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Diversity of aflatoxin-producing fungi and their impact on food safety in sub-Saharan Africa

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ABSTRACT

Crops frequently contaminated by aflatoxins are important sources of revenue and daily nourishment in many portions of sub-Saharan Africa. In recent years, reports have associated aflatoxins with diminished human health and export opportunities in many African Nations. Aflatoxins are highly carcinogenic metabolites mainly produced by members of *Aspergillus* sect. *Flavi*. The current study examined aflatoxin-producing fungi associated with maize grain intended for human consumption in 18 sub-Saharan African countries. 4469 *Aspergillus* sect. *Flavi* isolates were obtained from 339 samples. The majority (75%) of isolates belonged to the L strain morphotype of *A. flavus*. Minor percentages were *A. tamarii* (6%), *A. parasiticus* (1%), and isolates with S strain morphology (3%). No *A. bombycis* or *A. nomius* isolates were detected. Phylogenetic analyses of partial sequences of the nitrate reductase gene (*niaD*, 1.3 kb) and the aflatoxin pathway transcription factor gene (*aflR*, 1.7 kb) were used to verify isolate assignments into species and lineages. Phylogenetics resolved S strain isolates producing only B aflatoxins into two lineages fully supported by sizes of deletions in the gene region spanning the aflatoxin biosynthesis genes *cypA* (aflU) and *norB* (aflF). One lineage was the *A. flavus* S strain with either 0.9 or 1.5 kb deletions. The second lineage, recently described from Kenya, has a 2.2 kb deletion. Taxa with S strain morphology differed in distribution with strain S_{BG} limited to West Africa and both *A. minisclerotigenes* and the new lineage from Kenya in Central and East Africa. African *A. flavus* L strain isolates formed a single clade with L strain isolates from other continents. The sampled maize frequently tested positive for aflatoxins (65%), fumonisins (81%), and deoxynivalenol (40%) indicating the presence of fungi capable of producing the respective toxins. Percentage of samples exceeding US limits for total aflatoxins (regulatory limit), fumonisins (advisory limit), and deoxynivalenol (advisory limit) were 47%, 49%, 4%, respectively.

1. Introduction

Maize is a staple grown and eaten throughout Africa (McCann, 2005). Mycotoxin contamination of maize increases health risks for both humans and domestic animals (Wild et al., 1991; Peers et al., 1976) and frequently diminishes crop value (Khlangwiset et al., 2011). Aflatoxins are highly toxic with aflatoxin B_1 listed by the International Agency for Research on Cancer (IARC) as carcinogenic to humans (International Agency for Research on Cancer, 2002). The risks of human aflatoxicosis is high in countries where strict aflatoxin regulations are not in force and crops are consumed without monitoring (Shephard, 2003; Williams et al., 2004). Reduced growth in children (Gong et al., 2004, 2008), impaired immune systems (Turner et al., 2003), and severe liver damage (Turner et al., 2000) are consequences of dietary aflatoxin exposure. Fumonisins, first reported from moldy maize in South Africa (Syndenham et al., 1990), compromise human (e.g. neural tube defects in newborn children) and animal health by interfering with sphingolipid, phospholipid and fatty acid metabolism. Fumonisin B1 is possibly carcinogenic to humans and was classified by IARC as a Group 2B carcinogen (International Agency for Research on Cancer, 2002). Like fumonisins, deoxynivalenol (DON), also known as Vomitoxin, was first reported from moldy maize grain from southern Africa (Vesonder et al., 1973). Exposure to DON, a type B trichothecene, can cause typical symptoms of food-borne illness including vomiting, diarrhea and dizziness in both animals and humans (Pestka and Smolinski, 2005; Pestka, 2007). DON was implicated in one of the largest outbreaks of human mycotoxicosis in India in 1987 affecting 50,000 people (Bath et al., 1989).

Structures of fungal communities associated with crops heavily influence the severity of aflatoxin contamination (Cotty et al., 2008; Grubisha and Cotty, 2010). The contamination process is complex and starts in the field where crops first 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 to fungal growth (e.g. temperatures above 28 °C) increase susceptibility of crops to infection (Cotty et al., 1994, 2008). Contamination continues after crop maturation when the crop is exposed to warm temperature and

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humidity, both in the field and during storage (Cotty et al., 1994, 2008). Aflatoxin levels may increase and new infections become established until crops are ultimately consumed (Cotty et al., 1994). In order to estimate risks associated with communities of aflatoxin-producing fungi resident on maize both aflatoxin-producing potentials and frequencies of occurrence need to be considered (Cotty et al., 2008; Probst et al., 2010).

Most fungi capable of producing aflatoxins belong to *Aspergillus* sect. *Flavi*. Members of this section vary widely in many characteristics including aflatoxin production and ability to infect and decay crops (Cotty, 1997; Horn, 2007). *A. flavus* is the most common causal agent of contamination (Klich, 2007). The species can be delineated into two major morphotypes, the L strain (produces copious amounts of conidia and relatively few, large sclerotia, average diameter >400 μm) and the S strain (produces few conidia and copious amounts of small sclerotia, average diameter <400 μm) (Cotty, 1989). On average, isolates of the L strain morphotype produce less aflatoxin than isolates of the S strain morphotype (Cotty, 1989; Probst et al., 2010). Atoxigenic (absence of aflatoxin production) isolates of the L strain morphotype are frequently isolated from crops and soils (Atehnkeng et al., 2008; Cotty, 1997). Increased frequencies of atoxigenics reduce the average aflatoxin-producing potential of fungal communities and, as a result, the aflatoxin content of crops (Cotty et al., 2008).

Molecular phylogenetics has increased the number of described species that produce aflatoxins (Feibelman et al., 1998; Ito et al., 2001; Peterson et al., 2001; Ehrlich et al., 2002; Pildain et al., 2008). However, the importance of newly described species to crop contamination remains unclear. Since recent outbreaks of acute human aflatoxicosis in Kenya (CDC, 2004), aflatoxin contamination of food crops in Africa has received increased attention (Integrated Regional Information Networks, 2010; Probst et al., 2010; Atser, 2011). Contamination events resulting in severe aflatoxicoses in Kenya have been attributed to a recently described fungal lineage with S strain morphology within section *Flavi* (Probst et al., 2007). This lineage is distinguished by a 2.2 kb deletion in the aflatoxin biosynthesis gene *cypA*. Aflatoxin-producing fungi with similar morphologies include the S strain of *A. flavus*, the aflatoxin B and G producing species *A. minisclerotigenes*, and an unnamed *Aspergillus* species common in West Africa (commonly referred to as strain S_{BG}) (Donner et al., 2010; Probst et al., 2012). Knowledge of the distribution of these aflatoxin producers in sub-Saharan Africa is limited (Cotty and Cardwell, 1999; Cardwell and Cotty, 2002; Kaaya and Kyamuhangire, 2006; Bandyopadhyay et al., 2007; Atehnkeng et al., 2008; Probst et al., 2010) and detailed comparisons among African nations on any single crop are lacking. Even though fungi with S strain morphology are associated with the most deadly known episodes of aflatoxicosis, such taxa have only been sporadically described in Africa and distributions across Africa are largely unexplored. The current study provides insights into both distributions of aflatoxin-producing fungi across the continent and causal agents of aflatoxin contamination of maize of potential regional importance.

