FUNGI ASSOCIATED WITH AFLATOXIN CONTAMINATION IN AFRICA

by

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DEDICATION

„So eine Arbeit wird eigentlich nie fertig,
man muß sie für fertig erklären,
wenn man nach Zeit und Umständen
das mögliche getan hat.“

Johann Wolfgang von Goethe, 1787

Für meinen Mann Pedro, der mir zeigte was wahre Liebe ist und mir zwei wunderbare

Kinder geschenkt hat, die mein Leben vervollständigen.

Te amo!

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ABSTRACT

Aflatoxins are secondary metabolites produced by members of the fungal genus *Aspergillus*. Immunosuppressive and carcinogenic activities of these toxins negatively impact human health especially in developing countries. Severity of contamination is influenced by both fungal community structure and the environment to which the crop is exposed either prior to or after harvest.

In 2004, a severe episode of lethal human aflatoxicosis occurred in the Eastern Province of Kenya. Analysis of fungal community structure revealed that this event was caused by a previously unknown fungal lineage closely resembling the S strain morphotype of *Aspergillus flavus*. Fungal communities associated with maize produced in affected regions of Kenya were invariably dominated by the new fungal lineage and its incidence was strongly correlated with maize aflatoxin content. Analyses of fungal communities of maize grown in adjacent Kenyan provinces showed that incidences of the new lineage are limited outside the Eastern Province where the aflatoxicoses outbreaks occurred. Multi-locus phylogenetic analyses suggest the newly identified Kenyan lineage is closely related to the B and G aflatoxin producing species *A. minisclerotigenes*, and more distantly related to both the *A. flavus* S strain and an unnamed taxon with similar morphology endemic in West Africa (strain S_{BG}). Sequence analyses of the *cypA* aflatoxin biosynthesis gene identified a previously unknown 2.2 kb deletion unique to the Kenyan lineage and coherent with its phylogenetic placement. A polyphasic approach was used to study aflatoxin-producing

fungal communities, with emphasis on occurrence of fungi with S strain morphology, in Sub-Saharan Africa. Four phylogenetically distinct groups of fungi with S strain morphology were identified with restrictions to West Africa (strain S_{BG}) or Central and East Africa (*A. flavus* S strain, *A. minisclerotigenes*, the new lineage).

Aflatoxin production in synthetic media was a poor predictor of aflatoxin production in viable maize grain. An *in vitro* assay was developed to predict the aflatoxin-producing potential of fungal isolates in maize. This screen was used to identify atoxigenic isolates of *A. flavus* with potential value for biological control within highly toxic *Aspergillus* communities associated with maize production in Kenya. These atoxigenic isolates have potential value for mitigating aflatoxin contamination in Kenya.

CHAPTER 1 - OUTBREAK OF AN ACUTE AFLATOXICOSIS IN KENYA IN 2004: IDENTIFICATION OF THE CAUSAL AGENT

Applied and Environmental Microbiology (2007), Vol. 73 (8), p. 2762-2764

Abstract

In 2004, Kenya experienced a severe outbreak of acute aflatoxin poisoning (aflatoxicosis). Although lethal aflatoxicoses have occurred in repeatedly since 1981, the fungi contaminating the maize with aflatoxins have not been characterized. We analyzed 104 maize samples collected during the 2004 outbreak in order to identify the causative fungi. Over 97% of the 1,232 *Aspergillus* section *Flavi* isolates recovered from the maize were *Aspergillus flavus* and the remaining 3% were *Aspergillus parasiticus*. Most (72%) of the observed *A. flavus* isolates belonged to the S strain morphotype, which was not previously known in Africa. Kenyan S strain isolates produced much greater quantities of aflatoxins than the 28% of *A. flavus* isolates belonging to the L strain morphotype. Furthermore, maize aflatoxin content was strongly correlated ($r = 0.89$; $p = 0.016$) with S strain incidence. Indeed, the S strain occurred in the poisonous maize at higher proportions than previously observed on any crop from any location. The results suggest that dominance of the S strain in the Kenyan maize led to both extreme aflatoxin levels and the resulting severe toxicity.

Introduction

The 2004 outbreak of acute aflatoxicosis in Kenya was one of the most severe episodes of human aflatoxin poisoning in history. A total of 317 cases were reported by 20 July 2004, with a case fatality rate of 39% (Centers for Disease Control and Prevention, 2004; Azziz-Baumgartner *et al.*, 2005). This epidemic resulted from ingestion of contaminated maize (Lewis *et al.*, 2005). However, identities of the fungi causing the contamination remain unclear.

Aflatoxins are carcinogenic metabolites produced by several *Aspergillus* species (Bennett and Klich, 2003; Williams *et al.*, 2004). Aflatoxin-producing fungi vary widely in many characteristics, including virulence for crops and aflatoxin-producing capacity (Cotty, 1989). *A. flavus* and *A. parasiticus* are most commonly implicated as causal agents of aflatoxin contamination. *A. flavus* has two morphotypes, the typical or L strain (sclerotia of $\geq$ 400 µm in diameter) and the S strain (sclerotia of $\leq$ 401 µm in diameter) (Cotty, 1989; Horn, 2003). S strain isolates produce more aflatoxins than L strain isolates, on average (Cotty, 1989). Many L strain isolates produce no aflatoxins ("atoxigenic") (Cotty, 1997). All members of *A. flavus* lack the ability to synthesize G aflatoxins due to a 0.8 to 1.5 kb deletion in the 28-gene aflatoxin biosynthesis cluster (Ehrlich *et al.*, 2004). In contrast to cases in the United States, studies conducted in West Africa found that an unnamed taxon (sometimes called strain S_{BG}) is commonly implicated in contamination events (Cotty and Cardwell, 1999). Strain S_{BG} is morphologically similar to the S strain of *A. flavus*, but DNA-based phylogenies reveal strain S_{BG} to be a distinct species ancestral to both *A.*

flavus and *A. parasiticus* (Egel *et al.*, 1994; Ehrlich *et al.*, 2005b). In order to determine the primary causal agent(s) of the 2004 contamination events in Kenya, we considered both fungal aflatoxin-producing potential and frequency of occurrence in the contaminated crop (Cotty, 1997).

Methods and Results

Representative maize samples were collected from major agricultural markets and storage facilities of the most affected Kenyan districts by the National Public Health Laboratory Services in Nairobi, Kenya, during the 2004 outbreak (Muture and Ogana, 2005). Samples were screened for aflatoxin content, and only B aflatoxins were detected (Lewis *et al.*, 2005; Muture and Ogana, 2005). Subsamples (n = 103; average weight = 87.5 g; range of contamination = 0.27 to 4,400 ppb total aflatoxin) were imported to the United States from the National Public Health Laboratory Services for fungal analyses. Fungi were isolated from the maize by using the dilution plate technique on modified rose Bengal agar (Cotty, 1994a). Isolates were classified into species and strains by observing colony characteristics and sclerotial and conidial morphologies after subculturing on 5/2 agar (5% V8 juice; 2% agar; pH 5.2) (Cotty, 1989). Isolations were repeated two to four times to verify results. Isolates from each sample were collected from at least two isolations. Quantities of *Aspergillus* section *Flavi* isolates in maize were expressed as the number of CFU per mg. A total of 1,232 isolates (10 to 18 per sample) were recovered from the maize, saved, and stored at 4°C. *A. flavus* was recovered from all samples (97.9% of isolates); 15 samples also contained *A. parasiticus* (2.1% of isolates). Other aflatoxin producers were not detected. All *A. flavus* isolates were assigned to either the L strain or the S strain, 28.2% and 71.8%, respectively (Cotty, 1989).

Both simple linear and quadratic regression analyses ($b_0 + b_1x$ and $b_0 + b_1x + b_2x^2$, respectively) were performed for aflatoxin content as a function of S strain

incidence, *A. parasiticus* incidence, or *A. flavus* quantity (CFU/mg) using SAS 8.0 software (SAS Institute, Cary, NC). Maize aflatoxin content and S strain incidence were highly correlated. When maize samples were sorted into groups based on aflatoxin content, the incidence of the S strain increased with average maize aflatoxin content from 69% in samples with < 20 ppb total aflatoxins to 94% in samples with > 1,000 ppb (Table 1.1; Figure 1.1). Only S strain isolates were recovered from five of six samples with >1,000 ppb (the sixth sample was 66.7% S strain). *A. parasiticus* was not recovered from any sample with > 260 ppb, and its incidence was not correlated with aflatoxin content (Table 1.1).

Aflatoxin production by representative *A. flavus* isolates (26 S strain isolates; 26 L strain isolates) was measured to assess variance in aflatoxin production among strains. Fermentations were carried out in the medium of Mateles and Adye (Mateles and Adye, 1965) with 22.5 mM urea as the sole nitrogen source exactly as described previously (Cotty and Cardwell, 1999; Cardwell and Cotty, 2002). This medium allows detection of the strain S_{BG} phenotype. Reference isolates from the United States (*A. flavus* S strain AF70 and L strain AF13) and West Africa (strain S_{BG} isolates BN008R and BN038G) were included for comparison. Fermentations were replicated three times. S strain isolates produced more (mean = 356.46 µg aflatoxin B_1/ g mycelium) aflatoxins than L strain isolates (mean = 37.55 µg aflatoxin B_1/ g mycelium). Fifty percent (n = 13) of the L strain isolates were atoxigenic. Similar disparities in aflatoxin production by S and L strain isolates have been reported from other continents (Saito *et al.*, 1986; Novas and Cabral, 2002; Jaime-Garcia and Cotty, 2006b).

An additional 100 *A. flavus* S strain isolates were screened in similar fermentations. The examined isolates produced only B aflatoxins (mean = 488.95 µg aflatoxin B_1/ g mycelium); this excludes the possibility that any of the tested isolates belong to strain S_{BG}, previously reported from West Africa (Cardwell and Cotty, 2002).

To further investigate the potential of Kenyan S strain isolates to contaminate maize, 20 S strain isolates were inoculated onto living maize kernels surface sterilized in hot water (80°C, 45 s). Kernels were adjusted to 25% moisture and incubated for 7 days (31°C), and aflatoxin was quantified as described previously (Brown *et al.*, 1993). Inoculated maize developed 95,000 ppb to 212,000 ppb aflatoxin B_1. G aflatoxins were not detected.

Table 1.1 Quantity of *Aspergillus* section *Flavi* and incidences of *A. parasiticus* and the S strain of *A. flavus* in Kenyan maize containing various amounts of aflatoxins [a,b,c,d,e,f]

Region of isolation	Amt of Aflatoxin (ppb)	No. of samples[g]	S (%)	AP (%)	No. of CFU/mg
Machakos District	> 1,000	1	100	0	41.9
	301 to 1,000	3	93	0	5.9
	21 to 300	10	70	13	5
	2 to 19	3	67	3	0.1
	0.5 to 2	3	66	2	1.3
Total		**20**	**75**	**7**	**5.5**
Makueni District	> 1,000	3	89	0	15.4
	301 to 1,000	4	75	0	3
	21 to 300	19	73	2	2
	2 to 19	3	71	2	2.9
	0.5 to 2	8	67	0	1.5
Total		**37**	**73**	**1**	**3.2**
Kitui District	> 1,000	2	100	0	56.6
	301 to 1,000	4	86	0	4.9
	21 to 300	15	70	0	3.3
	2 to 19	6	58	1	4.9
	0.5 to 2	11	75	4	1.5
Total		**38**	**73**	**1**	**7.1**
All samples	> 1,000	6	94	0	33.6
	301 to 1,000	12	84	0	7.5
	21 to 300	49	68	3	3.2
	2 to 19	12	63	2	3.2
	0.5 to 2	24	69	2	1.4
Total		**103**	**71**	**2**	**5**

[a] S (%), percentage of *Aspergillus* section *Flavi* isolates belonging to the S strain of *A. flavus*; AP (%), percentage of *Aspergillus* section *Flavi* isolates belonging to *Aspergillus parasiticus*; no. of CFU/mg, no. of CFU of *Aspergillus* section *Flavi* per mg of maize.

[b] Trends are as follows. For Machakos District, S (%), positive; AP (%), negative; no. of CFU/mg, positive. For Makueni District, S (%), positive; AP (%), negative; no. of CFU/mg, positive. For Kitui District, S (%), positive; AP (%), negative; no. of CFU/mg, positive. For all samples, S (%), positive; AP (%), negative; no. of CFU/mg, positive.

[c] Linear r^2 values are as follows. For Machakos District, S (%), 0.89; AP (%), 0.229; no. of CFU/mg, 0.897. For Makueni District, S (%), 0.95; AP (%), 0.279; no. of CFU/mg, 0.905. For Kitui District, S (%), 0.774; AP (%), 0.215; no. of CFU/mg, 0.991. For all samples, S (%), 0.889; AP (%), 0.595; no. of CFU/mg, 0.965.

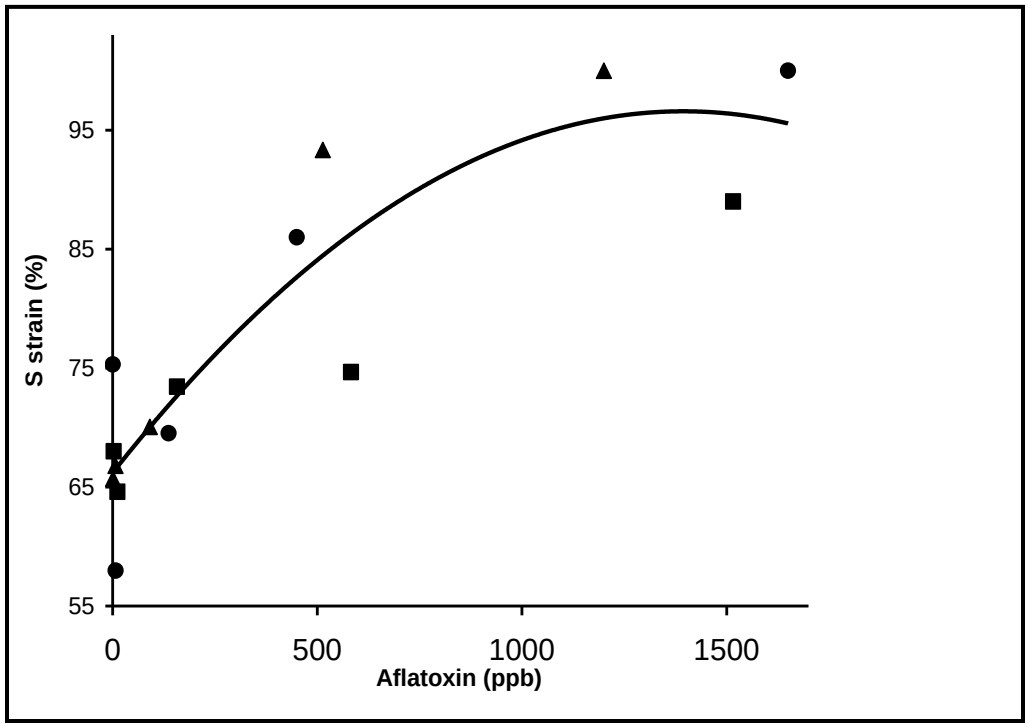
[d] Linear *P* values are as follows. For Machakos District, S (%), 0.016; AP (%), 0.415; no. of CFU/mg, 0.0144. For Makueni District, S (%), 0.0048; AP (%), 0.3598; no. of CFU/mg, 0.0127. For Kitui District, S (%), 0.0492; AP (%), 0.4314; no. of CFU/mg, 0.0004. For all samples, S (%), 0.0163; AP (%), 0.1268; no. of CFU/mg, 0.0028.

[e] Quadratic r^2 values are as follows. For Machakos District, S (%), 0.975; AP (%), 0.741; no. of CFU/mg, 0.932. For Makueni District, S (%), 0.956; AP (%), 0.279; no. of CFU/mg, 0.979. For Kitui District, S (%), 0.979; AP (%), 0.341; no. of CFU/mg, 0.991. For all samples, S (%), 0.988; AP (%), 0.703; no. of CFU/mg, 0.989.

[f] Quadratic *P* values are as follows. For Machakos District, S (%), 0.0245; AP (%), 0.2593; no. of CFU/mg, 0.0682. For Makueni District, S (%), 0.0436; AP (%), 0.359; no. of CFU/mg, 0.021. For Kitui District, S (%), 0.0202; AP (%), 0.6591; no. of CFU/mg, 0.0088. For all samples, S (%), 0.0119; AP (%), 0.297; no. of CFU/mg, 0.0102.

[g] Ten to fifteen individuals were isolated from each sample and examined.

Figure 1.1 Incidence of the S strain of *Aspergillus flavus* increased with aflatoxin content in maize samples collected in Kenya during 2004.



Discussion

Characterization of causal agents is an important initial step for development of management procedures. Attribution of specific etiologies to aflatoxin contamination episodes is complicated by variability in aflatoxin-producing capacity among species, strains, and isolates (Cotty *et al.*, 1994a). The maize contamination event that led to the 2004 outbreak of acute aflatoxicosis in Kenya is a particular important contamination episode, because it led to deaths of more than 100 people. Results of the current study suggest that the Kenyan outbreak was caused by the S strain of *A. flavus*.

This is supported by the following. (i) The S strain, which was not previously found in Africa (Cardwell and Cotty, 2002), was repeatedly isolated from all 103 maize samples from all affected districts. Communities of aflatoxin-producing fungi associated with highly contaminated maize were invariably dominated by the S strain of *A. flavus*, which occurred in the most toxic Kenyan maize at proportions greater than those previously observed on any crop from any location (Novas and Cabral, 2002; Jaime-Garcia and Cotty, 2006a). (ii) S strain isolates from the Kenyan maize consistently produced large amounts of aflatoxins in both liquid medium and living maize. (iii) Only S strain isolates were recovered from five out of six samples with > 1,000 ppb total aflatoxin. (iv) The S strain incidence was strongly correlated with maize aflatoxin content. (v) The incidence of no other aflatoxin-producing fungus was correlated with contamination.

Identification of factors leading to S strain dominance in semiarid regions of Kenya may result in management procedures effective in both Kenya and other regions

where the S strain is an important etiological agent of aflatoxin contamination. Currently, atoxigenic *A. flavus* isolates are used to competitively exclude aflatoxin producers during crop infection and thereby limit contamination in U.S. agriculture (Cotty, 1994b; Dorner, 2004). Such atoxigenic strains are highly effective against the S strain. Deployment of similar technologies in Africa could provide a promising strategy for prevention of future aflatoxicosis in East Africa while enhancing export possibilities for maize (Bankole and Adebanjo, 2003).

Representative isolates (A1168, A1169, A1170, and A1171) have been deposited at the Fungal Genetics Stock Center, St. Louis, MO.

CHAPTER 2 - IMPACT OF *ASPERGILLUS* SECTION *FLAVI* COMMUNITY STRUCTURE ON THE DEVELOPMENT OF LETHAL LEVELS OF AFLATOXINS IN KENYAN MAIZE (*ZEA MAYS*)

Journal of Applied Microbiology (2010), Vol. 108, p. 600-610

Abstract

Aims: Evaluate the potential role of fungal community structure in predisposing Kenyan maize to severe aflatoxin contamination by contrasting aflatoxin-producing fungi resident in the region with repeated outbreaks of lethal aflatoxicosis to those in regions without a history of aflatoxicosis.

Methods and Results: Fungi belonging to *Aspergillus* section *Flavi* were isolated from maize samples from three Kenyan provinces between 2004 and 2006. Frequencies of identified strains and aflatoxin-producing abilities were assessed and the data was analysed by statistical means. Most aflatoxin-producing fungi belonged to *Aspergillus flavus*. The two major morphotypes of *A. flavus* varied greatly between provinces, with the S strain dominant in both soil and maize within aflatoxicosis outbreak regions and the L strain dominant in non-outbreak regions.

Conclusions: *Aspergillus* community structure is an important factor in the development of aflatoxins in maize in Kenya and, as such, is a major contributor to development of aflatoxicosis in the Eastern Province.

Significance and Impact of the Study: Since 1982, deaths due to aflatoxin contaminated maize have repeatedly occurred in the Eastern Province of Kenya. The current study characterized an unusual fungal community structure associated with the lethal contamination events. The results will be helpful in developing aflatoxin management practices to prevent future outbreaks in Kenya.

Introduction

Today, 500 years after its introduction, maize (*Zea mays*) is the most widely grown staple food in Africa (McCann, 2005). Maize consumption is a primary avenue through which humans in Africa become exposed to aflatoxins (Egal *et al.*, 2005; Shephard, 2008). Aflatoxins are metabolites produced by several *Aspergillus* species. These metabolites are highly toxic to humans and domestic animals. In order to minimize potential human exposure, the aflatoxin content of food and feed is strictly regulated in most of the world (van Egmond and Jonker, 2004; Shephard, 2008). However, these standards have little relevance to poor, small-scale farmers in Africa, who often rely on maize for daily nutrition and income.

Aflatoxin contamination of maize may be caused by several species in *Aspergillus* section *Flavi*. These fungi vary widely in both ability to infect and decay crops and in aflatoxin-producing capacity (Cotty, 1989). Thus, the potential of these fungi to contaminate crops with aflatoxin also varies. Aflatoxin-producing members of section *Flavi* also differ in morphology, physiology, and ecology (Cotty, 1989; Cotty *et al.*, 1994a; Bock *et al.*, 2004). In general, the process of crop contamination with aflatoxins begins in the field during crop development and may continue after crop maturation until the grain is ultimately consumed (Cotty *et al.*, 1994a). Contamination is strongly influenced by abiotic factors such as temperature and humidity as well as biotic factors including insects and the average aflatoxin-producing fungi potential of the fungal community associated with crops (Cotty, 1997; Cotty *et al.*, 2008). *Aspergillus parasiticus* and *A. flavus* are the species most commonly implicated as

causal agents of aflatoxin contamination (Klich, 2007; Cotty *et al.*, 2008). *A. flavus* is delineated into two morphotypes called the S and L strains (Cotty, 1989). The S strain produces many small sclerotia (≤ 400 μm in diameter), relatively few conidia and consistently high levels of aflatoxin. The L strain produces fewer, larger sclerotia (≤ 401 μm in diameter), more conidia and, on average, less aflatoxin than the S strain. A significant percent of L strain isolates produce no aflatoxin. Several of these atoxigenic isolates are the principal active agents in biocontrol products used to manage aflatoxin contamination (Cotty and Bhatnagar, 1994; Dorner, 2004).

The most common aflatoxin, aflatoxin B_1, is a genotoxin known to be carcinogenic and teratogenic for both humans and animals (Wang and Tang, 2004; McKean *et al.*, 2006). This aflatoxin was first listed as a human carcinogen in the *First Annual Report on Carcinogens* in 1980 by the National Toxicology Program of the Department of Health and Human Services (NTP, 1980). To date, aflatoxin B1 is the only mycotoxin classified as a Group 1a human carcinogen by the International Agency for Research on Cancer (International Agency for Research on Cancer, 2002). Intake of low daily doses of aflatoxins over long periods may result in chronic aflatoxicosis expressed as impaired food conversion, stunting in children (Gong *et al.*, 2004), immune suppression, cancer, and reduced life expectancy (Gong *et al.*, 2004; Williams *et al.*, 2004; Cardwell and Henry, 2006; Farombi, 2006). Ingestion of high concentrations of aflatoxin results in rapid development of acute aflatoxicosis characterized by severe liver damage leading to jaundice, hepatitis and, when most severe, death (Williams *et al.*, 2004). Outbreaks of acute aflatoxicosis have never been

reported for developed countries but have occurred in several developing countries (Krishnamachari *et al.*, 1975; Tandon *et al.*, 1977; Ngindu *et al.*, 1982; Lye *et al.*, 1995; Probst *et al.*, 2007). However, only in India and Kenya have epidemics of acute aflatoxicosis been repeatedly reported.

In Kenya, maize is the staple that dominates food security considerations. It has a per capita consumption of 98 kg per annum and accounts for about 40% of the daily calorie intake (www.fao.org). As a direct consequence, Kenyans are exposed to regular doses of aflatoxins through maize ingestion. The first reported outbreak of acute aflatoxicosis in Kenya occurred in 1982 in the Eastern Province (Ngindu *et al.*, 1982). More outbreaks were officially reported in 2001, 2004-2006 and 2008 (Shephard, 2003; Centers for Disease Control and Prevention, 2004). The outbreaks occurred exclusively in only four of the 71 Kenyan districts. The affected districts are adjacent to each other and located in Kenya's Eastern and Central Provinces. The districts Kitui (Eastern), Machakos (Eastern), Makueni (Eastern) and Thika (Central) were affected, with Kitui and Machakos reporting the highest death rates in all years. Kenya is the only African nation with recurrent outbreaks of acute aflatoxicosis. The 2004 outbreak was one of the most severe episodes of human aflatoxin poisoning in history and was caused by ingestion of homegrown maize (Lewis *et al.*, 2005; Muture and Ogana, 2005). Analysis of maize samples collected during the 2004 outbreak by the National Public Health Laboratory Services in Nairobi and the Center for Disease Control and Prevention (CDC) suggests that the fungal community structure was an underlying contributor to the 2004 aflatoxicosis outbreak (Probst, 2005; Probst *et al.*, 2007). The

primary causal agent was determined to be the S strain of *Aspergillus flavus*. These conclusions were supported by the high frequency of S strain isolates in highly contaminated maize, by the consistently high aflatoxin production by these S strain isolates in vitro and vivo, and by the strong positive correlation between percentage of the S strain in the infecting *A. flavus* community and the maize aflatoxin content (Probst *et al.*, 2007). This study was the first to link a particular fungal taxon to an aflatoxicosis epidemic. Identification of the precise causal agent is an important initial step in development of management practices (Cotty *et al.*, 2008). However, information on how fungal communities vary between the districts with and without histories of acute aflatoxicosis is unknown and corroborating evidence for dominance of the S strain on maize from the affected districts in years other than 2004 is lacking.

The present study sought to compare communities of aflatoxin-producing fungi on maize in the affected Kenyan districts during the aflatoxicosis outbreak years of 2005 and 2006 with those previously described for 2004 and with fungal communities on maize in adjacent districts with no histories of aflatoxicosis epidemics. In the process, a body of evidence was developed that convincingly implicates fungal community composition as an important factor predisposing the affected districts to increased incidences of acute aflatoxicosis.

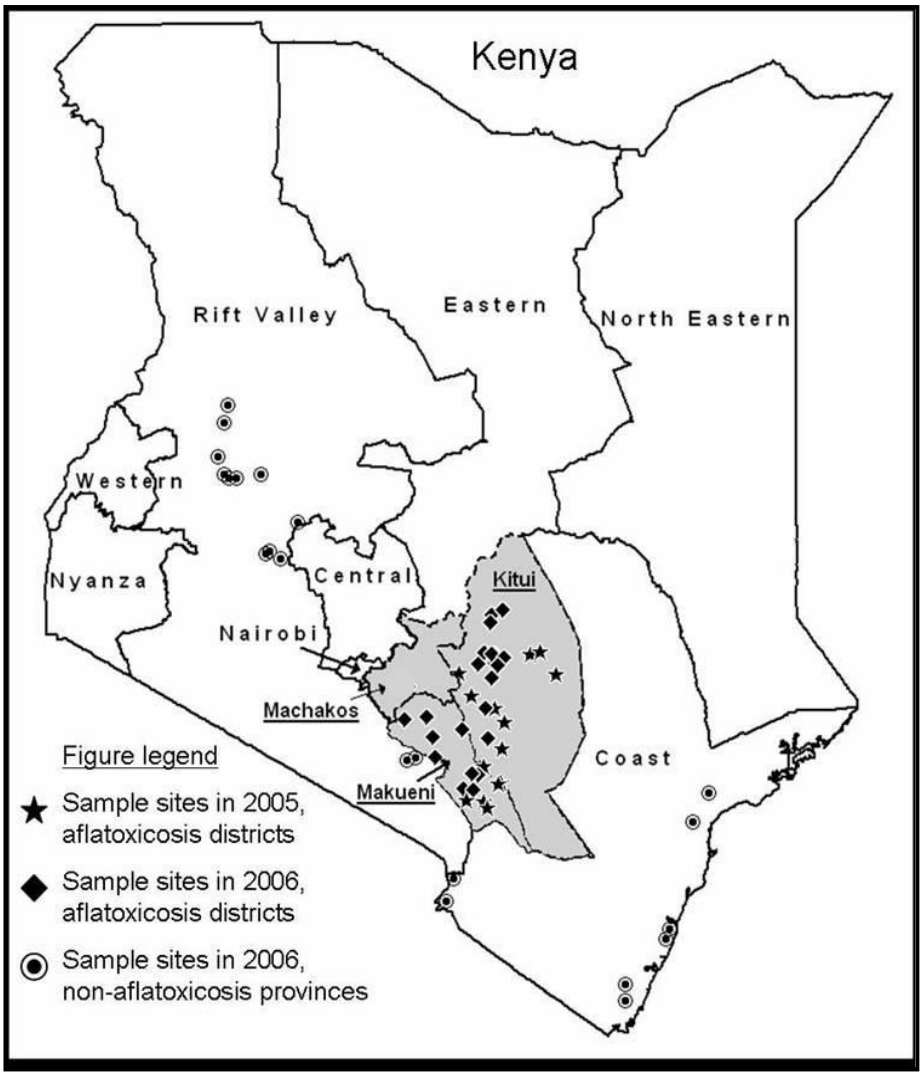
Materials and methods

Sampling

In 2005, ground maize and soil samples were collected in Kitui district of Eastern Province, Kenya at locations where lethal aflatoxicosis had been reported (Figure 2.1). The Eastern Province is characterized by its semi-arid midlands and bimodal rain patterns. The elevation of this province is between 400 and 1800 m. Maize samples were taken from household storage, and soil was collected in the fields in which the sampled maize was produced. Each soil sample was a composite of eight to sixteen subsamples taken from the top 2 cm of the soil from locations at least 4 m apart. Additional maize samples were collected from farmers and local markets in Kitui. In 2006, additional ground maize samples were collected in the Eastern Province (Makueni and Kitui Districts) (Figure 2.1) and in two provinces, the Rift Valley Province and the Coast Province, adjacent to the Eastern Province but with no history of lethal aflatoxicosis (Figure 2.1). Six of the Coast Province samples were taken along the coast at elevations between 12 m and 145 m. Two samples were taken inland along the maize supply route from Tanzania at about 1,000 m. Those areas differ in climate and maize production. The southern coast is warm and semi-humid with bimodal rain patterns and some maize production. In contrast, the north coast is warmer and drier with very little maize production but high import rates. All maize samples from the Rift Valley originated from high elevation areas (1026 m to 2412 m) in the central and eastern parts of the province (Figure 2.1). The central region of the Rift Valley is characterized by its cool and humid climate with only one long rain season and

intensive maize production. Most of the maize for local markets and export is grown in this area. The humid eastern parts have a bimodal rain pattern and sufficient maize production for local consumption. Soil and maize samples were imported to the USDA, ARS, Laboratory for Aflatoxin Reduction in Crops, at the University of Arizona, Tucson under permits issued by the USDA Animal and Plant Health Inspection Service.

