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Population structure and Aflatoxin production by *Aspergillus* Sect. *Flavi* from maize in Nigeria and Ghana

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ABSTRACT

Aflatoxins are highly toxic carcinogens that contaminate crops worldwide. Previous studies conducted in Nigeria and Ghana found high concentrations of aflatoxins in pre- and post-harvest maize. However, little information is available on the population structure of *Aspergillus* Sect. *Flavi* in West Africa. We determined the incidence of *Aspergillus* Sect. *Flavi* and the level of aflatoxin contamination in 91 maize samples from farms and markets in Nigeria and Ghana. *Aspergillus* spp. were recovered from 61/91 maize samples and aflatoxins B_1 and/or B_2 occurred in 36/91 samples. Three samples from the farms also contained aflatoxin G_1 and/or G_2. Farm samples were more highly contaminated than were samples from the market, in terms of both the percentage of the samples contaminated and the level of mycotoxin contamination. One-hundred-and-thirty-five strains representative of the 1163 strains collected were identified by using a multilocus sequence analysis of portions of the genes encoding calmodulin, β-tubulin and actin, and evaluated for aflatoxin production. Of the 135 strains, there were 110 – *Aspergillus flavus*, 20 – *Aspergillus tamarii*, 2 – *Aspergillus wentii*, 2 – *Aspergillus flavofurcatus*, and 1 – *Aspergillus parvisclerotigenus*. Twenty-five of the *A. flavus* strains and the *A. parvisclerotigenus* strain were the only strains that produced aflatoxins. The higher contamination of the farm than the market samples suggests that the aflatoxin exposure of rural farmers is even higher than previously estimated based on reported contamination of market samples. The relative infrequency of the *A. flavus* S_{BG} strains, producing small sclerotia and high levels of both aflatoxins (B and G), suggests that long-term chronic exposure to this mycotoxin are a much higher health risk in West Africa than is the acute toxicity due to very highly contaminated maize in east Africa.

1. Introduction

Maize (*Zea mays* L.) is the most widely grown staple food in Africa. In parts of West Africa, it may be consumed three times per day (Adebajo et al., 1994). Maize and peanuts are the primary means by which humans are exposed to aflatoxins in Africa (Shephard, 2008). In Nigeria, from 1992 to 1996 the total maize cultivation area increased from 1.8 million to 4.0 million ha (Manyong et al., 1996), with total maize production increasing from 4–5 to 7–7.5 million tons in the last decade (FAO, 2012). Maize is produced in all of the agro-ecological zones of Nigeria except the Sahel Savannah, with the greatest area of cultivation in the Northern Guinea Savannah (Manyong et al., 1996). In Ghana, maize production has been stable for the last decade at 1.3–1.5 million tons per year. Maize is the most widely consumed staple food in Ghana, based on a nationwide survey in 1990, with 94% of all households reporting that they consumed maize during an arbitrarily selected two-week period (Alderman and Higgins, 1992; FAO, 2012).

Maize worldwide is vulnerable to degradation by toxigenic fungi, but in the developing world, and particularly in sub-Saharan Africa, official monitoring of mycotoxin contamination levels is rare. In West Africa, 98% of the population was serologically positive for aflatoxin exposure (Wild et al., 1990), and in Bénin, Nigeria and Ghana high concentrations of aflatoxins have been found in both pre- and post-harvest maize (Hell et al., 2000; Udoh et al., 2000; James et al., 2007).

Aflatoxins are secondary metabolites synthesized by several *Aspergillus* species and are highly toxic to humans and animals when ingested at high concentrations. They may cause severe liver

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damage accompanied by jaundice, hepatitis and death. Aflatoxin $B_1$is genotoxic, carcinogenic and teratogenic for both humans and animals. Daily consumption of foods contaminated with low levels of aflatoxin B_1 can result in chronic aflatoxicosis with stunting in children, immune suppression, cancer and reduced life expectancy (Shephard, 2008). Aflatoxin contamination of crops may be caused by several species in *Aspergillus* Sect. *Flavi*, but *Aspergillus flavus* and *Aspergillus parasiticus* are the species most commonly implicated as problematic.

A. flavus is the species most responsible for aflatoxin contamination of maize worldwide (Donner et al., 2009; Horn, 2007). Based on morphological, genetic and physiological criteria, *A. flavus* can be divided into two strain types. S-type strains produce numerous small sclerotia (<400 μm diameter) and high levels of the B-series aflatoxins. L-type strains produce fewer, larger sclerotia, and, on average, lower levels of aflatoxins. *A. flavus* isolates produce only B series aflatoxins due to a 1.5–2.2 kb deletion in the aflatoxin biosynthetic gene cluster, which results in loss of the gene *cypA*, a gene whose product is required for G series aflatoxin biosynthesis (Ehrlich and Cotty, 2004). An additional as yet unnamed *Aspergillus* taxon, informally termed S_{BG}, is morphologically similar to *A. flavus* S-type strains and produces small sclerotia, but also can synthesize large amounts of both B- and G-aflatoxins. The S_{BG}, strain type has a more limited distribution but is suspected as an important source of aflatoxin contamination in West Africa (Cardwell and Cotty, 2002; Probst et al., 2007). S_{BG} strains are phylogenetically divergent from the *A. flavus* S_B group (Ehrlich et al., 2003). Strains of the recently described *Aspergillus minisclerotigenes* have many characters that are similar to those exhibited by strains in the S_{BG} group (Pildain et al., 2008). Isolates that share morphological characters with S_{BG} have been reported from Thailand, Argentina and Australia, but the exact taxonomic designation of S_{BG} remains unclear (Cotty and Cardwell, 1999; Saito and Tsuruta, 1993; Donner et al., 2009).