The study objectives were: i) to characterize *Aspergillus* sect. *Flavi* communities on maize from countries in East, West and Southern sub-Saharan Africa, ii) to assess relationships of the identified fungi to described aflatoxin-producers, iii) to define distributions of aflatoxin-producing fungi with S strain morphology on maize across sub-Saharan Africa, and iv) to relate characteristics of *Aspergillus* communities to concentrations of aflatoxins in the examined maize. In addition, other mycotoxins (fumonisins and deoxynivalenol) common in maize and of concern for human health were quantified to assess importance of aflatoxins in relation to other mycotoxins.

2. Material and methods

2.1. 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 (Fig. 1). To ensure that grains were produced locally, maize grain was sampled from individual farmers' fields or small local markets. Generally, 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 water content (HB43 Halogen Moisture Analyzer; Mettler Toledo, Columbus, OH, USA), 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 further analyses. The maize was finely ground in a laboratory hammer mill (IKA Labortechnik, Heitersheim, Germany), and fungal isolates were recovered as described below.

2.2. Fungal isolation and quantification

Aspergillus sect. *Flavi* colonies were obtained by dilution plate technique on modified Rose Bengal agar (Cotty, 1994). 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 of an appropriate dilution (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. *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, pH 5.2), incubated unilluminated for 5 to 7 days at 31 °C, and identified as to species and, if applicable, strain on the basis of colony, conidia and sclerotia morphologies (Klich and Pitt, 1988; Cotty, 1989; Probst et al., 2012). Fungal isolations were conducted at least twice for each sample to yield a total of 15 to 20 isolates. Representative isolates for each morphotype from each country were subjected to DNA based phylogenetic analyses to verify assignments based on morphological criteria.

2.3. In vitro aflatoxin production

Ability to produce either no aflatoxins, B and G aflatoxins, or only B aflatoxins was determined on autoclaved maize grain for the same isolates subjected to phylogenetic analyses utilizing the aflatoxin production assay previously described (Probst and Cotty, 2012). Briefly, healthy, undamaged maize kernels (10 g/250 ml Erlenmeyer flask) were autoclaved for 60 min at 121 °C. 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. Maize was inoculated with a conidia suspension of the tested isolate (10^6 conidia/ml). The quantity of conidia was determined by turbidity using a turbidity meter (Model 965-10; Orbeco-Hillige, Farmingdale, NY) and concentrations were calculated [Nephelometric turbidity unit (NTU) versus colony forming unit (CFU) curve (Y = 49,937X; X = NTU, Y = conidia per ml)]. 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/10 g maize). 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 visualized using thin-layer chromatography (TLC) and quantified with a scanning densitometer (TLC Scanner 3, Camag Scientific Inc., Wilmington, NC).

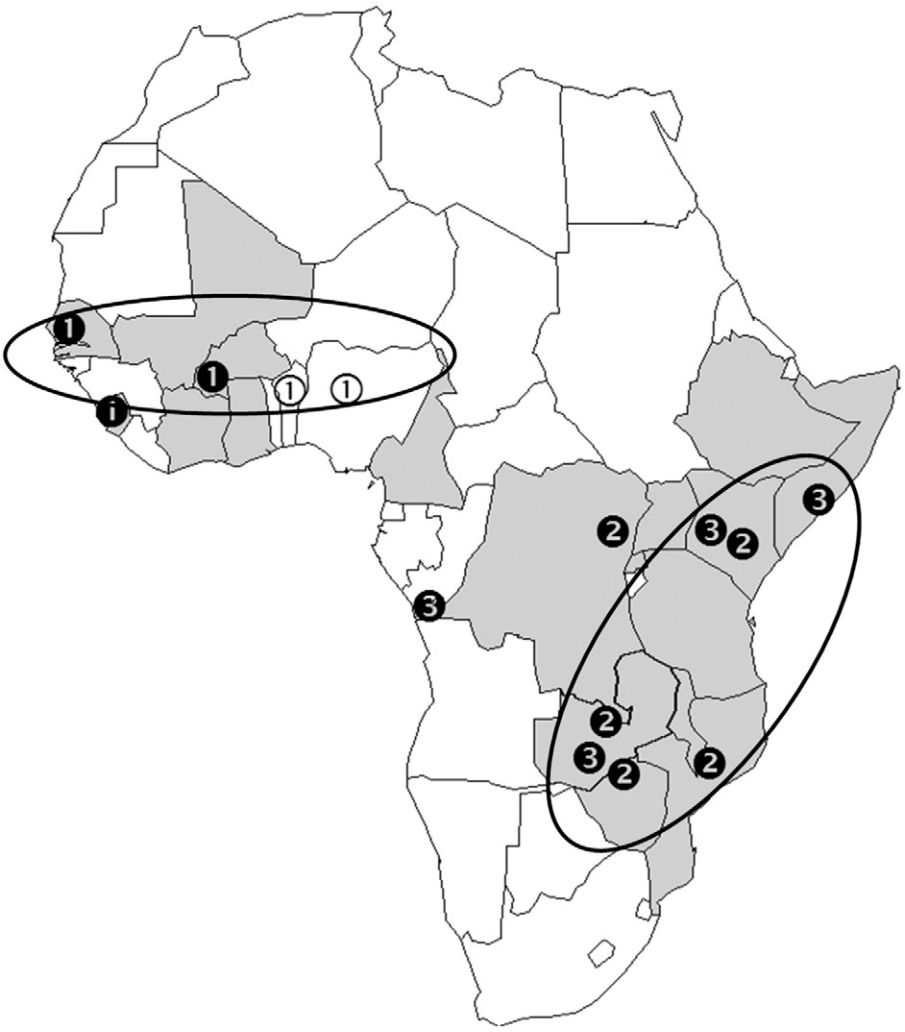

Fig. 1. Map of Africa indicating sampled countries and incidences of isolates with S strain morphotypes. 1: Strain S_{BG}, 2: new fungal lineage, 3: *A. minisclerotigenes*. Incidences for the individual countries were as follows: Burkina Faso (1, n = 11; 2, n = 0; 3 n = 0), Senegal (1, n = 7; 2, n = 0; 3, n = 0), Sierra Leone (1, n = 3; 2, n = 0; 3, n = 0), DR Congo (1, n = 0; 2, n = 1; 3, n = 1), Kenya (1, n = 0; 2, n = 5; 3, n = 2), Somalia (1, n = 0; 2, n = 0; 3, n = 1), Mozambique (1, n = 0; 2, n = 1; 3, n = 0), Zambia (1, n = 0; 2, n = 4; 3, n = 2), Zimbabwe (1, n = 0; 2, n = 3; 3, n = 0). Regions with semi-arid and sub-humid climates are circled.