Figure 2.1 Map of Kenya indicating sample sites. Districts of the Eastern province that had reported aflatoxicosis outbreaks are highlighted grey. Names of districts are underlined. Names of Provinces are not underlined. Symbol may stand for more than one sample.



Culture medium

Modified rose Bengal agar (M-RB), a defined, semi-selective medium for *Aspergillus* section *Flavi* (Cotty, 1994a) was used for isolations. For culture maintenance, 5/2 agar (5% V8-juice; 2% Agar; adjusted to pH 5.2 prior autoclaving) was used.

Fungal isolation and quantification

Prior to analysis, maize and soil samples were homogenized. Soil samples were hammered to break-up soil clods prior to homogenization. Maize samples were finely ground in a laboratory mill. Both ground maize and powdered soil were vigorously shaken to ensure proper mixing. Samples were also weighed, analyzed for moisture content (HB43 Halogen Moisture Analyzer, Mettler Toledo), dried to 5-8% moisture to prevent fungal growth, and stored for up to four weeks at 4°C until further analysis. Maize samples were between 110 g and 433 g (mean = 291 g).

Fungal isolates were recovered by dilution plate technique on M-RB (Cotty, 1994). Sample material (about 1 g) was mixed by inverting in a 15 ml test tube containing 5 ml sterile distilled water for approximately 20 min, and aliquots (100 µl per plate) of the resulting suspension were spread on M-RB plates (n = 3). After incubation (3 d, 31°C, dark) *Aspergillus* section *Flavi* colonies were enumerated (Colony Forming Units (CFU) g-1). Up to ten discrete colonies were aseptically transferred to 5/2-agar and incubated (5 to 7 d, 31°C). *Aspergillus* species (Klich and Pitt, 1988, Kurtzman et al., 1987) and strains (Cotty, 1989) were identified by both macroscopic and microscopic characters. Isolations were performed two to four times

to verify results. A total of 15 *Aspergillus* section *Flavi* isolates were stored long-term as three-mm plugs of sporulating culture in sterile distilled water at 4°C. Because *A. tamarii* has been repeatedly reported to be atoxigenic, isolates of *A. tamarii* were identified, enumerated, and discarded after initial verification of the atoxigenicity of *A. tamarii* isolates from Kenya.

Quantification of aflatoxins in ground maize

A USDA/GIPSA certified Enzyme Linked ImmunoSorbent Assay (ELISA; MycoChek, Strategic Diagnostics, Inc, Newark, DE) was used to detect and quantify aflatoxins in the maize samples. Ground maize samples were mixed thoroughly and a 50 g sub-sample was blended with 250 ml 70% aqueous methanol and the aflatoxin content determined according to the manufacturer's instructions.

Aflatoxin production in maize kernels

The aflatoxin-producing abilities of 126 random L strain isolates that originated from three Kenyan provinces in 2006 were compared. An aflatoxin assay in maize kernels was conducted to assess toxin production in the host. The experiment was conducted twice, with 84 isolates in the first experiment and 42 isolates in the second experiment, and aflatoxin values were calculated as an average from three repetitions. Undamaged maize kernels (10 g/ 250 ml Erlenmeyer flasks) were autoclaved (60 min), cooled to room temperature, and adjusted to 25% moisture. Flasks were seeded with freshly prepared spore suspensions (2 ml containing 1.9x106 to 2.0x106 spores) from five day old cultures and incubated for seven days at 31°C in the dark. Maize cultures

were blended in 80% methanol (50 ml) until evenly homogenized and maize-methanol slurry was filtered through Whatman No. 4 paper. Culture filtrate was spotted directly onto thin-layer chromatography (TLC) plates (Silica gel 60, EMD, Darmstadt, Germany) adjacent to aflatoxin standards (Aflatoxin Mix Kit-M, Supelco) containing a mixture of aflatoxins B_1, B_2, G_1 and G_2. Plates were developed in ethyl ether-methanol-water, 96:3:1, air dried and aflatoxins were visualized under 365-nm UV light. Aflatoxins were quantified directly on TLC plates with a scanning densitometer (TLC Scanner 3, Camag Scientific Inc, Wilmington, N.C.). Filtrates initially negative for aflatoxins were partitioned twice with methylene chloride and concentrated prior to quantification (limit of detection 1 ng g-1 mycelium) as previously described (Cotty, 1997). Each isolate was subjected to three replications, and each experiment was performed twice.

Production of spores by *A. flavus* isolates on maize kernels was determined with a turbidity meter (Model 965-10, Orbeco-Hillige, Farmingdale, NY). After inoculation and growth as described above, kernels were washed with 50 ml methanol, 1 ml of the resulting spore suspension was diluted in 19 ml ETOH:H2O, the turbidity measured and spore concentration calculated with the NTU (Nephelometric Turbidity Unit) versus CFU (Colony Forming Unit) curve Y = 49937X (X = NTU, Y = spores ml-1).

Data analysis

Mean comparisons were subjected to either Student's t-test or, for multiple comparisons, Analysis of Variance and Tukey's HSD Test as implemented in either Stata 9.2 (StataCorp, College Station, TX) or SAS 8.0 (SAS Institute, Cary, NC).

Results

Isolation and quantification of Aspergillus section Flavi from ground maize

In the current study, 2,256 isolates of *Aspergillus* section *Flavi* were examined from a total of 165 ground maize samples obtained in 2005 and 2006 (Table 2.1). Results for 2004 were previously reported (Probst *et al.*, 2007). In total, *A. flavus* made up 98% of *Aspergillus section Flavi* isolates from maize samples that originated in the affected Kenyan districts. On the basis of colony characteristics and sclerotial morphology, 76% of the *A. flavus* isolates from the affected areas belonged to the S strain morphotype and 22% to the L strain morphotype. Incidences of the morphotypes did not differ ($P = 0.05$) among 2004, 2005 and 2006. *A. parasiticus* was only present in 26 samples and made up 2% of the isolates. Other members of *Aspergillus* section *Flavi* made up less than 1% of the total isolates (Table 2.1).

In stark contrast to the Eastern Province, maize samples from the Coast Province (n = 8) mainly contained the L strain of *A. flavus* (88% of *Aspergillus* section *Flavi*) and no S strain isolates were recovered. The remaining 12% of *Aspergillus* section *Flavi* isolates were the atoxigenic species *A. tamarii* (Table 2.1). Maize from the Rift Valley was also predominantly infected by *A. flavus* (mean = 94%) with the L strain dominant (mean = 91%) and with the S strain composing up 13% of the *Aspergillus* section *Flavi* fungi (mean = 3%). Other *Aspergillus* section *Flavi* species were minor components of the examined fungal communities (Table 2.1). *A. parasiticus* was not detected in either the Coast or Rift Valley Provinces (Table 2.1). Compositions of *A. flavus* communities associated with maize from the aflatoxicosis

outbreak region differed significantly ($P < 0.05$) from those associated with both the Rift Valley and Coast Provinces (Table 2.1).

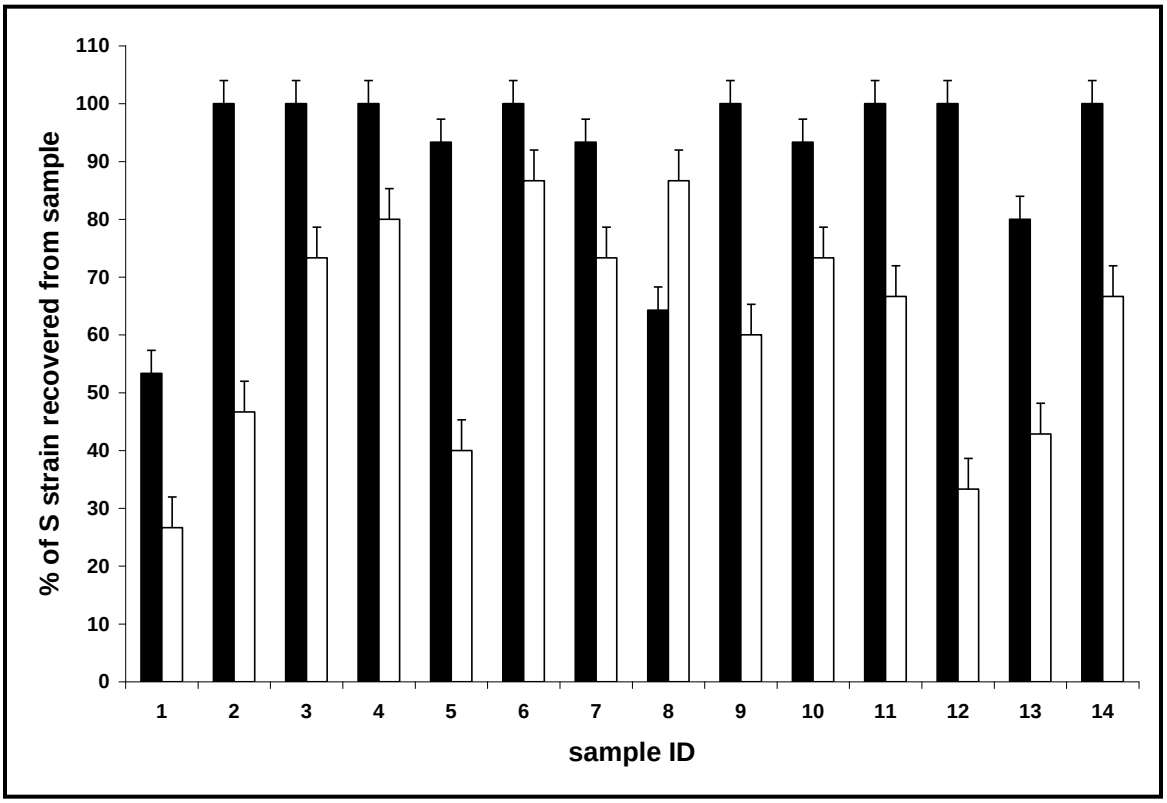
Table 2.1 Incidences of *Aspergillus* sect. *Flavi* species and strains on maize grown in three Provinces of Kenya

Sampling Year	Kenyan Province, District	Aflatoxicosis Outbreaks	Number of samples	Number of isolates	Total aflatoxin (µg kg $^{-1}$)	*A. flavus*		*A. parasiticus*	*A. tamarii*	Other
						S (%)	L (%)	(%)	(%)	(%)
2005	Eastern, Kitui	Yes	39	585	426.3 a	83 a	15 a	2 a	0 a	0 a
2006	Eastern, Kitui	Yes	45	540	219.6 a	75 a	25 a	0 a	0 a	0 a
2006	Eastern, Makueni	Yes	60	791	375.9 a	70 a	25 a	4 a	0 a	1 a
2006	Coast, Taita Taveta	No	2	37	0.1 b	0 b	81 b	0 a	19 a	0 a
2006	Coast, Kwale	No	2	40	120.4 b	0 b	81 b	0 a	19 a	0 a
2006	Coast, Tana River	No	2	32	10.9 b	0 b	90 b	0 a	10 a	0 a
2006	Coast, Kilifi	No	2	30	1.8 b	0 b	100 b	0 a	0 a	0 a
2006	Rift Valley, Marakwet	No	2	32	0 b	13 b	84 b	0 a	0 a	3 a
2006	Rift Valley, Baringo	No	3	47	0 b	2 b	88 b	0 a	2 a	8 a
2006	Rift Valley, Keiyo	No	2	30	13.4 b	0 b	90 b	0 a	0 a	10 a
2006	Rift Valley, Kajiado	No	2	31	6.6 b	3 b	90 b	0 a	7 a	0 a
2006	Rift Valley, Nakuru	No	2	31	5.6 b	0 b	97 b	0 a	3 a	0 a
2006	Rift Valley, Laikipia	No	2	32	3.1 b	0 b	94 b	0 a	3 a	3 a

Aspergillus section Flavi in paired soil and maize samples from small stakeholder farms in Kitui District, Eastern Province

Fifteen *Aspergillus* section *Flavi* isolates were recovered from each maize and soil sample (14 pairs total) from affected households in Kitui district. The only aflatoxin-producing species detected were *A. flavus* and *A. parasiticus*. The latter species was present in only one maize and six soil samples where it composed 0.5% to 13% of the *Aspergillus* section *Flavi*. *A. flavus* was the most common aflatoxin producer in all 28 samples. In maize 91.3% of the *Aspergillus* section *Flavi* isolates belonged to the S strain and 8.3% to the L strain; only 0.5% belonged to *A. parasiticus*. Incidence of the S strain was significantly ($P \leq 0.001$) less (61% versus 91%) in soil than in maize while incidences of both the L strain and *A. parasiticus* were greater (Figure 2.2). Additionally, the non aflatoxin-producing species *A. tamarii* was present in 9% of the soil samples, but was not detected in any maize sample. There were significantly more ($P \leq 0.005$, Student T-test) *Aspergillus* section *Flavi* propagules in maize (mean = 487 CFU g-1) than in the soil (mean = 9.4 CFU g-1).

Figure 2.2 Percent of *Aspergillus* sect. *Flavi* composed of the S strain in maize (black bars) and soil samples (white bars) obtained at 14 locations in Kitui District (Eastern Province, Kenya) in 2005. Error bars indicate standard errors of the mean. The means of the maize and soil samples were significantly different at the $P \leq 0.001$ (paired t-test).



Aflatoxin content in maize

Aflatoxin content in maize, as determined by ELISA, differed significantly ($P \leq 0.01$, Student's T-test) among provinces with the greatest concentrations of aflatoxins found in maize from the Eastern Province (mean = 340 ppb, range of annual means = 219 to 426). The aflatoxin content of maize from the Eastern Province did not differ significantly between 2005 and 2006 (Table 2.1). Only 41% of the Eastern Province samples were below 20 ppb compared to 75% percent of maize from the Coast and 100% of the samples from the Rift Valley (Table 2.1). Only one sample recovered from Kwale district in the Coast Province was highly contaminated with 240 ppb total aflatoxin.

Aflatoxin production in maize kernels

Aflatoxin-producing ability of L strain isolates from three Kenyan Provinces (total of 42 isolates per district) was assessed on maize kernels (Table 2.2). The results obtained from both experiments were consistent with each other. L strain isolates from Eastern Province and Rift Valley Province did not differ significantly. Both aflatoxin-production and sporulation on maize were similar. On the other hand, L strain isolates from the Coast Province consistently produced the lowest concentration of aflatoxin B1 and had the highest incidence of atoxigenic strains (Table 2.2). Isolates from the Coast Province also produced significantly ($P<0.05$) more spores on maize than isolates from the Eastern Province, but differences with isolates from the Rift Valley were only detected in the second experiment.

Aflatoxin B1 production by 22 toxigenic *A. flavus* isolates from the Eastern Province (11 L strain and 11 S strain isolates) was compared (Table 2.3). Isolates were obtained from maize collected in the Eastern province in 2004. Aflatoxin B1 production by L and S strains differed significantly ($P \leq 0.001$). L strain isolates produced 4 ppb to 15 ppm aflatoxin B1. In contrast, S strain isolates produced up to 233 ppm aflatoxin B1 (Table 2.3).

Table 2.2 Comparison of aflatoxin and spore production by *Aspergillus flavus* L strain isolates from three adjacent Provinces in Kenya.

Kenyan Province	Experiment No.	Number of tested L strain isolates	Avg. aflatoxin (ug kg^{-1})	Atoxigenic (%)	Avg. spores ml^{-1}
Eastern	1	28	13200[a]	54	1.2 $X10^{7}$ [a]
Rift Valley	1	28	12000 [a]	61	1.4 $X10^{7}$ [a b]
Coast	1	28	1200 [b]	82	1.5 $X10^{7}$ [b]
Eastern	2	14	27800 [a]	57	1.2 $X10^{7}$ [a]
Rift Valley	2	14	12200 [a]	79	1.3 $X10^{7}$ [a]
Coast	2	14	4800 [b]	93	1.6 $X10^{7}$ [b]

Avg., average value of three repetitions; means followed by the same letter in each column are not significantly different ($P \leq 0.05$) by Tukey's Studentized Range Test. Different isolates were used in the two experiments.

Table 2.3 Aflatoxin B_1 production on maize kernels by *Aspergillus flavus* S and L strain isolates from the Eastern Province of Kenya.

	Average aflatoxin B_1 (µg kg^{-1})	
Isolate #	***A. flavus* L strain**	***A. flavus* S strain**
1	4^{c}	7520^{h}
2	6^{c}	14666^{h}
3	9^{c}	18368^{h}
4	16^{c}	34096^{h}
5	$6261^{b,c}$	$59680^{g,h}$
6	$6411^{b,c}$	$101151^{f,g}$
7	6950^{b}	$115725^{f,g}$
8	7263^{b}	$126047^{e,f}$
9	7361^{ab}	$136896^{e,f}$
10	10946^{a}	$179243^{d,e}$
11	15108^{a}	233029^{d}
Mean	**5485**	**93311**

Values (averages of three replicates) followed by the same letter are not significantly different ($P \leq 0.05$) from each other (Tukey-Kramer HSD test). L and S strain means differ significantly ($P \leq 0.001$, t-test).

Discussion

The lethal aflatoxicosis outbreak in the Eastern Province of Kenya in 2004 resulted in widespread interest within the international food safety community. Although epidemiological explanations for the contamination were discussed (Azziz-Baumgartner *et al.*, 2005; Lewis *et al.*, 2005), efforts to precisely describe the etiologic agent lagged until an association of the S strain *of A. flavus* with the most severely contaminated maize was found (Probst *et al.*, 2007). The precise etiology of aflatoxin-contamination events is difficult to describe because aflatoxin-producing fungi exist in communities composed of individuals that vary widely in both virulence to plants and aflatoxin-producing ability (Cotty, 1989; Brown *et al.*, 1992; Shieh *et al.*, 1997; Cotty *et al.*, 2008). Thus, both the incidence of a particular fungus in the affected crop and the aflatoxin-producing capacity of the fungus must be taken into consideration. The S strain of *A. flavus* was both very common in maize associated with the 2004 epidemic and capable of producing very high concentrations of aflatoxins. Aflatoxin content of the maize was directly correlated with the proportion of the infecting fungi belonging to the S strain (Probst *et al.*, 2007). The current study supports attribution of the S strain as the primary cause of the aflatoxicosis outbreaks in Kenya by describing dominance of the *A. flavus* S strain among fungi infecting maize in regions where aflatoxicosis outbreaks were reported during 2005 and 2006 (Table 2.1). Furthermore maize from neighboring Provinces (Rift Valley and Coast) without histories of lethal aflatoxicosis (Figure 2.1) was shown to have low (Rift Valley Province) to no (Coast Province) incidences of the *A. flavus* S strain. Aflatoxin-production assays confirmed

high aflatoxin-producing potentials of S strain isolates from Kenya. All isolates consistently produced much higher quantities of aflatoxin B1 than L strain isolates (Averages = 93 ppm vs. 548 ppm, Table 2.3). This result is similar to observations from other continents (Saito *et al.*, 1986; Novas and Cabral, 2002). S strain incidence was previously correlated with crop aflatoxin content(Jaime-Garcia and Cotty, 2006a).

To determine if L strain isolates contribute similarly to aflatoxin-producing potential of fungal communities within and adjacent to outbreak areas, we compared aflatoxin-production by L strain isolates from each of the three Kenyan Provinces studied. L strain isolates from the Coast Province produced less aflatoxin than those from either the Eastern or Rift Valley Provinces. Reduced aflatoxin-producing potential in the Coast Province was associated with high frequencies (93%) of atoxigenic isolates. No S strain isolates were found in Coast Province, and the only other member of *Aspergillus* section *Flavi* found was *A. tamarii*, an atoxigenic species. Although L strain isolates did vary in aflatoxin-producing ability among the districts, average aflatoxin production by L strain isolates from all three provinces was consistently below that observed for S strain isolates.

When environmental conditions favor contamination, crops become associated with and infected by complex communities of aflatoxin-producing and closely related fungi (Horn, 2003; Jaime-Garcia and Cotty, 2007; Cotty *et al.*, 2008). Even when only *A. flavus* is present, individual seeds become infected with multiple strains and/or vegetative compatibility groups (VCG) that vary in aflatoxin producing capacity (Novas and Cabral, 2002; Pildain *et al.*, 2004). Atoxigenic strains typically make up

significant percentages (Horn and Dorner, 1999; Vaamonde *et al.*, 2003; Donner *et al.*, 2009) of infecting *A. flavus* communities and greatly modulate the extent to which crops become contaminated (Cotty *et al.*, 2008). Indeed, this is one mechanism through which atoxigenic strain biocontrol agents reduce contamination in treated crops (Bayman and Cotty, 1993; Dorner, 2004). The dominance of the S strain and the paucity of atoxigenic *A. flavus* L strain isolates is the most likely explanation for the very high levels of aflatoxin seen in the affected districts of the Eastern Province.

Factors that lead to dominance of the S strain in this area remain unclear. Cultural practices during cultivation, harvest and storage and/or climatic factors may support this dominance but roles of specific factors need to be investigated.

In addition to aflatoxin, several other highly toxic compounds are known to be concentrated within sclerotia of *Aspergillus* (Wicklow and Cole, 1982). Isolates of the S strain produce greater quantities of sclerotia than other *A. flavus* (Jaime-Garcia and Cotty, 2006a), and S strain sclerotia may form both on crop surfaces and within developing seeds. Sclerotia, particularly those formed within crop tissues, might not be readily evident during hand sorting. Furthermore, during milling, the tiny S strain sclerotia would be cryptically incorporated into the flour. As such, sclerotial production by S strain isolates might contribute toxicity beyond that expected from aflatoxins alone.

Currently management is directed at cultural practices (e.g. harvest procedures, irrigation, and storage) and development of resistant cultivars (Brown *et al.*, 1993; Bruns, 2003; Turner *et al.*, 2005). The identification of the causal fungi may be an

initial step in interrupting the aflatoxin contamination processes in Kenya. The S strain of *A. flavus* is ecologically and physiologically different from other aflatoxin producers (Cotty and Mellon, 2006) and responds to crop rotations and seasons differently than the L strain isolates (Bock *et al.*, 2004; Jaime-Garcia and Cotty, 2006a). Thus, the S strain life cycle should be taken into consideration when designing interventions. Furthermore, it is not clear that cultivars respond similarly to L and S strain isolates. When screening cultivars for reduced susceptibility to contamination, incorporating the actual causal agent into screens would be the wisest course. Alternative methods of management should also be considered including use of atoxigenic strains of *A. flavus* as biocontrol agents.

The aflatoxin-producing potential of fungal communities can be reduced through application of native atoxigenic strains of *A. flavus* (Brown *et al.*, 1991; Cotty and Bayman, 1993; Dorner, 2004). Two atoxigenic strains are registered for aflatoxin management in the U.S. (Dorner, 2004; Ehrlich and Cotty, 2004) and certain atoxigenic strains are known to be particularly effective against the S strain (Garber and Cotty, 1997; Cotty and Antilla, 2003). Atoxigenic strain applications shift fungal community composition towards dominance of atoxigenic fungi and, as a direct consequence, reduce the aflatoxin content of infected crops (Cotty and Bayman, 1993; Dorner *et al.*, 1999; Cotty *et al.*, 2008). Implementation of biocontrol techniques for West Africa are currently under development (Atehnkeng *et al.*, 2008b; Cotty *et al.*, 2008).

Aflatoxin contamination of maize in Coast Province was detected in the current study at levels considered to be unsafe for human consumption. Thus, the current results indicate that the environment of Coast Province is sufficient to support contamination of maize to unsafe levels by fungal communities lacking the S strain and with relatively low aflatoxin-producing potentials. Establishment of the S strain in maize producing areas of the Coast Province could be expected to result in increased incidences and severities of contamination.

L strain isolates from the Coast Province produce more spores on maize kernels than L strain isolates from districts affected by acute aflatoxicosis. High sporulating isolates from the Coast Province may be well suited to competitively exclude the S strain during maize production and, as such, atoxigenic isolates among these may be good candidates for biocontrol agents directed at preventing future episodes of lethal aflatoxicosis in the Eastern Province through competitive exclusion of the S strain.

In West Africa an unnamed taxon (frequently called strain S_{BG}) absent from North America but morphologically similar to the S strain of *A. flavus* is common (Cotty and Cardwell, 1999). DNA based phylogenies indicate strain S_{BG} is a distinct species that groups outside both *A. flavus* and *A. parasiticus* (Egel *et al.*, 1994; Ehrlich *et al.*, 2005b). Morphological similarities between the S strain and strain S_{BG} makes differentiation based on macroscopic or microscopic characteristics intractable. However, the *A. flavus* S strain can be readily separated from strain S_{BG} by aflatoxin production. All strains of *A. flavus* produce only B aflatoxins as a result of a 0.8 to 1.5 kb deletion in the 28 gene aflatoxin biosynthesis cluster (Ehrlich *et al.*, 2004). In

contrast, strain S_{BG} produces both B and G aflatoxins. Both strain S_{BG} and *A. flavus* are common within communities of aflatoxin-producing fungi in West Africa. However, all *A. flavus* isolates from West Africa belong to the L strain morphotype (Cotty and Cardwell, 1999; Cardwell and Cotty, 2002; Atehnkeng *et al.*, 2008a). Indeed, the *A. flavus* S strain had not been detected in Africa prior to the initial report on maize produced in the Eastern Province of Kenya during 2004 (Probst *et al.*, 2007). Our current findings support these observations. Strain S_{BG} was not isolated from any maize or soil samples collected from the Eastern Province in Kenya in 2005 or 2006. *A. flavus* was the dominant species throughout Kenya with the S strain dominant in the Eastern Province and the L strain dominant in the Coast and Rift Valley Provinces.

In the Eastern Province the S strain was a more important component of the *A. flavus* community infecting maize than the A. flavus community resident in the soil in which the maize was produced (91% of *A. flavus* in maize was S strain whereas 61% was S strain in soil). This is surprising because the S strain produces relatively few spores compared to the L strain during maize infection. Apparently, in the Eastern Province, there are factors that favor S strain movement to maize from soil and subsequent colonization and infection.

It is not clear from where the *A. flavus* S strain isolates originated. The possibility that they were introduced into Kenya, as was maize, from the Americas should be investigated. It is possible that when maize was distributed over the world, the S strain was also inadvertently introduced in several regions. Similar introductions of pests of maize in Kenya have been reported in the literature. For example, the larger

grainborer *Prostephanus truncates* (Horn) (Coleoptera: Bostrichidae) was introduced from Meso-America into East Africa in the early 1980s, and has been reported from maize deficit areas in the dry mid-altitudes of Kenya since the early 1990s (Hodges *et al.*, 1983; Hodges *et al.*, 1996).

Incidences of the *A. flavus* S strain remained high from 2004 through 2006 in the Eastern Province, a period during which outbreaks of acute aflatoxicosis recurred leading hundreds of deaths. Intervention is urgently needed.

CHAPTER 3- IDENTIFICATION OF ATOXIGENIC *ASPERGILLUS FLAVUS* ISOLATES TO REDUCE AFLATOXIN CONTAMINATION OF MAIZE IN EASTERN KENYA

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Abstract

Aspergillus flavus has two morphotypes, the S strain and the L strain, that differ in aflatoxin-producing ability and other characteristics. Fungal communities on maize dominated by the S strain of *A. flavus* have repeatedly been associated with acute aflatoxin poisonings in Kenya where management tools to reduce aflatoxin levels in maize are needed urgently. *A. flavus* isolates (n = 290) originating from maize produced in Kenya and belonging to the L strain morphotype were tested for aflatoxin-producing potential. A total of 96 atoxigenic isolates was identified from four provinces sampled. The 96 atoxigenic isolates were placed into 53 vegetative compatibility groups (VCGs) through complementation of nitrate non-utilizing mutants. Isolates from each of 11 VCGs were obtained from more than one maize sample; isolates from 10 of the VCGs were detected in multiple districts; and isolates of four VCGs were found in multiple provinces. Atoxigenic isolates were tested for potential to reduce aflatoxin concentrations in viable maize kernels that were co-inoculated with highly toxigenic S strain isolates. The 12 most effective isolates reduced aflatoxin levels by >80%. Reductions in aflatoxin levels caused by the most

effective Kenyan isolates were comparable to those achieved with a United States isolate (NRRL-21882) used commercially for aflatoxin management. This study identified atoxigenic isolates of *A. flavus* with potential value for biological control within highly toxic *Aspergillus* communities associated with maize production in Kenya. These atoxigenic isolates have potential value in mitigating aflatoxin outbreaks in Kenya, and should be evaluated under field conditions.