Interest in the variation of aflatoxin production by strains of *Aspergillus* Sect. *Flavi* has increased recently because atoxigenic strains of *A. flavus* are being used as biological control agents to reduce the risk of aflatoxin contamination (Atehnkeng et al., 2008a; Wu and Khlangwiset, 2010). In West Africa (Benin, Nigeria and Senegal), L strains of *A. flavus* are the most common strains in the agroecosystems (Cardwell and Cotty, 2002; Atehnkeng et al., 2008b; Diedhiou et al., 2011). At least four phylogenetically distinct groups with S strain morphological characters are or have been assigned to *A. flavus* (Probst et al., 2012). One of these groups has been described as *A. minisclerotigenes* (S_{BG}). A second is the highly toxic Kenyan S_B group (Probst et al., 2007), and a third is the S_B group from elsewhere in the world, *e.g.*, Thailand, the United States, and the Philippines. Finally, there is the S_{BG} strain group from Nigeria. Members of the S_{BG} group are found mainly in locations where high levels of aflatoxin contamination occur. S_B strains are not found in West Africa, so even though S_{BG} strains may represent only a small proportion of *Aspergillus* Sect. *Flavi*, they could be most important cause of aflatoxin contamination in Nigeria (Donner et al., 2009).

The objective of this study was to assess the incidence of *Aspergillus* Sect. *Flavi* strain types and aflatoxin contamination in maize samples collected from markets and farms at harvest in Nigeria and Ghana. Our working hypotheses were: i) *A. flavus* is the most common *Aspergillus* species recovered, ii) atoxigenic strains outnumber toxigenic strains, and iii) aflatoxin contamination is more common in farm samples than in market samples. These results are important because they define the background populations that must be altered if biological control is to be successful, and because they indicate the importance of on-farm interventions to reduce the exposure of rural populations in Nigeria and Ghana to aflatoxins.

2. Materials and methods

2.1. Food samples

We evaluated 91 unprocessed African maize samples (sample sizes of 200–300 g). Samples were purchased in different production zones in 2003–2004, including 47 samples from Nigerian markets, 9 from Ghanaian markets, and 35 from subsistence farms at harvest time in Ghana (16 samples) and Nigeria (19 samples) during the 2002–2003 growing season. Samples were placed in sterile plastic bags and stored at 4 °C until processed. Each sample was divided equally into two sub-samples. One subsample was evaluated mycologically and the other was ground in a Model MLI-204 Bühler mill (Milan, Italy) for mycotoxin analysis. Sub-samples were stored in plastic bags at 4–6 °C until analyzed to prevent further fungal growth and mycotoxin biosynthesis prior to testing. Mycotoxin analyses were based on toxins present in the collected material and not on the potential of the identified fungi to produce mycotoxins.

2.2. Mycological analysis

From each sample, 100 g of grain was immersed in a 1% sodium hypochlorite solution for 3 min and then rinsed in three changes of sterile distilled water. Freshly prepared Dichloran Rose-Bengal Chloramphenicol Agar (DRBC, Oxoid) medium was used. Fifty seeds were plated for each sample. The plates were incubated at 28 ± 2 °C for 5–7 days, and examined daily. After incubation, the number of seeds with visible fungal growth was recorded and the incidence of fungal contamination determined for each sample.

2.3. Identification of species

Yellow-green *Aspergillus* colonies, presumably belonging to section *Flavi*, were subcultured for subsequent identification to species level. Species names were determined by following the taxonomic keys of Klich (2002). Cultures were grown on CYA (Czapek Yeast Extract Agar - $NaNO_3$ 3 g; K_2HPO_4 1 g; KCl 0.5 g; $MgSO_4{*}7H_2O$ 0.5 g; $FeSO_4{*}7H_2O$ 0.01 g; yeast extract 5 g; sucrose 30 g; agar 20 g and distilled water to 1 L) at 25 °C and 37 °C, CZ (Czapek Dox Agar-sucrose 30 g; $NaNO_3$ 3 g; K_2HPO_4 1 g; KCl 0.5 g; $MgSO_4{*}7H_2O$ 0.5 g; $FeSO_4{*}7H_2O$ 0.01 g; agar 15 g and distilled water to 1 L) and MEA (Malt Extract Agar-malt extract 30 g; mycological peptone 5 g; agar 15 g and distilled water to 1 L) at 25 °C to enable formation of diagnostic characters. All plates were incubated for 7 days.

2.4. Chemicals

Potassium bromide, sodium chloride, and standards of aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, and aflatoxin G_2, were purchased from Sigma–Aldrich (Milan, Italy). Acetonitrile and methanol (HPLC-grade) were purchased from Mallinckrodt Baker (Milan, Italy). Ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA). Immunoaffinity columns (Aflatest®P) were obtained from Vicam (Watertown, MA, USA). Glass microfiber filters (Whatman GF/A), paper filters (Whatman no. 4) and RC 0.2 μm (regenerated cellulose membranes) filter were obtained from Whatman (Maidstone, UK).