2.4. Mycotoxin extraction and analyses

Enzyme-linked immunosorbent assay (ELISA) tests were performed utilizing test kits (Strategic Diagnostics, Inc., Newark, DE; MycoChek® Test Kit) certified by the USDA, Grain Inspection, Packers and Stockyards Administration (GIPSA) to quantify concentrations of aflatoxins, fumonisins and DON within the maize. Mycotoxins were extracted from the maize matrix following the 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 manufacturer's instructions (http://www.sdix.com). Mycotoxin concentrations were quantified by measuring color intensity with a microplate reader (ELX800, Biotek Instruments Inc., Winooski, VT) at 650 nm and extrapolating from standard curves generated for each microplate.

2.5. Molecular analyses and phylogeny

Phylogenetic reconstructions using 1536 bp sequence from the nitrate reductase gene (*niaD*, on chromosome 4) were performed to verify assignment of isolates to specific *Aspergillus* species was consistent with isolates of the species previously described from other continents. In general, three *A. flavus* L strain isolates were randomly chosen from each African country. Reference isolates of *A. parasiticus*, *A. tamarii*, and isolates with S strain morphology producing both B and G aflatoxins (*A. minisclerotigenes* and strain S_{BG}) or only B aflatoxins (*A. flavus* and the new lineage from Kenya) were included: *Aspergillus* isolates from the Americas (USA and Argentina), Australia, Asia and Europe included reference isolates for the *A. flavus* S strain [isolates AF70 and AF42 (Cotty, 1989)], *A. flavus* L strain [isolates AF13 (Cotty, 1989) and NRRL3251 (Hesseltine et al., 1970)], *A. bombycis* [isolate 174 (Hesseltine et al., 1970)], *A. minisclerotigenes* [isolates A-11611, 4-2, 12-4, and 13-4 (Hesseltine et al., 1970; Pildain et al, 2008)], *A. parasiticus* [isolates NRRL 2999 (Hesseltine et al., 1970) and BNO09-E (Cotty and Cardwell, 1999)], the West African strain S_{BG} [isolates A-11612 (Hesseltine et al., 1970), BNO38-G and BNO40-B (Cotty and Cardwell, 1999)] and the unnamed new lineage from Kenya isolate [isolate K06 44-K (Probst et al, 2012)].

Isolates were grown and collected as previously described (Donner et al., 2010). DNA was isolated using the FastDNA SPIN Kit and the FastPrep Instrument following the 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 prior to PCR. Approximately 1.3 kb of the *niaD* gene was amplified with two sets of primers (Table 1). For isolates

Table 1
Primers and locus specific annealing temperature (T_a) used for PCR amplifications.

Forward primer		Reverse primer		T_a (°C)[a]	Product (bp)
niadF	CGGACGATAAGCAACAACAC	niadAR	GGATGAACACCCGTTAATCTG	52	795
niadBF	ACGGCCGACAGAAGTGCTGA	niad1R	GGTCCAGGGCCCAGTTCAAT	57	741
CP-5 F	GGGACCCTTTTCCGGTGCGG	CPR-R	GGCGGCCCCTCAGCAAACAT	62	3100
AP1729	GTGCCCAGCATCTTGGTCCACC	AP3551	AAGGACTTGATGATTCCTC	58	1700
aflR1F	CATGGCTGAGGATAGCTCGTG	aflR2R	ACGGTGGCGGGACTGTTGCTACA	60	2092
aflR1F[b]	CATGGCTGAGGATAGCTCGTG	aflR1R	TCGTGGAGGTGAGGAAGG AAT	–	729
aflRF[b]	GGAAACAAGTCTTTTCTGG	aflRR	CAGAGCGTGTGGTGGTTG	–	779
aflR2F[b]	GACTTCCGGCGCATAACACGT A	aflR2R	ACGGTGGCGGGACTGTTGCTACA	–	786

[a] Primer specific annealing temperature.
[b] Sequencing primer.

with S strain morphology (n = 37) the *aflR* gene was amplified with one set of primers (aflR1F-aflR2R) covering approximately 2.1 kb of the gene. Segments of the 2.1 kb *aflR* amplicon were sequenced using three pairs of sequencing primers each covering approximately 0.7 kb of the PCR product (Table 1). PCR reactions were performed in 20 μl using 2 μl genomic DNA and the PCR PreMix (AccuPower® HotStart) with: 5 min at 95 °C followed by 38 cycles of 20 s at 94 °C (denaturalization), 30 s at the locus-specific annealing temperature (Table 1), and 30 s at 72 °C (extension) and a final 10 min at 72 °C. Amplicons were sequenced at 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, corrected manually, and aligned using the MUSCLE algorithm with default settings in Geneious Pro Version 4.8.2 (Biomatters Ltd, Auckland, New Zealand).

Maximum likelihood (ML) analyses were conducted with Phylip Version 3.69 (Felsenstein, 1989) and maximum parsimony (MP) trees generated with PAUP* edition 4.0b10 (Swofford, 1998) were used to confirm tree topologies. Support for inferred topologies was evaluated with 1000 bootstrap replicates. Branches with less than 85% support were collapsed. Gaps were treated as missing data. Trees were mid-point rooted and visualized with FigTree v.1.3.1 (http://tree.bio.ed.ac.uk/).

2.6. Sequence deletions in cypA

Sizes of *cypA*/*norB* deletions in the aflatoxin biosynthesis gene cluster were determined for isolates that did not produce G aflatoxins. Deletion size is diagnostic for a new lineage of aflatoxin-producing fungi common in Kenya. PCR utilized previously described primer sets (Ehrlich et al., 2004; Table 1). Amplicons were sequenced as above and aligned with known sequences to confirm deletion size and position (Probst et al., 2012).

