of the active ingredients were characterized.

Results

Diversity of potential aflatoxin-producing Aspergillus section Flavi

A total of 7189 Aspergillus section Flavi fungal isolates were recovered from 629 maize field soils during the
long rains and the short rains seasons of 2012. Sampled fields spanned 10 agricultural areas in seven counties (Fig. 1). The fungi included 2812 isolates from *A. flavus* L-strain morphotype, 3,400 isolates of LAF (*LAF = Lethal Aflatoxicosis Fungus*; Kachapulula *et al.*, 2017; Singh *et al.*, 2020) from a high aflatoxin-producing unnamed species with S morphology associated with lethal aflatoxin contamination of maize produced in Kenya (Probst *et al.*, 2007; Probst *et al.*, 2012) and 986 *A. parasiticus* (Table 1). Each of these species was detected in all ten of the sampled agricultural areas across Kenya. However, LAF and the L morphotype of *A. flavus* were the most common. Similar frequencies of LAF occurred in all areas except Area-8 (Tana River County), where LAF was rarely detected (Table 1).

### SSR amplification, genotyping and clone correction

A total of 2744 isolates were clearly amplified at 17-SSR loci and included in this analysis (Table 2). SSR amplification across these isolates was free of PCR artefacts and only generated single peaks in the expected size range. There were no missing data and no null alleles. The number of alleles per locus was high and ranged from 9 to 72. High allelic diversity resulted in high genotypic diversity, with 2140 unique SSR haplotypes (Table 2).

Isolates were culled to only allow a single isolate with any given SSR haplotype from each soil. After this clone correction, 2408 isolates remained for analysing frequencies, distribution, adaptation and lineage relationships of the four KE01 active ingredients within naturally occurring fungal populations in Kenyan soil.

### Relationships between SSR haplotype and VCG of KE01 isolates

The four KE01 active ingredients (C6E, E63I, R7H and R7K) were separated by both VCG analysis and SSR typing (Table 3). Haplotypes of 24 soil isolates matched the R7K haplotype, and *nit* mutants of each of these isolates complemented an auxotrophic tester mutant of R7K but not any of the six tester mutants developed for the other three KE01 active ingredients. Complementation of an R7K tester mutant indicates membership in the R7K VCG. In a similar manner, all isolates matching the haplotypes of C6E (*n* = 15), E63I (*n* = 3) and R7H (*n* = 10) were shown to belong to the same VCG as the respective active ingredient. In addition, isolates differing from C6E (*n* = 5), E63I (*n* = 13) and R7H (*n* = 3) at only
| Agricultural area | District/location                           | County     | Soils<sup>a</sup> (###) | Isolates<sup>a</sup> (###) | A. flavus<sup>b</sup> (###) | A. parasiticus<sup>d</sup> (###) | Soils<sup>a</sup> (###) | Isolates<sup>a</sup> (###) | A. flavus<sup>b</sup> (###) | A. parasiticus<sup>d</sup> (###) |
|------------------|--------------------------------------------|------------|--------------------------|-----------------------------|-------------------------------|---------------------------------|--------------------------|-----------------------------|-------------------------------|-------------------------------|
| Area-1           | Embu East; Mbeere North                     | Embu       | 121                      | 1445                        | 368                           | 892                             | 185                     | 60                          | 169                           | 445                           |
| Area-2           | Kangundo; Kathiani; Matungulu               | Machakos   | 63                       | 749                         | 300                           | 291                             | 158                     | 32                          | 112                           | 131                           |
| Area-3           | Machakos; Makueni; Mbooni                  | Machakos;  | 93                       | 1118                        | 312                           | 690                             | 116                     | 70                          | 187                           | 385                           |
| Area-4           | Kitui Central; Nzambani                     | Kitui      | 40                       | 470                         | 185                           | 202                             | 83                      | 31                          | 335                           | 132                           |
| Area-5           | Mutomo                                     | Kitui      | –                        | –                           | –                             | –                               | –                      | –                           | 10                            | 86                            |
| Area-6           | Ikutha                                      | Kitui      | 14                       | 174                         | 149                           | 22                              | 3                      | 10                          | 77                            | 23                            |
| Area-7           | Makindu                                     | Makueni    | 19                       | 241                         | 160                           | 63                              | 18                     | 10                          | 36                            | 57                            |
| Area-8           | Tana River                                  | Tana River | 40                       | 471                         | 465                           | 1                               | 5                      | –                           | –                             | –                             |
| Area-9           | Homabay                                     | Homabay    | –                        | –                           | –                             | –                               | –                      | 8                           | 31                            | 38                            |
| Area-10          | Rongo                                       | Migori     | –                        | –                           | –                             | –                               | –                      | 8                           | 86                            | 5                             |