2.5. Preparation of standards

Standard solutions of each aflatoxin were prepared by dissolving the solid commercial toxin in toluene:acetonitrile (9:1,

v/v) to a concentration of 10 μg/ml. The exact concentration of aflatoxin in each standard solution was determined according to AOAC Official Method 971.22 (AOAC Official Methods of Analysis, 2000a). A stock solution was prepared by mixing the simple aflatoxin solution and diluting it with toluene:acetonitrile (9:1 v/v) into amber silanized vials to obtain a solution containing 1 μg/ml of aflatoxin B_1 or G_1 and 0.5 μg/ml of aflatoxin B_2 or G_2. Aliquots of the stock solution were transferred to 4 ml amber silanized glass vials and evaporated to dryness under a stream of nitrogen at 50 °C. The residue was dissolved with water:acetonitrile:methanol (64:13:23, v/v/v) to obtain final concentrations of 1.0, 2.0, 10, 20, 40, 100, 150 and 200 ng/ml of aflatoxin B_1 and G_1, or 0.5, 1.0, 5.0, 10, 20, 50, 75 and 100 ng/ml of aflatoxin B_2 and G_2. Standard solutions were stored at −20 °C and warmed to room temperature before use.

2.6. Apparatus

The HPLC apparatus consisted of a Perkin–Elmer (Norwalk, CT, USA) Series 200 LC binary pump equipped with a Rheodyne (Cotati, CA, USA) model 7125 injection valve, a Jasco (Tokyo, Japan) FP-1520 fluorometric detector, and a Turbochrom 4.0 data system (Perkin Elmer). The analytical column was a C12 Sinergy Max RP 4 μm (Phenomenex, Torrance, CA, USA), with a guard column inlet filter (0.5 μm × 3 mm diameter, Rheodyne Inc., CA, USA) and the mobile phase consisted of a mixture of water:acetonitrile:methanol (64:13:23, v/v/v) with 119 mg potassium bromide and 100 μl of concentrated nitric acid added per liter of mobile phase. Aflatoxins B_1 and G_1 were derivatized using Kobra Cell (Rhone Diagnostics Technologies Ltd., Lyon, France). The mobile phase flow rate was set at 0.8 ml/min. The fluorometric detector was set at wavelengths, ex = 360 nm and em = 440 nm.

2.7. Aflatoxin production

Aflatoxin production by the strains belonging to *Aspergillus* Sect. *Flavi* was assessed. The strains were grown for aflatoxin production for 7 days at 28 °C in 50 ml vials containing 10 ml of liquid YES (yeast extract 20 g; sucrose 150 g; $MgSO_4{}^*7H_2O$ 0.5 g; agar 20 g and distilled water to 1 L) medium. The contents of the vials were filtered through RC 0.2 μm syringe filters and then analyzed with an HPLC. Aflatoxins were measured by comparing peak areas with a calibration curve obtained with standard solutions. The detection limits were 0.05 μg/kg for aflatoxins B_1 and G_1, and 0.1 μg/kg for aflatoxins B_2 and G_2 (signal to noise ratio of 3:1).

2.8. Natural occurrence of aflatoxins

Aflatoxins were measured by following the protocol of 49.2.18 AOAC Official Method 991.31, with minor modifications (AOAC Official Methods of Analysis and Volume, 2000b).

Twenty-five grams of maize grain was finely ground in a mill (Model MLI-204 Bühler (Milan, Italy)), and transferred a blender jar containing with 5 g NaCl. Aflatoxins were extracted with 125 ml methanol:water (80:20, v/v) by blending at high speed for 2 min with a Sorvall Omnimixer (Dupont Instruments, Newtown, CT, USA). The extract was filtered through Whatman no. 4 filter paper. Ten ml of filtered extract was diluted with 40 ml of distilled water and filtered through a glass microfiber filter. Ten ml of the diluted extract was applied to an Aflatest immunoaffinity column. The column was washed with 10 ml distilled water. Aflatoxins were eluted with 1.5 ml methanol. Eluates were evaporated to dryness under a stream of nitrogen at 50 °C. The residues were dissolved in 250 μl water:acetonitrile:methanol (64:13:23, v/v/v) and 100 μl of the resulting solution was injected into a HPLC apparatus by using a full loop injection system for aflatoxins analysis. Aflatoxins B_1 and G_1 were derivatized with Kobra Cell (Rhone Diagnostics Technologies Ltd., Lyon, France).

2.9. Isolation and analysis of nucleic acids

Fungal strains were grown and DNA was extracted as previously described by Perrone et al. (2006). A portion of the gene encoding β-tubulin, *benA*, was amplified by using primers Bt2a and Bt2b (Glass and Donaldson, 1995). A portion of the gene encoding calmodulin (*CaM*) also was amplified by using primers CL1 and CL2A (O'Donnell et al., 2000). Primers Act512F and Act738R (Hong et al., 2005) were used to amplify a portion of the gene encoding actin, *ACT*. All PCR amplification products were purified by gel filtration through Sephadex G-50 (Amersham Pharmacia Biotech, Piscataway, NJ, USA), equilibrated in double-distilled water, and then sequenced with and analyzed by an "ABI PRISM 3100 Genetic Analyzer" (Applied Biosystems).

2.10. Analysis of sequence data

The partial β-tubulin, calmodulin and actin gene sequences were aligned by using BioNumerics 5.1 from Applied Maths (Sint-Martens-Latem, Belgium) and adjusted, manually, as necessary. Phylogenetic and molecular evolutionary analyses were made with MEGA version 5 (Tamura et al., 2011). Phylogenetic trees were prepared by the neighbor-joining method (Saitou and Nei, 1987). Evolutionary distances were calculated by using the Tamura-Nei method embedded in the MEGA package and are expressed in units of the number of base substitutions per site (Tamura and Nei, 1993). All positions containing gaps or missing data were eliminated from the dataset (Complete deletion option). Bootstrap values were calculated from 1000 replications of the bootstrap test in MEGA. DNA polymorphism was evaluated with DnaSP v5 (Librado and Rozas, 2009).