3. Results

3.1. Maize grain samples and fungal isolation

The 364 maize grain samples from 18 countries in sub-Saharan Africa collected from June 2006 through January 2008 had an average mass of 110 g (Table 3). Samples negative (n = 25) for the three analyzed mycotoxins and for which no microorganism was recovered were considered to have been degraded and were excluded from the study. A total of 4469 *Aspergillus* sec. *Flavi* isolates were obtained from 339 samples (Table 3). Most isolates belonged to the *A. flavus* L strain (84.2%), followed by *A. tamarii* (7.4%), isolates with S strain morphology (3.0%), and *A. parasiticus* (1.2%). Isolates with S strain morphology were assigned to one of four taxa based on aflatoxin production (production of B and G aflatoxins or only B aflatoxins), size of the *cypA*/*norB* sequence deletion (no deletion, 1.5 kb, 0.9 kb or 2.2 kb deletion), and phylogenetics. Assignments based on the *cypA* deletion agreed with assignments based on phylogeny (Fig. 2, Table 2). Frequencies of these taxa were as follows: the new lineage of aflatoxin B producers previously described only from Kenya (1.7%), strain S_{BG} (0.8%), *A. minisclerotigenes* (0.5%), and the S strain morphotype of *A. flavus* (0.02%). The *A. flavus* S strain was detected only in a single maize sample from Rwanda (isolate RW 54-P).

3.2. Mycotoxin analyses

ELISA was used to determine concentrations of total aflatoxins (μg/kg = ppb), fumonisins (μg/g = ppm) and deoxynivolenol (DON, μg/g) (Table 4). Samples were grouped based on mycotoxin content. Aflatoxins: 178 samples contained aflatoxin concentrations below the limit of detection (LoD = 1 μg/kg); 115 samples (34%) contained 2 to 19 μg/kg total aflatoxins (mean = 7.3 μg/kg); 23 samples (7%) ranged from 20 to 99 μg/kg total aflatoxins (mean = 42.4 μg/kg); 22 samples (6%) ranged from 100 to 999 μg/kg total aflatoxins (mean = 311.2 μg/kg); one sample had >1000 μg/kg total aflatoxins (1408.9 μg/kg, Table 4). The greatest proportions of maize grain samples contaminated with aflatoxin concentrations >100 μg/kg occurred in the Coast Province of Kenya (33%), followed by Somalia (22%), and Uganda (20%).

Fumonisins (Table 4): 64 samples contained fumonisin concentrations below the limit of detection (LoD = 0.5 μg/g); 110 samples (32%) had <1 μg/g total fumonisins (mean = 0.1 μg/g); 116 samples (34%) contained 1 to 4.9 μg/g total fumonisins (mean = 2.2 μg/g); 23 samples (7%) had 5 to 19 μg/g total fumonisins (mean = 8.7 μg/g); 14 samples (4%) contained 20 to 100 μg/g total fumonisins (mean = 49.5 μg/g); twelve samples (3%) had >100 μg/g total fumonisins (mean = 143.3 μg/g). All maize samples from Zimbabwe were contaminated with >10 μg/g total fumonisins, with 53% of the samples containing quantities >100 μg/g. Most of these samples (79%) contained aflatoxin levels below 4 μg/kg. This is in contrast to samples from the Coast Province in Kenya. Here, 50% of samples contained high levels of both fumonisins (mean = 32 μg/g) and aflatoxins (mean = 97 μg/kg). Aflatoxins and fumonisins both contaminated 35% of the maize. However, 77% of the maize contained at least one of these detrimental toxins.

DON (Table 4): 177 samples contained DON concentrations below the limit of detection (LoD = 0.1 μg/g); 107 samples (35%) had <1 μg/g total DON (mean = 0.23 μg/g), 22 samples (7%) contained 1 to 4.9 μg/g total DON (mean = 1.9 μg/g); and three samples (1%) had ≥5 μg/g total DON (mean = 9.1 μg/g). Two out of three samples with DON ≥5 μg/g originated in Zimbabwe.

Insufficient sample sizes prevented quantification of DON for maize from Burkina Faso and Zambia and fumonisin for 15 samples from Somalia.

3.3. Phylogenetic analyses

All isolates placed in the L strain morphotype of *A. flavus* formed a highly supported clade with both L and S strain *A. flavus* isolates from the Americas, Australia, and Asia (Fig. 2, Group 2). Phylogenetic analyses of the concatenated *niaD* and *aflR* sequences split isolates with S strain morphology and ability to produce aflatoxins B and G into two clades containing either strain S_{BG} (e.g. reference isolates A-11612, BNO38-G, and BNO40-B) or *A. minisclerotigenes* (e.g. isolate A-

Table 2
Aspergillus sect. *Flavi* isolates used for phylogenetic reconstructions.