Total (10 Areas, 16 locations; 7 Counties) 390 4668 1939 2161 568 239 2530 873 1239 418

<sup>a</sup> Number of fields sampled. A single composite soil sample composed of 30 to 40 subsamples along a 40 to 50 m transect was taken from each field.
<sup>b</sup> Total number of total isolates of Aspergillus section Flavi (A. flavus, LAF and A. parasiticus) isolates recovered.
<sup>c</sup> Number of isolates identified as Aspergillus flavus L morphotype.
<sup>d</sup> Number of isolates belonging to the Kenyan S morphotype referred to as the Lethal Aflatoxicosis Fungus (LAF; Kachapulula et al., 2017; Singh et al., 2020) which has been associated with contamination events that led to deaths in Kenya (Probst et al., 2007, 2012).
<sup>e</sup> Number of isolates identified as Aspergillus parasiticus.
a single SSR locus belonged to the same VCG as the nearly-matching active ingredient (Table 3). In no case did any of the active ingredients react with a tester mutant developed from a different active ingredient.

**Distributions of KE01 haplotypes**

In total, 118 haplotypes occurred in 2 to 23 soil samples (data not shown). The haplotypes of each of the four KE01 active ingredients were among the first ten most frequently occurring haplotypes (Table 4). These haplotypes occurred in multiple areas and both seasons. The haplotype of active ingredient R7K (H-1462) was detected in 3.8% (23 total) of soils and was the most widespread haplotype (occurring in eight of ten study areas) in both long rains and short rains seasons (Table 4). Haplotypes belonging to two other active ingredients (C6E and R7H) were found in 2.4% and 1.8% of the soils, respectively, and were both found in multiple areas (7 to 9 areas; Fig. 2). However, no member of any of the clonal groups of the four KE01 isolates was found in Area-8 (Fig. 2). The clonal groups of R7K, C6E, R7H and E63I were found in 7.2%, 4.1%, 4.5% and 5.1% of the soil samples respectively (Fig. 2). In total, clonal lineages of the active ingredients occurred within 21% of examined soils.

**Discussion**

The current results agree with previous observations that the *A. flavus* L morphotype is widely distributed in agricultural soils across Africa and including Kenya (Donner et al., 2009; Probst et al., 2014; Table 1). Populations of S-morphology fungi in Africa and North America are distinct (Cotty and Cardwell, 1999). Although the *A. flavus* S morphotype is common in the USA (Singh et al., 2020), it either very rare or does not occur in Kenya (Probst et al., 2007, 2012). However, there are common S-morphology fungi in Kenya, sometimes referred to as LAF (LAF = Lethal Aflatoxicosis Fungus; Kachapulula et al., 2017; Singh et al., 2020) that are phylogenetically distinct from *A. flavus* but still consistently produce high concentrations of aflatoxins in maize and were associated with lethal aflatoxicoses occurring in Kenya from 2004 to 2006 (Probst et al., 2012, 2014). The LAF is common in regions with histories of lethal aflatoxicosis, including Machakos, Makueni and Kitui counties (Probst et al., 2007, 2010, 2012). In the current study, LAF was only infrequent in Area-8 (Tana River County; Table 1) which is primarily the Tana River Irrigation Scheme near the town of Bura. Another high aflatoxin-producing species, *A. parasiticus*, composed more than 10% of the aflatoxin producers in four areas but was also not detected in Area-8. *A. parasiticus* and LAF may not be

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**Table 2.** Genetic diversity of *A. flavus* L-morphotype isolates in 629 maize field soils across ten agricultural areas from seven counties in Kenya during 2012.

| Area  | District/location | County       | Total isolates<sup>a</sup> | SC isolates<sup>b</sup> | Haplotypes<sup>c</sup> |
|-------|------------------|--------------|-----------------------------|------------------------|-----------------------|
| 1     | Embu East; Mbeere North | Embu         | 519                         | 479                    | 453                   |
| 2     | Kangundo; Kathiani; Matungulu | Machakos    | 389                         | 341                    | 326                   |
| 3     | Machakos; Makueni; Mbooni East | Machakos; Makueni | 490                         | 420                    | 383                   |
| 4     | Kitui Central; Nzambani | Kitui        | 322                         | 295                    | 281                   |
| 5     | Mutombo           | Kitui        | 86                          | 78                     | 77                    |
| 6     | Ikutha            | Kitui        | 224                         | 192                    | 188                   |
| 7     | Makindu           | Makueni      | 187                         | 154                    | 149                   |
| 8     | Tana River        | Tana         | 474                         | 402                    | 356                   |
| 9     | Homabay           | Homabay      | 31                          | 27                     | 27                    |
| 10    | Rongo             | Migori       | 22                          | 20                     | 20                    |
| Total |                  |              | 2744                        | 2408                   | 2140                  |