3. Results

3.1. Occurrence and distribution of Aspergillus Sect. Flavi

Forty-two of the 56 market samples and 21 of the 35 farm samples contained strains of *Aspergillus* Sect. *Flavi* (Table 1). The range of incidence of contamination (% infected kernels) varied in both the market and the farm samples, with a mean of 21% for both types of samples, and a median of 11.5 and 16%, respectively (Table 1). *A. flavus* was the most commonly isolated species (98.5%), followed by *Aspergillus tamarii* (1.4%). In addition, there were two strains of *Aspergillus flavofurcatus,* a sibling species of *Aspergillus tamari,* from Ikenne and one strain of *Aspergillus parvisclerotigenus* from Mokwa. No isolates of *A. parasiticus* were recovered (Table 2). Species distribution was homogenous across the locations, with *A. tamari* recovered at 8/17 locations. All of the *A. flavus* strains recovered were of the L-type.

Table 1
Incidence and distribution of *Aspergillus* Sect. *Flavi* on maize samples from Ghana and Nigeria.

Samples	Number of positive/ tested samples	% Kernels infected with *Aspergillus* sect *Flavi*		
		Range %	Mean %	Median
Market	42/56	1–85	21.3	11.5
Farm	21/35	1–65	21.4	16

Table 2
Incidence of species within *Aspergillus* Section *Flavi* isolated from maize kernels in different locations of Nigeria and Ghana.

Location	Number of positive/tested samples	Number of strains isolated	*A. flavus* (%)	*A. tamari* (%)
Nigeria				
Abuja	3/4	22	91	9
Ado-Ekiti	4/5	82	95.	5
Akwanga	2/5	11	100	0
Bida	4/5	58	100	0
Ikire	3/6	56	100	0
Ikenne[a]	1/2	60	98	2
Kogi	4/5	74	100	0
Lafia	5/7	110	96	4
Makurdi	5/8	38	97	3
Minna	3/6	22	100	0
Mokwa[b]	4/5	128	99	1
Ogbomosho	6/8	187	99	1
Ghana				
Brong-Ahafo	3/4	39	95	5
Kintampo	3/4	94	100	0
Kpalsogu	4/5	85	100	0
Lepusi	3/6	52	100	0
Tamale	4/6	45	98	2

[a] 2 strains of *A. flavofurcatus* (ITEM 7810 and 7811) within the *A. tamarii* clade.
[b] 1 strain of *A. parvisclerotigenus* (ITEM 7819) was isolated.

3.2. Natural occurrence of aflatoxins

Aflatoxin contamination of the maize kernels varied by location. More samples were infested with strains from *Aspergillus* Sect. *Flavi* than were contaminated with aflatoxins (Table 3), probably reflecting the high proportion of atoxigenic L strains recovered. All four aflatoxins, B_1, B_2, G_1 and G_2 were detected in the maize kernels collected from farms, but only aflatoxins B_1 and B_2 were found in the market samples (Table 3). The incidence of aflatoxin contamination was higher (54%) in farm samples than in market samples (30%), with three farm samples, Lafia in Nigeria and Kpalsogu and Kintampo in Ghana, containing more than 1 ppm of aflatoxin B_1 respectively. The most heavily contaminated samples were from the districts of Brong-Ahafo (83–290 μg/kg), Kpalsogu (4–1400 μg/kg) and Kintampo (1200 μg/kg) in Ghana, and Lafia (1200 μg/kg) and Mokwa (5–480 μg/kg), in Nigeria.

3.3. Distribution of toxigenic and atoxigenic strains of *A. flavus*

The relative proportions of toxigenic and atoxigenic strains of *A. flavus* differed between the market and the farm samples (Fig. 1), with the farm samples more heavily infested with toxigenic strains than the market samples. This result is consistent with the higher levels of aflatoxin contamination found in the farm samples than in the market samples (Table 3). In general, the incidence of atoxigenic strains was significantly higher than that of toxigenic strains, however, some samples from Akwanga, Bida, Lafia and Lepusi also had a high percentage of aflatoxigenic strains. Twenty-five of the 110 *A. flavus* strains produced aflatoxin B_1, but none produced any G series aflatoxins, and 16 of the 25 B_1 producing strains produced both B_1 and B_2 (Table 4). Neither the 20 *A. tamarii* strains nor the two *A. flavofurcatus* strains produced any aflatoxins, which is consistent with their previously reported atoxigenicity. The single strain of *A. parvisclerotigenus*, isolated from a farm in Nigeria, produced all four aflatoxins.

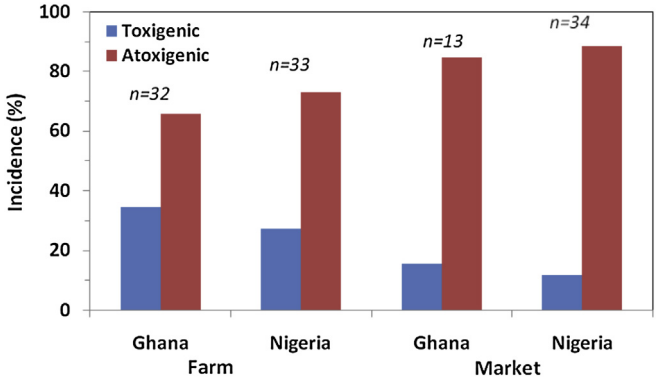

Fig. 1. Distribution of atoxigenic and toxigenic strains of *Aspergillus* Section *Flavi* in maize kernels from market and farm samples in Ghana and Nigeria. *N* = the sample size analyzed in of each farm and market sample type.