	Country	Substrate	Group[a]	Isolate	Species[b]	Morphotype[c]	Aflatoxins[d]	Deletion (kb)[e]
Africa	Benin	Soil	2	BN001-B	*A. flavus*	L	None	0.9
			2	BN011-N	*A. flavus*	L	B	0.9
			6	BN009-E	*A. parasiticus*	n/a	BG	0.0
			1	BN023-O	*A. tamarii*	n/a	None	0.0
			5	BNO38-G	Strain S_{BG}	S	BG	0.0
			5	BNO40-B	Strain S_{BG}	S	BG	0.0
	Burkina Faso	Maize	2	BF1-A	*A. flavus*	L	B	1.5
			2	BF4-B	*A. flavus*	L	B	0.9
			2	BF9-G	*A. flavus*	L	B	1.5
			5	BF3-H	Strain S_{BG}	S	BG	0.0
			5	BF13-H	Strain S_{BG}	S	BG	0.0
			5	BF22-N	Strain S_{BG}	S	BG	0.0
			5	BF28-F	Strain S_{BG}	S	BG	0.0
			5	BF35-E	Strain S_{BG}	S	BG	0.0
			5	BF38-G	Strain S_{BG}	S	BG	0.0
			5	BF45-K	Strain S_{BG}	S	BG	0.0
	Cameroon	Maize	2	Cam49-A	*A. flavus*	L	None	1.5
			2	Cam53-D	*A. flavus*	L	None	0.9
			2	CamMO-C	*A. flavus*	L	None	1.5
	Congo East	Maize	2	CONGO E1-E	*A. flavus*	L	None	1.5
			2	CONGO E15-A	*A. flavus*	L	None	0.9
			–	CONGO E5-A	Unidentified	n/a	BG	0.0
			4	CONGO E10-X	New lineage, S_B	S	B	2.2
	Congo, Bas	Maize	2	CONGO W7-A	*A. flavus*	L	B	0.9
			2	CONGO W8-X	*A. flavus*	L	B	0.9
			2	CONGO W10-A	*A. flavus*	L	B	1.5
			3	CONGO W8-D	*A. minisclerotigenes*	S	BG	0.0
	Ethiopia	Maize	2	ET48-B	*A. flavus*	L	None	1.5
			2	ET64-A	*A. flavus*	L	None	1.5
			2	ET78-D	*A. flavus*	L	B	1.5
			1	ET58-D	*A. tamarii*	n/a	None	0.0
			–	ET72-B	Unidentified	S	BG	0.0
	Ghana	Maize	2	GH1-C	*A. flavus*	L	B	1.5
			2	GH2-A	*A. flavus*	L	B	1.5
			2	GH3-B	*A. flavus*	L	B	1.5
	Ivory Coast	Maize	2	CI2-A	*A. flavus*	L	B	1.5
			2	CI3-I	*A. flavus*	L	B	0.9
			2	CI4-B	*A. flavus*	L	B	1.5
	Kenya	Maize	2	K06 4-I	*A. flavus*	L	B	–
			2	K06 24-D	*A. flavus*	L	None	1.5
			2	MW6-B	*A. flavus*	L	None	1.5
			2	MW15-L	*A. flavus*	L	None	1.5
			–	Rift 2-C	Unidentified	L	BG	0.0
			4	K06 44-K	New lineage, S_B	S	B	2.2
			4	K06 56-A	New lineage, S_B	S	B	2.2
			4	K05 59-M	New lineage, S_B	S	B	2.2
			4	K04 921-E	New lineage, S_B	S	B	2.2
			–	K06 111-D	Unidentified	S	BG	0.0
			3	Rift4-A	*A. minisclerotigenes*	S	BG	0.0
			3	Rift6-A	*A. minisclerotigenes*	S	BG	0.0
			3	Rift8-N	*A. minisclerotigenes*	S	BG	0.0
			4	Rift12-N	New lineage, S_B	S	B	2.2
	Malawi	Maize	2	MW13-B	*A. flavus*	n/a	B	1.5
			6	MW13-L	*A. parasiticus*	n/a	BG	0.0
	Mali	Maize	2	ML4-C	*A. flavus*	L	None	0.9
			2	ML6-B	*A. flavus*	L	None	1.5
	Mozambique	Maize	2	MZ7-A	*A. flavus*	L	None	1.5
			2	MZ2-A	*A. flavus*	L	None	1.5
			2	MZ35-L	*A. flavus*	L	None	1.5
			6	MZ31-L	*A. parasiticus*	n/a	BG	0.0
			4	MZ17-L	New lineage, S_B	S	B	2.2
	Nigeria	Soil	2	Mi18G-27	*A. flavus*	L	None	1.5
			2	Lo44G-19	*A. flavus*	L	B	1.5
			2	Ak29G-01	*A. flavus*	L	B	1.5
			3	A11611	*A. minisclerotigenes*	S	BG	0.0
			5	A11612	Strain S_{BG}	S	BG	0.0
	Rwanda	Maize	2	RW 47-F	*A. flavus*	L	B	1.5
			2	RW 4-A	*A. flavus*	L	B	0.9
			2	54-O	*A. flavus*	L	B	0.9
			2	54-P	*A. flavus*	S	B	0.9
	Senegal	Maize	2	SN 16-D	*A. flavus*	L	None	–
			2	SN 8-G	*A. flavus*	L	None	–
			5	SN 11-C	Strain S_{BG}	S	BG	0.0

(continued on next page)

Table 2 (*continued*)

	Country	Substrate	Group[a]	Isolate	Species[b]	Morphotype[c]	Aflatoxins[d]	Deletion (kb)[e]
	Senegal	Maize	5	SN 3-A	Strain S_{BG}	S	BG	0.0
			5	SN 2-A	Strain S_{BG}	S	BG	0.0
	Sierra Leone	Maize	2	SL 3-N	*A. flavus*	L	None	1.5
			2	SL 14-L	*A. flavus*	L	None	–
			2	SL 5-H	*A. flavus*	L	None	–
			2	So 7-A	*A. flavus*	L	None	–
			2	So 3a-I	*A. flavus*	L	None	0.9
			2	So 6-J	*A. flavus*	L	None	1.5
			1	SL 16-P	*A. tamarii*	n/a	None	0.0
	Somalia	Maize	1	So 19-G	*A. tamarii*	n/a	None	0.0
			3	So 6a-A	*A. minisclerotigenes*	S	BG	0.0
	Tanzania	Maize	2	TN 1-E	*A. flavus*	L	None	1.5
			2	TN 5-O	*A. flavus*	L	B	1.5
			2	TN 2-R	*A. flavus*	L	None	0.9
	Uganda	Maize	2	UG 4-D	*A. flavus*	L	None	0.9
			2	UG 8-G	*A. flavus*	L	None	–
			2	UG 10-D	*A. flavus*	L	None	–
		Soil	6	2999	*A. parasiticus*	n/a	BG	0.0
	Zambia	Maize	–	Z 1-K	Unidentified	n/a	BG	0.0
			2	Z15-M	*A. flavus*	L	None	–
			2	Z21-B	*A. flavus*	L	B	2.2
			5	Z20-K	Unidentified	n/a	BG	0.0
			–	Z19-D	Unidentified	n/a	B	–
			6	Z12-A	*A. parasiticus*	n/a	BG	0.0
			–	Z20-A	Unidentified	n/a	None	–
			6	Z 20-M	*A. parasiticus*	n/a	BG	0.0
			4	Z18-B	New lineage, S_B	S	B	2.2
			3	Z23-K	*A. minisclerotigenes*	S	BG	0.0
	Zimbabwe	Maize	2	ZW 15-P	*A. flavus*	L	B	2.2
			2	ZW 4-A	*A. flavus*	L	B	2.2
			2	ZW 11-G	*A. flavus*	L	None	2.2
			6	ZW 1-B	*A. parasiticus*	n/a	BG	0.0
			4	ZW 16-K	New lineage, S_B	S	B	2.2
			4	ZW 17-L	New lineage, S_B	S	B	2.2
Australia	Australia	Soil	2	A 1-9	*A. flavus*	L	B	0.9
			2	A 5-1	*A. flavus*	L	B	0.9
			4	1-22	New lineage, S_B	S	B	2.2
			3	4-2	*A. minisclerotigenes*	S	BG	0.0
			3	12-4	*A. minisclerotigenes*	S	BG	0.0
			3	13-4	*A. minisclerotigenes*	S	BG	0.0
Asia	Thailand	Soil	2	Sukkothai-16	*A. flavus*	L	B	1.5
	Philippines	Soil	2	L1E2	*A. flavus*	L	B	2.2
			2	MIZAN14	*A. flavus*	L	B	0.9
Europe	Italy	Maize	2	8117	*A. flavus*	L	None	–
			2	8111	*A. flavus*	L	B	–
	Spain	Soil	2	Turegano AQ	*A. flavus*	L	None	1.5
	Turkey	Pepper	2	TP 1-A	*A. flavus*	L	B	0.9
			2	TP 4-B	*A. flavus*	L	B	0.9
North America	US, Arizona	Soil	2	AF42	*A. flavus*	S	B	1.5
			2	AF70	*A. flavus*	S	B	1.5
			2	AF12	*A. flavus*	S	B	1.5
	US, California	Walnut	2	3251	*A. flavus*	S	B	1.5
	US, Texas	Maize	2	LD08-J	*A. flavus*	L	B	1.5
			2	TEC 2-B	*A. flavus*	L	B	1.5
			2	TBA1-A	*A. flavus*	L	B	1.5
			2	TWC 21-G	*A. flavus*	L	B	1.5
			2	LAT2-H	*A. flavus*	L	B	0.9
			2	LBC13-N	*A. flavus*	L	B	1.5
			2	LEC4-C	*A. flavus*	L	B	1.5
			2	LBY 1-L	*A. flavus*	L	B	1.5
			2	TBY 1-M	*A. flavus*	L	B	1.5
			2	TBC 2-F	*A. flavus*	L	B	1.5
			2	TDO4-D	*A. flavus*	L	B	1.5
			4	TX La Feria 2 F	New lineage, S_B	S	B	2.2
			4	TX04 A5-B	New lineage, S_B	S	B	2.2
			4	TX07 CB73-I	New lineage, S_B	S	B	2.2
South America	Argentina	Soil	3	TAR 4 N30	*A. minisclerotigenes*	S	BG	0.0