<sup>a</sup> Area locations are illustrated in Fig. 1.
<sup>b</sup> Total number of isolates after sample correction (removal of isolates with repeated haplotypes in the same soil sample).
<sup>c</sup> Number of isolates.
<sup>d</sup> Total number of SSR haplotypes.
Table 3. Relationship of vegetative compatibility group (VCG) to identical and closely related haplotypes of Asfla KE01 active ingredients.

| Asfla KE01 active ingredient | Haplotypes | No. of isolates | Mutational changes (distance) from KE01 Haplotype |
|------------------------------|------------|----------------|-----------------------------------------------|
|                             | ID         |                | VCG ID                                      |
| R7K                         | H-1462     | 24             | KN012                                       |
| C6E                         | H-0199     | 15             | KN00A                                       |
|                             | H-0197     | 3              | KN00A                                       |
|                             | H-0198     | 1              | 1 (One step) KN00A                         |
| E63I                        | H-1019     | 1              | KN00A                                       |
|                             | H-1017     | 13             | KN001                                       |
|                             | H-0212     | 10             | 1 (One step) KN001                         |
|                             | H-0214     | 3              | 1 (One step) KN011                         |

a. The 17 SSR loci are named (AF-28 to AF-55). Allele sizes indicate amplicon size in base pairs as called on an ABI 3730 DNA Analyzer with the LIZ500 standard (Applied Biosystems).

b. Asfla KE01 active ingredients registered for aflatoxin mitigation in Kenya. Active ingredient names and VCG names are from Probst et al. (2011).
Over long periods, mutation and clonal evolution may cause diversity at SSR loci within VCGs (Islam et al., 2018). This is evident in the current data set with three of the active ingredients. The VCGs of those active ingredients include one to three haplotypes different at one SSR locus (Table 3). Indeed, the fourth most common haplotype belongs to the VCG of active ingredient R7K, again suggesting better adaptation to Kenya’s agroecosystems than most VCGs not included in KE01.

Soil collections for this study were undertaken 6–7 years after harvest of the maize from which the active ingredients of the biocontrol product were collected (Probst et al., 2011) indicating persistence of these clonal lineages over long periods. The high frequencies, wide distributions (Table 4) and long persistence of the KE01 haplotypes and their close relatives (clonal lineages) across the study areas suggest that these genotypes are well adapted to Kenyan agroecosystems. Although haplotypes of the KE01 active ingredients are relatively common, and natural presence of these atoxigenic genotypes likely positively influence crop contamination (Cotty et al., 2008), the background levels of these genotypes is far below what would be required to prevent unhealthy levels of aflatoxins. Therefore, biocontrol treatments with KE01, which are expected to result in over 80% frequencies of the active ingredients on the crop (Bandyopadhyay et al., 2016), will likely provide benefit. The current work provides reference points from which to judge both single-season and long-term efficacies across portions of Kenya most in need of aflatoxin mitigation.

Table 4. Distributions of the ten most frequent Aspergillus flavus L-morphotype haplotypes identified in soils from Kenya. Haplotypes of all four Aflasafe KE01 active ingredients are included.

| Haplotype ID<sup>a</sup> | Area-1<sup>c</sup> | Area-2 | Area-3 | Area-4 | Area-5 | Area-6 | Area-7 | Area-8 | Area-9 | Area-10 |
|-------------------------|------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
|                         | N<sup>b</sup>   | S1<sup>d</sup> | S2<sup>e</sup> | S1 | S2 | S1 | S2 | S1 | S2 | S1 | S2 | S1 | S2 | S1 | S2 |
| H-1462 (R7K)<sup>a</sup> | 23 | 3 | 3 | 1 | 1 | 4 | 0 | 3 | 2 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| H-1354                  | 17 | 0 | 2 | 2 | 1 | 1 | 6 | 0 | 2 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 |
| H-0199 (C6E)<sup>a</sup> | 14 | 0 | 2 | 0 | 3 | 1 | 2 | 1 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| H-1017                  | 13 | 0 | 2 | 1 | 1 | 0 | 5 | 0 | 3 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| H-0212 (R7H)<sup>a</sup> | 10 | 0 | 1 | 0 | 1 | 0 | 4 | 1 | 2 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| H-0591                  | 8  | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 3 | 0 | 1 | 0 |
| H-0318                  | 6  | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 0 |
| H-0557                  | 5  | 0 | 3 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| H-1158                  | 4  | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| H-1019 (E63I)<sup>a</sup> | 3 | 1 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

<sup>a</sup> Haplotype of one of the four active ingredients of Aflasafe KE01, a registered biopesticide for aflatoxin management in Kenya. H = haplotype; unique haplotypes were numbered H0001 to H2710. Haplotypes were identified based on 17-multilocus SSR loci. Only the ten most common haplotypes across both seasons and all areas are included. Numbers in columns indicate the number of isolates with the indicated haplotype in each area (Area 1 to 10) and season (S1 & S2).