3.4. Analysis of genetic variation

We examined the genetic relatedness of 135 strains of *Aspergillus* Sect. *Flavi* (Table 4), by using nucleotide sequences of partial *benA*, *CaM* and *ACT* genes. Fifteen reference strains from *Aspergillus* Sect. *Flavi* in the ITEM Culture Collection (Fig. 2) were included, as was the type strain of *Aspergillus niger* (ITEM 4501), as an outgroup isolate. There were five haplotypes among the 110 strains of *A. flavus* from maize and only 6/1141 sites were polymorphic in the multilocus alignment of the three partial genes. Ninety-one strains belonged to haplotype 1, 14 belonged to haplotype 2, three belonged to haplotype 3, and one strain each belonged to haplotypes 4 and 5. There were two haplotypes amongst the 22 strains in the *A. tamarii* group based on the multilocus sequence alignment. One haplotype included the *A. tamarii* type strain (ITEM 9602) and 20/22 strains, while the second haplotype included the *A. flavofurcatus* type strain (ITEM 9603) and the other two field strains from the *A. tamarii* group. In a neighbor-joining phylogenetic analysis, all of the *A. flavus* strains clustered together, as did the *A. tamarii* strains. No strains of *A. parasiticus* were identified. A single strain, ITEM 7819, was identified as *A. parvisclerotigenus*, and it produced both B and G series aflatoxins (Table 4), as expected. Two strains (ITEM 7892 and 7899), initially placed in the *Flavi* Section were identified as *Aspergillus wentii*, a species that belongs to *Aspergillus* Sect. *Cremei*.

4. Discussion

In this study, we document the distribution and toxigenicity of species within *Aspergillus* Section *Flavi* from mature maize kernels

Table 3
Incidence and levels of aflatoxins in Maize samples from Ghana and Nigeria.

Samples	Number of positive/tested samples	Aflatoxin range (μg/kg)[a]				
		B_1	B_2	G_1	G_2	Total
Market	17/56	0.7–440 (74/5.3)[b]	0.1–71 (13/3.5)	–	–	0.5–480 (84/9)
Farm	19/35	0.1–1800 (290/80)	0.1–39 (11/6.4)	4.6–390 (113/31)	0.4–6.6 (1.9/0.5)	0.1–1900 (330/91)

[a] The range reported is related to the positive samples.
[b] The numbers in parenthesis represent the mean/median of aflatoxin contamination among the positive samples.

Table 4
Aspergillus Section *Flavi* strains isolated from maize characterized by sequence analysis and aflatoxins production.