[a] Group refers to phylogenetic groups indicated in Fig. 1.
[b] Species assignments were confirmed with phylogenetics. In some case species and consequently group numbers were not assigned (unidentified). Some of these unidentified species are closely related to known species (such as *A. parasiticus*, Group 6).
[c] n/a; classification into morphotypes not applicable.
[d] BG indicates ability to produce both B and G aflatoxins; B indicates ability to produce only B aflatoxins; none indicates that no aflatoxins were detected (limit of detection 1 μg/kg).
[e] Size of sequence deletion in the *cypA* gene. We were unable to obtain an amplicon for several isolates as indicated by en dash (–).

Table 3
Fungal incidences in maize grain obtained from 18 sub-Saharan countries in Africa.

Country, province	Year[a]	No. samples	No. isolates	Strain S_{BG}	*A. minisclerotigenes*	Species, morphotype (%)[b]					
						Unnamed new lineage	*A. flavus*, S strain	*A. flavus*, L strain	*A. parasiticus*	*A. tamarii*	Inconclusive
Burkina Faso	2007	51	763	2.5	0	0	0	96.7	0	0.7	0.1
Cameroon	2006	17	205	0.7	0	0	0	90.9	2.2	6.2	0
DR Congo, Bas	2006/07	10	243	0	4	0	0	65.3	1.2	29.5	0
DR Congo, East	2006/07	12	236	0	0	0.3	0	79.9	1	17.9	0.9
Ethiopia	2006/07	13	49	0	0	0	0	89.4	0	6.7	3.9
Ghana	2006	8	67	0	0	0	0	100	0	0	0
Ivory Coast	2007	4	48	0	0	0	0	100.0	0	0	0
Kenya, Coast	2006	8	140	0	0	0	0	89.9	0	10.1	0
Kenya, Rift Valley	2006	13	206	0	2.4	4.8	0	90.4	0	2.4	0
Malawi	2007	12	153	0	0	5.6	0	81.0	1.6	11.8	0
Mali	2007	13	170	0	0	0	0	100.0	0	0	0
Mozambique	2007/08	40	456	0	0	0.2	0	98.0	1.3	0.5	0
Rwanda	2007	16	55	0	0	0	1.3	98.7	0	0	0
Senegal	2007	19	305	10.3	0	0	0	86.7	0	3	0
Sierra-Leone	2006	16	232	3.4	0	0	0	81.9	0	14.7	0
Somalia	2007	27	443	0	0.2	0	0	77.8	0.5	21.5	0
Tanzania	2007	5	73	0	0	0	0	88.5	4.5	0	7
Uganda	2007	15	243	0	0	0	0	79.2	0	20.8	0
Zambia	2006/07	21	191	0	2.4	10.1	0	72.5	10.0	1.9	3.1
Zimbabwe	2007	19	191	0	0	12.6	0	84.6	1.8	0	1.0
	Sum	**339**	**4469**								

[a] Year samples were collected.
[b] Fungal isolates were assigned to species and morphotypes with metabolic, morphologic and DNA data. Percentages were calculated based on the total number of isolates obtained from each country. Inconclusive = isolates that could not be placed into any known aflatoxin-producing species.

11611 and Australian isolates 4-2, 12-4, and 13-4) (Figs. 2 and 3, Group 3 and 4). However, the 1.5 kb of *niaD* sequence alone was insufficient to separate B and G aflatoxin producing *A. minisclerotigenes* from the lineage from Kenya that produces only B aflatoxins (Fig. 2). ML trees based on *aflR* sequence (n = 37, 2.1 kb total sequence) alone successfully resolved these distinct groups of aflatoxin producers into distinct sister clades (Fig. 3). Many atoxigenic *A. flavus* L strain isolates have deletions covering all or part of *aflR* (Chang et al., 1995). Thus, *aflR* analyses were useful only for isolates with S strain morphology.

Isolates belonging to strain S_{BG} were limited to countries in West Africa (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 (The Democratic Republic of the Congo), East (Kenya and Somalia), and Southern Africa (Zambia) (Fig. 1).

3.4. Molecular analyses of cypA sequence deletions

As expected, sequence deletions were not detected in the *cypA* gene of isolates that produced G aflatoxins. All *A. flavus* isolates exhibited either the 0.9 kb or 1.5 kb sequence deletions (Table 2), characteristic of *A. flavus*. This included the sole *A. flavus* S strain isolate (RW 54-P) recovered from African maize in the current study (Table 2). The remaining isolates with S strain morphology and ability to produce only B aflatoxins

Table 4
Mycotoxin contamination of maize samples from 18 African nations.