<sup>b</sup> N = total number of isolates overlapped with same haplotypes.

<sup>c</sup> Locations Area 1 through Area 10 are indicated in Fig. 1.

<sup>d</sup> S1, Long Rain Season (May 2012).

<sup>e</sup> S2, Short Rain Season (November 2012).

Fig. 2. The 10 most frequently occurring clonal groups (CGs) of closely related haplotypes (sharing identical genotypes for at least 14 of the 17 SSR loci) of A. flavus including the groups of the four Aflasafe KE01 active ingredients (indicated with active ingredient name on top of the bars). Locations of the 10 agricultural areas (named Area-1 through Area-10) are indicated in Fig. 1. A. flavus populations were sampled in maize soils in both the long rains and short rains seasons during 2012. The results above combine the data of the two seasons.

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Certain VCGs and SSR haplotypes of *A. flavus* are naturally widely distributed. For instance, several VCGs of *A. flavus* isolated from agricultural soils occur across a large section of the United States (Horn and Dorner, 1998, 1999; Ehrlich et al., 2007; Grubisha and Cotty, 2010, 2015; Ogubayyo et al., 2013) and others occur across Africa (Ogubayyo et al., 2013). Dispersal of *A. flavus* is consistent with the production of large quantities of airborne conidia (Bock et al., 2004) and association with both insects (Stephenson and Russell, 1974) and human transported crop materials (Garber and Cotty, 2014; Shenge et al., 2019). Detection of the KE01 active ingredients in all areas except Area-8 (Tana River County) of the Tana River Irrigation Scheme near Bura is most likely due to either physical barriers to dispersal (i.e. stretches of arid land) or requirements for adaptive success differing among areas. Different requirements for niche residence in Area-8 may cause high frequencies of locally restricted haplotypes (Islam et al., 2018). In some cases, production areas with distinct agroecologies or aflatoxin aetiology may warrant the development of targeted biocontrol agents.

In conclusion, high genotypic diversity in the *A. flavus* population resident in Kenya is attributable to the size (very large) and age (ancient) of that population (Islam et al., 2018). The wide distribution and persistence of SSR haplotypes closely related to the four active ingredients of KE01, especially 6–7 years after initial isolation, indicate that these fungal genotypes and genetic lineages have been growing in and adapting to Kenyan agroecosystems for long periods. Thus, the KE01 active ingredients can be expected to perform and compete well against resident aflatoxin producers. The results of the current study suggest that these active ingredients are excellent selections for a Kenya-specific aflatoxin biocontrol product for which long-term efficacy, persistence and cumulative benefits are desired.

**Experimental procedures**

**Sample collection**

Soils from agricultural fields cropped to maize were sampled in seven counties (Embu, Machakos, Makueni, Kitui, Tana, Homabay and Migori) located across the regions of Kenya most vulnerable to aflatoxin contamination, as previously described (Islam et al., 2018). Sampling was performed during both the long rains (late April to Early June) and the short rains (November to December) seasons of 2012 (Table 1). The two seasons are separated by approximately six months. Five counties (Tana River, Makueni, Kitui, Machakos and Embu) were sampled just prior to the long rains season (May 2012), and six counties (Embu, Machakos, Makueni, Kitui, Homabay and Migori) were sampled just prior to the short season rains (November 2012) (Table 1). Fields were geo-referenced prior to sampling. Samples were collected from 629 fields across ten agricultural areas (Fig. 1), where 390 fields were sampled during the long rains season and 239 fields during the short season rains (Fig. 1 and Table 1) following a previously described method (Cotty, 1997). Briefly, in each field, a 30 to 40 m transect was sampled at 3- to 4-m intervals. At each point, four small scoops of soil (1 to 3 g each) were collected to a depth of 2 cm and combined with samples from other points. These pooled samples were homogenized and stored dry at room temperature. All samples were shipped to the USDA-ARS laboratory at the School of Plant Sciences of the University of Arizona, Tucson, under a permit from USDA-APHIS (permit to move live plant pests, noxious weeds, and soil). The active ingredients of KE01 (C6E, E63I, R7H and R7K) belong to the L morphotype and were originally isolated in the USDA-ARS laboratory at the University of Arizona from maize produced in Kenya during the lethal aflatoxicosis outbreaks of 2004 through 2006 (Probst et al., 2011).