Introduction

Aflatoxins are a series of highly toxic polyketides produced by several species of *Aspergillus* (Klich and Pitt, 1988; Sweeney and Dobson, 1998). The most commonly occurring aflatoxin, aflatoxin B_1, is a genotoxin known to be carcinogenic and teratogenic for both humans and animals (Wang and Tang, 2004; McKean *et al.*, 2006) and, to date, the only mycotoxin classified as a Group 1a human carcinogen by the International Agency for Research on Cancer (International Agency for Research on Cancer, 2002). Crops infected by aflatoxin-producing fungi frequently become contaminated with aflatoxins. Aflatoxin contamination results in reduced crop value, and diminished health of humans and domestic animals that consume the contaminated crops (Wu and Khlangwiset, 2010). The quantity of ingested aflatoxins determines if health effects are chronic (e.g., immune suppression, impaired child growth, abnormal fetal development, and cancer) or acute (e.g., hepatitis and jaundice, abdominal swellings, and death) (Gong *et al.*, 2004; Williams *et al.*, 2004; Egal *et al.*, 2005; Cardwell and Henry, 2006). To date, Kenya is the only nation with an extant population that has repeatedly experienced epidemics of acute aflatoxicosis (Ngindu *et al.*, 1982; Centers for Disease Control and Prevention, 2004; Muthomi *et al.*, 2009). These episodes resulted from consumption of highly contaminated, homegrown maize and have extended over two decades with the most recent occurring from 2004 through 2006 when several hundred Kenyans died from acute aflatoxin poisoning in several districts of the Eastern Province (Centers for Disease Control and Prevention, 2004). During these periods, many thousands of individuals were exposed to unsafe

aflatoxin levels (Muture and Ogana, 2005; Probst *et al.*, 2007). In 2010, another extensive epidemic occurred in Kenya with high frequencies of harvested maize lots containing levels of aflatoxins unfit for human consumption (Integrated Regional Information Networks, 2010).

Aspergillus flavus is the most frequently implicated causal agent of aflatoxin contamination of maize (Klich, 2007). This species has several morphotypes (commonly called strains), among which the L and S strains are most studied. These strains differ in several characteristics including production of sclerotia, conidia, and aflatoxins (Cotty, 1989). L strain isolates produce few, large sclerotia (average $\geq$ 400 µm) and highly variable quantities of aflatoxins, with some isolates (called atoxigenic isolates) entirely lacking the ability to produce aflatoxins. In contrast, S strain isolates produce numerous, small sclerotia (average $\leq$ 400 µm) and, on average, higher levels of aflatoxins than L strain isolates (Cotty, 1989). Each of the morphotypes is further subdivided into many vegetative compatibility groups (VCGs) delineated by a heterokaryon incompatibility system (Papa, 1986). There is also variability among VCGs in aflatoxin-producing ability. Thus, *A. flavus* exists in complex communities that vary widely in both strain/VCG composition and aflatoxin-producing ability. Fungal communities in Kenya associated with severe maize contamination and deaths in human populations have atypical structures, with the S strain of *A. flavus* highly dominant, and increasing incidence of the S strain associated with increasing contamination levels (Probst *et al.*, 2007; Probst *et al.*, 2010).

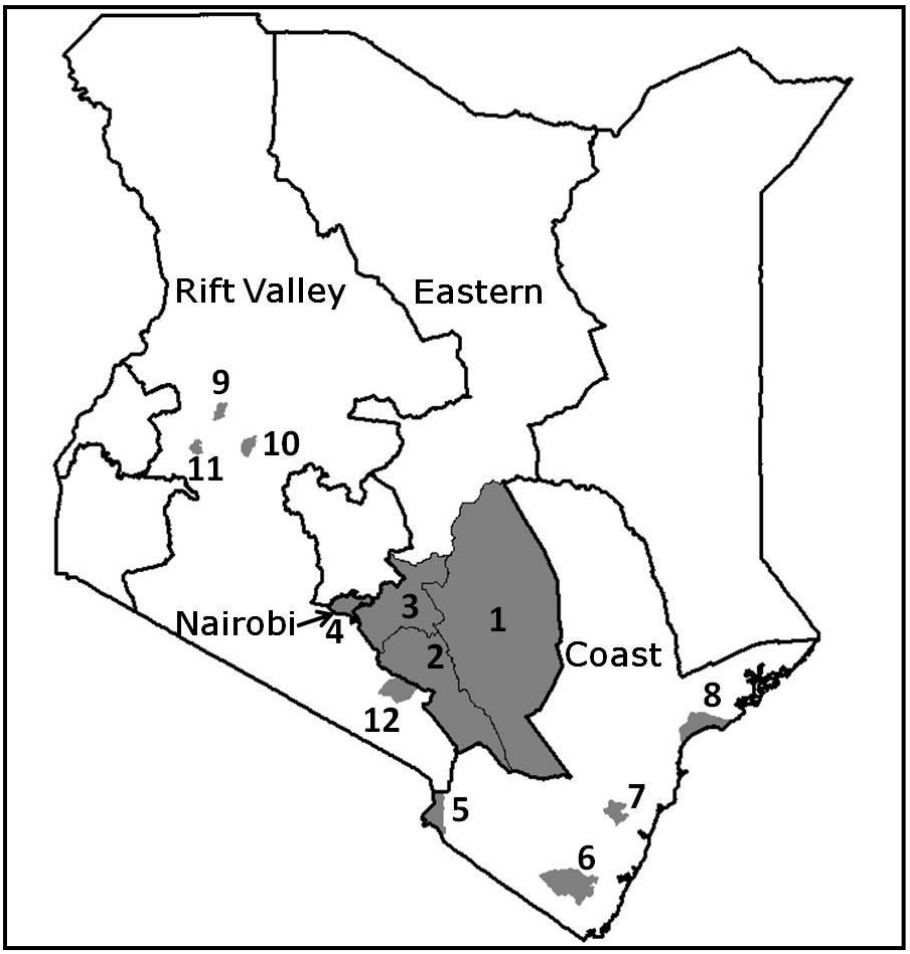
The influence of aflatoxins on human populations in Kenya over the past decade demonstrates a clear need for tools to manage contamination of locally produced maize. A highly promising method for aflatoxin management has been the use of atoxigenic isolates of *A. flavus* to competitively exclude aflatoxin producers and, thereby, reduce aflatoxin concentration in a crop (Dorner, 2004; Cotty and Mellon, 2006; Cotty *et al.*, 2007). Two atoxigenic isolates used commercially in the US are very effective at inhibiting aflatoxin contamination by the S strain of *A. flavus* (Garber and Cotty, 1997). Identification of atoxigenic isolates of *A. flavus* native to Kenya might provide an environmentally sound, ecologically adapted, native, biological resource useful in mitigating aflatoxin contamination of maize produced in Kenya. This study sought to determine whether atoxigenic isolates of *A. flavus* with potential value in biological control could be selected from highly toxic fungal communities in aflatoxin-contaminated maize produced in Kenya that had been associated with lethal aflatoxicosis.

Material and methods

Fungal isolation, maize samples, and fungal inoculum preparation

Isolates of *A. flavus* were collected during previous studies (Probst *et al.*, 2007; Probst *et al.*, 2010). Briefly, samples of maize kernels (n = 263) were collected from farmers in 12 districts in four provinces (Eastern, Coast, Rift Valley, and Nairobi) during 2004, 2005, and 2006 (Figure 3.1). Mean sample weight was 290 g (range of 111 to 430 g/ sample). The maize was finely ground in a laboratory hammer mill (IKA Labortechnik, Heitersheim, Germany), and fungal isolates were recovered by dilution plate technique on modified rose Bengal agar (M-RBA), as described previously (Cotty, 1994a). After cultivation at 31˚C for 5 days on 5/2-agar (5% V-8 vegetable juice, 2% agar, pH 5.2), *Aspergillus* section *Flavi* isolates were identified to species (Kurtzman *et al.*, 1987; Klich and Pitt, 1988) and strain based on macroscopic and microscopic characteristics, and stored in sterile water (four to six colonized, 3 mm diameter agar plugs added to 2.5 ml sterile water) at 8˚C for the working cultures and on silica gel at 8˚C for long-term storage. To produce fungal inoculum, each L strain isolate was grown on 5/2-agar for 5 days at 31˚C in the dark. Conidia were transferred to glass vials containing 20 ml sterilized, deionized water using cotton swabs. Conidial concentrations were measured with a turbidity meter (Model 965-10; Orbeco-Hillige, Farmingdale, NY), calculated using the nephelometric turbidity unit (NTU) vs. colony forming unit (CFU) curve: Y = 49,937X, where X = NTU and Y = conidia/ ml. The conidial concentration for each isolate was adjusted to 10^6 conidia/ml.

Figure 3.1 Sites in four provinces and 12 districts of Kenya from which maize samples were collected and isolates of *Aspergillus flavus* obtained. Maize samples from Eastern and Nairobi Provinces were associated with acute aflatoxicoses outbreaks in 2004 to 2006. Maize samples from the Rift Valley and Coast Provinces were collected in 2006 to evaluate *Aspergillus* communities in maize growing areas adjacent to the aflatoxicosis outbreak regions. Eastern Province: District 1 = Kitui, 2 = Makueni, and 3 = Machakos; Nairobi Province: District 4 = Nairobi; Coast Province: District 5 = Taita Taveta , 6 = Kwale , 7 = Kilifi; and 8 = Tana River; Rift Valley Province: District 9 = Marakwet; 10 = Baringo, 11 = Uasin Gishu, and 12 = Kajiado.



Identification of atoxigenic isolates associated with maize produced in Kenya

Aflatoxin-producing ability of each of the *A. flavus* L strain isolates was determined using autoclaved maize kernels (10 g/ 250 ml Erlenmeyer flask). Maize cultivar 33F88 (Pioneer Hi-Bred International Inc., Johnston, IA) was used for all experiments. Each flask was sealed with a BugStopper (Whatman, Piscataway, NJ) and autoclaved at 121˚C for 60 min. After autoclaving, maize moisture level was quantified with a HB43 Halogen Moisture Analyzer (Mettler Toledo, Columbus, OH) and adjusted to 25%. Each autoclaved maize sample was then inoculated with 1 ml of spore suspension (10^6 conidia/ml water) of the appropriate isolate, and incubated for 7 days at 31˚C in the dark. After incubation, the maize cultures of *A. flavus* were processed as described previously (Cotty, 1997; Probst *et al.*, 2010). Briefly, the colonized maize for each sample was blended in 80% methanol (50 ml) until evenly homogenized, and the blended mixture was filtered through Whatman #4 filter paper. Culture filtrates were spotted directly onto thin-layer chromatography (TLC) plates (Silica gel 60; EMD, Darmstadt, Germany) adjacent to aflatoxin standards (Aflatoxin Mix kit-M; Supelco, Bellefonte, PA). The plates were developed in ethyl ether:methanol:water (96:3:1), air-dried, and the aflatoxins visualized under 365 nm UV light. Aflatoxins were quantified directly on TLC plates with a scanning densitometer (TLC Scanner 3, Camag Scientific Inc, Wilmington, NC). Filtrates that initially tested negative for aflatoxins were combined with 50 ml water and extracted twice serially with methylene chloride (25 ml). Methylene chloride extracts were passed through a layer of anhydrous $NaSO_4$, evaporated to dryness, and dissolved in an appropriate volume of methylene chloride

for accurate densitometry. Aflatoxins were then separated on TLC plates and quantified as described above. Isolates that produced levels of aflatoxins below the limit of detection (0.5 ng aflatoxin B_1/ g) were considered atoxigenic and evaluated further as potential biocontrol agents. Each L strain isolate of *A. flavus* was tested for atoxigenicity at least three times.

Aflatoxin production during co-infection of viable maize kernels with toxigenic and atoxigenic strains of A. flavus

Each atoxigenic L strain isolate of *A. flavus* (n = 96) was initially evaluated on viable maize kernels for ability to interfere with aflatoxin production by a highly toxigenic *A. flavus* S strain isolate (FGSC A1169). Isolate FGSC A1169 was previously obtained from a highly contaminated maize sample associated with the 2004 outbreak of acute aflatoxicosis in Kenya (Probst *et al.*, 2007). Prior to inoculation, undamaged maize kernels of Pioneer 33F88 were surface-sterilized by submerging the kernels in hot water for 45 s at 80˚C (Mehl and Cotty, 2010), air dried for approximately 1 min on an aseptic surface in a biological safety cabinet, and distributed into sterilized glass flasks (10 g maize/ 250 ml flask). Flasks were plugged with BugStoppers to prevent humidity loss and allow gas exchange. Moisture content of the surface-sterilized maize kernels was determined as described above. Efficiency of surface-sterilization and ability of kernels to germinate were monitored by plating three randomly selected kernels from each flask onto a selective agar medium (M-RBA, Cotty 1994a), followed by incubation at 31˚C in the dark for 14 days. Greater

than 90% of the kernels germinated and fungal contaminants were not observed at the end of the incubation period. Equal inocula (2 x 10^5 conidia/isolate/flask) of S strain (aflatoxin-producing) and atoxigenic L strain isolates were mixed and added to the flasks (0.75 to 0.80 ml/flask dependent on kernel moisture content), which were then gently agitated to coat the kernels with the inoculum. Kernel moisture after inoculation was 25%. Each *A. flavus* L strain isolate was tested using four replicates. Inoculated maize was incubated at 31˚C for 7 days in the dark. The experiment was then terminated by addition of 50 ml 70% methanol, and aflatoxins were extracted following the protocol outlined above. Inhibition of aflatoxin contamination in these co-inoculation experiments was calculated as a percent of the aflatoxin content in kernels inoculated with only the aflatoxin producing isolate (FGSC A1169).

Initial evaluation of the 96 isolates was a screen to select isolates for further testing. Thus, the ability of each of the 96 atoxigenic L strain isolates to interfere with aflatoxin production by FGSC A1169 was tested in at least a single comparison of 15 to 20 isolates at a time. Some isolates were included in up to five comparisons. Each completely randomized experiment was replicated three times. Isolates (n = 13) that either performed well (> 80% less aflatoxin B_1 contamination than the control samples) in the initial screen, or that were associated with > 50% less aflatoxin contamination and were members of VCGs isolated from two or more locations in Kenya (n = 10), were evaluated further. The thresholds of 80 and 50% less aflatoxin contamination, respectively, were selected on an arbitrary basis to reduce the number of isolates for further evaluation in the preliminary experiments (Table 3.1).

Five *A. flavus* L strain isolates (C6-E, C8-F, E62-L, E63-I, and R1-N) most effective at reducing aflatoxin contamination of maize kernels in the preliminary experiments (Table 1) were tested further for consistency in efficacy against two S strain isolates, FGSC A1169 and ATCC MYA-384. This was done using the assay described above in two additional experiments; one with each S strain isolate, and each experiment included four replicates per treatment (Table 3.2). In these experiments, comparisons were made with NRRL-21882, the atoxigenic isolate that is the active ingredient in AflaGuard (Syngenta, Wilmington, DE), a biocontrol product currently registered for management of aflatoxins in maize in the United States.

Vegetative compatibility group (VCG) analysis

All 96 atoxigenic L strain isolates of *A. flavus* were subjected to VCG analyses in order to assess diversity among the atoxigenic isolates, and to determine which VCGs are most common in the Kenyan maize samples evaluated and, thus, potentially well adapted to maize cultivation in Kenya. In summary, mutations in any one of several genes in the nitrate-reductase pathway can produce nitrate non-utilizing mutants (*nit*$^-$). Mutants affected in different genes can complement each other when paired on starch complementation medium at 31˚C, and restore wildtype growth. In this study, *nit*$^-$ mutants were generated for each atoxigenic isolate on M-RBA medium containing chlorate, as described previously (Bayman and Cotty, 1993; Cotty, 1994b). Complementary tester mutants (*cnx*$^-$ and *niaD*$^-$) were developed (Bayman and Cotty, 1993), and the ability of a *nit*$^-$ mutant from each isolate to complement one or both

tester mutants was evaluated. Isolates yielding *nit*$^{-}$ mutants that did not complement one of the tester mutants within 10 days were considered to belong to a VCG other than the one defined by the tester pair, while those complementing one of the tester mutants were considered to belong to the same VCG as the tester pair.

Statistical analyses

Randomized complete block designs with three to four replicates were used in all experiments. Aflatoxin concentration was log transformed and subjected to analysis of variance (ANOVA). ANOVA was performed with the general linear model (GLM) procedure of SAS Version 9.2 (SAS Institute, Cary, NC). Mean separations were performed on data from experiments with statistically significant ($P \leq 0.05$) differences, using Tukey's Honestly Significant Difference (HSD) test . Mean differences in aflatoxin levels (% difference between inoculated maize and control maize treatments) were calculated as: [1 - (total aflatoxin content in maize co-inoculated with both toxigenic and atoxigenic isolates of *A. flavus*/ total aflatoxin content in maize inoculated with the S strain isolate alone)] * 100. Standard deviations of mean differences in aflatoxin levels were calculated as a measure of variability in efficacy. All analyses and calculations were performed with SAS.

Results

Identification of atoxigenic isolates of A. flavus

More than 3,400 *A. flavus* isolates were recovered from 263 ground maize samples collected in 2004 to 2006 from four provinces of Kenya. Detailed descriptions of the samples and general characteristics of the fungal isolates have been published (Probst *et al.*, 2007; Probst *et al.*, 2010). The *A. flavus* morphotypes were very unevenly distributed among provinces, with the L strain composing 27% of the *A. flavus* isolates from the Eastern Province compared to 88% of the isolates from the Rift Valley Province and 91% of the isolates from the Coast Province (Probst *et al.*, 2010). Greater numbers of isolates were obtained from the Eastern Province because of the long history of lethal aflatoxicosis in that province (Probst *et al.*, 2010) and the resulting urgent need for aflatoxin management options in that region. Of the 290 L strain isolates screened for aflatoxin-producing ability on autoclaved maize (222 from the Eastern Province, 32 from the Coast Province, and 36 from the Rift Valley Province), 96 were identified as atoxigenic based on a detection limit of 0.5 µg total aflatoxins/kg maize (data not shown). Aflatoxin B_1 production levels of the remaining 194 isolates ranged from 0.5 to > 400,000 µg aflatoxin B_1/ kg maize (ppb)**.**

Co-infection of viable maize kernels with toxigenic and atoxigenic isolates of A. flavus

All 96 atoxigenic *A. flavus* L strain isolates were associated with lower maize aflatoxin content when co-inoculated with a highly toxigenic *A. flavus* S strain isolate (FGSC A1169), compared to maize kernels inoculated with the S strain isolate alone (data not shown). Average reductions in aflatoxin B_1 concentration ranging from 7.1 ± 4.4% to 98.3 ± 8.0% (mean of 59.2 ± 22.9% for all 96 isolates) were observed in co-inoculated maize kernels compared with maize kernels inoculated with FGSC A1169 alone. Twenty-three isolates from the initial screens were chosen for further tests. Aflatoxin B_1 levels in co-inoculation experiments conducted with the 23 selected isolates were significantly lower in both experiments. Average aflatoxin B_1 levels were 63.6 to 89.6% less in co-inoculated treatments compared to the control treatments with an S strain alone (mean of 79.3% reduction for all 23 isolates; Table 3.1).

Co-inoculation of maize kernels with each of the five Kenyan atoxigenic L strain isolates selected for further evaluation with the atoxigenic isolate NRRL-21882, demonstrated significantly lower aflatoxin levels than in kernels inoculated with an S strain isolate alone (Table 3.2). The five isolates from Kenya performed similarly during the additional evaluations as in the preliminary experiments, causing an average of 87.4 ± 2.4% less aflatoxin in the preliminary tests (Table 3.1) and 84.4 ± 1.5% less aflatoxin in the additional evaluations (Table 3.2). All atoxigenic isolates were

statistically similar in ability to interfere with aflatoxin production in viable maize kernels regardless of which S strain isolate was used in the experiment (Table 3.2).

VCG analysis

All 96 atoxigenic *A. flavus* isolates obtained from maize produced in Kenya were successfully placed into a VCG by complementation of *nit*$^{-}$ auxotrophs. A total of 53 VCGs was detected. However, 41 of the VCGs were each represented by a single atoxigenic isolate. Fifty percent of the atoxigenic isolates belonged to one of 12 multi-member VCGs. Seven of the multi-member VCGs were detected in the Eastern Province, seven in the Coast Province, and four in the Rift Valley Province. Isolates in four of the VCGs were detected in two provinces, and one isolate (in VCG KN008) was detected in six districts, three provinces, and seven maize samples (Table 3.3). These included four districts that are targets for aflatoxin management because of recent health or economic impacts from aflatoxin contamination of maize: Kitui, Machakos, Makueni, and Tana River.

Table 3.1 Influence of co-inoculation with atoxigenic L strain isolates of *Aspergillus flavus* from four provinces in Kenya on the aflatoxin content of viable maize kernels infected by an aflatoxin-producing isolate of the S strain morphotype of *A. flavus*

	Isolate [u]	VCG[v]	Aflatoxin B1 (µg/g)		Avg. difference (mean ± stdev %)[y]
			Exp. 1	Exp. 2	
Eastern	E971-E		199 a [w]	253 a	
Coast	C6-E	KN00A	21 bc	41 de	86.6 ± **4.0**
	C2-J	KN00B	19 c	49 bcde	85.5 ± **7.0**
	C8-F	KN012	28 bc	37 de	85.7 ± **0.5**
	C5-K	KN006	28 bc	52 bcde	82.7 ± **4.5**
	C3-G	KN015	23 bc	66 bcd	81.2 ± **10.2**
Eastern	E63-I	KN001	17 c	31 e	89.6 ± **2.8**
	E62-L	KN00C	18 c	37 de	88.2 ± **4.0**
	E266-D	KN004	nd[x]	48 de	81.0 ± **5.3**
	E836-A	KN00D	39 bc	54 bcde	79.5 ± **1.5**
	E138-A	KN015	49 abc	53 bcde	77.2 ± **2.8**
	E987-H	KN00E	38 bc	72 bcd	76.2 ± **6.8**
	E103-G	KN00F	54 abc	53 bcde	76.0 ± **4.4**
	E54-I	KN002	50 abc	62 bcde	75.2 ± **0.5**
	E916-O	KN008	57 abc	81 bcd	69.7 ± **2.3**
	E804-C	KN00G	nd	92 b	63.6 ± **18.0**
Rift Valley	R1-N	KN00H	21 bc	40 de	86.8 ± **3.8**
	R7-K	KN012	nd	39 de	84.6 ± **7.0**
	R8-F	KN00I	nd	44 cde	82.6 ± **11.5**
	R1-J	KN00J	31 bc	62 bcde	80.0 ± **6.3**
	R7-H	KN011	50 abc	55 bcde	76.6 ± **2.7**
	R5-R	KN00K	51 abc	55 bcde	76.3 ± **2.7**
	R8-P	KN007	nd	69 bcd	72.7 ± **25.5**
	R8-C	KN007	94 ab	64 bcde	63.7 ± **15.4**

[u] All isolates originated from maize samples collected in Kenya from 2004 to 2006. Isolates did not produce aflatoxins except for isolate E971-E (FGSC A1169) which produced >100 ng aflatoxin B_1/g maize and belongs to the S strain morphotype of *A. flavus*. The results for E971-E indicate the aflatoxin value for kernels inoculated with that isolate alone. Other isolates were co-inoculated with E971-E to assess capacity to interfere with aflatoxin contamination during co-infection.

[v] VCG = Vegetative Compatibility Group. VCGs with a letter (A to K) indicate isolates that belonged to a single-member VCG (VCGs for which only one isolate was detected). VCGs with a number (KN001 to KN0015) indicate isolates that belonged to multiple-member VCGs (VCGs for which more than one isolate was detected).

[w] Isolates with mean aflatoxin B_1 values followed by some of the same letters within a column do not differ significantly according to Tukey's Honestly Significant Difference test ($P \leq 0.05$) (56).

[x] nd = no data available. Isolate was not included in Experiment 1.

[y] Average difference in aflatoxin levels (%) = [1- (total aflatoxin content in maize co-inoculated with both toxigenic and atoxigenic isolates of *A. flavus*/total aflatoxin content in maize inoculated with only isolate E971-E)] *100. Standard deviations for isolates included in both tests were calculated based on the difference in aflatoxin levels (%) obtained in the two experiments. Standard deviations for the five isolates that were only included in Experiment 2 (E266-D, E804-C, R7-K, R8-F, and R8-P) were calculated based on differences in aflatoxin levels (%) for the four replicates of each treatment.

Table 3.2 Aflatoxin B_1 levels produced following co-infection of viable maize kernels by atoxigenic L strain isolates of *Aspergillus flavus* that originated from Kenya or the US (NRRL-218820), and two highly toxigenic S strain isolates from Kenya.

Isolate co-inoculated	**Aflatoxin B_1 (µg/g)**				**Average difference (mean % ± stdev)[z]**	
	Experiment 1		**Experiment 2**			
None[x]	105.4	a[y]	109.2	a		
62-L	8.4	b	19.0	b	87.3	**± 6.7**
C6-E	8.8	b	20.6	b	86.5	**± 7.4**
NRRL-21882	9.3	b	23.1	b	85.1	**± 8.7**
R1-N	11.5	b	25.0	b	83.1	**± 8.4**
63-I	12.4	b	25.3	b	82.6	**± 8.1**
C8-F	12.7	b	26.8	b	81.7	**± 8.8**

[x] Two S strain isolates of *A. flavus* were used. Experiment 1: Isolate FGSC A1169, Experiment 2: Isolate ATCC MYA-384. The corresponding aflatoxin concentration was obtained from maize kernels inoculated with the S strain isolate alone.

[y] Mean values followed by a common letter within a column do not differ significantly according to Tukey's Honestly Significant Difference test ($P \leq 0.05$) (56).

[z] Average difference in aflatoxin concentrations (%) = [1 - (total aflatoxin content in maize co-inoculated with both toxigenic and atoxigenic isolates of *A. flavus*/ total aflatoxin content in maize inoculated with the S strain isolate alone)] * 100.

Table 3.3 Occurrence of single and multiple member vegetative compatibility groups (VCGs) of *Aspergillus flavus* isolates obtained from maize samples collected from four maize growing provinces in Kenya in 2004 to 2006

		Vegetative Compatibility Group					
Province	**District**[w]	**KN001**	**KN002**	**KN004**	**KN005**	**KN006**	**KN007**
Eastern	Kitui	+[z]	+	+	+		
	Machakos				+		
	Makueni	+	+	+			
Nairobi	Nairobi			+			
Coast	Taita Taveta					+	
	Kwale						
	Tana River					+	
	Kilifi						
Rift Valley	Marakwet						+
	Baringo						+
	Kajiado						+
	Uasin Gishu						+
No. of samples[x]		**2**	**2**	**4**	**2**	**3**	**4**
No. of atoxigenic isolates[y]		**3**	**5**	**4**	**2**	**3**	**11**

Table 3.3 - *continued*

Vegetative Compatibility Group						
KN008	**KN010**	**KN011**	**KN012**	**KN013**	**KN015**	**single**
+					+	+
+						+
+						+
						+
	+	+		+		+
					+	+
+		+				+
+			+			+
			+			+
						+
+						+
		+	+			+
7	**1**	**3**	**5**	**2**	**3**	**33**
7	**2**	**4**	**10**	**2**	**3**	**41**

[w] District within the province. See Figure 3.1 for a map of the district and province locations.

[x] Number of maize samples from which the indicated VCG of *A. flavus* was isolated. Total number of samples was 156.

[y] Number of atoxigenic *A. flavus* isolates detected in the indicated VCG.

[z] Presence (+) of isolates of specific multiple-member VCGs (KN001 to KN015, VCGs for which more than one isolate was detected) and single-member VCGs (41 VCGs for which only a single isolate was detected) in each district.

Discussion

Aflatoxins impact both the safety and value of foods and feeds and, as such, management of aflatoxin contamination of food and feed is required both to capture optimal markets and to maintain food security (Wu and Khlangwiset, 2010). In developing nations, economic incentives for management of aflatoxin contamination are typically reduced due to less stringent regulatory oversight (Wu and Khlangwiset, 2010). However, in recent years public awareness campaigns, news reports of human health effects, and episodes of crop destruction have greatly increased incentives for aflatoxin management even in very low income regions (Egal *et al.*, 2005; Integrated Regional Information Networks, 2010). Nowhere have aflatoxins been more in the public eye than in Kenya, where severe aflatoxin contamination of maize has caused human deaths repeatedly over the past three decades (Ngindu *et al.*, 1982; Centers for Disease Control and Prevention, 2004). In Kenya, crop destruction is a devastating aflatoxin management option. Crops may be a farmers' only possession of cash value, and crop destruction can result in impoverishment and malnourishment. Farmers in food-scarce regions are in urgent need of affordable management strategies to protect consumers from the harmful effects of aflatoxins, and to optimize crop value.