Item#	Species	Source	Location	Aflatoxins (ng/g)			
				B_1	B_2	G_1	G_2
7764	*A. flavus*	Farm (1)	Lepusi, Ghana	510	35	–	–
7765; 7766; 7767; 7768	*A. tamarii*	Farm (1)	Lepusi, Ghana	–	–	–	–
7769; 7770; 7771; 7772; 7773	*A. flavus*	Farm (2)	Bida, Nigeria	–	–	–	–
7774	*A. flavus*	Farm (3)	Lepusi, Ghana	–	–	–	–
7775; 7776	*A. flavus*	Farm (4)	Lepusi, Ghana	–	–	–	–
7777	*A. flavus*	Farm (5)	Lepusi, Ghana	–	–	–	–
7778	*A. flavus*	Farm (5)	Lepusi, Ghana	48	2	–	–
7779	*A. flavus*	Farm (5)	Lepusi, Ghana	1.3	0.1	–	–
7780	*A. flavus*	Farm (5)	Lepusi, Ghana	1	0.1	–	–
7781; 7784; 7785	*A. flavus*	Farm (6)	Lepusi, Ghana	–	–	–	–
7782	*A. flavus*	Farm (6)	Lepusi, Ghana	0.7	–	–	–
7783	*A. flavus*	Farm (6)	Lepusi, Ghana	0.8	–	–	–
7786	*A. flavus*	Farm (7)	Kintampo, Ghana	0.8	–	–	–
7787	*A. flavus*	Farm (7)	Kintampo, Ghana	0.7	–	–	–
7788	*A. flavus*	Farm (7)	Kintampo, Ghana	0.7	–	–	–
7789	*A. flavus*	Farm (7)	Kintampo, Ghana	0.7	–	–	–
7790	*A. flavus*	Farm (8)	Kintampo, Ghana	0.4	–	–	–
7791; 7792; 7793; 7794; 7795	*A. flavus*	Farm (9)	Kpalsogu, Ghana	–	–	–	–
7796; 7797; 7798	*A. flavus*	Farm (10)	Kpalsogu, Ghana	–	–	–	–
7799; 7800; 7801; 7802; 7803	*A. flavus*	Farm (11)	Kpalsogu, Ghana	–	–	–	–
7804; 7805; 7806; 7807; 7808; 7809	*A. flavus*	Farm (12)	Ikenne, Nigeria	–	–	–	–
7810; 7811	*A. tamarii*	Farm (12)	Ikenne, Nigeria	–	–	–	–
7812; 7813; 7814; 7815; 7816	*A. flavus*	Farm (13)	Ogbomosho, Nigeria	–	–	–	–
7817	*A. flavus*	Farm (14)	Mokwa, Nigeria	0.6	–	–	–
7818	*A. flavus*	Farm (14)	Mokwa, Nigeria	–	–	–	–
7819	*A. parvisclerotigenus*	Farm (14)	Mokwa, Nigeria	120	2.4	210	2.9
7820	*A. flavus*	Farm (15)	Minna, Nigeria	10	0.3	–	–
7821; 7822	*A. flavus*	Farm (15)	Minna, Nigeria	–	–	–	–
7823; 7824	*A. flavus*	Farm (16)	Abuja, Nigeria	–	–	–	–
7825; 7826	*A. tamarii*	Farm (16)	Abuja, Nigeria	–	–	–	–
7827	*A. flavus*	Farm (17)	Akwanga, Nigeria	350	17	–	–
7828	*A. flavus*	Farm (17)	Akwanga, Nigeria	740	47	–	–
7829	*A. flavus*	Farm (17)	Akwanga, Nigeria	2	0.1	–	–
7830	*A. flavus*	Farm (18)	Lafia, Nigeria	1.6	0.1	–	–
7831	*A. flavus*	Farm (18)	Lafia, Nigeria	15	0.5	–	–
7832	*A. flavus*	Farm (18)	Lafia, Nigeria	310	15	–	–
7833	*A. tamarii*	Farm (18)	Lafia, Nigeria	–	–	–	–
7834; 7835	*A. flavus*	Farm (19)	Makurdi, Nigeria	–	–	–	–
7836	*A. tamarii*	Farm (19)	Makurdi, Nigeria	–	–	–	–
7837	*A. flavus*	Farm (20)	Ado-Ekiti, Nigeria	–	–	–	–
7838	*A. flavus*	Market (1)	Kpalisogu, Ghana	0.37	0.07	–	–
7839	*A. flavus*	Market (1)	Kpalisogu, Ghana	–	–	–	–
7844; 7845	*A. flavus*	Market (2)	Kintampo, Ghana	–	–	–	–
7859; 7860	*A. flavus*	Market (3)	Brong-Ahafo, Ghana	–	–	–	–
7871	*A. flavus*	Market (4)	Tamale, Ghana	–	–	–	–
7881; 7882; 7883; 7884	*A. flavus*	Market (5)	Tamale, Ghana	–	–	–	–
7885	*A. tamarii*	Market (5)	Tamale, Ghana	–	–	–	–
7886	*A. flavus*	Market (6)	Tamale, Ghana	1.2	0.1	–	–
7887	*A. flavus*	Market (6)	Tamale, Ghana	–	–	–	–
7888	*A. tamarii*	Market (6)	Tamale, Ghana	–	–	–	–
7893	*A. flavus*	Market (7)	Ogbomosho, Nigeria	–	–	–	–
7894	*A. flavus*	Market (8)	Ogbomosho, Nigeria	–	–	–	–
7895	*A. flavus*	Market (9)	Ogbomosho, Nigeria	–	–	–	–
7898	*A. flavus*	Market (10)	Ogbomosho, Nigeria	–	–	–	–
7900	*A. tamarii*	Market (10)	Ogbomosho, Nigeria	–	–	–	–
7905	*A. flavus*	Market (11)	Ogbomosho, Nigeria	0.3	–	–	–
7911	*A. flavus*	Market (12)	Mokwa, Nigeria	–	–	–	–
7913	*A. flavus*	Market (13)	Mokwa, Nigeria	–	–	–	–
7916	*A. flavus*	Market (14)	Bida, Nigeria	210	9.7	–	–
7917	*A. flavus*	Market (14)	Bida, Nigeria	52	2.8	–	–
7918	*A. flavus*	Market (14)	Bida, Nigeria	38	3.1	–	–
7919; 7920	*A. flavus*	Market (14)	Bida, Nigeria	–	–	–	–
7923	*A. flavus*	Market (15)	Bida, Nigeria	–	–	–	–
7925: 7926; 7927	*A. flavus*	Market (16)	Minna, Nigeria	–	–	–	–
7946	*A. flavus*	Market (17)	Lafia, Nigeria	–	–	–	–
7947	*A. tamarii*	Market (17)	Lafia, Nigeria	–	–	–	–
7951; 7952	*A. flavus*	Market (18)	Lafia, Nigeria	–	–	–	–
7953; 7954; 7955	*A. tamarii*	Market (18)	Lafia, Nigeria	–	–	–	–
7958; 7959; 7960	*A. flavus*	Market (19)	Makurdi, Nigeria	–	–	–	–
7961	*A. tamarii*	Market (19)	Makurdi, Nigeria	–	–	–	–
7969	*A. flavus*	Market (20)	Makurdi, Nigeria	–	–	–	–
7975	*A. flavus*	Market (21)	Kogi, Nigeria	–	–	–	–
7977; 7978	*A. flavus*	Market (22)	Kogi, Nigeria	–	–	–	–

Table 4 (*continued*)

Item#	Species	Source	Location	Aflatoxins (ng/g)			
				B_1	B_2	G_1	G_2
7979; 7980	*A. flavus*	Market (23)	Kogi, Nigeria	–	–	–	–
7984	*A. flavus*	Market (24)	Ado-Ekiti, Nigeria	–	–	–	–
7987	*A. flavus*	Market (25)	Ado-Ekiti, Nigeria	–	–	–	–
7989; 7990	*A. flavus*	Market (26)	Ado-Ekiti, Nigeria	–	–	–	–
7991; 7992; 7993; 7994	*A. tamarii*	Market (26)	Ado-Ekiti, Nigeria	–	–	–	–
7995	*A. flavus*	Market (27)	Abuja, Nigeria	–	–	–	–
8050	*A. flavus*	Market (28)	Abuja, Nigeria	–	–	–	–

collected from farms and markets of the major maize producing regions of Nigeria and Ghana. The farm samples were more highly contaminated with aflatoxins than were the market samples. *A. flavus* was the species most commonly isolated.