**Isolation of Aspergillus section Flavi fungi**

Soil samples were subjected to the dilution plate technique on the semi-selective medium modified Rose Bengal agar (M-RB) (Cotty, 1994a,b). Briefly, 5 to 10 g of soil was suspended in 250 ml sterile distilled water with 0.1% TWEEN-80 solution by stirring for 20 min at 200 rpm. Aliquots of the resulting suspension were spread on three M-RB plates (200 g per plate). When necessary, adjustments to aliquot volume and soil quantities were made to obtain no more than 12 *Aspergillus* section *Flavi* colonies per plate. After incubation at 31°C for three days, *Aspergillus* section *Flavi* colonies were subcultured onto 5/2 agar ([5% V-8 juice (Campbell Soup Company, Camden, NJ), 20 g l⁻¹ bacto-agar, pH 6.0] at 31°C (Cotty, 1989). A total of 10 to 15 *Aspergillus* section *Flavi* isolates were cultured from each soil sample, and specific fungal species or morphotypes were identified on the basis of colony characteristics and spore morphology. All isolates belonging to the *A. flavus* L morphotype (Table 1) were included for further analyses. Cultures were stored as 3-mm plugs of 5-day-old cultures on 5/2 agar in sterile distilled water at 4°C for further use. For long-term storage, select cultures were stored on silica gel. The active ingredients (C6E, E63I, R7H and R7K) of KE01 were cultured from silica storage maintained at the Tucson location. One piece of silica was subcultured on 5/2 agar as previously (Cotty, 1989). Sporulating cultures (5–7 days old) were stored as 3-mm plugs on 5/2 agar in sterile distilled water at 4°C until further use.
**DNA extraction and SSR amplification**

DNA was extracted from active ingredients (C6E, E63I, R7H and R7K) of KE01, and all A. flavus L-morphotype isolates recovered from soil samples (Table 1). A previously described protocol was followed for DNA extraction (Callicott and Cotty, 2015). Briefly, isolates were plated onto 5/2 agar (Cotty, 1989) and incubated at 31°C for 8–10 days. Conidia were then suspended in 500 µl lysis buffer and incubated with agitation in a Thermomixer 5436. DNA was ethanol precipitated, dissolved in sterile water, quantified with a spectrophotometer (Nanodrop, Wilmington, DE) and stored at −20°C until use.

Variability was determined at 17 SSR loci distributed across all eight A. flavus chromosomes, as previously described (Islam et al., 2018). Briefly, five multiplex PCR panels optimized for high-throughput SSR amplification and genotyping. Multiplex PCRs were carried out using 0.08 µmol l⁻¹ of each primer, 1X AccuStart II PCR SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) and 5 ng of genomic DNA in a final reaction volume of 10 µl. PCR conditions were as follows: 94°C for 1 min, 26 cycles of 94°C for 30 s, 57°C for 90 s, 72°C for 30 s and 30 min at 60°C. PCR amplicons were sized at the University of Arizona Core Genomics Laboratory with the LIZ500 standard and an ABI 3730 analyzer (Applied Biosystems, Waltham, Massachusetts, USA). Allele sizes were called with GeneMarker version 2.6 (SoftGenetics LLC, State College, Pennsylvania, USA).

**SSR genotyping and clone correction**

Allelic data from 17-SSR markers were combined for each of the study isolates, and haplotypes (genotypes) were determined using the program HAPLOTYPE-ANALYSIS V 1.04 (Eliades and Eliades, 2009). In order to construct a clone-corrected data set for each sample, each haplotype was only included in the dataset once for each soil sample (Table 2). Clone-corrected data sets were used as the basis for further analysis.

**Analysis of vegetative compatibility group (VCG)**

In order to test whether isolates with an SSR haplotype matching one of the active ingredients of KE01 belonged to that active ingredient’s VCG, isolates were typed using VCA based on complementation of nitrate non-utilizing auxotrophs (Bayman and Cotty, 1991). All isolates with haplotypes either matching an active ingredient of KE01 or closely related to an active ingredient’s haplotype (differing at only one SSR locus) were typed with VCA. Briefly, isolate auxotrophs (nit⁻ mutants) were generated for each isolate on chlorate containing media (Cotty, 1994a,b). Complementary tester auxotrophic mutants (one cnx⁻ and one niaD⁻) from each of the four active ingredients of KE01 were previously developed (Probst et al., 2011). A nitrate auxotroph (nit⁻) from each isolate was paired with both tester auxotrophs. Isolate auxotrophs that complemented one or both tester auxotrophs were classified as belonging to the VCG defined by that tester pair.