Development of technologies to prevent or reduce aflatoxin contamination of maize has been a complex and frustrating goal for over three decades. Proper drying and storage of grains like maize can prevent postharvest increases in aflatoxin content (Wyllie, 1990; Turner *et al.*, 2005), but driers and storage facilities are not available to

all growers, and timely harvest and drying of grain can sometimes be prevented by adverse weather conditions that promote contamination of harvested grains. Furthermore, significant quantities of aflatoxins can develop in grain prior to harvest. Cultivars resistant to aflatoxin contamination by *A. flavus* have been sought for at least 30 years (Widstrom *et al.*, 1984; Mehan *et al.*, 1988; Brown *et al.*, 2001; Henry *et al.*, 2009). However, commercially available maize cultivars labeled as aflatoxin resistant still are not available in the United States, where such cultivars have been sought intensively (De Leon *et al.*, 1995; Guo *et al.*, 2001; Henry *et al.*, 2009). A biological control strategy that utilizes naturally occurring, atoxigenic isolates of *A. flavus* to competitively exclude aflatoxin producers was originally a controversial proposal (Cotty, 1992; Kilman, 1993) thought to lack practical value. However, this strategy has received farmer acceptance and support in the United States (Smith, 2009; Smith, 2010), and currently provides the only preharvest management tool with documented efficacy in commercial agriculture (Cotty and Antilla, 2003; Abbas *et al.*, 2006; Atehnkeng *et al.*, 2008b; Dorner, 2009). In North America, this biological control strategy is currently the basis for the only aflatoxin management products registered for commercial preharvest use on any crop (Jones, 2003; Dorner, 2004). Although commercial use of atoxigenic *A. flavus* began in the United States (Cotty, 1992), developing nations with reduced infrastructure and high consumption of maize and groundnut may be where biocontrol of aflatoxins has the greatest potential. Indeed, atoxigenic isolate formulations have long-term stability under non-refrigerated

conditions, a trait useful in regions where refrigeration is unavailable or expensive (Bock and Cotty, 1999a) .

Aspergillus communities associated with acute aflatoxicosis and human death in the Eastern Province of Kenya have some of the highest aflatoxin-producing potentials ever reported (Probst *et al.*, 2007; Probst *et al.*, 2010). This study demonstrated that, even within these highly toxic fungal communities, there reside atoxigenic isolates of potential value in the management of aflatoxins. Communities composed of highly toxigenic fungi have proportionally fewer atoxigenic isolates (Probst *et al.*, 2010) and, thus, screening for atoxigenic isolates from such communities may be time- and cost-intensive. Nonetheless, 48 of the 96 identified atoxigenic L strain isolates of *A. flavus* were isolated from highly contaminated maize samples (up to 4,400 µg total aflatoxins/ kg maize) originating from acute aflatoxicosis outbreak regions in Kenya in 2004 to 2006. These isolates represent a small fraction of the L strain population of *A. flavus* which, likewise, comprise a small fraction of the total *Aspergillus* community in the Eastern Province of Kenya (Probst *et al.*, 2010). Experience in the United States has shown that appropriately timed application of similarly rare, atoxigenic isolates of *A. flavus* results in fungal communities dominated by the applied atoxigenic isolates, resulting in greatly reduced aflatoxin-producing potential of the affected fungal communities (Cotty, 2006; Cotty *et al.*, 2007). Since 2007, hundreds of isolates of *A. flavus* have been screened, first by morphological criteria to remove highly toxigenic S strain isolates, then by quantifying aflatoxin production during *in vitro* culture on sterilized maize kernels and, finally, by testing the

ability of the atoxigenic isolates to interfere with aflatoxin contamination of viable maize kernels by toxigenic isolates in laboratory assays. These selected atoxigenic isolates are now ready for field testing. Successful collection of atoxigenic isolates of potential value in aflatoxin management from maize samples associated with lethal aflatoxicosis in the Eastern Province suggests that valuable atoxigenic isolates may similarly be found in *Aspergillus* communities resident in other areas where improved aflatoxin management is of concern.

A. flavus is composed of many VCGs (Bayman and Cotty 1991). VCGs evolve as clonal lineages (Ehrlich *et al.*, 2007a; Grubisha and Cotty, 2010) and aflatoxin production is more similar within VCGs than among VCGs. Some VCGs of *A. flavus* are composed of only atoxigenic members (Bayman and Cotty, 1993). The VCGs reported in this study provide potentially valuable fungal germplasm for development of biological control agents directed at limiting contamination of maize in Kenya. Atoxigenic isolates are frequently monitored in the environment by VCG analyses (Pildain *et al.*, 2004; Donner *et al.*, 2010). In order to perform VCG analyses in this study, auxotroph tester pairs were developed and are now available for atoxigenic isolate monitoring during field testing in Kenya. Although the atoxigenic isolates represent 53 locally adapted genetic groups of *A. flavus*, they may represent a small subset of the overall genetic diversity of atoxigenic isolates of *A. flavus* endemic to Kenya. Diverse fungal germplasm may be of value in overcoming problems with biocontrol agents (e.g., loss of efficacy) or for assembling complex mixtures of atoxigenic isolates for increased efficacy across complex cropping systems and for

long-term displacement of highly toxigenic *Aspergillus* isolates. A comprehensive collection of *A. flavus* isolates from across Kenya would increase genetic resources for development of such strains.

Atoxigenic isolates of *A. flavus* can prevent unacceptable aflatoxin levels in crops both through direct interference with aflatoxin production during co-infection and modification of *A. flavus* population composition by competitive exclusion. The first process involves both competitive exclusion during host tissue invasion and disruption of aflatoxin biosynthesis through an, as yet, undefined process (Cotty and Bayman, 1993; Mehl and Cotty, 2010). In this study, atoxigenic isolates were compared for interference with aflatoxin contamination of maize kernels co-infected by a highly toxigenic S strain isolate of *A. flavus*. This type of evaluation has been used (Brown *et al.*, 1991; Abbas *et al.*, 2006; Atehnkeng *et al.*, 2008b) to compare efficacy of atoxigenic isolates since such isolates were first evaluated as potential biocontrol agents (Cotty, 1990). However, this type of screen may not provide the most useful insights for selecting optimal atoxigenic isolates of *A. flavus* for biocontrol because competitive exclusion of aflatoxin producers during crop production contributes to both single season and long-term efficacy (Cotty *et al.*, 2007). Competitive exclusion is dependent on isolate reproduction and competition in the local environment (Cotty *et al.*, 2008). Isolates of *A. flavus* that produce the most conidia dominate *A. flavus* communities and have proportional influences on contamination regardless of ability to compete during host tissue invasion (Mehl and Cotty, 2010). Competitive exclusion of aflatoxin producers during epidemic increases allows atoxigenic isolates to compensate

for uneven application and to have an influence beyond treated areas, potentially influencing compositions of fungal communities in nearby, untreated fields (Cotty and Antilla, 2003). This is a biocontrol benefit not shared with chemical pesticide applications.

Atoxigenic isolates of *A. flavus* in VCGs identified in this study all belong to the L strain morphotype of *A. flavus* (Cotty, 1989), similar to L strain VCGs from other regions (Cotty, 1989; Pildain *et al.*, 2004). However, *A. flavus* VCGs can evolve independently for long periods (Ehrlich *et al.*, 2007a; Grubisha and Cotty, 2010). Even within morphotypes, there is great variability among VCGs in adaptive traits (Bayman and Cotty, 1993; Cotty and Mellon, 2006), including aflatoxin producing ability, virulence, competitiveness, sensitivity to antibiotics, tolerance to soil conditions, and epidemiology. Phenotypic variability suggests differential adaptation among isolates of different VCGs to various ecological niches (Cotty *et al.*, 1994b; Cotty and Mellon, 2006). The diversity of niches to which isolates of specific VCGs identified in this study are adapted is not yet known. Introduction and application of exotic microorganisms can bear considerable risk to animals, plants and to the environment (Simberloff and Stiling, 1996; van Lenteren *et al.*, 2006). It has been suggested that atoxigenic *A. flavus* isolates originating from North America be introduced to various African countries for aflatoxin management because the North American isolates are components of biopesticides currently registered with the United States Environmental Protection Agency (EPA) and, as such, have proven safe for agricultural uses. However, given the large number of atoxigenic isolates of *A. flavus* native to

Kenya identified in this study, and similar diversity documented in West Africa (Atehnkeng *et al.*, 2008a), justification for such introductions remains questionable.

In addition to the processes described above through which atoxigenic isolates may influence aflatoxin contamination of crops, the ability of a fungal isolate to persist within the local agroecosystems in the absence of a target crop is important. Such environmental fitness is particularly important for atoxigenic isolates being developed for low income regions where farmers consume most of the crop and have limited economic incentive to apply aflatoxin mitigation measures. In these regions, responsibility may fall to public organizations to apply atoxigenic isolates to crops. Adaptation of atoxigenic isolates to the target agroecosystem favors the use of native atoxigenic isolates of *A. flavus* for reducing aflatoxin contamination of crops. Among the three VCGs of *A. flavus* studied to date, divergence appears to have occurred 18,000 to 63,000 years ago (Grubisha and Cotty, 2010). Since this divergence, variation among VCGs has developed in diverse characteristics as isolates of the VCGs adapted to ecological niches. Isolates of native VCGs are adapted to compete for local resources and local cropping systems (including crop rotations and co-cropping). Native isolates should allow wider and more persistent displacement of aflatoxin-producing strains than exotic isolates. Native atoxigenic isolates are also genetic resources of the source countries, where the governments have the responsibility to manage such resources for maximum benefit of the population, as outlined in the Kenyan Environmental Management and Co-ordination Act of 2006 (Kenyan Ministry for Environment and Natural Resources, 2006; Schroeder and Pogge, 2009).

In this study, atoxigenic *A. flavus* isolates belonging to 12 different VCGs were found in multiple maize samples in the Eastern Province of Kenya. Recovery of an atoxigenic VCG from the target crop at multiple locations in a region potentially indicates a successful phenotype with competitive advantage in that agroecosystem. Isolates of such VCGs may be the best candidates for biocontrol products designed to provide long-term reductions in aflatoxin contamination. The number of VCGs detected within multiple samples in this study was surprising. A single atoxigenic isolate was collected from each of 37 of the 51 maize samples evaluated. These VCGs represent an important start for developing commercial biological control agents for maize aflatoxin contamination in Kenya. The large number of atoxigenic VCGs detected provides opportunity to utilize VCG mixtures similar to those developed for West Africa (Atehnkeng *et al.*, 2010). Isolate mixtures may compete effectively in a greater diversity of environmental niches than individual isolates. Furthermore, modified *Aspergillus* communities with multiple atoxigenic VCGs are expected to have greater stability than those formed with single VCGs. This is important in regions where low income farmers consume their crops directly, and where annual applications of a biological control product may not be feasible economically.

CHAPTER 4- RELATIONSHIPS BETWEEN *IN VIVO* AND *IN VITRO* AFLATOXIN PRODUCTION: RELIABLE PREDICTION OF FUNGAL ABILITY TO CONTAMINATE MAIZE WITH AFLATOXINS

Abstract

Aflatoxins are highly carcinogenic mycotoxins frequently produced by *Aspergillus flavus*. Contamination of maize with aflatoxins imposes both economic and health burdens in many regions. Identification of the most important etiologic agents of contamination is complicated by mixed infections and varying aflatoxin-producing potential of fungal species and individuals. To predict the potential of an isolate to cause a contamination event, the ability of the isolate to produce aflatoxins on the living host must be determined. Aflatoxin production *in vitro* (synthetic and natural media) was contrasted with *in vivo* (viable maize kernels) to determine ability of *in vitro* techniques to predict the relative importance of causal agents to maize contamination events. Several media types and fermentation techniques (aerated, non-aerated, fermentation volume) were compared. Isolates that produced aflatoxins on viable maize frequently failed to produce detectable (limit of detection = 1 ppb) aflatoxin concentrations in synthetic media. We found no correlation between aflatoxin production in viable maize and production in any of the liquid fermentation media using any of the fermentation techniques tested here. However, aflatoxin production on autoclaved maize kernels was highly correlated with production on viable maize kernels. The results have important implications for researchers seeking

to either identify causal agents of contamination events or characterize atoxigenic isolates for biological control.

Introduction

Aflatoxins are highly carcinogenic, teratogenic and immunosuppressive polyketides produced by members of the fungal genus *Aspergillus* (Eaton and Groopman, 1994; Klich, 2007). *Aspergillus flavus*, a generalist ascomycete fungus, is most frequently associated with aflatoxin contamination events of agricultural commodities (Cotty *et al.*, 1994a). Based on cultural and genetic characteristics, *A. flavus* can be delineated into large (L) and small (S) sclerotial morphological types (commonly referred to as the S and L strain morphotypes) (Cotty, 1989). Both morphotypes are frequently found in nature and are able to co-infect crops and co-exist in various environmental niches. However, they differ in a variety of characteristics including aflatoxin synthesis (Cotty, 1989; Bayman and Cotty, 1993; Orum *et al.*, 1997) : isolates of the L strain morphotype produce, on average, less aflatoxins than those of the S strain morphotype, and some L strain isolates are atoxigenic (i.e. produce no aflatoxins). Isolates of the S strain morphotype consistently produce high levels of aflatoxins (Cotty, 1994b). Atoxigenic isolates occur rarely within the S morphotype group and have not been well described (Cotty *et al.*, 1994a; Cotty, 1997).

The aflatoxin contamination process can be divided into two phases based on crop maturity (Bock and Cotty, 1999b; Cotty and Jaime-Garcia, 2007; Cotty *et al.*, 2008). The first phase occurs during crop development and is associated with physical damage and plant stress; the second phase occurs after seed maturity when the mature crop is exposed to environmental conditions conducive to aflatoxin-producing fungi (Russell *et al.*, 1976; Cotty, 2001; Jaime-Garcia and Cotty, 2003). During both stages

fungal community structure greatly influences concentrations of aflatoxins in crops (Cotty, 1990; Horn, 2005; Atehnkeng *et al.*, 2008a; Mehl and Cotty, 2010). Aflatoxin management strategies must address both phases of contamination in order to optimize efficacy and reliability.

A clear understanding of disease etiology is necessary to direct management strategies. The process of identifying the most important causal agents of aflatoxin contamination is complicated by varying aflatoxin producing potential of species, morphological types, and isolates associated with affected crops. The incidence of a causal agent is an important measure. However, equally important is the ability of a fungal strain to contaminate the specific crop of concern: a high aflatoxin producer that is rare may contribute more to contamination than a low aflatoxin producer that is common (Cotty *et al.*, 2008).

Traditionally, liquid fermentations are used to measure aflatoxin-producing ability of *Aspergillus* isolates (Zuber *et al.*, 1987; Cotty and Cardwell, 1999; Ehrlich *et al.*, 2007b; Reddy *et al.*, 2009). This is intended to reveal the relative ability of isolates to contaminate a substrate but is complicated by the various fermentation media and methods available (Mateles and Adye, 1965; Davis *et al.*, 1966; Shih and Marth, 1972; Dyer and McCammon, 1994; Norton, 1995). Furthermore, relationships between aflatoxin production *in vitro* and *in vivo* are not well defined. Fungal isolates able to produce high concentrations of aflatoxins in laboratory media may not be highly toxigenic during crop infection.

The current study sought to determine the extent to which *in vitro* aflatoxin production assays reflect the aflatoxin-producing ability of *A. flavus* isolates in viable maize (*in vivo*). The results provide guidance for the use of *in vitro* techniques and have important implications for researchers seeking to either identify causal agents of aflatoxin contamination events or to characterize atoxigenic isolates for biological control.

Materials and methods

For all experiments, ultrapure water provided by a Millipore Milli-Q-System (Billerica, MA) was used. $MgSO_4*7H_2O$ was purchased from EMD Chemicals Inc. (Gibbstown, NJ). All other chemicals were obtained in analytical grade or better from Mallinckrodt Baker (Phillipsburg, NJ) and VWR (West Chester, PA).

Fungal isolates and inocula preparation

Thirty-seven isolates of *A. flavus* were included in this study (Table 4.1). Sixteen isolates belonged to the S strain morphotype and 21 isolates belonged to the L strain morphotype. Isolates were previously obtained from highly contaminated maize from the Eastern Province in Kenya (Probst *et al.*, 2007). These isolates were chosen for the current study because they were associated with an aflatoxin contamination event on maize. Fungal isolates were cultivated in the dark for 5 to 7 d (31 ˚C) on 5/2-agar (5% V-8 vegetable juice, 2% agar, pH 5.2). After cultivation, conidia were transferred with sterile cotton swabs into glass vials containing 20 ml sterile ultrapure water. Conidial concentrations were measured with a turbidity meter (Model 965-10; Orbeco-Hillige, Farmingdale, NY) and calculated with a Nephelometric turbidity unit (NTU) versus colony forming unit (CFU) curve (Y = 49 937X; X = NTU, Y = conidia per ml). Conidial suspensions for all fungal inocula were adjusted to 10^6 conidia /ml.

Effect of liquid fermentation method and media on aflatoxin production

The following media types were assessed: yeast extract sucrose (YES) medium (Davis *et al.*, 1966), and several forms of Adye and Mateles (A&M) medium (Mateles

and Adye, 1965) (Table 4.2). A&M medium was amended with one of the following as the sole nitrogen source and adjusted to the indicated pH with NaOH and HCl: 22.5 mM glutamine ($C_5H_{10}N_2O_3$), pH 6.5; 22.5 mM ammonium sulfate ($(NH_4)_2SO_4$), pH 4.75; and 22.5 mM urea (NH_2CONH_2), pH 4.75. Urea and glutamine were filter-sterilized and added aseptically to autoclaved media. Ammonium sulfate was added prior to autoclaving the medium.

All four media were evaluated with three fermentation methods. 70 ml aerated: 70 ml fermentation medium (per 250 ml Erlenmeyer flask), and constant agitation on a rotary shaker (160 rpm); 70 ml stationary: 70 ml fermentation medium (per 250 ml Erlenmeyer flask), stationary; 5 ml stationary: 5 ml fermentation medium (per 20 ml glass vial), stationary. All fermentations were incubated in a refrigerated benchtop incubator shaker (Model 4629, Lab Line Scientific Instruments, Maharashtra, India) for 7 days at 31 ˚C in the dark.

Liquid media were aseptically seeded with conidial suspension (10^6 conidia /ml, 100 µl per flask) and incubated. Each liquid medium was evaluated in a separate experiment. Experiments were conducted twice, each with three replicates and two independent sets of fungal isolates. At the end of the incubation period, pH was measured and fermentations were terminated by addition of acetone (50 ml per 70 ml fermentation; 5 ml per 5 ml fermentation) as previously described (Cotty, 1997; Cotty and Cardwell, 1999). Cultures were allowed to rest for 1 h at room temperature to allow for fungal cell lysis and release of aflatoxins contained within the mycelium. Flask contents were filtered through Whatman No. 4 filter paper (Whatman,

Piscataway, NJ), mycelia were dried at 45 ˚C for 48 h, and fungal biomass was weighed. Filtrates free of mycelia were spotted directly onto thin-layer chromatography (TLC) plates (Silica gel 60, EMD, Darmstadt, Germany) adjacent to aflatoxin standards (Aflatoxin Mix Kit-M, Supelco, Bellefonte, PA). Plates were developed in ethyl ether-methanol-water (96:3:1) and air-dried. Aflatoxins were visualized under 365-nm UV light and quantified with a scanning densitometer (TLC Scanner 3, Camag Scientific Inc, Wilmington, NC). Filtrates initially negative for aflatoxins were partitioned twice with methylene chloride and concentrated prior to quantification (limit of detection 1 ng g^{-1} mycelium) as previously described (Cardwell and Cotty, 2002).

Assessment of aflatoxin production in viable (in vivo) and autoclaved (in vitro) maize grain

All experiments were performed twice, each with four replicates and two independent sets of fungal isolates. All isolates were tested in both liquid fermentation and grain assays. Maize grain (cultivar Pioneer 33F88) was supplied by Pioneer Hi-Bred International Inc. (Johnston, IA).

For *in vivo* assays, undamaged, healthy maize kernels (10 g) were surface disinfected by immersion in hot water (85 ˚C, 45 s) and placed in pre-sterilized Erlenmeyer flasks (250 ml). Flasks were plugged with BugStoppers (Whatman, Piscataway, NJ) to prevent humidity loss but allow gas exchange. Efficiency of surface disinfection and ability of kernels to germinate was monitored by plating three randomly selected representative kernels per flask on modified rose Bengal medium,

M-RB , followed by incubation at 31 ˚C in the dark for 21 days. Greater than 90% of kernels surface disinfected in this manner germinated and kernels were free of fungal contaminants.

For *in vitro* assays, maize kernels were autoclaved prior to inoculation. In each 250 ml Erlenmeyer flask, 10 g of healthy, undamaged maize kernels were autoclaved for 60 min at 121 °C. As described above, a representative set of three autoclaved kernels per flask was placed on modified rose Bengal medium (MR-B, Cotty 1994a) to monitor effectiveness of sterilization and confirm that all kernels used in the experiments were free of fungal contaminants and not viable.

Maize water content was determined with a HB43 Halogen Moisture Analyzer (Mettler Toledo, Columbus, OH) after sterilization and adjusted to 25% with sterile, ultrapure water containing 10^6 conidia per flask. The quantity of spores was determined by turbidity as described above. Inoculated maize was incubated for 7 d at 31 ˚C in the dark. At the end of the incubation period, maize kernels were washed with 80% methanol (50 ml). The maize-methanol mixture was homogenized in a laboratory grade Waring Blender (Seven-speed laboratory blender, Waring Laboratory, Torrington, CT) for 30 s on speed seven, filtered through Whatman No. 4 paper, and aflatoxins were quantified as summarized above and previously reported (Probst *et al.*, 2007).

Data analysis

Analysis of variance (ANOVA) was performed using the General Linear Model (GLM) procedure of SAS (version 9.1; SAS Institute, Cary, NC), which uses the least

squares method to fit data to a general linear model. Means were separated with Tukey's honest significant difference test ($P \leq 0.05$). Aflatoxin B_1 data were transformed to the natural logarithm prior to statistical analyses. Factorial ANOVA with linear models were performed to evaluate interactions between fungal isolate and type of medium for each fermentation method. Regression analyses were used to evaluate relationships between aflatoxin B_1 production in stationary 5 ml and 70 ml fermentations as well as *in vivo* and *in vitro* grain assays.

Table 4.1 Aflatoxin B_1 production by isolates of *Aspergillus flavus* on viable maize

Test 1				Test 2			
Strain	**Isolate**	**Aflatoxin B_1 (µg g^{-1} medium)[a]**		**Strain**	**Isolate**	**Aflatoxin B_1 (µg g^{-1} medium)[a]**	
L	910-E	140	bcde	L	54-A	0.08	d
L	951-K	38	abc	L	45-C	0.10	d
L	953-E	13	bcd	L	53-F	0.11	cd
L	971-L	111	ab	L	23-B	0.13	cd
L	804-H	205	ab	L	13-C	49	ab
L	782-C	0	d	L	41-A	52	ab
L	810-L	317	a	L	12-G	57	ab
L	792-B	0	d	L	43-B	73	ab
L	930-A	10	cde	L	42-A	80	ab
L	903-L	831	a	L	25-P	88	a
L	854-K	0	d				
S	854-E	337	a	S	34-A	26	b
S	782-G	375	a	S	58-A	95	a
S	930-B	420	a	S	52-F	154	a
S	953-K	516	a	S	56-A	167	a
S	976-K	634	a				
S	903-I	722	a	S	27-G	105	a
S	910-I	747	a	S	33-M	137	a
S	792-C	787	a	S	44-K	131	a
S	971-E	1099	a				
S	935-A	1139	a				

[a] Results are averages of four replicates. Values for a aflatoxin value within a column followed by a common letter are not significantly different based on Tukey's HSD test ($P \leq 0.05$).

Table 4.2 Composition of liquid and solid media

Media	Composition (per liter)
Liquid media	
Yeast Extract Sucrose (YES)	20 g yeast extract, 15 g sucrose, pH 6.5
Adye and Mateles (A&M)	50 g sucrose, 10 g KH_2PO_4, 2 g $MgSO_4*7H_2O$, 1 ml micronutrient solution, pH 4.75 or 6.5 depending on nitrogen source
Solid substrates	
Maize	10 g, 25% moisture
Supplements	
Micronutrient solution	0.11 g $MnS0_4*H2O$, 0.5 g $(NH_4)_6 Mo_7O_{24}*4 H_2O$, 17.6 g $ZnSO_4*7 H_2O$, 0.7 g $Na_2B_4O7*10 H_2O$ 0.3 g $CuSO_4*5 H_2O$

Results

Effect of liquid fermentation method and media on aflatoxin production

Both agitation and fermentation volume affected aflatoxin B_1 production by the tested isolates. On average, less aflatoxin B_1 was produced when cultures were shaken than under stationary incubation (mean = 1,534 ppb aflatoxin B_1 vs. mean = 7,903 ppb aflatoxin B_1, $P = 0.021$). In stationary fermentations, statistically higher aflatoxin B_1 concentrations were produced in 5 ml than in 70 ml fermentations in all liquid media types (mean = 11,459 ppb aflatoxin B_1 vs. mean = 3,298 ppb aflatoxin B_1, $P \leq 0.001$). The relationship between aflatoxin B_1 production in stationary 5 ml and 70 ml fermentations was assessed for each media type using linear regressions. In A&M medium, coefficients of determination were moderate to strong depending on the nitrogen source ($r^2 = 0.64$, 0.68, or 0.85 for glutamine, ammonium sulfate or urea, respectively). In YES medium, no linear relationship among aflatoxin B_1 production in 5 ml and 70 ml stationary fermentations was observed.

Media composition also affected aflatoxin B_1 production. Overall, more aflatoxin B_1 was produced in YES medium than in A&M medium with any nitrogen source (Table 4.3). Comparisons of aflatoxin B_1 production by *A. flavus* showed significant differences ($P \leq 0.001$) between isolates of the S and L strain morphotypes. S strain isolates produced on average more aflatoxin B_1 than L strain isolates in all liquid media (Table 4.3). Isolates that did not produce detectable quantities of aflatoxins (limit of detection = 1 ng g^{-1}) on viable maize kernels were considered atoxigenic. The number of isolates identified falsely as atoxigenic (false negatives)

increased from four to eleven depending on the liquid medium and method used (Table 4.4). Isolates identified falsely as negative produced from 12 ppb to 84,000 ppb aflatoxin B_1 on viable maize.

Effect of fermentation method and media composition on pH

Changes in pH were monitored for all liquid fermentations. In all cases but one, there was a drop in pH during fermentation. Media composition influenced the extent to which pH changed and differences between media were significant ($P \leq 0.001$) (Figure 4.1). The greatest decrease in pH (4.75 to 2.31) occurred in A&M medium with ammonium sulfate. An interaction between medium composition and the influence of method on pH was observed. For example in 5 ml stationary fermentation pH did not change significantly in A&M medium with urea but the greatest drop in pH among treatments occurred in A&M with ammonium (Figure 4.1).

The L and S strain morphotypes of *A. flavus* modified pH differently (data not presented). L strain isolates dropped pH of A&M media significantly more than S strain isolates ($P \leq 0.001$). However, the opposite was true for YES medium. Here, S strain isolates dropped pH significantly ($P \leq 0.0001$) more than L strain isolates (pH 4.89 and 5.04, respectively).

Aflatoxin production and sporulation in viable (in vivo) and autoclaved (in vitro) maize

Although there were significant differences among isolates (data not shown), even within the same morphotype, most isolates produced statistically similar amounts of conidia on autoclaved and viable maize. Sporulation by isolates of L and S strain morphotypes differed significantly ($P \leq 0.05$) with S strain isolates consistently producing fewer conidia than L strain isolates (total yield of $4.6*10^7$ and $3.1*10^8$, respectively). Aflatoxin B_1 production also differed significantly ($P \leq 0.001$) with S strain isolates producing more aflatoxin B_1 than L strain isolates (Table 4.3). The viability of maize did not significantly affect aflatoxin B_1 production by isolates of either strain (Table 4.3). The range of aflatoxin B_1 production by the tested *A. flavus* isolates on viable maize grain is given in Table 4.1. In general, L strain isolates are highly variable aflatoxin-producers on viable maize grain with some isolates failing to produce detectable quantities of aflatoxins (limit of detection 1 ppb) and others highly toxigenic (up to 1,139 µg g^{-1} aflatoxin B_1). Aflatoxin-producing abilities of certain L strain isolates were statistically similar to the aflatoxin-producing abilities of certain S strain isolates (Table 4.1).

Relationship between aflatoxin B_1 production in vitro and in vivo

Aflatoxin B_1 production was significantly higher on maize than in any of the liquid media used in this study (Table 4.3). Linear regressions between aflatoxin B_1 production *in vivo* and production in liquid media were weak to moderate (r^2 ranges from 0.002 to 0.554) (Table 4.4). In contrast to liquid media, aflatoxin production on

autoclaved maize was highly predictive of aflatoxin production in viable maize kernels with a high coefficient of determination ($r^2 = 0.982$, $P \leq 0.0001$) for the linear relation between aflatoxin production on autoclaved maize kernels and production on viable maize kernels (Figure 4.2).