The limited sequence diversity among of the *A. flavus* strains is consistent with the hypothesis that the *A. flavus* L strain population from maize cultivated in Ghana and Nigeria is relatively young. This conclusion is based on the very limited genetic variation present in these populations, as it appears that these populations have recently been through a population bottleneck that reduced the variation present. Neither sexual reproduction nor mutation has operated at a sufficient level to generate many multilocus genotypes (recombination; Grubisha and Cotty, 2010) or variation at numerous nucleotide sites (mutation). Aflatoxin production has been postulated not to be advantageous for L strains of *A. flavus* in crop-associated niches (Donner et al., 2010).

Modern agricultural management may create unique ecological niches that select for toxigenic fungi and influence the relative proportion of toxigenic and atoxigenic strains in a population (Bilgrami et al., 1981). The structure of *A. flavus* populations and the aflatoxin contamination they produce can potentially be modulated by the availability of various nutrients in the environment (Mehl and Cotty, 2013). Highly competitive environmental conditions, such as those found in non-agricultural settings, might stabilize aflatoxin production ability and other wild-type characters in *A. flavus*, but be selectively disadvantageous in nutritionally rich

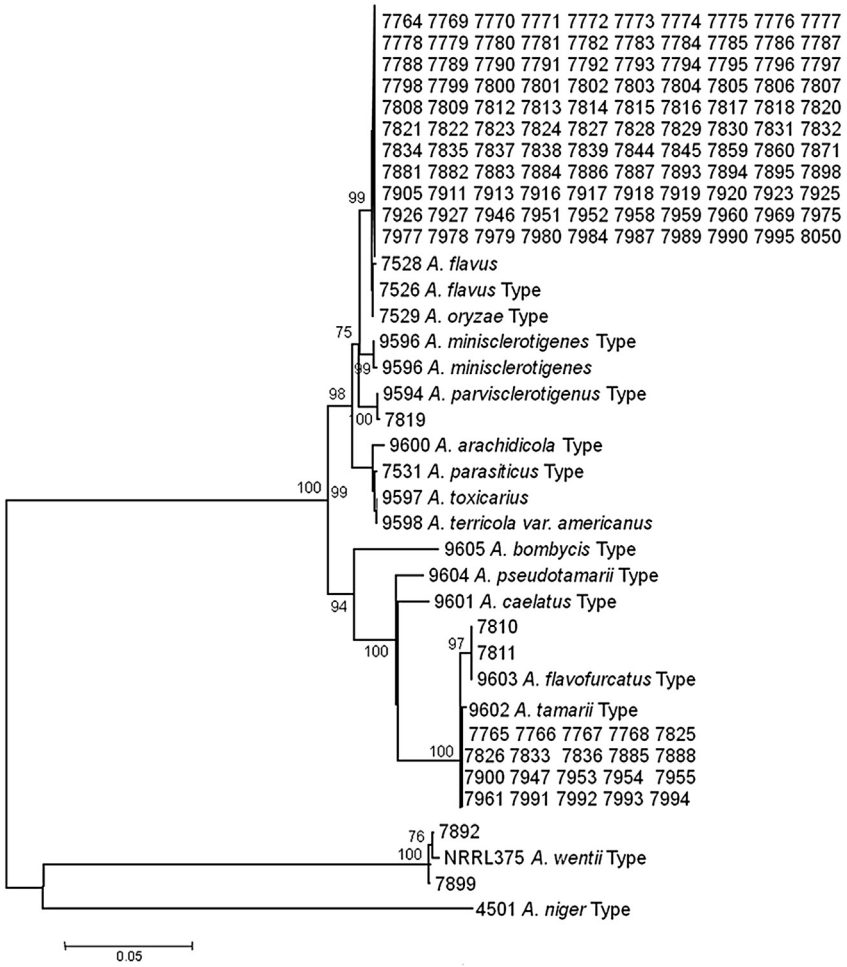

Fig. 2. Phylogenetic trees produced from the combined sequence data of the three loci (*CaM*, *benA*, *Act*) of 152 strains listed with ITEM collection reference numbers. On the branches only bootstrap values >70% are indicated (the sum of branch length is 0.6279). The evolutionary history was inferred using the Neighbor-Joining method.

environments, such as monoculture crop agroecosystems (Bilgrami et al., 1988; Horn and Dorner, 2002). In essence, polyketide metabolites, *e.g.*, aflatoxins, may increase fungal survival in soil, but be an unnecessary vestigial function in carbon-rich environments (Perrone et al., 2007). Adaptation by *A. flavus* to cultivated crops, in particular those that are carbon-rich, could facilitate loss of function and/or inactivation of genes that would provide a competitive edge in a more diverse environment. Many isolates that cannot produce aflatoxins carry multiple mutations in the aflatoxin gene cluster (Chang et al., 2005).