**Analysis of genotypic diversity and distributions**

Haplotypic diversity, frequencies and distributions were estimated for both cropping seasons (long and short rains) for the year 2012, both individually and together. Area-wide haplotypic diversity, including the number of haplotypes and frequencies of repeated haplotypes displayed by two or more isolates, was measured using the program haplotype-analysis V 1.04. Frequencies of the most common haplotypes across the study areas were statistically tested with analysis of variance (ANOVA) based on the general linear model procedure in sas (version 9.2; SAS Institute, Cary, NC). Mean separations were performed on data from tests with statistically significant differences (*P* = 0.05) using Tukey’s honestly significant difference test (Pagano and Gauvreau, 2000).

**Analysis of closely related lineages of the biocontrol product**

Closely related lineages (closely related haplotypes are presumed to have evolved through clonal reproduction and mutation) of the active ingredients of KE01 were identified based on principles used for determining clonal lineages of the asexually reproducing bacteria with the program eburst v3 (http://eburst.mlst.net/) (Feil et al., 2004). A user-defined group definition was set to include those groups that shared identical genotypes for at least 14 of the 17 SSR loci.

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**Conflict of interest**

All authors declare no conflict of interest.

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References

Agbetiameh, D., Ortega-Beltran, A., Awuah, R.T., Atehnkeng, J., Elzein, A., Cotty, P.J., and Bandyopadhyay, R. (2020) Field efficacy of two atoxigenic biocontrol products for mitigation of aflatoxin contamination in maize and groundnut in Ghana. Biol Control 150: 104351.

Agbetiameh, D., Ortega-Beltran, A., Awuah, R.T., Atehnkeng, J., Islam, M.S., Callicott, K.A., et al. (2019) Potential of atoxigenic Aspergillus flavus vegetative compatibility groups associated with maize and groundnut in Ghana as biocontrol agents for aflatoxin management. Front Microbiol 10: 2069.

Atehnkeng, J., Donner, M., Oljamoso, P.S., Ikotun, B., Augusto, J., Cotty, P.J., and Bandyopadhyay, R. (2016) Environmental distribution and genetic diversity of vegetative compatibility groups determine biocontrol strategies to mitigate aflatoxin contamination of maize by Aspergillus flavus. Microb Biotechnol 9: 75–88.

Atehnkeng, J., Oljamoso, P.S., Cotty, P.J., and Bandyopadhyay, R. (2014) Field efficacy of a mixture of atoxigenic Aspergillus flavus Link: Fr vegetative compatibility groups in preventing aflatoxin contamination in maize (Zea mays L.). Biol Control 72: 62–70.

Azizi-Baumgartner, E., Lindblade, K., Gieseke, K., Rogers, H.S., Kieszak, S., Njapau, H., et al. (2005) Case-control study of an acute aflatoxicosis outbreak, Kenya, 2004. Environ Health Persp 113: 1779–1783.

Bandyopadhyay, R., and Cotty, P.J. (2013) Biological controls for aflatoxin reduction. In Aflatoxins: Finding Solutions for Improved Food Safety. Unnevehr, L., and Grace, D. (eds). Washington, DC: International Food Policy Research Institute.

Bandyopadhyay, R., Ortega-Beltran, A., Akande, A., Mutegi, C., Atehnkeng, J., Kaptoge, L., et al. (2016) Biological control of aflatoxins in Africa: current status and potential challenges in the face of climate change. World Mycotoxin J 9: 771–789.

Bayman, P., and Cotty, P.J. (1991) Vegetative compatibility and genetic diversity in the Aspergillus flavus population of a single field. Can J Bot 69: 1707–1711.

Bayman, P., and Cotty, P.J. (1993) Genetic diversity in Aspergillus flavus: association with aflatoxin production and morphology. Can J Bot 71: 23–31.

Berbegal, M., Ortega, A., Jimenez-Gasco, M.M., Olivares-Garcia, C., Jimenez-Diaz, R.M., and Armengol, J. (2010) Genetic diversity and host range of Verticillium dahliae isolates from artichoke and other vegetable crops in Spain. Plant Dis 94: 396–404.

Bock, C.H., Mackey, B., and Cotty, P.J. (2004) Population dynamics of Aspergillus flavus in the air of an intensively cultivated region of south-west Arizona. Plant Pathol 53: 422–433.

Callicott, K.A., and Cotty, P.J. (2015) Method for monitoring deletions in the aflatoxin biosynthesis gene cluster of Aspergillus flavus with multiplex PCR. Lett Appl Microbiol 60: 60–65.