Table 4.3 Effect of media composition on aflatoxin B_1 production by *A. flavus* isolates

		Aflatoxin B_1 (μg g^{-1} medium)[a]					
		Maize		**Media**			
Test	Strain	viable	autoclaved	YES	A&M, Glutamine	A&M, Urea	A&M, Ammonium
1	S	670 a	848 a	26 b	6 cd	5 d	12 bc
	L	130 a	90 a	3 b	0.8 b	2 b	0.6 c
	All	**384 a**	**434 a**	**12 b**	**2 c**	**3 c**	**6 bc**
2	S	117 a	148 a	207 a	13 b	16 b	6 b
	L	40 a	29 a	45 b	1 c	0.5 c	0.5 c
	All	**91 a**	**78 a**	**101 a**	**6 b**	**6 b**	**3 b**

[a] Results are averages of four (maize) or three (media) replicates. Values for a variable within a row followed by a common letter are not significantly different based on Tukey's HSD test ($P \leq 0.05$).

Table 4.4 Relationship of aflatoxin B_1 production in viable maize kernels (*in vivo*) to various *in vitro* culture media and fermentation methods

Medium	# False neg.	Method		r^2	P	Trend
A&M, Glutamine	4	5 ml	stationary	0.041	0.23	positive
	4	70 ml	shaking	0.007	0.63	positive
	4	70 ml	stationary	0.002	0.81	positive
A&M, Urea	9	5 ml	stationary	0.019	0.41	positive
	5	70 ml	shaking	0.476	<.0001	positive
	10	70 ml	stationary	0.002	0.77	positive
A&M, Ammonium	11	5 ml	stationary	0.554	<.0001	positive
	6	70 ml	shaking	0.554	<.0001	positive
	6	70 ml	stationary	0.473	0.0023	positive
YES	4	5 ml	stationary	0.018	0.42	negative
	5	70 ml	shaking	0.326	0.0002	positive
	4	70 ml	stationary	0.423	<.0001	positive
Maize	0		Autoclaved	0.982	<.0001	positive

[a] # False neg., number of false negatives isolates (= produced no detectable aflatoxins in the respective media, but were aflatoxigenic on autoclaved maize).

Figure 4.1 Culture pH values after 7d fermentations. Bars represent mean pH values (n = 111) for each fermentation method within the indicated medium. Black bar, 5 ml and stationary fermentations; White bar, 70 ml and shaking; Grey bar, 70 ml and stationary. Differences between media types were significant ($P \leq 0.001$). Asterisk indicates significant differences in pH changes among methods within each medium. Horizontal line indicates initial pH prior fermentation.

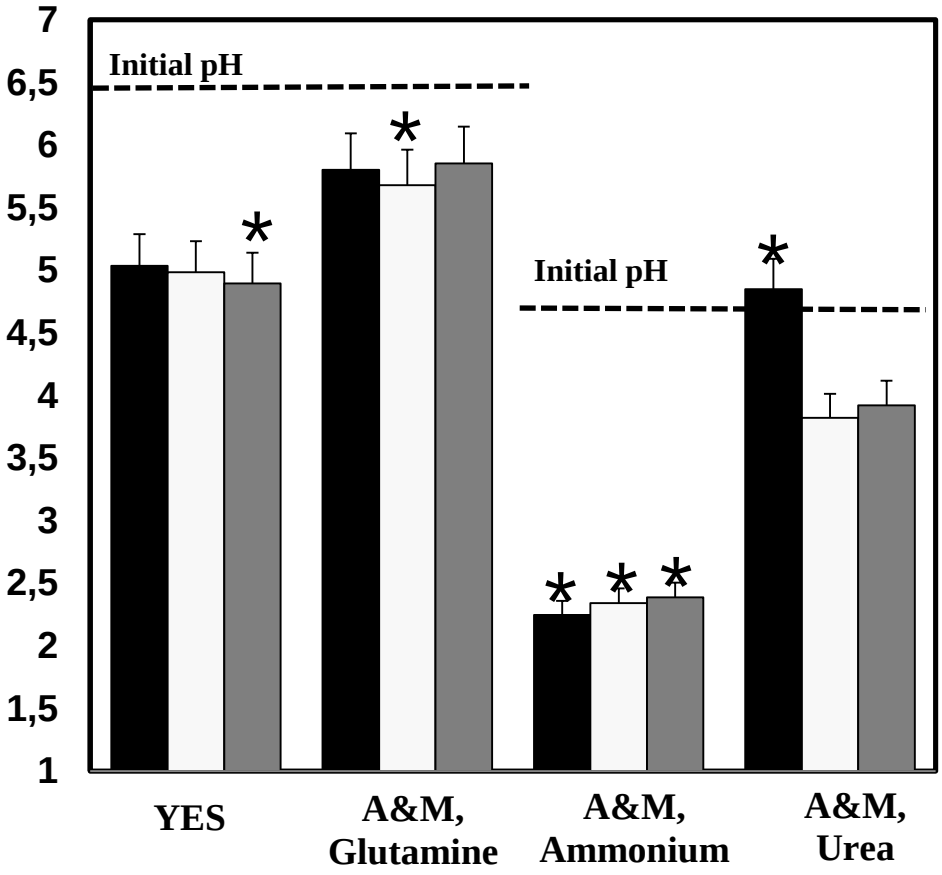
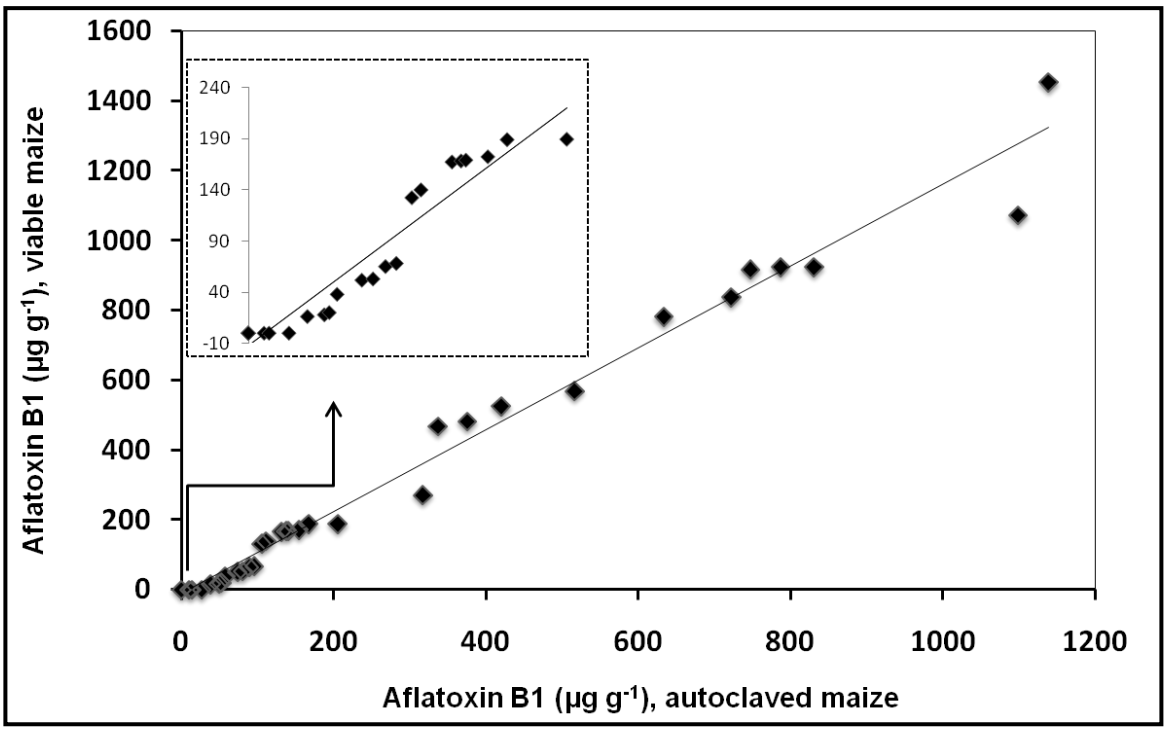


Figure 4.2 Relationship between quantities of aflatoxin B_1 produced during infection of viable maize grain by 37 *A. flavus* isolates and aflatoxin production on autoclaved maize by the same isolates (Y = 1.17x- 11.19 and r^2 = 0.98). Each value is the mean of four replicates.



Discussion

Etiology has been a central focus for plant pathologists since Anton deBary identified *Phytophthora infestans* as the causal agent of the Irish potato famine and Koch first put forth his postulates in 1890 (Agrios, 2004). In the case of aflatoxin contamination events, identification of the most significant causal agents is often complicated by complexity of the fungal community infecting crops. Communities of aflatoxin-producing fungi frequently are interactive mixtures composed of many fungal species and morphological types that belong to multiple vegetative compatibility groups (VCGs) differing in competitive ability, virulence, and aflatoxin-production (Bayman and Cotty, 1991; Cotty *et al.*, 2008; Mehl and Cotty, 2010). Many members of the community may have potential to produce some contamination. However, identification of the agent(s) primarily responsible for a contamination event requires taking into consideration both the frequency of various agents in the contaminated tissues and the ability of the agents to produce aflatoxins.

Isolates of *A. flavus* vary widely in aflatoxin-producing ability (Schroeder and Boller, 1973; Cotty, 1997; Probst *et al.*, 2007) with some isolates producing 10 ppb or less aflatoxin B_1 in maize grain tissues, and others producing over 200,000 ppb (Brown *et al.*, 1993; Probst *et al.*, 2007). This variability during maize infection is illustrated by the results in the current study (Table 4.1). If an isolate that contaminates the crop with 200,000 ppb aflatoxin B_1 infects only 0.1% of the seed (0.01 x 200,000 ppb = 2,000 ppb), it is still a more important causal agent than one that infects 90% of the seed but only produces 10 ppb (0.9 x 10 ppb = 9 ppb). An approach of this type was taken to

identify fungi with the S strain morphotype as the causal agent of the aflatoxin contamination events that led to the lethal aflatoxicoses outbreaks in Kenya in 2004, 2005, and 2006 (Probst *et al.*, 2007; Probst *et al.*, 2010). Here an unusual fungal community structure has led to repeated development of lethal levels of aflatoxins in maize (Probst *et al.*, 2010). The present study evaluated the relative value of several laboratory assays (liquid and solid media) for identification of causal agents of aflatoxin contamination events. The results suggest sterile forms of the crop of concern may be the best medium to employ during investigation of the etiologies of future aflatoxin outbreaks. Indeed, aflatoxin production on autoclaved maize grain was highly predictive of aflatoxin production during infection of viable grain. Fermentation media were originally developed to stimulate aflatoxin production (Mateles and Adye, 1965; Davis *et al.*, 1966) and basic requirements for aflatoxin-production by *Aspergilli* are well understood. Generally, aflatoxin production is favored by availability of organic nitrogen and simple sugars (Davis and Diener, 1968; Bhatnagar *et al.*, 1986), growth temperatures between 28˚ C and 31˚ C (Taber and Schroeder, 1967) and a slightly acidic pH (Reddy *et al.*, 1979; Cotty, 1988). All liquid media tested fulfilled these basic requirements and triggered aflatoxin production by most of the examined isolates, but all media also support aflatoxin levels much less than the host material. Even with only support for low aflatoxin levels, synthetic media would still be useful for identifying the most important causative agents of contamination if aflatoxin production in the medium correlates well with aflatoxin production in the host tissue of interest. In the current study aflatoxin production in all liquid fermentation media and

with each of the three fermentation methods was poorly correlated with ability of fungal isolates to infect and contaminate viable maize with aflatoxins. Of the four liquid media tested, A&M supplemented with ammonium sulfate best predicted aflatoxin production in viable maize. However, coefficients of determination were not very high even for this medium suggesting aflatoxin production in A&M with ammonium sulfate only explains about 50% of variability among *A. flavus* isolates in aflatoxin production *in vivo*. Among isolates that produced aflatoxins in viable maize grain, one or more failed to produce aflatoxins in each of the examined fermentation media. A&M medium with ammonium sulfate was the medium with the greatest number of these false negatives. Influence of ammonium sulfate on aflatoxin development through rapid decrease of pH generally stimulates aflatoxin production by *A. flavus* (Cotty, 1988; Ehrlich *et al.*, 2005a). Results of the current study suggest pH drops associated with ammonium sulfate utilization can also suppress aflatoxin biosynthesis. Contrary to results with liquid fermentations, the 37 *A. flavus* isolates examined in the current study produced on autoclaved maize grain aflatoxin levels very similar to those produced during infection of viable maize grain. There were no false negative isolates on autoclaved maize. This has particular importance for research directed towards identification of atoxigenic isolates, as in the case of development of agents for aflatoxin biocontrol (Cole and Cotty, 1990; Abbas *et al.*, 2006; Probst *et al.*, 2011).

Aflatoxin-producing potential of fungal strains and isolates may also be influenced by host resistance mechanisms. Maize varieties with increased resistance

against aflatoxin-producing fungi may influence the aflatoxin-producing potential of fungal isolates. In the present study, aflatoxin-producing potential of *A. flavus* isolates was tested on a maize cultivar susceptible to aflatoxin contamination. This cultivar supports fungal synthesis of high levels of aflatoxins. For the identification of causative agents, it is not very important to evaluate the range of which each isolate produces aflatoxins; production on crops and cultivars of direct concern only need to be considered. Choosing grain from the cultivar actually contaminated may be the best option, when such grain is obtainable. However, maize grain used for aflatoxin assays has to be aflatoxin free and sterilized before any treatment.

Aflatoxin synthesis is also influenced by aeration/ agitation (shaking vs. stationary) and culture volume (70 ml vs. 5ml in the current study) (Hayes *et al.*, 1966; Shih and Marth, 1974; Buchanan and Ayres, 1975; Cotty, 1988; Cotty and Cardwell, 1999). Our results are in general accordance with these studies. Aflatoxin production was higher in stationary cultures than in shaking cultures. Sclerotia did form on submerged mycelia of stationary cultures and a relationship between aflatoxin production and sclerotia formation exists (Cotty, 1988). Increased aflatoxin production in stationary culture may be related to the interrelationship between sclerotial morphogenesis and aflatoxin biosynthesis (Cotty, 1988).

For identification of both causative agents and atoxigenics, assays to determine fungal ability to produce aflatoxins should be performed on both the host tissue of interest and autoclaved maize. Autoclaved maize is easier to manipulate than surface sterilized viable maize grain and, in the current study, autoclaved maize supported the

highest levels of aflatoxin production by all the examined fungi. Autoclaved maize grain also exceeds synthetic liquid media in the ease of preparation and cost and is ideal for laboratories with less access to chemicals and financial support.

CHAPTER 5- DEADLY STRAINS OF KENYAN *ASPERGILLUS* ARE DISTINCT FROM OTHER AFLATOXIN PRODUCERS

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Abstract

Aflatoxin contamination of crops is a world-wide problem. Lethal aflatoxicosis of humans has been associated with maize produced in Kenya for over three decades. The S strain morphotype of *Aspergillus flavus* was identified as the primary cause of aflatoxin contamination events occurring between 2004 and 2006 in Kenya. Because the S strain was first described from North America, it was suggested that the agent causing lethal levels of aflatoxins was introduced to Kenya with maize. DNA sequence comparisons among 68 S strain isolates from Kenya, the Americas, Asia, and Australia suggest the Kenyan isolates are distinct from those causing aflatoxin contaminations in North America. Analyses of 4.06 kb representing three loci from distinct chromosomes indicate that most S strain isolates from North America resolved into a clade distinct from one containing the 30 Kenyan isolates S strain isolates were more closely related to the recently described species *A. minisclerotigenes* than to *A. flavus*. Furthermore, failure of the Kenyan isolates to produce G aflatoxins was attributed to a previously undescribed deletion in the *cypA* gene, suggesting that different deletion events led to loss of G aflatoxin production in North American and Kenyan S strain isolates. Thus, although the Kenyan isolates have S strain morphology and produce large quantities of only B aflatoxins like *A. flavus* S strain isolates, these isolates are phylogenetically divergent from those described from other regions. The

molecular characteristics of the Kenyan S strain isolates described herein are valuable tools to identify and track these highly aflatoxigenic fungi.

Introduction

Aspergillus flavus is a cosmopolitan fungus that thrives on living and dead organic matter. The fungus is a weak plant pathogen and mainly known for its ability to produce aflatoxins, some of the most potent, naturally occurring liver carcinogens currently known. Aflatoxin contamination of crops limits economic viability of agriculture and can lead to severe health impairment when present in staples required to meet human caloric needs (Wu and Khlangwiset, 2010). Health effects range from immune suppression and growth retardation to cancer and, in cases of severe poisonings, liver failure and subsequent death (Williams *et al.*, 2004). In developed nations, strict regulations and law enforcement prevent aflatoxins from entering the food chain. This is in stark contrast to most developing countries where aflatoxin contaminated crops may be the only food sources available and aflatoxin-related health problems are reported frequently . Kenya is the country most severely affected by aflatoxin contaminated crops, with human fatalities reported repeatedly since 1981 (Ngindu *et al.*, 1982; Centers for Disease Control and Prevention, 2004).

Attribution of aflatoxin contamination episodes to specific fungi is complicated by the complexity of *Aspergillus* section *Flavi* communities associated with crops and the propensity among fungal genotypes to produce widely varying levels of aflatoxins. Genotypes that produce very high concentrations of aflatoxins in crop tissues may be important etiologic agents of contamination, even when only infecting a small

proportion of the crop (Cotty *et al.*, 2008). The average aflatoxin-producing potential of a fungal community is an important determinant of the extent to which crops become contaminated. Crops associated with communities having high average aflatoxin-producing potential are much more likely to become contaminated than crops associated with communities with low average aflatoxin producing potential (Probst *et al.*, 2007). Indeed, aflatoxins can be effectively managed in agricultural fields by reducing the average aflatoxin-producing potential of fungi associated with crops through the application of atoxigenic strains of *A. flavus* (Cotty and Mellon, 2006; Cotty *et al.*, 2007).

The causal agent of the aflatoxin contamination events leading to the lethal aflatoxicosis in Kenya was identified as the S strain of *A. flavus* based on morphological and physiological characteristics (Probst *et al.*, 2007; Probst *et al.*, 2010). Isolates belonging to the S strain morphotype of *A. flavus* were first described in 1970 (Hesseltine *et al.*, 1970). The three morphologically similar isolates (isolates 3251, A-11611, and A-11612) produce both very high concentrations of aflatoxins and numerous small sclerotia. This morphotype would be later associated with aflatoxin contamination in North America and named the S strain morphotype (Cotty, 1989). Isolates with S strain morphology may produce only B aflatoxins (sometimes referred to as S_B isolates) (Cotty and Cardwell, 1999) (Ehrlich *et al.*, 2007b) or both B and G aflatoxins (sometimes referred to as S_{BG} isolates).

Recently, S strain isolates from Thailand were named as a new variety (*A. flavus* var. *parvisclerotigenus* M.Saito et Tsuruta) on the basis of sclerotial morphology

(Saito and Tsuruta, 1993). The variety was described with two chemotypes: S_B, producing only B aflatoxins, and S_{BG}, producing B and G aflatoxins. Phylogenetic comparisons later placed S_{BG} isolates from Thailand into a clade with *A. nomius,* and S_B isolates from Thailand in clades with L and S strain isolates of *A. flavus* from North America (Ehrlich *et al.*, 2007b). Isolates with the S strain morphotype from North America are uniformly S_B whereas those from West Africa are uniformly S_{BG} (Hesseltine *et al.*, 1970; Cotty and Cardwell, 1999; Cardwell and Cotty, 2002; Atehnkeng *et al.*, 2008a). Both morphotypes have been shown to be phylogenetically distinct from each other (Egel *et al.*, 1994; Ehrlich *et al.*, 2003). Phylogenetic comparisons have similarly been used to resolve S strain isolates into two groups: Group I is closely related to *A. flavus* L strain isolates and contains only B aflatoxin producers, whereas Group II contains both S_B and S_{BG} isolates (Geiser *et al.*, 2000). Recently, S_{BG} isolates from Argentina were named to a new species, *Aspergillus minisclerotigenes*, on the basis of morphology, phylogenetic comparison, and extrolite production (Pildain *et al.*, 2008). This new species includes one of the first described S_{BG} isolates, A-11611 (Hesseltine *et al.*, 1970). However, relationships are unknown between any of the above taxa and the S strain isolates causing the severe aflatoxin contamination episodes in the Eastern Province of Kenya from 2004 until present. In particular, it is unclear if the Kenyan S strain isolates were introduced from North America or independently evolved and adapted to maize cropping in East Africa.

Aflatoxins are produced by a complex biosynthetic pathway by more than 25 genes located in a 65 to 70 kb gene cluster (Ehrlich and Yu, 2010). Four naturally

occurring aflatoxins (B_1, B_2, G_1, and G_2) have been described (Ellis *et al.*, 1991). Some aflatoxin producing fungi (e.g. *A. flavus)*, have lost the ability to produce G aflatoxins through inactivation of a putative oxidative enzyme-encoding gene (*cypA*) required for aflatoxin G formation (Ehrlich *et al.*, 2004). Inactivation results from a sequence deletion removing both the promoter region and the translational start site. Two deletion events have been detected which are associated with the *A. flavus* L strain morphotype (0.8 kb) and the *A. flavus* S strain morphotype and *A. oryzae* (1.5 kb) (Ehrlich *et al.*, 2004). It is believed that loss of G aflatoxin production has occurred independently multiple times in ancestors to extant *Aspergillus* species (Ehrlich and Yu, 2010).

The current study sought to determine if the isolates in the S strain morphotype that repeatedly caused severe aflatoxin contamination of maize in Kenya are readily distinguishable from S strain isolates from other regions. Multigene phylogenetic analyses were used to compare Kenyan S strain isolates with isolates displaying similar morphology and aflatoxin-producing capacity from the Americas, Australia, and Southeast Asia. In addition, deletions in the aflatoxin biosynthesis cluster known to cause loss of G aflatoxin production in North American S strain isolates was characterized for Kenyan isolates in order to determine if identical deletion events led to loss of G aflatoxin production in Kenyan and North American isolates. Our analyses suggest S strain isolates from the Eastern Province of Kenya are distinct from the predominant S strain isolates from North America. Etiologic agents of aflatoxin contamination differ in potential to cause severe crop contamination. S strain isolates

from Kenya have been implicated in severe aflatoxin contamination events and present a risk for increased contamination of maize upon distribution outside of their current range. Molecular characteristics presented here provide a basis for monitoring movement of these dangerous fungi across the globe.

Materials and methods

Fungal isolates and aflatoxin production

Seventy isolates with S strain morphology and four isolates of related *Aspergillus* species (*A. bombycis*, *A. nomius* and *A. parasiticus*) were chosen based both on availability from personal (Laboratory for Research to Reduce Aflatoxins in Crops, School of Plant Sciences, University of Arizona, Tucson, AZ) and public (USDA-ARS) culture collections and use in previous studies (Hesseltine *et al.*, 1970; Rambo *et al.*, 1974; Kurtzman *et al.*, 1987; Egel *et al.*, 1994; Geiser *et al.*, 2000; Pildain *et al.*, 2004; Ehrlich *et al.*, 2007b; Probst *et al.*, 2010). Geographic origins and sources of the 74 isolates are shown in Table 5.1.

Aflatoxin-producing potential of the isolates was confirmed on autoclaved maize kernels (cultivar Pioneer 33F88, Pioneer Hi-Bred International Inc., Johnston, IA, USA). Undamaged maize kernels (10 g) in 250 ml Erlenmeyer flasks were autoclaved for 60 min at 121 °C. Maize moisture was measured with a HB43 Halogen Moisture Analyzer (Mettler Toledo, Columbus, OH, USA) and adjusted to 25% with sterile deionized water. Flasks were seeded with 200 µl of spore suspension (10^6 conidia/ ml) of the respective fungal isolate and incubated at 31 °C for 7 days in the dark. At the end of the incubation period, fungal growth was discontinued by addition of 70% methanol, and aflatoxins were quantified as described previously (Probst *et al.*, 2010).

DNA extraction, primers, and PCR amplification

Isolates were cultured in Potato Dextrose Broth (Difco, Detroit, MI, USA) at 31 °C for 48 h. Mycelium was harvested by vacuum filtration, and DNA was isolated using the FastDNA SPIN Kit and the FastPrep Instrument following manufacturer's instructions (MP Biomedicals LLC, Santa Ana, CA, USA). DNA was quantified by using a spectrophotometer (model ND-1000, NanoDrop, Wilmington, DE, USA) and diluted to a final concentration of 5 - 10 ng/ µl prior to PCR. Three regions of the genome were analyzed. The calmodulin (*cmd* on chromosome 2) and nitrate reductase (*niaD* on chromosome 4) genes were each amplified with two sets of primers covering approximately 1.3 kb of each gene. The gene for aflatoxin pathway transcription factor (*aflR* on chromosome 3) was amplified with one set of primers (aflR1F-aflR2R) covering approximately 2.1 kb of the gene. Segments of the 2.1 kb *aflR* gene amplicon were sequenced using three pairs of sequencing primers each covering approximately 0.7 kb of the amplified PCR product. Primer sequences and annealing temperatures are listed in Table 5.2. PCR reactions were performed in a 20 µl volume using 2 µl genomic DNA and the PCR PreMix (AccuPower® HotStart, Bioneer, Alameda, CA, USA) with the following protocol: 1 cycle of 5 min at 95 °C followed by 38 cycles of 20 s at 94 °C (denaturation), 30 s at locus-specific annealing temperature (Table 5.2), 30 s at 72 °C (extension), and finally, 1 cycle of 10 min at 72 °C. Amplicons were subjected to ExoSAP-IT (Affymetrix, Santa Clara, CA, USA), to eliminate unincorporated primers and dNTPs, and subsequently sequenced by the University of Arizona Genetics Core sequencing facility (UAGC, Tucson, AZ, USA).

Preparation of sequence data and phylogenetic analyses

Bidirectional sequences of the genes *aflR* (1.6 kb) and *niaD* (1.4 kb) from 74 and *cmd* (1.1 kb) from 68 *Aspergillus* section *Flavi* isolates were used for phylogenetic reconstruction. Consensus sequences were created by assembly of either 4 (*cmd*, *niaD*) or 6 (*aflR*) reads per gene, visually inspected and aligned using the MUSCLE algorithm with the default settings in Geneious Pro Version 4.8.2. (Biomatters Ltd, Auckland, New Zealand). Refinements to alignments were performed manually.

Phylogenetic analyses of the sequence data for the three loci were performed individually with maximum likelihood (ML) using dnaml from the Phylip v. 3.69 suite of programs (Felsenstein, 1989). Data sets were bootstrapped with 1000 replicates to generate branch confidence values, and bootstrap values <85% were omitted. Gaps were treated as missing data, and no outgroup was applied. Additionally, maximum parsimony (MP) trees were constructed to confirm tree topologies using PAUP* edition 4.0b10 (Swofford, 1998). Here, nodal support was tested by bootstrap analyses (1000 replicates). ML and MP trees were visualized using FigTree v.1.3.1. (http://tree.bio.ed.ac.uk/). In parallel, we performed ML (using dnaml) and Bayesian inference analyses using MrBayes (Huelsenbeck and Ronquist, 2001) for the concatenated sequences. Conflict among loci was tested both by creating trees from each locus separately and by comparing the likelihood of separate clades for the Kenyan S isolates and for isolates of *A. minisclerotigenes* for each locus and for the concatenated data using the usertree option of dnaml in PHYLIP.

Sequence analyses of norB and cypA

To determine size and position of deletions in the chromosome region containing the *norB* and *cypA* genes, approximately 1.8 kb of these genes was amplified by PCR using the primer set AP1729 and AP3551 (Ehrlich *et al.*, 2004). Because amplification was unsuccessful for all isolates in the phylogenetic clade that contained the Kenyan isolates, two PCR primers (CP-R: GGCGGCCCCTCAGCAAACAT; CP-5F: GGGACCCTTTTCCGGTGCGG), spanning approximately 3.1 kb and specific for the Kenyan S strain isolates were designed. Amplification was conducted at an annealing temperature of 62 °C. PCR and sequencing of the resulting amplicons was conducted as described above. Multiple sequence alignments for S strain isolates that produce only B aflatoxins from the Americas, Southeast Asia, Australia (isolate 1-22) and Africa were created and subsequently aligned with the sequence for an annotated *A. parasiticus* isolate (GenBank accession number AY371490, http://www.ncbi.nlm.nih.gov/genbank/). Alignments were adjusted manually to ensure proper determination of the size and position of deletions involving portions of cypA for all isolates that produce only B aflatoxins (Figure 5.2).