The widespread occurrence of L-type strains of *A. flavus* in Nigeria and Ghana on maize is consistent with previous reports of the dominance of this group of *A. flavus* isolates on maize from other continents/countries, *e.g.*, the United States (Cotty, 1997), Thailand (Ehrlich et al., 2007); Argentina (Nesci and Etcheverry, 2002), and Italy (Giorni et al., 2007). Atoxigenic strains of *A. flavus* were recovered more frequently than toxigenic strains at all locations and in both sample types (market or farm). Isolates of *A. tamarii* were identified in 15/50 samples collected (Table 4) at frequencies of 1–9% (Table 2). The distribution of strains of *Aspergillus* Sect. *Flavi* on maize varies by country and continent with one or more of *A. flavus* (L and S strains), *A. parasiticus*, *Aspergillus nomius*, and *A. tamarii* usually recovered. In most previous surveys, as in this one, ≥90% of the isolates are *A. flavus* L-strains. Our results are consistent with those of Atehnkeng et al. (2008b) who also evaluated the distribution of *Aspergillus* from maize in Nigeria, although they also recovered *A. parasiticus* (0.2–1%) and *A. flavus* S_{BG} strains (0.2–2.7%) at low levels while we did not. However, we recovered *A. parvisclerotigenus*, which could be the same as the previously unidentified S_{BG} group (Frisvad et al., 2005; Donner et al., 2009). Based on multilocus analysis of three genes of the aflatoxin biosynthetic cluster, some S_{BG} strains belong to *A. minisclerotigenes* (Probst et al., 2012), and others to an unknown taxon/group from Nigeria. Unfortunately, representatives of *A. parvisclerotigenus* were not included in this analysis.

The substrate and geographic distribution of recently described *Aspergillus* species warrant further investigation, but these species probably are not significant sources of aflatoxin contamination of foods and feeds due to their rare occurrence and limited distribution. *A. minisclerotigenes*, but not *A. parvisclerotigenus*, was isolated from maize in Portugal (Soares et al., 2012). The identification of *A. parvisclerotigenus* from maize in our study is a first. The need remains (Pildain et al., 2008) for a more detailed phenotypic study of isolates of *A. parvisclerotigenus* to determine if there are other phenotypic differences between the described taxa that produce small sclerotia, the extent of the aflatoxin contamination attributable to these species, and their relationship to strains with the S_{BG} phenotype that have not been assigned to a species.

The high frequencies of *A. flavus* compared to other members of *Aspergillus* section *Flavi* may be explained by the high frequency of this species in soil, plant debris and insects (Horn and Dorner, 1999; Jaime-Garcia and Cotty, 2004; Nesci and Etcheverry, 2002), which act as reservoirs of inoculum for maize infection in the field. Most of the *A. flavus* isolates we evaluated were atoxigenic, with toxigenic strains recovered more frequently from farm samples than from market samples (Fig. 1). The proportion of aflatoxigenic strains amongst the L strains in our survey is lower (23%) than the 40% previously reported for Nigeria (Atehnkeng et al., 2008b) or the 56% reported by Donner et al. (2009). A similarly broad range in the frequency of toxigenic strains recovered is known in other locations as well. For example, in the southern United States (Cotty, 1997; Horn and Dorner, 1999) most isolates of *A. flavus* produced aflatoxins, while in Argentina, only 29% of the *A. flavus* isolates were toxigenic (Vaamonde et al., 2003). Since the L type of *A. flavus* was the most common *Aspergillus* contaminant of maize, management strategies to reduce aflatoxin contamination should be directed at this species first.

Differences in aflatoxigenic fungal community structure are reflected in the relative abundance of the B and G series aflatoxins contaminating crops produced in different geographic areas (Cotty, 1997). In particular, S_B strains of *A. flavus*, are associated with acute aflatoxin contamination of maize in Africa (Probst et al., 2010; Donner et al., 2009). The relative infrequency of S_B and S_{BG} strains in West Africa suggests that the major problems associated with aflatoxin contamination in foods in West Africa will be due to long-term chronic exposure to the toxin, rather than the more sensational acute toxicity reported from Kenya and East Africa. The pattern of contamination of maize samples we detected, reinforces previous reports of aflatoxin risk in pre- and post-harvest maize from West Africa, and reaffirms that rural Africans are regularly and routinely exposed to aflatoxin levels that could have serious long-term health effects including liver cancer and suppressed immunity.

In summary, we found that atoxigenic strains of *A. flavus* dominated the *Aspergillus* populations recovered from maize in Nigeria and Ghana. Although some S_{BG} strains were recovered, most of the strains produced relatively low levels of aflatoxins. These results are similar to those from other surveys of maize (primarily market samples) from West Africa. The difference between the samples collected on-farm and those from the markets is of particular importance. The on-farm samples were generally more heavily contaminated than were the samples from the markets. The on-farm samples generally were freshly harvested, and their significant contamination and colonization emphasizes the importance of in-field, pre-harvest contamination to the overall contamination problem. In other words, aflatoxin contamination and *Aspergillus* colonization of maize are not just post-harvest problems. Samples from the markets may represent the best estimate for risk from inadequate storage, as only the best grain is sold off farm. Thus, the data in the present study suggest that the exposure of rural inhabitants in Africa to aflatoxins is even higher than estimated in earlier studies, which use aflatoxin levels primarily from market samples to estimate risks.

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