Chang, S.W., Jo, Y.K., Chan, T., and Jung, G. (2014) Evidence for genetic similarity of vegetative compatibility groupings in Sclerotinia homoeocarpa. Plant Pathol J 30: 384–396.
Aspergillus flavus vegetative compatibility groups. FEMS Microbiol Lett 268: 231–236.
Eliades, N.G., and Eliades, D.G. (2009) HAPLOTYPE ANALYSIS: software for analysis of haplotype data. Forest Goettingen (Germany): Genetics and Forest Tree Breeding, Georg-August University Goettingen.
Feil, E.J., Li, B.C., Aanensen, D.M., Hanage, W.P., and Spratt, B.G. (2004) eBURST: Inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol 186: 1518–1530.
Garber, N.P., and Cotty, P.J. (2014) Aspergillus parasiticus communities associated with sugarcane in the Rio Grande Valley of Texas: Implications of global transport and host association within Aspergillus section Flavi. Phytopathology 104: 462–471.
Gong, Y.Y., Wilson, S., Mwatha, J.K., Routledge, M.N., Castelino, J.M., Zhao, B., et al. (2012) Aflatoxin exposure may contribute to chronic hepatomegaly in Kenyan school children. Environ Health Persp 120: 893–896.
Grubisha, L.C., and Cotty, P.J. (2010) Genetic isolation among sympatric vegetative compatibility groups of the aflatoxin-producing fungus Aspergillus flavus. Mol Ecol 19: 269–280.
Grubisha, L.C., and Cotty, P.J. (2015) Genetic analysis of the Aspergillus flavus vegetative compatibility group to which a biological control agent that limits aflatoxin contamination in US crops belongs. Appl Environ Microbiol 81: 5889–5899.
Hadrich, I., Makni, F., Ayadi, A., and Ranque, S. (2010) Microsatellite typing to trace Aspergillus flavus infections in a hematology unit. J Clin Microbiol 48: 2396–2401.
Horn, B.W. (2003) Ecology and population biology of aflatoxigenic fungi in soil. J Toxicol Toxin Rev 22: 351–379.
Horn, B.W., and Dorner, J.W. (1998) Soil populations of Aspergillus species from section Flavi along a transect through peanut-growing regions of the United States. Mycologia 90: 767–776.
Horn, B.W., and Dorner, J.W. (1999) Regional differences in production of aflatoxin B(1) and cyclopiazonic acid by soil isolates of Aspergillus flavus along a transect within the United States. Appl Environ Microbiol 65: 1444–1449.
Horn, B.W., Greene, R.L., Sobolev, V.S., Dorner, J.W., Powell, J.H., and Layton, R.C. (1996) Association of morphology and mycotoxin production with vegetative compatibility groups in Aspergillus flavus, A. parasiticus, and A. tamarii. Mycologia 88: 574–587.
Islam, M.S., Callicott, K.A., Mutegi, J., Bandyopadhyay, R., and Cotty, P.J. (2018) Aspergillus flavus resident in Kenya: High genetic diversity in an ancient population primarily shaped by clonal reproduction and mutation-driven evolution. Fungal Ecol 35: 20–33.
Kachapulula, P.W., Akello, J., Bandyopadhyay, R., and Cotty, P.J. (2017) Aspergillus section Flavi community structure in Zambia influences aflatoxin contamination of maize and groundnut. Int J Food Microbiol 261: 49–56.
Leslie, J.F. (1993) Fungal vegetative compatibility. Annu Rev Phytopathol 31: 127–150.
Liu, Y., and Wu, F. (2010) Global burden of aflatoxin-induced hepatocellular carcinoma: A risk assessment. Environ Health Persp 118: 818–824.