Table 5.1 *Aspergillus* section *Flavi* isolates used in the current study

Isolate	Assignments	Origin	Source	AF	Gap (kb)
S morphotype					
1-22	1, 2	Australia	Soil	B	2.2
4-2	1, 2	Australia	Soil	B,G	0.0
12-4	1, 2	Australia	Soil	B,G	0.0
13-4	1, 2	Australia	Soil	B,G	0.0
L1A3	6	Asia, Philippines	Soil	B	0.9
L1D2	6	Asia, Philippines	Soil	B	0.9
L2E1	6	Asia, Philippines	Soil	B	0.9
MIN I C4	6	Asia, Philippines	Soil	B	0.9
V2D2	6	Asia, Philippines	Soil	B	0.9
Sukkothai19	3	Asia, Thailand	Soil	B	0.9
Sanpatong22	3	Asia, Thailand	Soil	B	0.9
Ubon3	3	Asia, Thailand	Soil	B	0.9
Yuin 20	3	Asia, Thailand	Soil	B	0.9
K04 771-B	5	Africa, Kenya	Maize	B	2.2
K04 854-I	5	Africa, Kenya	Maize	B	2.2
K04 921-E	5	Africa, Kenya	Maize	B	2.2
K05 109-B	5	Africa, Kenya	Maize	B	2.2
K05 153-G	5	Africa, Kenya	Maize	B	2.2
K05 206-J	5	Africa, Kenya	Maize	B	2.2
K05 211-A	5	Africa, Kenya	Soil	B	2.2
K05 212-O	5	Africa, Kenya	Maize	B	2.2
K05 258-I	5	Africa, Kenya	Maize	B	2.2
K05 273-H	5	Africa, Kenya	Soil	B	2.2
K05 286-N	5	Africa, Kenya	Maize	B	2.2
K05 288-F	5	Africa, Kenya	Soil	B	2.2
K05 289-K	5	Africa, Kenya	Maize	B	2.2
K05 289-M	5	Africa, Kenya	Maize	B	2.2
K05 28-A	5	Africa, Kenya	Maize	B	2.2
K05 315-K	5	Africa, Kenya	Maize	B	2.2
K05 37-K	5	Africa, Kenya	Maize	B	2.2
K05 46-I	5	Africa, Kenya	Maize	B	2.2
K05 50-K	5	Africa, Kenya	Maize	B	2.2
K05 94b-N	5	Africa, Kenya	Maize	B	2.2
K05 9-I	5	Africa, Kenya	Maize	B	2.2

Table 5.1 – *continued*

K05 9-M	5	Africa, Kenya	Maize	B	2.2
K06 108-H	5	Africa, Kenya	Maize	B	2.2
K06 108-L	5	Africa, Kenya	Maize	B	2.2
K06 44-K	5	Africa, Kenya	Maize	B	2.2
K06 46-E	5	Africa, Kenya	Maize	B	2.2
K06 56-A	5	Africa, Kenya	Maize	B	2.2
K06 57-L	5	Africa, Kenya	Maize	B	2.2
K06 58-A	5	Africa, Kenya	Maize	B	2.2
3251	7	North America, USA	Walnut	B	1.5
AF12	4	North America, USA	Soil	B	1.5
AF42	4	North America, USA	Soil	B	1.5
AF70	4	North America, USA	Soil	B	1.5
AL1-17	6	North America, USA	Soil	B	1.5
AL3-22	6	North America, USA	Soil	B	1.5
MISS2-5	6	North America, USA	Soil	B	1.5
MISS4-4	6	North America, USA	Soil	B	1.5
STV20-6	6	North America, USA	Soil	B	1.5
STV4-28	6	North America, USA	Soil	B	1.5
TX A10-A	6	North America, USA	Soil	B	1.5
TX A35-K	6	North America, USA	Soil	B	0.9
TX C.C. 1-3-J	6	North America, USA	Soil	B	1.5
TX D7-D	6	North America, USA	Soil	B	1.5
TX EC08 BY1-J	6	North America, USA	Maize	B	1.5
TX LaFeria 2-F	6	North America, USA	Soil	B	2.2
TX04 A5-B	6	North America, USA	Soil	B	2.2
TX06 CB 36-B	6	North America, USA	Maize	B	1.5
TX06 CB 9-G	6	North America, USA	Maize	B	0.9
TX07 CB 73-I	6	North America, USA	Maize	B	2.2
TX08 ECBYI-G	6	North America, USA	Maize	B	1.5
TX08 ECD06-H	6	North America, USA	Maize	B	1.5
LLF29	n/a	South America, Argentina	Peanut	B	2.2
LLF37	8	South America, Argentina	Peanut	B,G	0.0
TAR3N43	n/a	South America, Argentina	Peanut	B,G	0.0
TAR4N30	n/a	South America, Argentina	Peanut	B,G	0.0
A-11611	1, 2, 7	Africa, Nigeria	Soil	B,G	0.0
A-11612	1, 7	Africa, Nigeria	Soil	B,G	0.0

Table 5.1- *continued*

Other					
NRRL2999	*parasiticus*	Africa, Uganda	Peanut	B,G	0.0
BN009-E	*parasiticus*	Africa, Benin	Soil	B,G	0.0
174	*bombycis*	South America, Brazil	Brazil Nut	B,G	0.0
NRRL 13137	*nomius*	North America, USA	Wheat	B,G	0.0

Assignments:

1. Placed in *Aspergillus flavus* group II composed of isolates with S strain morphology and mostly B & G aflatoxin production by Geiser, et al. (2000);

2. Placed in *A. minisclerotigenes* during initial description of taxon by Pildain, et al. (2008);

3. Placed in *A. flavus* based on multigene phylogenetic analyses by Ehrlich, et al. (2007b);

4. Placed in the S strain morphotype of *A. flavus* by Cotty (1989);

5. Placed in the S strain of *A. flavus* by Probst, et al. (2007, 2010);

6. Placed in the S strain morphotype of *A. flavus* during previous unpublished work in the Cotty lab;

7. Placed in a new unnamed taxon composed of just NRRL 3251, A-11611, and A-11612 based on S strain morphology by Hesseltine, et al. (1970);

8. Placed in *A. flavus* based on S strain morphology and mycotoxin production by Pildain, et al. (2004); n/a, not available, isolates were kindly provided by M.B. Pildain.

AF = Aflatoxin production

Gap = refers to the size of the sequence deletion in the *cypA* gene of the aflatoxin biosynthesis cluster. Loss of *cypA* expression causes loss of G aflatoxin production in *A. flavus* (Ehrlich, et al. 2004).

Table 5.2 Primers used for phylogenetic reconstructions

Gene	Forward primer	Reverse primer	T_a (°C)	Product (bp)	Use
cmd	cmd42: GGCCTTCTCCCTATTCGTAA	cmd637: CTCGCGGATCATCTCATC	56	613	PCR, sequencing
cmd	cmd2F: GGCTGGATGTGTGTAAATC	cmd2R: ATTGGTCGCATTTGAAGGG	48	811	PCR, sequencing
niaD	niaDF: CGGACGATAAGCAACAACAC	niaDAR: GGATGAACACCCGTTAATCTG	52	795	PCR, sequencing
niaD	niaDBF: ACGGCCGACAGAAGTGCTGA	niaDBR: GGTCCAGGGCCCAGTTCA AT	57	741	PCR, sequencing
aflR	aflR1F: CATGGCTGAGGATAGCTCGTG	aflR2R: ACGGTGGCGGGACTGTTGCTACA	60	2092	PCR only
aflR	aflR1F: CATGGCTGAGGATAGCTCGTG	aflR1R:TCG TGG AGG TGA GGA AGG AAT	--	825	Sequencing only
aflR	aflRF: GGAAACAAGTCTTTTCTGG	aflRR: CAG AGC GTG TGG TGG TTG	--	779	Sequencing only
aflR	aflr2F: GACTTCCGGCGCATAACACGTA	aflr2R: ACG GTG GCG GGA CTG TTG CTA	--	745	Sequencing only

T_a = Annealing temperature;

cmd = calmodulin;

niaD = nitrate reductase;

aflR = aflatoxin pathway regulator gene

Results

Aflatoxin production

Ability of the isolates to produce only aflatoxins B or aflatoxins B and G is illustrated in Table 5.1. All S strain isolates from North America, Southeast Asia and Kenya produced only aflatoxins B when inoculated on maize. Isolates from West Africa (A-11611, A-11612), as well as three out of four isolates from Argentina (TAR3N43, TAR4N30, LLF37) and Australia (4-22, 12-4, and 13-4) produced both aflatoxins B and G (Table 5.1), as previously reported by Hesseltine et al. (1970), Pildain *et al.* (2004) and Geiser *et al.* (2000).

Phylogenetic relationships among the isolates

Sequencing of the three loci resulted in a total of 4063 unambiguously aligned characters: 1627 bp for *aflR*, 1119 bp for *cmd* and 1317 bp for *niaD*. The concatenated tree with the support values is given in Figure 5.1. ML and MP for the individual sequences yielded highly similar topologies for *aflR* and *niaD*, and after removing weakly supported clades (bootstrap support ≤ 85%), there were no inconsistencies in topology. Phylogenetic analysis of the *cmd* region yielded similar groupings but with moderate bootstrap support (some groups in the *aflR* and *niaD* trees were supported with bootstrap support at 75%). S strain isolates that produce only B aflatoxins were split into two distinct lineages: one contained *A. flavus* S strain isolates from North America and Asia, whereas the other, a previously undescribed lineage, contained isolates with S strain morphology from Kenya, Australia (isolate 1-22), Argentina

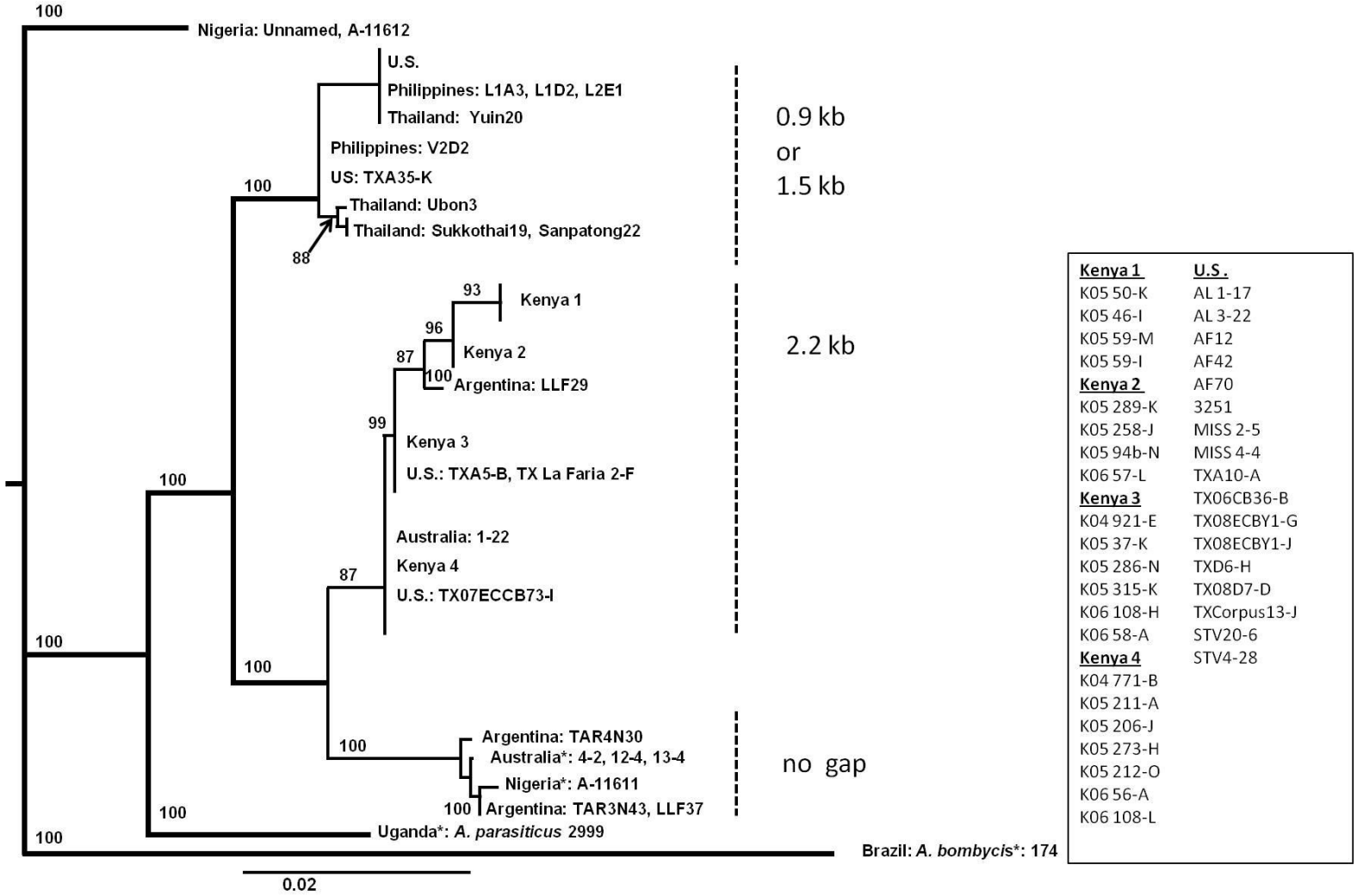
(isolate LLF29) and Texas (isolates TX CB73-I, TX04 A5-B, and TX LaFeria2-F). Isolates previously identified as *A. minisclerotigenes* that produce both aflatoxins B and G (isolates A-11611, 4-2, 12-4, 13-4, TAR4N30, TAR3N-43, LLF37) formed a sister clade to the new lineage of S strains, and these two groups formed a sister clade to the *A. flavus* S strain isolates (Figure 5.1). Groups had strong bootstrap support in ML and MP analyses. Concordance among loci of the major groups within the concatenated phylogeny was also tested by comparing the likelihood of the new lineage of Kenyan S strains and *A. minisclerotigenes* comingling in a single clade. The two clade structure was significantly more likely for the concatenated data ($P = 0.000$) and for *aflR* ($P = 0.000$). For *niaD* the two clade structure was more likely than a single mixed clade, but not significantly so ($P = 0.132$). For *cmd*, both results were equally likely (data not shown).

Deletions in the cypA/norB region

The protein encoded by the *cypA* gene is required for aflatoxin G production and partial deletions of *cypA* are the reason *A. flavus* lacks capacity to produce G aflatoxins (Ehrlich *et al.*, 2004). Alignment of sequences from isolates originating in the Americas, Africa, Southeast Asia and Australia with the *A. parasiticus* reference isolate AY371490 revealed that each isolate capable of producing only B aflatoxins had one of three deletions in the *cypA*/*norB* region. These deletions were 0.9 kb, 1.5 kb and 2.2 kb in size (Figure 5.2). Both the 0.9 kb and 1.5 kb deletions, previously described for isolates of *A. flavus* and *A. oryzae* (Ehrlich *et al.*, 2004), were detected in S strain isolates from North America and Southeast Asia. None of the S strain isolates

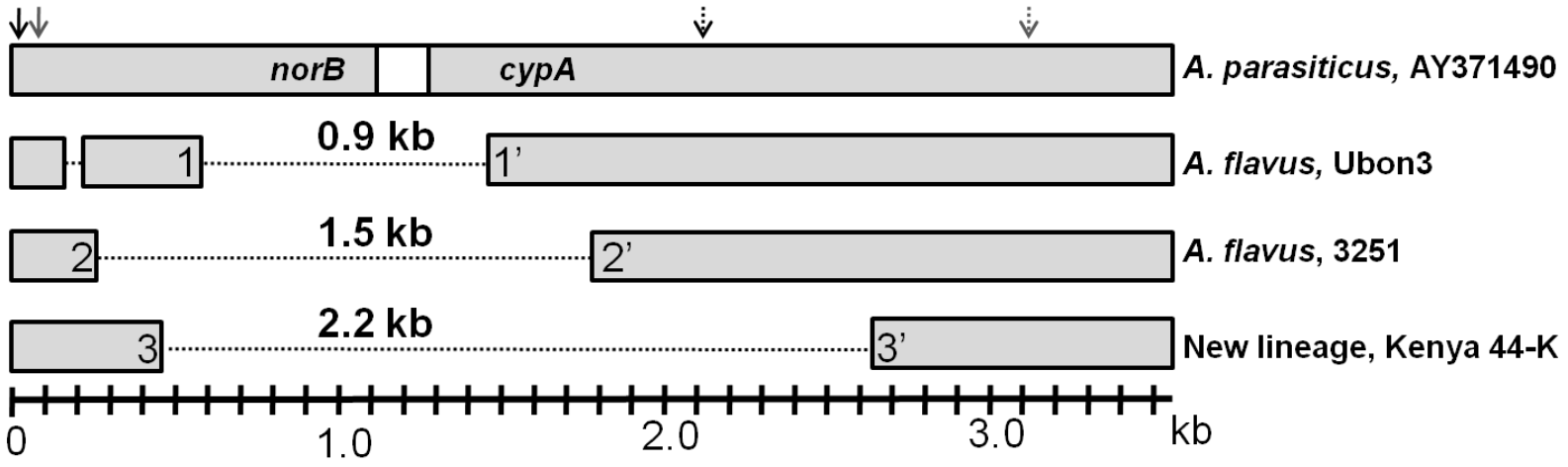
from these areas had the 2.2 kb deletion, which has not been previously reported. All S strain isolates from Kenya contained the 2.2 kb deletion as did isolate 1-22 from Australia, isolate LLF29 from Argentina and three isolates from Texas, U.S. (isolates TX07 CB73-I, TX04 A5-B, and TX LaFeria 2-F). This large sequence deletion starts approximately 0.5 kb downstream of the reverse primer AP1729 extending into the *cypA* gene and deleting the region where forward primer AP3551 is located (Figure 5.2). The newly designed primers (CP-R, CP-5F) span a 3.1 kb region of AY371490 making these primers useful in detecting this large deletion. Isolates exhibiting the 2.2 kb sequence deletion form a well supported monophyletic clade in phylogenetic trees based on concatenated partial gene sequences from the *aflR*, *niaD*, and *cmd* genes (Figure 5.1). Sequence analysis of the aflatoxin B and G producing isolates from Argentina, Australia and Nigeria confirmed that these isolates align to the full length *cypA*/ *norB* region of reference isolate SRRC143 without deletions. These isolates can all be placed by phylogenetics into either to *A. minisclerotigenes* (isolates A-11611, 4-2, 12-4, 13-4, TAR4N30, TAR3N-43, LLF37) or the unnamed taxon S_{BG} (isolate A-11612).

Figure 5.1 Midpoint rooted maximum likelihood tree showing the relationships between putative S strain isolates from Africa, Asia, Australia, and the Americas based on the concatenated *aflR, cmd,* and *niaD* DNA sequences (4.06 kb total sequence). Numbers above nodes represent bootstrap support > 85% from 1,000 replicates. Nodes supported at 75% in all individual ML trees are double thick. A star (*) after the country name indicates production of both B and G aflatoxins.



Kenya 1	U.S.
K05 50-K	AL 1-17
K05 46-I	AL 3-22
K05 59-M	AF12
K05 59-I	AF42
Kenya 2	AF70
K05 289-K	3251
K05 258-J	MISS 2-5
K05 94b-N	MISS 4-4
K06 57-L	TXA10-A
Kenya 3	TX06CB36-B
K04 921-E	TX08ECBY1-G
K05 37-K	TX08ECBY1-J
K05 286-N	TXD6-H
K05 315-K	TX08D7-D
K06 108-H	TXCorpus13-J
K06 58-A	STV20-6
Kenya 4	STV4-28
K04 771-B	
K05 211-A	
K05 206-J	
K05 273-H	
K05 212-O	
K06 56-A	
K06 108-L	

Figure 5.2 Schematic illustration of the *norB/ cypA* sequences of aflatoxin B (isolates Ubon3, 3251, K44-K) producing *Aspergillus* species. Flanking regions of the deletion are indicated in the box. Size and position of the sequence deletions was determined by sequence comparison with the annotated *A. parasiticus* reference isolate AY371490. Arrows indicate position of primers (Black solid arrow: primer AP1729, black dotted arrow: primer AP3551; grey solid arrow: primer CP-R, grey dotted arrow: primer CP5-F). Coding regions of the genes are presented as solid-grey and non coding regions as white bars.



1: -TTTGCCTGG……1': TCTGAGAAA-
2: -CACTTGCAA……2': TACGGTCAC-
3: -GACACATGG……...3': GAAGACCTT-

Discussion

Previously we used culture based approaches to describe and monitor community structures of aflatoxin-producing and closely related fungi on maize cultivated in the Eastern, Rift Valley and Coast Provinces of Kenya from 2004 to 2006 (Probst *et al.*, 2010). Isolates with the S strain phenotype were identified as the causal agent of the human aflatoxicosis outbreak in Kenya that caused over 100 fatalities in 2004 (Probst *et al.*, 2007). These isolates were originally recognized as members of *A. flavus*. However, phylogenetic analyses in the current study showed that the Kenyan S strain isolates form a clade distinct from the North American and Asian *A. flavus* isolates with S strain morphology. The isolates from Kenya are more closely related to the B and G aflatoxin producing species *A. minisclerotigenes*. These isolates, which are genetically divergent from other isolates with S strain morphology, are endemic in some districts of the Eastern Province of Kenya. In the southern Eastern Province, this new lineage dominates fungal communities on maize, causing great losses due to high levels of aflatoxin contamination (Probst *et al.*, 2010).

Additionally, a 2.2 kb sequence deletion has been identified as a characteristic of the unnamed Kenyan species with S strain morphology. This deletion is distinct from sequence deletions previously described from other aflatoxin B producing (e.g. *A. flavus*) or atoxigenic (e.g. *A. oryzae*) fungi. Isolates with S strain morphology that produce only B aflatoxins and with genetic similarities to the Kenya S strain isolates were detected from diverse regions of the world. Several isolates from Australia (isolate 1-22), Argentina (isolate LLF29), and the U.S. (isolates TX07 CB73-I, TX04

A5-B, and TX LaFeria 2-F) also exhibited the 2.2 kb deletion and were included in the lineage that contained all the B aflatoxins producing S strain isolates from Kenya. However, from the literature, it is clear that in none of these regions the new lineage dominates in a manner similar to that observed in the Eastern Province of Kenya (Geiser *et al.*, 2000; Probst *et al.*, 2007; Pildain *et al.*, 2008; Probst *et al.*, 2010). Both the geographic region of origin and the pattern of distribution and migration of this newly recognized lineage are currently unresolved. Deletions causing inactivation of *cypA*, a gene found near the distal terminus of the aflatoxin biosynthesis gene cluster, cause the inability of *A. flavus* to produce G aflatoxins (Ehrlich *et al.*, 2004). One of two distinct sequence deletions in *A. flavus* L and S strains (0.8 kb and 1.5 kb, respectively) were determined to be responsible for the inactivation of the gene. In our analyses, both deletions were detected in the *A. flavus* S strain morphotype. Indeed, all examined S strain isolates from Southeast Asia carry the smaller sequence deletion previously associated with only the L strain. This may indicate that either the *A. flavus* L or S strain morphotypes evolved multiple times after the two deletions had formed. However, our study did not find *A. flavus* S strain isolates carrying sequence deletions of sizes other than the two previously described. This is in contrast to the new lineage of S strain isolates that formed a highly supported monophyletic group and for which all members contained a previously unreported 2.2 kb sequence deletion.

Hawksworth *et al*. (1995) defined a ‘morphotype’ as a ‘group of morphologically differentiated individuals of a species of unknown or of no taxonomic significance’ (Hawksworth *et al.*, 1995). Our current and previous studies showed that

Aspergillus sect. *Flavi* contains phylogenetically diverse members with S strain morphology and that these fungi can be assigned to at least four distinct phylogenetic groups. The best example is given with the three originally described S strain isolates (Hesseltine *et al.*, 1970) which are now characterized as three separate fungal species (isolate 3251, *A. flavus* (Egel *et al.*, 1994); isolate A-11611 , *A. minisclerotigenes* (Pildain *et al.*, 2008); and isolate A-11612, the West African unnamed taxon S_{BG} (Egel *et al.*, 1994)). Thus, the S strain morphotype is useful in that it identifies groups of fungi that uniformly produce very high concentrations of aflatoxins. However, the S strain morphotype does not correspond to a single monophyletic group. Instead, this morphotype is shared by several morphologically similar but phylogenetically divergent taxa. In the case of the new lineage of isolates with S strain morphotype endemic in Kenya, this distinct phylogenetic group of fungi has considerable biological significance since those isolates dominate the fungal community structure on maize associated with the most significant aflatoxin contamination events reported to date(Probst *et al.*, 2007; Probst *et al.*, 2010).

The causes for dominance of the new lineage of S strain isolates on maize in specific districts of the Eastern Province of Kenya are unknown. However, in other regions, communities of *Aspergillus* section *Flavi* are deliberately modified to reduce the average aflatoxin-producing potential and thereby decrease crop aflatoxin content. Such aflatoxin management strategies have been successfully used in commercial production of several crops, including maize, in North America (Cotty *et al.*, 2007) and West Africa (Atehnkeng *et al.*, 2010). Similar biological control strategies utilizing

native atoxigenic isolates of the *A. flavus* L strain have been suggested for aflatoxin management in maize grown in Kenya (Probst *et al.*, 2011). Together with ongoing programs for better post harvest handling and storage, biocontrol with atoxigenic *A. flavus* may provide sufficient aflatoxin reductions to finally eliminate human aflatoxicosis in the Eastern Province of Kenya.

Members of *Aspergillus* section *Flavi* with S strain morphology caused the severe aflatoxin contamination events that led to lethal aflatoxicosis in Kenya. The current study identified these fungi as phylogenetically distinct from other S strain isolates. This unnamed taxon is endemic in several maize growing districts of the Eastern Province of Kenya and can be clearly distinguished from *A. flavus* and taxa that produce B and G aflatoxins by sequence comparison of the *cypA* locus. Although several isolates phylogenetically similar to the new lineage of S strain isolates were detected outside Africa, incidences of these fungi outside Kenya were low. The distribution of these highly toxic fungi across the globe has not yet been investigated.

CHAPTER 6- DIVERSITY OF AFLATOXIN-PRODUCING FUNGI ON MAIZE IN SUB-SAHARAN AFRICA

Abstract

Crops frequently contaminated by aflatoxins are the only source of revenue and daily nourishment in many portions of sub-Saharan Africa. Aflatoxins are highly carcinogenic metabolites mainly produced by members of *Aspergillus* sect. *Flavi*. The current study examined aflatoxin-producing fungi associated with maize grain produced in 18 sub-Saharan African countries. 4,469 *Aspergillus* sect. *Flavi* isolates were obtained from 339 samples. The majority (75%) of isolates belonged to the L strain morphotype of *A. flavus*. A minor percentage of fungi were *A. tamarii* (6%), *A. parasiticus* (1%), and isolates with S strain morphology (2.6%). Isolates with S strain morphology that produced only B aflatoxins were divided with into two lineages: those with either a 0.9 or 1.5 kb deletion in the *cypA*/ *norB* aflatoxin biosynthesis genes and those with a 2.2 kb deletion. S strain isolates were assigned to one of four taxa based on aflatoxin production, phylogenetic, and sequence deletion data: *A. flavus* (0.02%), *A. minisclerotigenes* (0.4%), the West African strain S_{BG} (0.8%), and a distinct lineage of aflatoxin B producing fungi previously associated with aflatoxin contamination of maize in Kenya (1.5%). There were differences among the taxa with S strain

morphology in distribution with strain S_{BG} limited to West Africa and both *A. minisclerotigenes* and the Kenya lineage distributed in Central and East Africa. Phylogenetic analyses of partial sequences from the nitrate reductase (*niaD*, 1.3 kb) and aflatoxin pathway transcription factor (*aflR*, 1.7 kb) genes were conducted to verify isolate assignments into species and lineages. African *A. flavus* L strain isolates formed a clade with L strain isolates from other continents. No *A. bombycis* or *A. nomius* isolates were identified. Commercial ELISA kits were used to quantify mycotoxin (fumonisin, aflatoxin, deoxynivalenol) levels in maize. Most samples tested positive for either fumonisins (81%), aflatoxins (65%), or deoxynivalenol (40%) indicating that fungi capable of producing the respective toxins are widely distributed on maize in Africa. The percentage of samples exceeding regulatory limits for total fumonisins, aflatoxins, and deoxynivalenol were 49%, 47%, 4%, respectively.

Introduction

Maize is a staple crop in Africa and is grown and eaten throughout the continent (McCann, 2005). Aflatoxin contamination of maize nearly always results in diminished crop value and increased health risks for both domestic animals and humans. Aflatoxins are highly toxic secondary metabolites with aflatoxin B_1 listed by the International Agency for Research on Cancer (IARC) as carcinogenic to humans (Group 1) (International Agency for Research on Cancer, 2002). The risks of human aflatoxicosis is high in countries, such as many countries in Africa, where strict aflatoxin regulations are not in force and crops are consumed without being inspected for the detrimental toxins (Williams *et al.*, 2004). Impaired growth in children (Gong *et al.*, 2004), immune system modifications (Turner *et al.*, 2003), and severe liver damage (Turner *et al.*, 2000) are some of the long-term health consequences of chronic dietary aflatoxin exposure.

The composition of the fungal community structure present during both early crop development and maturation heavily influences the severity of aflatoxin contamination (Cotty *et al.*, 2008). The contamination process is complex and starts in the field where during early developmental growth stages crops become infected by *Aspergilli* that reside in the soil and on decaying plant residues. Plant stress (e.g. physiological stress, insect damage) and an environment conducive for fungal growth (e.g. hot and arid conditions) will increase the susceptibility of crops to fungal infections (Cotty *et al.*, 1994b; Cotty *et al.*, 2008). These timely stages of fungal infection lead to an early onset of aflatoxin contamination. A later phase of

contamination occurs after crop maturation. Here, high temperatures and humidity, both in the field and during storage, are in particular contributing to fungal growth and aflatoxin development. Aflatoxin levels may increase and new infections become established until crops are finally consumed (Cotty *et al.*, 1994b). In order to estimate the risks associated with the communities of aflatoxin-producing fungi that are resident on maize both the aflatoxin-producing potential of fungi present and frequencies with which they occur need to be considered (Probst, Chapter 4).

The majority of aflatoxin-producing are members of *Aspergillus* sect. *Flavi* and known to vary widely in many characteristics for example in their ability to produce aflatoxins and to infect and decay crops. *A. flavus* is most frequently found in nature and has been implicated as the major contaminant of crops (Klich, 2007). The species can be delineated into two major morphotypes, the L strain morphotype (produces copious amounts of conidia and no or few, large sclerotia, diameter > 400 µm) and the S strains (produces few conidia and copious amounts of small sclerotia, diameter < 400 µm) (Cotty, 1989). On average, isolates of the L strain morphotype produce less aflatoxins than isolates of the S strain morphotype (Cotty, 1989).