Country, region	No. of Samples	Aflatoxin (μg/kg)[a]			Fumonisin (μg/g)[a]			Deoxynivalenol (μg/g)[a]		
		Avg.[b]	Min.[c]	Max.[d]	Avg.	Min.	Max.	Avg.	Min.	Max.
Burkina Faso	50	25	0	609	1	0	4	n/a[e]	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] Mycotoxin concentrations were determined by ELISA. Limit of detections are 1 μg/kg (aflatoxins), 0.5 μg/g (fumonisins), and 0.1 μg/g (DON).
[b] Avg., average concentration.
[c] Min., minimum concentration.
[d] Max., maximum concentration.
[e] n/a; data not available.

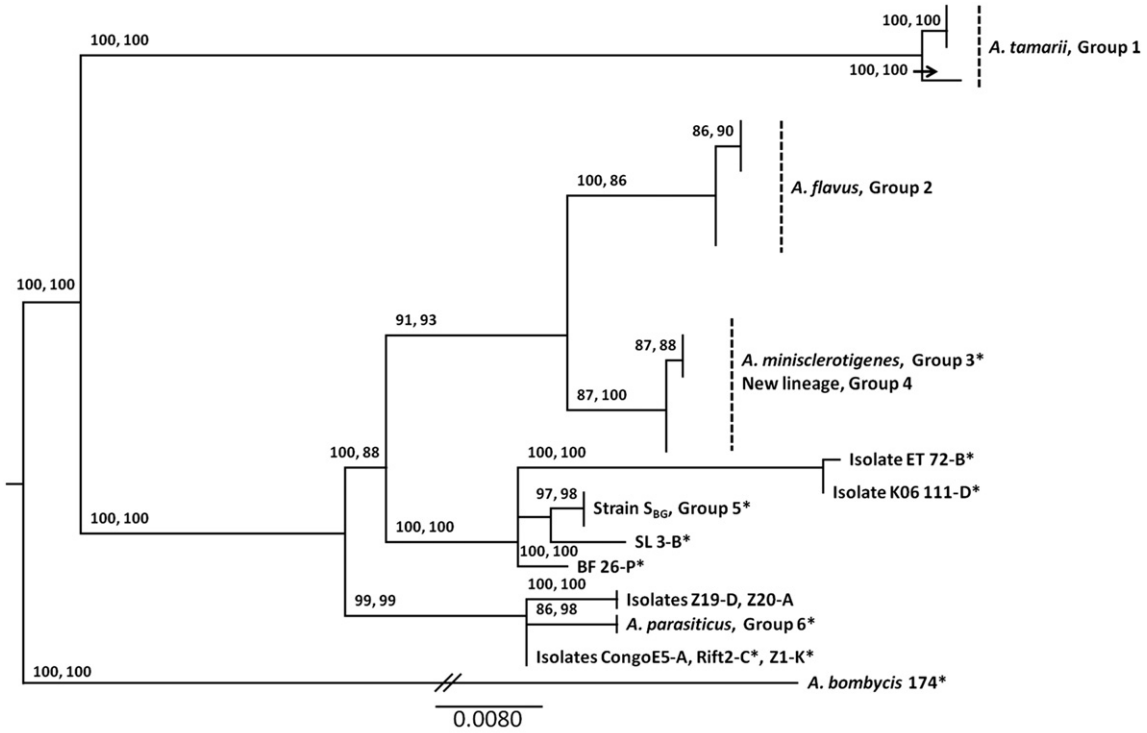

Fig. 2. Mid-point rooted maximum likelihood tree for 143 *Aspergillus* isolates obtained from Africa, Australia, Asia, Europe, and the Americas. Tree is based on *niaD* sequence data (1.5 kb). Values above nodes indicate bootstrap values from 1000 replicates derived from maximum likelihood analysis (before comma) and maximum parsimony analysis (after comma). Asterisks indicate ability of groups to produce both B and G aflatoxins. Group assignments: Group 1 = *Aspergillus tamarii*; Group 2 = *A. flavus*; Group 3 = *A. minisclerotigenes*; Group 4 = the new fungal lineage described from Kenya; Group 5 = West African strain S_{BG}; Group 6 = *A. parasiticus*. Isolates belonging to groups are listed in Table 2.

had a 2.2 kb deletion and were placed by phylogenetic analyses in the same well supported clade as previously reported isolates with S strain morphology from Kenya (Probst et al., 2012). Amplification of the *cypA* gene region in the atoxigenic species *A. tamarii* was unsuccessful.

4. Discussion

A. flavus was delineated into the L and S strain morphotypes more than 20 years ago (Cotty, 1989). Recently several closely related but