Mehl, H.L., and Cotty, P.J. (2013) Influence of plant host species on intraspecific competition during infection by Aspergillus flavus. Plant Pathol 62: 1310–1318.
Mehl, H.L., Jaime, R., Callicott, K.A., Probst, C., Garber, N.P., Ortega-Beltran, A., et al. (2012) Aspergillus flavus diversity on crops and in the environment can be exploited to reduce aflatoxin exposure and improve health. Ann NY Acad Sci 1273: 7–17.
Moral, J., Garcia-Lopez, M.T., Camilletti, B.X., Jaime, R., Michailides, T.J., Bandyopadhyay, R., and Ortega-Beltran, A. (2020) Present status and perspective on the future use of aflatoxin biocontrol products. Agronomy 10: 491.
Mutegi, C., Cotty, P., and Bandyopadhyay, R. (2018) Prevalence and mitigation of aflatoxins in Kenya (1960-to date). World Mycotoxin J 11: 341–357.
Obura, A. (2013) Aflatoxicosis: evidence from Kenya. In Aflatoxins: Finding Solutions for Improved Food Safety. Unnevehr, L., and Grace, D. (eds). Washington, DC: International Food Policy Research Institute.
Ogunbayo, T., Callicott, K., Probst, C., and Cotty, P. (2013) An atoxigenic vegetative compatibility group of Aspergillus flavus widely adapted to maize production in Africa and North America. Phytopathology 103: 105–106.
Ortega-Beltran, A., Callicott, K.A., and Cotty, P.J. (2020) Founder events influence structures of Aspergillus flavus populations. Environ Microbiol 22: 3522–3524.
Ortega-Beltran, A., and Cotty, P. (2018) Frequent shifts in Aspergillus flavus populations associated with maize production in Sonora, Mexico. Phytopathology 108: 412–420.
Pagano, M., and Gauvreau, K. (2000) Principles of biostatistics, 2nd edn. Pacific Grove, CA, USA: Duxbury, pp. 349–352.
Papa, K.E. (1986) Heterokaryon incompatibility in Aspergillus flavus. Mycologia 78: 98–101.
Picot, A., Doster, M., Islam, M.-S., Callicott, K., Ortega-Beltran, A., Cotty, P., and Michailides, T. (2018) Distribution and incidence of atoxigenic Aspergillus flavus VCG in tree crop orchards in California: A strategy for identifying potential antagonists, the example of almonds. Int J Food Microbiol 265: 55–64.
Probst, C., Bandyopadhyay, R., and Cotty, P.J. (2014) Diversity of aflatoxin-producing fungi and their impact on food safety in sub-Saharan Africa. Int J Food Microbiol 174: 113–122.
Probst, C., Bandyopadhyay, R., Price, L.E., and Cotty, P.J. (2011) Identification of atoxigenic Aspergillus flavus isolates to reduce aflatoxin contamination of maize in Kenya. Plant Dis 95: 212–218.
Probst, C., Callicott, K.A., and Cotty, P.J. (2012) Deadly strains of Kenyan Aspergillus are distinct from other aflatoxin producers. Eur J Plant Pathol 132: 419–429.
Probst, C., Njapau, H., and Cotty, P.J. (2007) Outbreak of an acute aflatoxicosis in Kenya in 2004: Identification of the causal agent. Appl Environ Microbiol 73: 2762–2764.
Probst, C., Schulthess, F., and Cotty, P.J. (2010) Impact of Aspergillus section Flavi community structure on the development of lethal levels of aflatoxins in Kenyan maize (Zea mays). J Appl Microbiol 108: 600–610.
Senghor, L., Ortega-Beltran, A., Atehnikeng, J., Callicott, K., Cotty, P., and Bandyopadhyay, R. (2020) The atoxigenic biocontrol product Asfasafe SN01 is a valuable tool to mitigate aflatoxin contamination of both maize and groundnut cultivated in Senegal. Plant Dis 104: 510–520.

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Shenge, K.C., Adhikari, B.N., Akande, A., Callicott, K.A., Atehnkeng, J., Ortega-Beltran, A., *et al.* (2019) Monitoring *A. flavus* with pyrosequencing monitoring *Aspergillus flavus* genotypes in a multi-genotype aflatoxin biocontrol product with quantitative pyrosequencing. *Front Microbiol* **10**: 2529.

Singh, P., Callicott, K.A., Orbach, M.J., and Cotty, P.J. (2020) Molecular analysis of S-morphology aflatoxin producers from the United States reveals previously unknown diversity and two new taxa. *Front Microbiol* **11**: 1236.

Stephenson, L.W., and Russell, T.E. (1974) The association of *Aspergillus flavus* with hemipterous and other insects infesting cotton bracts and foliage. *Phytopathology* **71**: 359–362.

Sweany, R.R., Damann, K.E., and Kaller, M.D. (2011) Comparison of soil and corn kernel *Aspergillus flavus* populations: evidence for niche specialization. *Phytopathology* **101**: 952–959.

Tran-Dinh, N., Kennedy, I., Bui, T., and Carter, D. (2009) Survey of Vietnamese peanuts, corn and soil for the presence of *Aspergillus flavus* and *Aspergillus parasiticus*. *Mycopathologia* **168**: 257–268.

World Health Organization (WHO) (2011b) *Mycotoxins. Children’s Health and the Environment*. WHO Training Package for the Health Sector World Health Organization (WHO), pp. 1–42.

Williams, J.H., Phillips, T.D., Jolly, P.E., Stiles, J.K., Jolly, C.M., and Aggarwal, D. (2004) Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *Am J Clin Nutr* **80**: 1106–1122.

Yard, E.E., Daniel, J.H., Lewis, L.S., Rybak, M.E., Paliakov, E.M., Kim, A.A., *et al.* (2013) Human aflatoxin exposure in Kenya, 2007: a cross-sectional study. *Food Addit Contam A* **30**: 1322–1331.