In recent years, the number of described aflatoxin-producing fungi has increased due to advances in molecular techniques (Feibelman *et al.*, 1998; Ito *et al.*, 2001; Peterson *et al.*, 2001; Ehrlich *et al.*, 2002; Pildain *et al.*, 2008) . However, the importance of these newly described species in natural contamination events remains unclear. Since the outbreak of acute human aflatoxicosis in Kenya in 2004 (Centers for Disease Control and Prevention, 2004), aflatoxin contamination of food crops in Africa

has received increased attention (Integrated Regional Information Networks, 2010; Probst *et al.*, 2010; Atser, 2011). Research on the fungal community structure on maize in Kenya demonstrated the existence of a putative new fungal lineage (Probst et al., Chapter 5), originally described as the S strain of *Aspergillus flavus*, to be the causal agent of contamination in the aflatoxicosis affected Kenyan districts (Probst *et al.*, 2007). In culture, this lineage, in which all members produce only aflatoxins B and have a 2.2 kb sequence deletion in the aflatoxin biosynthesis gene *cypA*, is morphologically indistinguishable from the S strain morphotype of *A. flavus* as well as the aflatoxin B and G producing species *A. minisclerotigenes*, and a group of unnamed *Aspergilli* found in West Africa (commonly referred to as strain S_{BG}) (Probst et al., Chapter 5).

Studies on the occurrence of aflatoxin-producing fungi in Sub-Saharan Africa are limited to few countries (Cotty and Cardwell, 1999; Kaaya and Kyamuhangire, 2006; Bandyopadhyay *et al.*, 2007; Atehnkeng *et al.*, 2008a; Essono *et al.*, 2009; Probst *et al.*, 2010). A detailed comparison of fungal communities found on a specific crop and aflatoxin exposure in most African countries are lacking. Regional co-occurrences of strain S_{BG} and the new lineage found in Kenya have not been reported yet which raises the question about geographic boundaries of these fungi.

The objectives of the current study were: i) to identify and characterize *Aspergillus* sect. *Flavi* communities endemic on maize in eighteen countries in Sub-Saharan Africa, ii) to assess their relationships to described aflatoxin-producers from other continents, iii) to assess incidences and regional occurrences of aflatoxin-

producing fungi with small sclerotial (S strain) morphology, and iv) to determine mycotoxin exposure from maize (aflatoxins, fumonisins, deoxynivalenol).

Material and Methods

Maize samples

Maize samples were collected in Burkina Faso (n = 50), Cameroon (n =16), DR Congo (n = 22), Ethiopia (n =81), Ghana (n =7), Ivory Coast (n = 4), Kenya (n = 22), Malawi (n = 9), Mali (n = 7), Mozambique (n = 42), Rwanda (n = 16), Senegal (n = 20), Sierra-Leone (n = 17), Somalia (n = 21), Tanzania (n = 5), Uganda (n = 17), Zambia (n = 28) and Zimbabwe (n = 19) during 2006 and 2007. To ensure grains were produced locally, maize grain was sampled from individual fields of farmers or small local markets. Ideally, a 1 to 2 kg sample from a single location was obtained, mixed thoroughly and a 100 to 200 g subsample was imported to the United States Department of Agriculture (USDA), Agricultural Research Service (ARS), at the University of Arizona, Tucson under permits issued by the USDA Animal and Plant Health Inspection Service (APHIS). Upon arrival, maize samples were weighed, analyzed for moisture content (HB43 Halogen Moisture Analyzer; Mettler Toledo, Columbus, OH), dried to under 8% moisture in a forced air drying oven (40 °C) if necessary, and stored for up to 4 weeks at 4 °C prior to being subjected to further analyses. The maize was finely ground in a laboratory hammer mill (IKA Labortechnik, Heitersheim, Germany), and fungal isolates were recovered by dilution plate technique on modified rose Bengal agar, as described below.

Fungal isolation and quantification

The *Aspergillus section Flavi* communities were characterized on all maize samples. *Aspergillus* sect. *Flavi* colonies were obtained by dilution plate technique on modified rose Bengal agar (MRBA, Cotty, 1994a). Ground maize (about 1 g) was suspended in 15-ml test tubes containing 5 ml sterile-distilled water and mixed by inverting for approximately 20 min. Aliquots (100 µl per plate) of the resulting suspension were spread on modified rose Bengal agar plates (n = 3) and incubated for 3 days at 31 °C in the dark. At the end of incubation, *Aspergillus* sect. *Flavi* colonies were microscopically identified and enumerated [Colony Forming Units (CFU)/ g]. Up to 10 discrete colonies per isolation were aseptically transferred to 5/2-agar plates (5% V8-juice, 2% agar, and pH 5.2), incubated for 5–7 days at 31 °C in the dark, and identified to species and, if applicable, strain on the basis of colony, spore and sclerotia morphologies (Klich and Pitt, 1988; Cotty, 1989; Ito *et al.*, 2001; Peterson *et al.*, 2001; Probst *et al.*, 2011). Fungal isolations were conducted at least twice for each sample to yield a total of 15 to 20 isolates. Representative isolates for each country and morphology type were subjected to DNA based phylogenetic analyses (below) to verify assignments made on morphological criteria.

In vitro aflatoxin production

The ability to produce aflatoxins was determined for the same isolates subjected to phylogenetic analyses. These assays determined just the ability of the isolate to produce either: no aflatoxins (limit of detection 1 ppb), B and G aflatoxins, or only B

aflatoxins (Table 6.1). Aflatoxin assays on autoclaved maize were conducted exactly as described previously (Probst *et al.*, 2011).

Mycotoxin extraction and analyses

Enzyme-linked immunosorbend assay (ELISA) tests were performed to assess maize aflatoxins, fumonisins and deoxynivalenol (DON) concentrations. The test kits (Strategic Diagnostics, Inc.,Newark, DE; MycoChek® Test Kit) are certified by the USDA, Grain Inspection, Packers and Stockyards Administration (GIPSA). Mycotoxins were extracted from their maize matrix following manufacturer's instructions with 70% methanol (for aflatoxins and fumonisins) or water (for DON) by blending 50 g ground maize with 250 ml of the appropriate solution. The slurry was shaken on a rotary shaker for 2 min and allowed to settle for an additional 2 min. The supernatant was filtered through Whatman No. 1 (Whatman, Piscataway, NJ) filter paper and immediately subjected to competitive ELISA analyses according to the manufacture's instructions (http://www.sdix.com). Mycotoxins were quantified by measuring the developing color intensity with a microplate reader (ELX800, Biotek Instruments Inc, Winooski, VT) at 650 nm. Aflatoxin concentrations were extrapolated from standard curves generated for each microplate.

Molecular analyses and phylogeny

Phylogenetic reconstructions using 1536 bp sequence data from the nitrate reductase gene (*niaD*, on chromosome 4) were performed to verify that assignment of isolates derived from maize grown in Africa to specific *Aspergillus* species was correct

and consistent with those from other continents. In general, three *Aspergillus flavus* L strain isolates were randomly chosen from each African country. This set was supplemented with isolates of *A. parasiticus, A. tamarii,* isolates displaying S strain morphology producing either either B and G aflatoxins or only B aflatoxins. *Aspergillus* isolates from the Americas (USA and Argentina), Australia, Asia and Europe, including reference isolates for each species (*A. flavus* S strain isolates AF70, AF42, *A. flavus* L strain isolates AF13 and 3251; *A. bombycis* isolate 174; *A. minisclerotigenes* isolates A-11611, 4-2, 12-4, and 13-4; *A. parasiticus* isolates 2999 and BNO09-E; West African strain S_{BG} isolates A-11612, BNO38-G and BNO40-B; and Kenyan strain S_B isolate K06 44-K) were included in the analyses as isolates with known affiliation.

Isolates were grown and were collected as previously described (Probst, Chapter 5). DNA was isolated using the FastDNA SPIN Kit and the FastPrep Instrument following manufacturer's instructions (MP Biomedicals LLC, Santa Ana, CA). DNA was quantified with a spectrophotometer (model ND-1000, NanoDrop) and diluted to a final concentration of 5-10 ng/µl before conducting PCR. Approximately 1.3 kb of the nitrogen reductase gene (*niaD*, on chromosome 4) was amplified with two sets of primers (Table 6.1). The gene for the aflatoxin pathway transcription factor (*aflR* on chromosome 3) was amplified with one set of primers (aflR1F-aflR2R) covering approximately 2.1 kb of the gene. Segments of the 2.1 kb *aflR* gene amplicon were sequenced using three pairs of sequencing primers each covering approximately 0.7 kb of the amplified PCR product (Table 6.2). PCR reactions were performed in a

20 µl volume using 2 µl genomic DNA and the PCR PreMix (AccuPower® HotStart) with the following protocol: 1 cycle of 5 min at 95 °C followed by 38 cycles of 20 s at 94 °C (denaturalization), 30 s of locus-specific annealing temperature (Table 6.2), 30 s at 72 °C (extension), and finally, 1 cycle of 10 min at 72 °C. Amplicons were sequenced in the University of Arizona Genetics Core sequencing facility (UAGC, Tucson, AZ). Consensus sequences were created by assembly of 2 reads (reverse and forward) per amplicon per gene, corrected manually, and aligned using the MUSCLE algorithm with the default settings in Geneious Pro Version 4.8.2. (Biomatters Ltd, Auckland, New Zealand).

Maximum likelihood (ML) analyses were conducted using Phylip Version 3.69 (Felsenstein, 1989). Additionally, maximum parsimony (MP) trees were generated using PAUP* edition 4.0b10 (Swofford, 1998) to confirm tree topologies. To evaluate the support for inferred topologies, both analyses were bootstrapped with 1000 replicates and branches with less than 85% support were collapsed. Gaps were treated as missing data and no outgroup was applied. Trees were mid-point rooted and visualized with FigTree v.1.3.1. (http://tree.bio.ed.ac.uk/).

Determination of sequence deletions in the cypA gene

Sequence deletions of various sizes have been described for *A. flavus* L and S strain isolates (0.9 or 1.5 kb) and the putative new lineage from Kenya (2.2 kb). Deletion patterns in extant aflatoxin B producing species have been recently explored as diagnostic tools for recognition of specific aflatoxin producing lineages (Probst, Chapter 5). PCR was conducted with the primer sets described by Ehrlich *et al*. (2004),

specific for *A. flavus*, and by Probst (Chapter 5) for the dissertation maybe put the chapter number), specific for the putative new lineage (Table 6.2). Molecular sequence data was generated as described for *niaD* and *aflR* above and subsequently aligned with an annotated *A. parasiticus reference* strain (GenBank accession number AY371490). Alignments were corrected manually to determine deletion positions and sizes. Sequences were aligned with those from isolates for which the sequences and deletions were previously described to confirm deletion sizes and positions (Probst, Chapter 5).

Table 6.1 *Aspergillus* sect. *Flavi* isolates used for phylogenetic reconstructions

Continent	Country	Substrate	Assignment[a]	Isolate	Species	Morphotype[b]	Aflatoxins[c]	Deletion (kb)[d]
Africa	Benin	Soil	Group 2b	BN001-B	*A. flavus*	L	none	0.9
			Group 2b	BN011-N	*A. flavus*	L	B	0.9
			Group 5	BN009-E	*A. parasiticus*	n/a	BG	0.0
			Group 1a	BN023-O	*A. tamarii*	n/a	none	0.0
			Group 4	BNO38-G	strain S_{BG}	S	BG	0.0
			Group 4	BNO40-B	strain S_{BG}	S	BG	0.0
	Burkina Faso	Maize	Group 2b	BF1-A	*A. flavus*	L	B	1.5
			Group 2b	BF4-B	*A. flavus*	L	B	0.9
			Group 2b	BF9-G	*A. flavus*	L	B	1.5
			Group 4	BF3-H	strain S_{BG}	S	BG	0.0
			Group 4	BF13-H	strain S_{BG}	S	BG	0.0
			Group 4	BF22-N	strain S_{BG}	S	BG	0.0
			Group 4	BF28-F	strain S_{BG}	S	BG	0.0
			Group 4	BF35-E	strain S_{BG}	S	BG	0.0
			Group 4	BF38-G	strain S_{BG}	S	BG	0.0
			Group 4	BF45-K	strain S_{BG}	S	BG	0.0
	Cameroon	Maize	Group 2a	Cam49-A	*A. flavus*	L	none	1.5
			Group 2b	Cam53-D	*A. flavus*	L	none	0.9
			Group 2b	CamMO-C	*A. flavus*	L	none	1.5
	Congo East	Maize	Group 2a	CONGO E1-E	*A. flavus*	L	none	1.5

Table 6.1 - *continued*

		Group 2a	CONGO E15-A	*A. flavus*	L	none	0.9
		CONGO E5-A	CONGO E5-A	n/a	n/a	BG	0.0
		Group 3b	CONGO E10-X	new lineage, S_B	S	B	2.2
Congo, Bas	Maize	Group 2a	CONGO W7-A	*A. flavus*	L	B	0.9
		Group 2a	CONGO W8-X	*A. flavus*	L	B	0.9
		Group 2a	CONGO W10-A	*A. flavus*	L	B	1.5
		Group 3b	CONGO W8-D	*A. minisclerotigenes*	S	BG	0.0
Ethiopia	Maize	Group 2b	ET48-B	*A. flavus*	L	none	1.5
		Group 2a	ET64-A	*A. flavus*	L	none	1.5
		Group 2a	ET78-D	*A. flavus*	L	B	1.5
		Group 1a	ET58-D	*A. tamarii*	n/a	none	0.0
		ET 72-B	ET72-B	n/a	S	BG	0.0
Ghana	Maize	Group 2b	GH1-C	*A. flavus*	L	B	1.5
		Group 2b	GH2-A	*A. flavus*	L	B	1.5
		Group 2b	GH3-B	*A. flavus*	L	B	1.5
Ivory Coast	Maize	Group 2b	CI2-A	*A. flavus*	L	B	1.5
		Group 2a	CI3-I	*A. flavus*	L	B	0.9
		Group 2a	CI4-B	*A. flavus*	L	B	1.5
Kenya	Maize	Group 3a	K06 4-I	*A. flavus*	L	B	--
		Group 2a	K06 24-D	*A. flavus*	L	none	1.5
		Group 2b	MW6-B	*A. flavus*	L	none	1.5
		Group 2a	MW15-L	*A. flavus*	L	none	1.5
		Rift 2-C	Rift 2-C	n/a	L	BG	0.0
		Group 3b	K06 44-K	new lineage, S_B	S	B	2.2
		Group 3a	K06 56-A	new lineage, S_B	S	B	2.2
		Group 3a	K05 59-M	new lineage, S_B	S	B	2.2

Table 6.1 - *continued*

		Group 3b	K04 921-E	new lineage, S_B	S	B	2.2
		K06 111-D	K06 111-D	n/a	S	BG	0.0
		Group 3b	Rift4-A	*A. minisclerotigenes*	S	BG	0.0
		Group 3b	Rift6-A	*A. minisclerotigenes*	S	BG	0.0
		Group 3b	Rift8-N	*A. minisclerotigenes*	S	BG	0.0
		Group 3a	Rift12-N	new lineage, S_B	S	B	2.2
Malawi	Maize	Group 2a	MW13-B	*A. flavus*	n/a	B	1.5
		Group 5	MW13-L	*A. parasiticus*	n/a	BG	0.0
Mali	Maize	Group 2b	ML4-C	*A. flavus*	L	none	0.9
		Group 2a	ML6-B	*A. flavus*	L	none	1.5
Mozambique	Maize	Group 2b	MZ7-A	*A. flavus*	L	none	1.5
		Group 2a	MZ2-A	*A. flavus*	L	none	1.5
		Group 2a	MZ35-L	*A. flavus*	L	none	1.5
		Group 5	MZ31-L	*A. parasiticus*	n/a	BG	0.0
		Group 3a	MZ17-L	new lineage, S_B	S	B	2.2
Nigeria	Soil	Group 2a	Mi18G-27	*A. flavus*	L	none	1.5
		Group 2a	Lo44G-19	*A. flavus*	L	B	1.5
		Group 2b	Ak29G-01	*A. flavus*	L	B	1.5
		Group 3a	A11611	*A. minisclerotigenes*	S	BG	0.0
		Group 4	A11612	strain S_{BG}	S	BG	0.0
Rwanda	Maize	Group 2b	RW 47-F	*A. flavus*	L	B	1.5
		Group 2b	RW 4-A	*A. flavus*	L	B	0.9
		Group 2b	54-O	*A. flavus*	L	B	0.9
		Group 2a	54-P	*A. flavus*	S	B	0.9
Senegal	Maize	Group 2b	SN 16-D	*A. flavus*	L	none	--
		Group 2b	SN 8-G	*A. flavus*	L	none	--

Table 6.1 - *continued*

		Group 4	SN 11-C	strain S_{BG}	S	BG	0.0
		Group 4	SN 3-A	strain S_{BG}	S	BG	0.0
		Group 4	SN 2-A	strain S_{BG}	S	BG	0.0
Sierra Leone	Maize	Group 2a	SL 3-N	*A. flavus*	L	none	1.5
		Group 2a	SL 14-L	*A. flavus*	L	none	--
		Group 2a	SL 5-H	*A. flavus*	L	none	--
		Group 2b	So 7-A	*A. flavus*	L	none	--
		Group 2b	So 3a-I	*A. flavus*	L	none	0.9
		Group 2b	So 6-J	*A. flavus*	L	none	1.5
		Group 1b	SL 16-P	*A. tamarii*	n/a	none	0.0
Somalia	Maize	Group 1a	So 19-G	*A. tamarii*	n/a	none	0.0
		Group 3b	So 6a-A	*A. minisclerotigenes*	S	BG	0.0
Tanzania	Maize	Group 2a	TN 1-E	*A. flavus*	L	none	1.5
		Group 2b	TN 5-O	*A. flavus*	L	B	1.5
		Group 2a	TN 2-R	*A. flavus*	L	none	0.9
Uganda	Maize	Group 2b	UG 4-D	*A. flavus*	L	none	0.9
		Group 2a	UG 8-G	*A. flavus*	L	none	--
		Group 2b	UG 10-D	*A. flavus*	L	none	--
	Soil	Group 5	2999	*A. parasiticus*	n/a	BG	0.0
Zambia	Maize	Z 1-K	Z 1-K	*A. flavus*	L	BG	0.0
		Group 2a	Z15-M	*A. flavus*	L	none	--
		Group 2a	Z21-B	*A. flavus*	L	B	2.2
		Group 5	Z20-K	n/a	n/a	BG	0.0
		Z19-D	Z19-D	n/a	n/a	B	--
		Group 5	Z12-A	*A. parasiticus*	n/a	BG	0.0
		Z20-A	Z20-A	n/a	n/a	none	--
		Group 5	Z 20-M	*A. parasiticus*	n/a	BG	0.0

Table 6.1 - *continued*

			Group 3a	Z18-B	new lineage, S_B	S	B	2.2
			Group 3a	Z23-K	*A. minisclerotigenes*	S	BG	0.0
	Zimbabwe	Maize	Group 2a	ZW 15-P	*A. flavus*	L	B	2.2
			Group 2b	ZW 4-A	*A. flavus*	L	B	2.2
			Group 2b	ZW 11-G	*A. flavus*	L	none	2.2
			Group 5	ZW 1-B	*A. parasiticus*	n/a	BG	0.0
			Group 3a	ZW 16-K	new lineage, S_B	S	B	2.2
			Group 3a	ZW 17-L	new lineage, S_B	S	B	2.2
Australia	Australia	Soil	Group 2b	A 1-9	*A. flavus*	L	B	0.9
			Group 2b	A 5-1	*A. flavus*	L	B	0.9
			Group 3a	1-22	new lineage, S_B	S	B	2.2
			Group 3b	4-2	*A. minisclerotigenes*	S	BG	0.0
			Group 3b	12-4	*A. minisclerotigenes*	S	BG	0.0
			Group 3b	13-4	*A. minisclerotigenes*	S	BG	0.0
Asia	Thailand	Soil	Group 2b	Sukkothai-16	*A. flavus*	L	B	1.5
	Philippines	Soil	Group 2a	L1E2	*A. flavus*	L	B	2.2
			Group 2b	MIZAN14	*A. flavus*	L	B	0.9
Europe	Italy	Maize	Group 2b	8117	*A. flavus*	L	none	--
			Group 2a	8111	*A. flavus*	L	B	--
	Spain	Soil	Group 2a	Turegano AQ	*A. flavus*	L	none	1.5
	Turkey	Pepper	Group 2b	TP 1-A	*A. flavus*	L	B	0.9
			Group 2b	TP 4-B	*A. flavus*	L	B	0.9
North America	US, Arizona	Soil	Group 2a	AF42	*A. flavus*	S	B	1.5
			Group 2a	AF70	*A. flavus*	S	B	1.5
			Group 2a	AF12	*A. flavus*	S	B	1.5
	US, California	Walnut	Group 2a	3251	*A. flavus*	S	B	1.5

Table 6.1 - *continued*

	US, Texas	Maize	Group 2a	LD08-J	*A. flavus*	L	B	1.5
			Group 2b	TEC 2-B	*A. flavus*	L	B	1.5
			Group 2b	TBA1-A	*A. flavus*	L	B	1.5
			Group 2b	TWC 21-G	*A. flavus*	L	B	1.5
			Group 2b	LAT2-H	*A. flavus*	L	B	0.9
			Group 2b	LBC13-N	*A. flavus*	L	B	1.5
			Group 2a	LEC4-C	*A. flavus*	L	B	1.5
			Group 2b	LBY 1-L	*A. flavus*	L	B	1.5
			Group 2b	TBY 1-M	*A. flavus*	L	B	1.5
			Group 2b	TBC 2-F	*A. flavus*	L	B	1.5
			Group 2b	TDO4-D	*A. flavus*	L	B	1.5
			Group 3a	TX La Feria 2F	new lineage, S_B	S	B	2.2
			Group 3a	TX04 A5-B	new lineage, S_B	S	B	2.2
			Group 3a	TX07 CB73-I	new lineage, S_B	S	B	2.2
South America	Argentina	Soil	Group 3b	TAR 1N33	n/a	L	BG	0.0
			Group 3b	TAR 4N30	*A. minisclerotigenes*	S	BG	0.0

[a] Assignments refer to phylogenetic groups as indicated in Fig1.

[b] n/a; classification into species or morphotypes not applicable.

[c] BG indicates ability to produces aflatoxins B and G; B indicates ability to produce aflatoxins B only; none indicates that no aflatoxins were detected (limit of detection 1ppb).

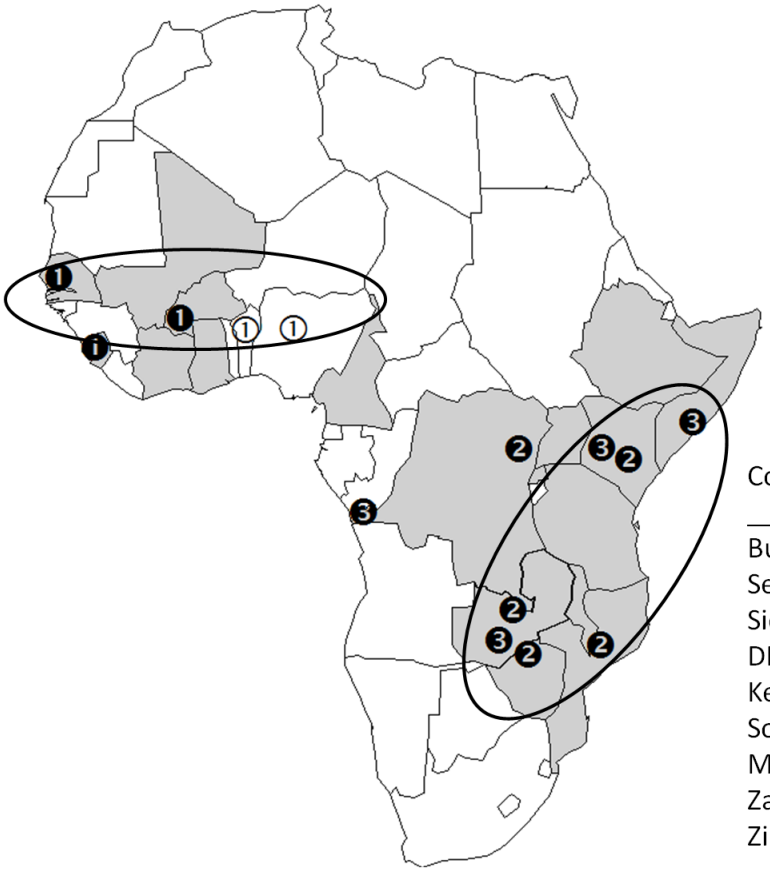
[d] Size of sequence deletion in the *cypA* gene. Amplification for some isolates was unsuccessful as indicated with lines (--).

Table 6.2 Primers and locus specific annealing temperature (T_a) used for PCR amplifications

Forward primer		Reverse primer		T_a (°C)	Product (bp)	Use
niadF	CGG ACG ATA AGC AAC AAC AC	niadAR	GGA TGA ACA CCC GTT AAT CTG	52	795	PCR, sequencing
niadBF	ACG GCC GAC AGA AGT GCT GA	niad1R	GGT CCA GGG CCC AGT TCA AT	57	741	PCR, sequencing
CP-5F	GGG ACC CTT TTC CGG TGC GG	CPR-R	GGC GGC CCC TCA GCA AAC AT	62	3100	PCR, sequencing
AP1729	GTG CCC AGC ATC TTG GTC CA	AP3551	AAG GAC TTG ATG ATT CCT C	58	1700	PCR, sequencing
aflR1F	CAT GGC TGA GGA TAG CTC	aflR2R	ACG GTG GCG GGA CTG TTG CTA	60	2092	PCR only
aflR1F	CAT GGC TGA GGA TAG CTC	aflR1R	TCG TGG AGG TGA GGA AGG A	--	729	Sequencing only
aflRF	GGA AAC AAG TCT TTT CTG G	aflRR	CAG AGC GTG TGG TGG T	--	779	Sequencing only
aflR2F	GAC TTC CGG CGC ATA ACA C	aflR2R	ACG GTG GCG GGA CTG TTG CTA	--	786	Sequencing only

[a] Primer specific annealing temperature

Figure 6.1 Map of Africa indicating sampled countries (shaded) and incidences of isolates with S strain morphotypes. Solid circles with numbers indicate observations of isolates with S strain morphology from the current study; empty circles with numbers indicate observations from previously published studies (Cotty and Cardwell, 1999; Donner et al, 2009). 1: strain S_{BG} (Cotty and Cardwell, 1999; Ehrlich et al, 2003), 2: the new *Aspergillus* lineage associated with aflatoxicoses in Kenya (Probst, Chapter 5), 3: *A. minisclerotigenes.*
Circles indicate regions with semi-arid and sub-humid climate.



Country	❶ n	❷ n	❸ n
Burkina Faso	11	--	--
Senegal	7	--	--
Sierra-Leone	3	--	--
DR Congo	--	1	1
Kenya	--	5	2
Somalia	--	--	1
Mozambique	--	1	--
Zambia	--	4	2
Zimbabwe	--	3	--

Results

Maize grain samples and fungal isolation

364 maize grain samples were collected from June 2006 through January 2008 in 18 countries in Sub-Saharan Africa (Table 6.3). The number of samples ranged from four (Ivory Coast) to 50 (Burkina Faso) with an average weight of 110 g. Samples that tested negative (n = 25) for presence of any mycotoxin and for which no microorganism could be recovered were considered to have been sterilized after collection and excluded from the study. Samples that tested positive (n= 25) for at least one of the analyzed mycotoxins but no aflatoxin-producing fungi were recovered were included in the study. A total of 4,469 *Aspergillus* sec. *Flavi* isolates were obtained from 339 samples (Table 6.3). The majority of the isolates belong to the *A. flavus* L strain (75.4%) followed by *A. tamarii* (6.1%), isolates with S strain morphology (2.8%), and *A. parasiticus* (1.0%). Isolates with S strain morphology were assigned to one of four taxa based on aflatoxin production (production of aflatoxins B and G vs. aflatoxins B only), presence and size of the *cypA* sequence deletion (no deletion, 1.5 kb or 0.9 kb deletion, and 2.2 kb deletion). These assignments agreed with assignments based on phylogenetic grouping (Figure 6.2, Table 6.1). These taxa were as follows: the putative new lineage of aflatoxin B producers previously described from Kenya (Probst, Chapter 5; 1.5%), strain S_{BG} (Cotty and Cardwell, 1999; 0.8%), *A. minisclerotigenes* (Pildain et al., 2008; 0.4%), and the *A. flavus* S strain morphotype (Cotty, 1989, 0.02%). Surprisingly, the *A. flavus* S strain was only detected in a single maize grain sample in Rwanda (isolate RW 54-P).

Mycotoxin analyses

ELISA tests were used to determine total aflatoxins (ppb), fumonisins (ppm) and DON (ppm) concentrations in the maize grain (Table 6.4). Samples were grouped based on mycotoxin content. Aflatoxins: 178 samples were free of aflatoxins (limit of detection 1 ppb); 115 samples (34%) ranged between 2 - 19 ppb total aflatoxins (mean = 7.3 ppb); 23 samples (7%) ranged between 20 – 99 ppb total aflatoxins (mean = 42.4 ppb); 22 samples (6%) ranged between 100 - 999 ppb total aflatoxins (mean = 311.2 ppb); one sample had >1000 ppb total aflatoxins (1408.9 ppb, Table 6.4).

Fumonisins: In 64 samples fumonisins were not detected (limit of detection 0.5 ppm); 110 samples (32%) had <1 ppm total fumonisins (mean = 0.1 ppm); 116 samples (34%) ranged between 1 - 4.9 ppm total fumonisin (mean= 2.2 ppm); 23 samples (7%) ranged between 5 – 19 ppm total fumonisins (mean = 8.7 ppm); 14 samples (4%) ranged between 20 – 100 ppm total fumonisins (mean = 49.5 ppm); twelve samples (3%) had > 100 ppm total fumonisins (mean = 143.3 ppm) (Table 6.4).

DON: In 177 samples DON was not detected (limit of detection 0.1 ppm); 107 samples (35%) had <1 ppm total DON (mean = 0.23 ppm), 22 samples (7%) range between 1 and 4.9 ppm total DON (mean = 1.9 ppm); and three samples (1%) had > 5 ppm total DON (mean = 9.1 ppm) (Table 6.4). Two out of three samples with DON > 5 ppm originated from Zimbabwe. Quantifications of DON concentrations for samples

from Burkina Faso and Zambia and fumonisins concentrations for 15 samples from Somalia were not possible, because the sample sized were not sufficient.

The greatest number of maize grain samples contaminated with aflatoxin concentrations > 100 ppb occurred in the Coast Province of Kenya (33%), followed by Somalia (22%), and Uganda (20%). All maize samples from Zimbabwe were contaminated with > 10 ppm total fumonisins (100%), with 53% of the samples containing quantities > 100 ppm. 55% of samples (55%) from the Coast Province, Kenya, were also contaminated with > 10 ppm total fumonisins.

Phylogenetic analyses

The majority of aflatoxin-producing fungi obtained in the current study from maize grain produced in Africa were assigned to the *A. flavus* L strain morphotype through the above criteria. Phylogenetic data confirmed this placement as all African *A. flavus* isolates form a clade with isolates from the Americas (e.g. reference isolates 3251, AF70, AF42), Australia, and Asia (Figure 6.2). Isolates with S strain morphology and ability to produce aflatoxins B and G formed two separate clades with reference isolates previously described for strain S_{BG} (e.g. isolates A-11612, BNO38-G, and BNO40-B) or *A. minisclerotigenes* (e.g. isolate A-11611 and Australian isolates 4-2, 12-4, and 13-4). Both, partial *niaD* and *aflR* sequence data were able to separate isolates belonging to either one of these two taxa (Figures 6.2 and 6.3). However, partial *niaD* sequence data was insufficient to separate between *A. minisclerotigenes* and the new lineage of aflatoxins B producers, primarily found in Kenya (Figure 6.2). To obtain higher resolution among isolates with S strain morphology, phylogenetic

reconstructions with *aflR* sequence data (n = 37, 2.1 kb total sequence) were performed. ML tree obtained with *aflR* sequences grouped isolates of *A. minisclerotigenes* and the new lineage into sister clades (Figure 6.3). This is concordant to results from other studies (Probst, Chapter 5). Based on the high number of atoxigenic *A. flavus* L strain isolates and the likelihood that some of these would have deletions covering *aflR* (Chang *et al.*, 1995; Donner *et al.*, 2010), we refrained from performing the *aflR* analyses for all 143 isolates.

In the current study, isolates belonging to strain S_{BG} were limited to countries in West Africa (here Benin, Burkina Faso, Nigeria, Senegal, and Sierra-Leone) while isolates with similar aflatoxin-producing ability and identified by phylogenetics as *A. minisclerotigenes* were found in Central (here in the Democratic Republic of the Congo), East (here in Kenya, Somalia), and Southern Africa (here Zambia) (Figure 6.1).

Molecular analyses of the cypA sequence deletions

No sequence deletion in the *cypA* gene has been detected in aflatoxin B and G producing fungal taxa. Sequence amplification in the atoxigenic species *A. tamarii* was unsuccessful, *A. flavus* L strain isolates exhibited either 0.9 kb or 1.5 kb sequence deletions (Table 6.1), characteristic for *A. flavus* (Ehrlich *et al.*, 2004). The aflatoxin B producing S strain isolate from Rwanda (RW 54-P) also exhibits a 0.9 kb sequence deletion (Table 6.1). Phylogenetic data, confirmed the utility of the *cypA* deletion size in identifying isolate RW 54-P as the only member of the *A. flavus* S strain morphotype detected in the current study (Figures 6.2 and 6.3). The remaining isolates

with S strain morphology and producing only B aflatoxins exhibited a 2.2 kb deletion thought to be diagnostic for the new lineage of aflatoxin-producing S strains, first identified in Kenya. Phylogenetic analyses confirmed the utility of this deletion for identifying members of this physiologically distinct *Aspergillus* lineage that is closely related to *A. minisclerotigenes* (Figures 6.2 and 6.3).

Table 6.3 Fungal incidences in maize grain obtained from 18 Sub-Saharan countries in Africa

				Species, Morphotype (%)						
Country	Year	No samples	No isolates	Strain S_{BG}	*A. minisclerotigenes*	Unnamed New lineage	*A. flavus*, S strain	*A. flavus*, L strain	*A. parasiticus*	*A. tamarii*
Burkina Faso	2007	51	763	2.5	0	0	0	96.8	0	0.7
Cameroon	2006	17	205	0.7	0	0	0	85.5	2	6
DR Congo, Bas	2006/07	10	243	0	4	0	0	59.0	1	27
DR Congo, East	2006/07	12	236	0	0	0.3	0	79.9	1	17.9
Ethiopia	2006/07	13	49	0	0	0	0	89.4	0	6.7
Ghana	2006	8	67	0	0	0	0	87.5	0	0
Ivory Coast	2007	4	48	0	0	0	0	100.0	0	0
Kenya, Coast	2006	8	140	0	0	0	0	89.9	0	10.1
Kenya, Rift Valley	2006	13	206	0	2.4	4.8	0	90.4	0	2.4
Malawi	2007	12	153	0	0	5.6	0	81.0	1.6	11.8
Mali	2007	13	170	0	0	0	0	100.0	0	0
Mozambique	2007/08	40	456	0	0	0.1	0	93.1	1.3	0.5
Rwanda	2007	16	55	0	0	0	0.4	30.9	0	0
Senegal	2007	19	305	10.3	0	0	0	86.7	0	3
Sierra-Leone	2006	16	232	3	0	0	0	71.7	0	12.8
Somalia	2007	27	443	0	0.2	0	0	77.8	0.5	21.5
Tanzania	2007	5	73	0	0	0	0	88.5	4.5	0
Uganda	2007	15	243	0	0	0	0	79.2	0	20.8
Zambia	2006/07	21	191	0	1.9	8.2	0	58.7	8.1	1.6
Zimbabwe	2007	19	191	0	0	10.6	0	71.2	1.5	0

Table 6.4 Mycotoxin contamination of maize samples from 18 countries in Africa

	No. of	Aflatoxin (µg/kg)			Fumonisin (µg/g)			Deoxynivalenol (µg/g)		
Country, Region	samples	Avg. [a]	Min.[b]	Max.[c]	Avg.	Min.	Max.	Avg.	Min.	Max.
Burkina Faso	50	25	0	609	1	0	4	n/a[d]	n/a	n/a
Cameroon	16	15	0	122	1	0	4	0	0	0
DR Congo, East	12	63	0	393	2	0	9	1	0	4
DR Congo, Bas	10	12	0.1	57	1	0	5	0.1	0	0.4
Ethiopia	81	3	0	23	5	0	150	0.4	0	3
Ghana	7	0.1	0.2	0.2	0.4	0	1	0	0	0
Ivory Coast	4	7	2	21	0.2	0	0.5	0.2	0	0.4
Kenya, Coast	9	102	0	525	18	0.5	50	0.2	0	0.4
Kenya, Rift Valley	13	11	0	87	1	0	4	0.3	0	1
Malawi	9	12	5	20	2	1	9	0	0	0
Mali	7	4	0	6	1	0	3	0	0	0
Mozambique	42	0	0	0	2	0	10	0	0	0
Rwanda	16	0	0	0.3	0.3	0	1	0	0	0.5
Senegal	20	47	0.3	395	2	0	9	0.1	0	0.5
Sierra-Leone	17	23	2	162	0.1	0	1	0.1	0	0.5
Somalia	6	133	1	1407	5	0	9	0.2	0	1
Tanzania	5	2	0	7	1	0	5	0	0	0
Uganda	17	95	0	435	2	0	19	0.8	0	8
Zambia	28	7	0	108	2	0	21	n/a	n/a	n/a
Zimbabwe	19	9	0	123	105	36	159	1	0.0	12

[a] Avg., average aflatoxin concentrations;

[b] Min., minimum aflatoxin concentrations;

[c] Max., maximum aflatoxin concentrations

[d] n/a; data not available

Figure 6.2 Mid-point rooted maximum likelihood tree of 143 *Aspergillus* isolates obtained from Africa, Australia, Asia, Europe, North America and South America. Tree is based on *niaD* sequence data (1.5 kb). Values above nodes indicate bootstrap values from 1000 replicates derived from ML analysis (before comma) and MP analysis (after comma). Asterisk behind group/isolate name indicates ability to produce aflatoxins B and G, with exception of group 3 which also includes aflatoxin B producing isolates. Group assignments; Group 1, *Aspergillus tamarii*; Group 2, *A. flavus*; Group 3, new lineage and *A. minisclerotigenes*; Group 4, strain S_{BG}; Group 5, *A. parasiticus*. Isolates found in individual groups are indicated in Table 6.1.

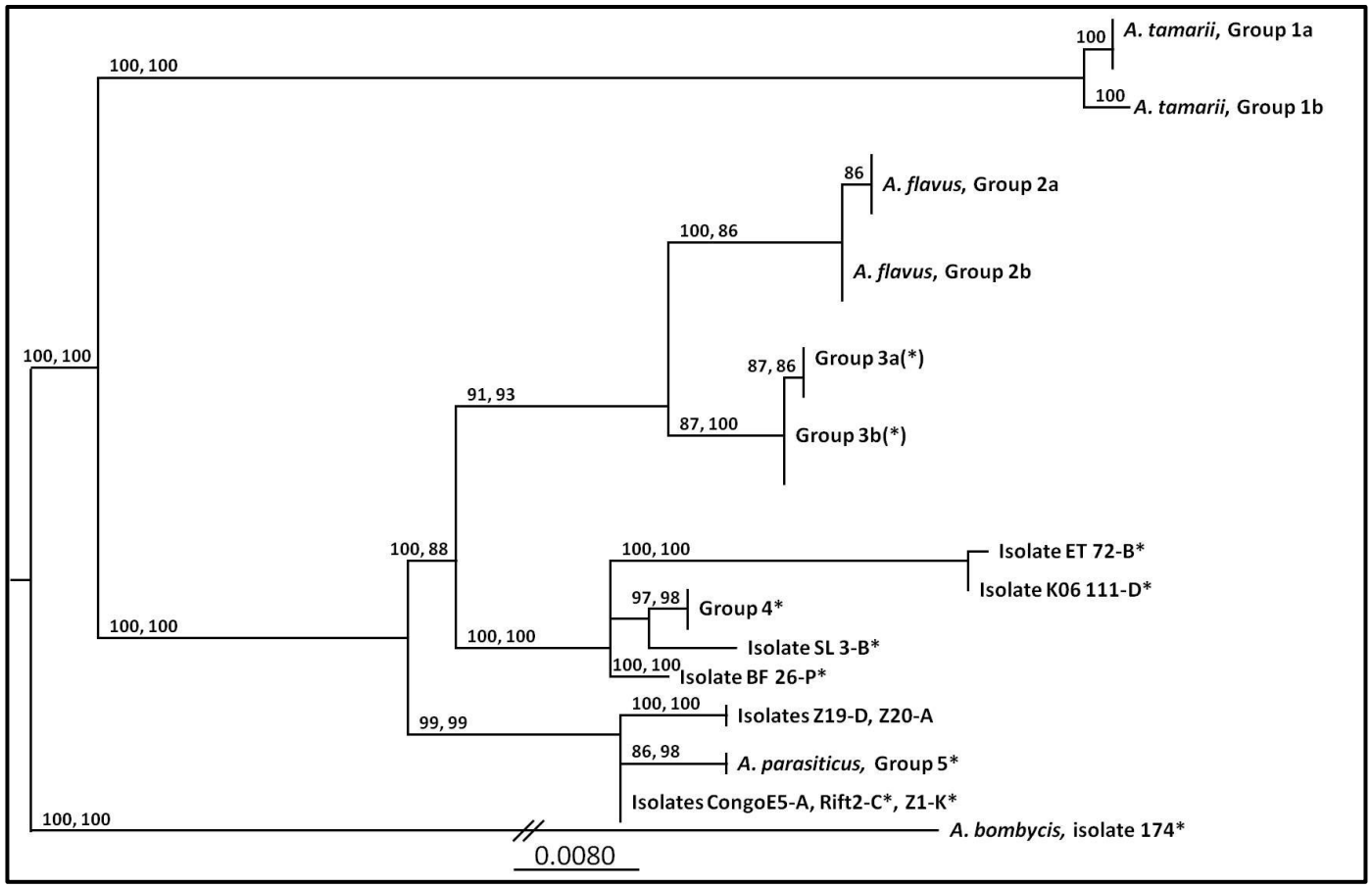
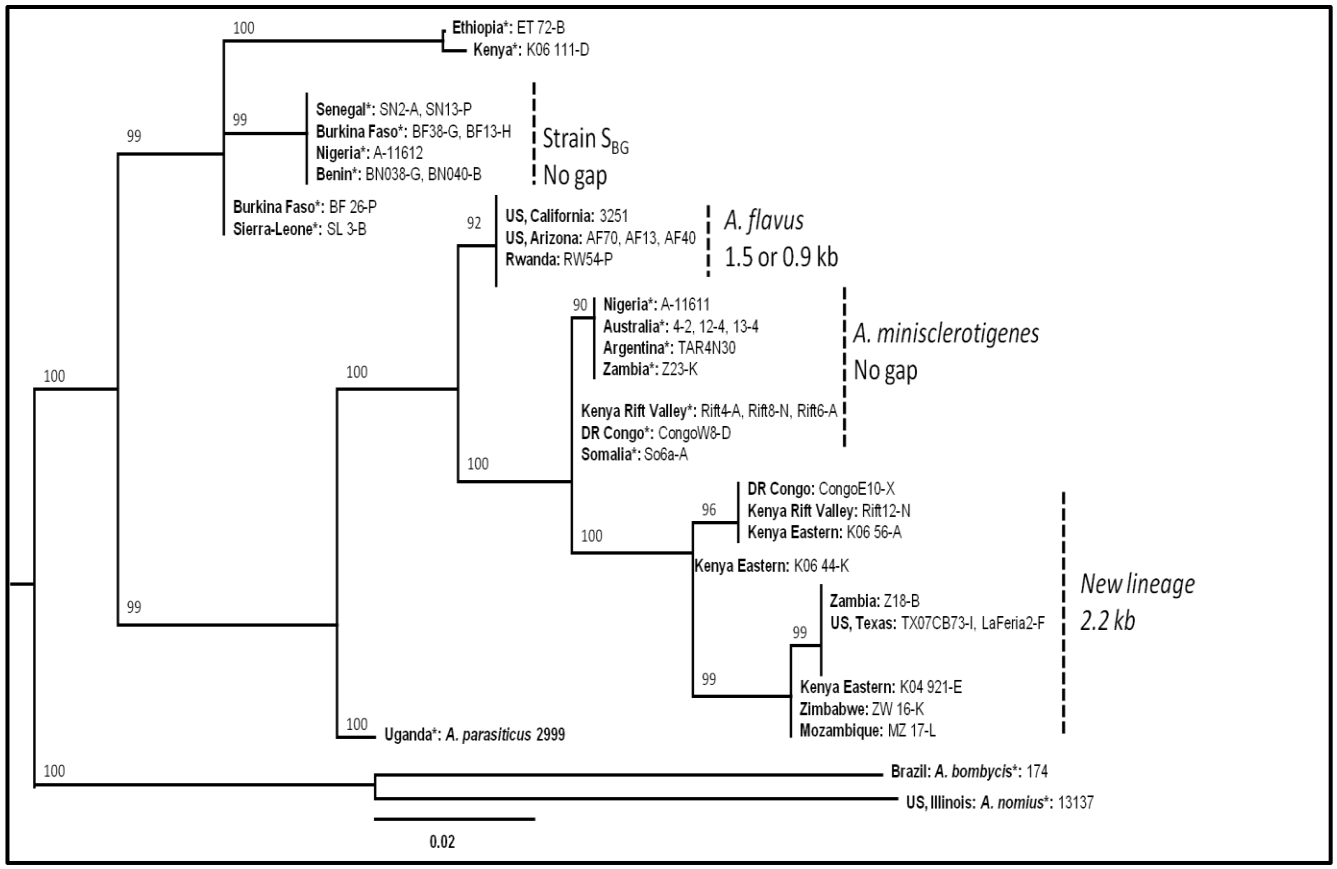

100
A. tamarii, Group 1a
100, 100
100
A. tamarii, Group 1b
86
A. flavus, Group 2a
100, 86
A. flavus, Group 2b
100, 100
87, 86
Group 3a(*)
91, 93
87, 100
Group 3b(*)
100, 88
100, 100
Isolate ET 72-B*
Isolate K06 111-D*
97, 98
Group 4*
100, 100
Isolate SL 3-B*
100, 100
Isolate BF 26-P*
100, 100
100, 100
Isolates Z19-D, Z20-A
99, 99
86, 98
A. parasiticus, Group 5*
Isolates CongoE5-A, Rift2-C*, Z1-K*
100, 100
A. bombycis, isolate 174*
0.0080

Figure 6.3 Mid-point rooted maximum likelihood tree of 37 *Aspergillus* isolates with S strain morphology and three isolates of related *Aspergillus* species (*A. parasiticus*, *A. nomius*, *A. bombycis*). Tree is based on *aflR* sequence data (2.1 kb total sequence). Values above nodes indicate bootstrap values from 1000 replicates. Asterisk behind country/ species name designate ability to produce both B and G aflatoxins.


100
Ethiopia*: ET 72-B
Kenya*: K06 111-D
99
Senegal*: SN2-A, SN13-P
Burkina Faso*: BF38-G, BF13-H
Nigeria*: A-11612
Benin*: BN038-G, BN040-B
Strain S BG
No gap
99
Burkina Faso*: BF 26-P
Sierra-Leone*: SL 3-B
92
US, California: 3251
US, Arizona: AF70, AF13, AF40
Rwanda: RW54-P
A. flavus
1.5 or 0.9 kb
90
Nigeria*: A-11611
Australia*: 4-2, 12-4, 13-4
Argentina*: TAR4N30
Zambia*: Z23-K
A. minisclerotigenes
No gap
100
100
Kenya Rift Valley*: Rift4-A, Rift8-N, Rift6-A
DR Congo*: CongoW8-D
Somalia*: So6a-A
100
96
DR Congo: CongoE10-X
Kenya Rift Valley: Rift12-N
Kenya Eastern: K06 56-A
100
Kenya Eastern: K06 44-K
Zambia: Z18-B
US, Texas: TX07CB73-I, LaFeria2-F
New lineage
2.2 kb
99
99
99
Kenya Eastern: K04 921-E
Zimbabwe: ZW 16-K
Mozambique: MZ 17-L
100
Uganda*: A. parasiticus 2999
100
Brazil: A. bombycis*: 174
US, Illinois: A. nomius*: 13137
0.02

Discussion

Aflatoxin contamination of food crops has been from central concern in Africa for decades (Gong *et al.*, 2008; Kpodo and Bankole, 2008). Impairment in child development and severe liver damage are only two of the many health consequences of chronic aflatoxin intake (Turner *et al.*, 2003; Gong *et al.*, 2004). An outbreak of acute and lethal aflatoxin poisonings in Kenya during 2004 (Centers for Disease Control and Prevention, 2004) invigorated the world's attention on the ongoing threat aflatoxins pose to world food security and the everyday lives of people in Africa (Atser, 2011). Even with these concerns, studies contrasting communities of aflatoxin producing fungi across Africa have been lacking. The current study provides insights into both how these communities vary across the continent and potentially important causal agents of maize aflatoxin contamination in various regions.

The majority of aflatoxin-producing fungi are found in *Aspergillus* sect. *Flavi* (Horn, 2003; Frisvad et al., 2005). Not all of these fungi are equally important etiologic agents of aflatoxin contamination events (Cotty *et al.*, 2008). Frequencies in the environment, aflatoxin-producing potential, competitive ability, and virulence on host plants influence the importance of these fungi as causative agents of crop contamination (Cotty, 1989; Horn, 2003; Mehl and Cotty, 2010). Under natural conditions, mixtures of different aflatoxigenic and atoxigenic fungi are present on crops and the balance between these greatly influences the severity of contamination events (Probst *et al.*, 2010). If frequencies of atoxigenic or low aflatoxigenic fungi are high, less aflatoxin will develop and, hence, more crops will be fit for human

consumption and export. This is in stark contrast to cases where highly aflatoxigenic fungi dominate the fungal community (Jaime-Garcia and Cotty, 2004; Probst *et al.*, 2007). In such cases, aflatoxins impose great risk for human and animal health and diminish income of farmers. Morphological and molecular identification of major aflatoxin-producing fungi combined with data on aflatoxin-producing potential, ideally assessed on sterilized host material (Probst, Chapter 4), and the relative frequencies of the obtained fungi are first steps in determining the relative potential for fungal communities to contaminate crops. Analyzing the aflatoxin-producing potential of isolates associated with highly contaminated maize is a first step in finding causal agent of contamination. A similar approach was employed in the present study to identify communities of aflatoxin-producing fungi in Africa. We found incidences of the *A. flavus* L strain to be high in all countries. Even though aflatoxin-producing ability was only conducted for isolates used for phylogenetic reconstructions, both highly toxigenic and atoxigenic isolates were found (Table 6.1).

The general dominance of *A. flavus* is in agreement with findings from studies on aflatoxin-producing fungi in other continents and generally confirms that *A. flavus* is the predominant aflatoxin-producing species on crops (Horn and Dorner, 1998; Barros *et al.*, 2003; Atehnkeng *et al.*, 2008a). Less frequently, were the highly aflatoxigenic fungi with the S strain morphotype. In Africa, at least three fungal taxa exhibit S strain morphology: Taxon 1, consists of a lineage of aflatoxin B and G producing fungi (strain S_{BG}) endemic in West Africa (Cotty and Cardwell, 1999; Atehnkeng *et al.*, 2008a); Taxon 2, includes aflatoxin B and G producing fungi

recently described as *A. minisclerotigenes* (Pildain et al., 2008) common in eastern and southern Africa and occurring on other continents ; and Taxon 3, contains a lineage of aflatoxin B producing fungi that have been identified as the causal agents of the acute outbreak of aflatoxicosis in Kenyan in 2004 and occur in low frequencies in Texas, Argentina and Australia (Figure 1, current study; Probst, Chapter 5; Probst et al., 2007). Previously, occurrence of Taxon 1 (strain S_{BG}) with drier agroecological zones in Bènin with its average incidences increasing from 3 to 60% between the humid Coastal Savannah and the semi-arid Sudan Savannah (Cardwell and Cotty, 2002). Our findings are in concordance with this study, with most isolates of strain S_{BG} obtained from semi-arid and sub-humid parts of West Africa. Fungi with both similar morphology and similar aflatoxin-producing potential found in Central, East, and Southern Africa were found to be members of Taxon 2 (*A. minisclerotigenes*), a species also found in Argentina and Australia (Pildain *et al.*, 2008). All isolates with S strain morphology and capable of producing only B aflatoxin, with the exception of one isolate from Rwanda, had the 2.2 kb sequence deletion in *cypA* characteristic of Taxon 3 (Probst, Chapter 5). This is the first report about the occurrence of Taxon 3 in other African countries. Incidences of Taxon 3 were generally low and limited to just on or few isolates per region. Like strain S_{BG}, most isolates of Taxa 2 and 3 were obtained from semi-arid and sub-humid regions which suggests that drier climates may favor aflatoxin producing fungi with S strain morphology.

For human health concerns, the three most important mycotoxins contaminating grain are fumonisins, aflatoxins, and deoxynivalenol. However, the occurrence of

these in maize, Africa's most important staple, has been not widely investigated. The current study found in the majority of maize in the 13 sampled countries to contain one or more of these human toxins. The vast majority of maize (81%) had detectable levels of fumonisins and/ or aflatoxins (65%). The regulatory limit of the European Union for total aflatoxins in food is 4 ppb (Agriculture and Consumer Protection, 2004) and a total of 124 samples, including all samples from Malawi and more than 50% of samples obtained from Sierra-Leone, Somalia, Uganda, Kenya (Rift Valley), DR Congo (Bas) and Cameroon, exceeded this limit. This finding exemplifies that aflatoxin contamination is wide spread in Sub-Saharan Africa, diminishing export opportunities for farmers and raising health concerns for resident populations that consume large proportions of their daily caloric intake as maize. Another potential health concern was found with maize contamination with fumonisins in Zimbabwe where all obtained sampled maize tested positive for fumonisins at levels greater than 10 times the provisional maximum tolerable intake level of 2 ppm proposed by the FAO/WHO Committee on Food Additives (Agriculture and Consumer Protection, 2004). This is of particularly concern since maize is the number one staple consumed by the Zimbabwe population. Clearly, studies on fumonisin-producing fungi associated with maize in Zimbabwe are needed. The third mycotoxin, deoxynivalenol, previously detected in maize in Zambia and South Africa (Marasas *et al.*, 1977) was found in 40% of all samples indicating maize infection by trichothecene producing *Fusarium* species. In the current study, 10% of samples had deoxynivalenol levels exceeding the action level proposed by the European Union of 0.75 ppm (Agriculture

and Consumer Protection, 2004). Based on the results of the current study, of the three mycotoxins studied, aflatoxins contamination warrants the greatest management effort.

In recent years, efforts have been made to develop aflatoxin management strategies to decrease contamination levels and increase food security in many countries (Atehnkeng *et al.*, 2010; Cotty and Bandyopadhyay, 2010; Probst *et al.*, 2011). Tools based on atoxigenic strains of *A. flavus* have been central components of such strategies. Atoxigenic strains are used to competitively displace aflatoxin-producing fungi. These biological control agents, such as AflaSafe (Atehnkeng *et al.*, 2010), have been shown capable of decreasing aflatoxin contamination in treated crops in Africa over 90% both before harvest and during storage (Bandyopadhyay *et al*., personal communication). Crop management practices (e.g. crop rotations, decreased storage humidity and temperatures) are also known to slow down fungal growth and, consequently, reduce aflatoxin development (Hell *et al.*, 2003; Jaime-Garcia and Cotty, 2006b; Kaaya and Kyamuhangire, 2006). Knowledge about dominant aflatoxin-producing fungi will help to identify potential hotspots of contamination and to implement prevention strategies. In the present study, fungal communities on maize in Africa were mainly composed of the L strain of *A. flavus*, a morphotype that responds well to competitive replacement practices and in which atoxigenic isolates are frequently found. Additionally, even though the S strain morphotypes were found as minor components of the fungal communities, their importance in aflatoxin contamination events should not be underestimated. A high aflatoxin producer that is rare may contribute more to contamination than a low aflatoxin producer that is

common (Probst, Chapter 4; Cotty *et al.*, 2008). However, mitigation of these highly toxigenic fungi with native atoxigenic *A. flavus* L strain isolates has been demonstrated successful in several studies (Dorner, 2009; Atehnkeng *et al.*, 2010; Probst *et al.*, 2011). Identification of native atoxigenic *A. flavus* L strain isolates is a promising route to control aflatoxin contamination caused by any of the identified aflatoxin-producing fungi in the respective countries.

Due to recent advances in and decreased costs of molecular methods our understanding of genetic diversity within populations of aflatoxin-producing fungi and the relationships among species has been increased. New aflatoxin-producing fungi have been identified (Pildain *et al.*, 2008), species have been reclassified (Ehrlich *et al.*, 2007b), and genetically isolated subgroups have been recognized (Grubisha and Cotty, 2010). In the past, most homothallic species were identified based on the morphological species concept (Samson and Varga, 2009). Many 'true' *Aspergillus* species have been identified with this functional species identification system. Morphological criteria used to place isolates in *A. flavus* and *A. parasiticus* are reliable even when applied to isolates from greatly different continents as confirmed by phylogenetic comparisons of fungi in the current study. However, the morphological species concept failed to separate between the several species of *Aspergilli* displaying the S strain morphotype. Assessment of aflatoxin production is frequently used to separate species of aflatoxin B and G producers and sole producers of aflatoxins B. The current study showed that aflatoxin-producing ability is an insufficient measure to separate between species with similar aflatoxin-producing ability and morphology,

such as isolates of strain S_{BG} and *A. minisclerotigenes*, or, isolates of the *A. flavus* S strain and the new lineage. While information on *cypA* gene deletions is useful to distinguish between aflatoxin B producing fungi (here: *A. flavus* and the new lineage; Probst, Chapter 5) a similar tool is missing for aflatoxin B and G producing species. Phylogenetic analysis supports the utility of the other criteria for placing isolates of aflatoxin B producing S strains into appropriate species and clades, and, is the only tool available to reliably distinguish among aflatoxins B and G producing S strains.

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