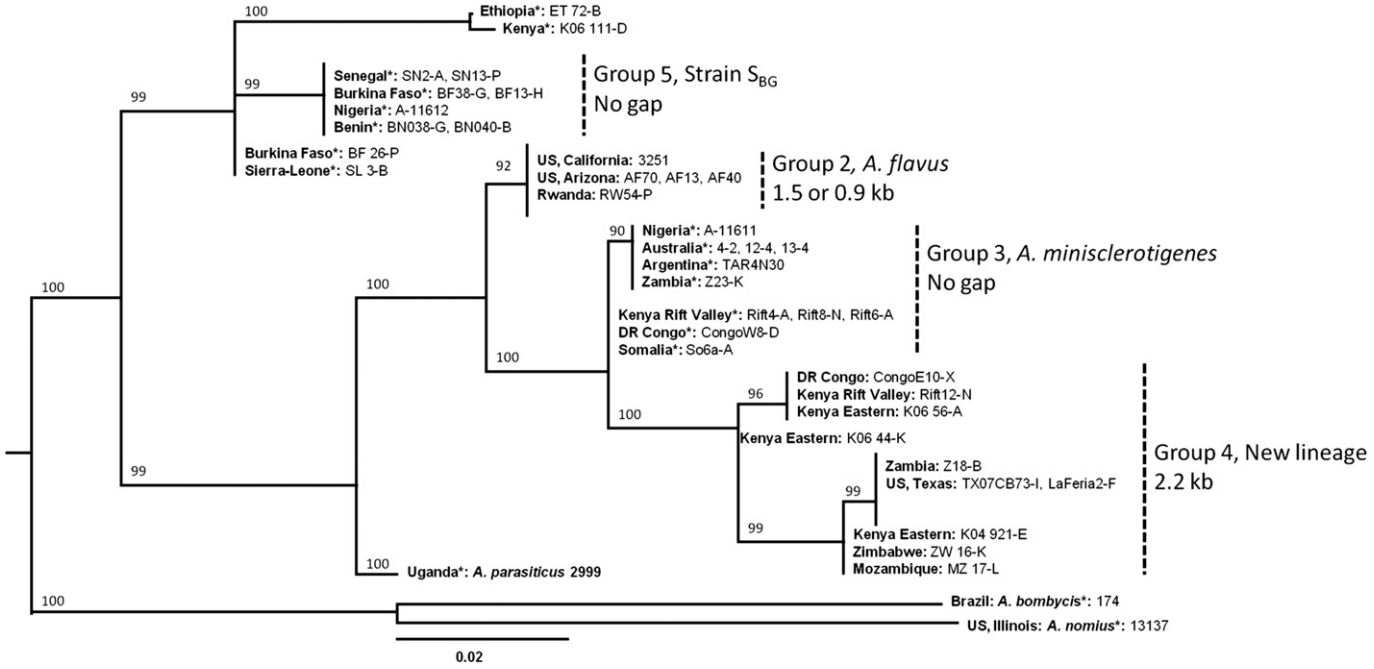

Fig. 3. Mid-point rooted maximum likelihood tree of 37 *Aspergillus* isolates with S strain morphology and three isolates of related *Aspergillus* species (*A. parasiticus*, *A. nomius*, and *A. bombycis*). Tree is based on *aflR* sequence data (2.1 kb total sequence). Values above nodes indicate bootstrap values from 1000 replicates. Asterisks indicate ability to produce both B and G aflatoxins.

phylogenetically distinct fungi with S strain morphology have been reported (Pildain et al., 2008; Donner et al., 2009; Probst et al., 2012). These fungi are indistinguishable with morphology but can be separated with molecular (e.g. *cypA* deletion pattern), phylogenetic, and physiologic criteria (e.g. aflatoxin production) (Probst et al., 2012). In the US, the *A. flavus* S strain is common in soil and crop samples (Cotty, 1989, 1997; Orum et al., 1997; Horn, 2003; Jaime-Garcia and Cotty, 2004) and was dominant on maize in north central Texas during years with high levels of contamination. This is in stark contrast to sub-Saharan Africa, where in the current study only one *A. flavus* S strain isolate was found. In this region, fungi with S morphology distinct from *A. flavus* are more common. This contrasts with the distribution of the L strain morphotype of *A. flavus* which was universally detected. The current work detected distinct geographical distributions across sub-Saharan Africa among the four taxa with S strain morphology. However, the underlying mechanisms through which these distinct distributions are maintained remain unknown.

The majority of aflatoxin-producing fungi belong to *Aspergillus* sect. *Flavi* (Horn, 2003; Frisvad et al., 2005). Not all aflatoxin-producers are equally important etiologic agents of contamination (Horn, 2007; Cotty et al., 2008). Frequencies in crops, aflatoxin-producing potential, competitive ability, and virulence to host plants influence their importance as causative agents (Cotty, 1989; Horn, 2003; Cotty et al., 2008; Mehl and Cotty, 2010, 2011). Diverse mixtures of various aflatoxigenic and atoxigenic members of *Aspergillus* sect. *Flavi* are present on crops and the average aflatoxin-producing potential greatly influences severity of contamination (Cotty et al., 2007). If frequencies of atoxigenic or low aflatoxigenic fungi are high, less aflatoxin will develop and, hence, more crops will be fit for human consumption (Cotty et al., 2007). Morphological and molecular tools aid quantification of specific frequencies of aflatoxin-producing and closely related fungi. When combined with data on aflatoxin-producing potential (Probst and Cotty, 2012), the abilities of fungal communities to contaminate crops can be compared.

In the current study, the *A. flavus* L strain was common in all countries and across the sampled regions; *A. parasiticus* was relatively rare comprising on average only 1.2% of the *Aspergillus* section Flavi examined and only comprised large proportions of section Flavi communities in a few samples. This is in agreement with findings from studies on aflatoxin-producing fungi on other continents and supports that *A. flavus* is the predominant aflatoxin-producing species on maize across much of sub-Saharan Africa (Horn and Dorner, 1998; Barros et al., 2003; Ehrlich et al., 2007; Atehnkeng et al., 2008). Aflatoxin-producers with S strain morphology were less frequently encountered. However, as a result of their greater aflatoxin-producing potential these fungi may be regionally important etiologic agents (Probst et al., 2007; Cotty et al., 2008; Probst et al., 2010; Probst et al., 2012). Detected taxa with S strain morphology include a) the recently described *A. minisclerotigenes* common in eastern and southern Africa, b) the S strain of *A. flavus* was found (one isolate) for the first time on the African continent in the current study, c) an unnamed B and G aflatoxin producing taxon (strain S_{BG}), common in West Africa and d) the unnamed taxon originally described from Kenya where it was associated with lethal aflatoxicoses. In the current study, this last taxon was detected outside of Kenya for the first time. Occurrence was confined to East and Southern Africa. All isolates had the characteristic 2.2 kb deletion in the *cypA* biosynthesis gene and were phylogenetically distinct from other taxa with S strain morphology; this supports hypotheses (citation) that the Kenyan S strain isolates comprise a new species of aflatoxin-producing fungi.

Strain S_{BG} has been associated with drier agroecological zones in Bènin with average incidences increasing from 3 to 60% between the humid Coastal Savannah and the semi-arid Sudan Savannah (Cardwell and Cotty, 2002). Similarly, in the current study, strain S_{BG} had the greatest frequencies in crops 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 assigned by phylogenetics to *A. minisclerotigenes*, a species also found in Argentina and Australia (Pildain et al., 2008; Samson and Varga, 2009). Like strain S_{BG}, most isolates of *A. minisclerotigenes* were obtained from semi-arid and sub-humid regions. Similarly, the S strain of *A. flavus* is favored by warm, dry conditions (Orum et al., 1997; Bock et al., 2004).

Three mycotoxins of frequent concern (aflatoxins, fumonisins, and deoxynivalenol) (Kpodo and Bankole, 2008) were found in the majority of analyzed samples in the current study. The vast majority of samples (81%) had detectable levels of aflatoxins (65%) and/or fumonisins. The regulatory limit of the European Union for total aflatoxins for ready-to-eat maize is 4 μg/kg (Agriculture and Consumer Protection, 2004; The European Commission, 2010). 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 the EU limit. High frequencies of aflatoxin contamination in sub-Saharan Africa documented in the current report, diminish export opportunities and raises health concerns.

Fumonisins in maize also impose economic losses and jeopardize human and animal health. Maize is the most consumed staple in Zimbabwe. Results from the current study suggest humans in Zimbabwe are exposed to highly unacceptable quantities of fumonisins. All maize samples from Zimbabwe contained at least 10 times the provisional maximum tolerable intake level of 2 μg/g (Agriculture and Consumer Protection, 2004). Fumonisin levels detected in this study are known to cause severe symptoms in humans and domestic animals. Deoxynivalenol, which is known in maize produced in South Africa (Marasas et al., 1977), was found in 40% of samples in the current study indicating infection by trichothecene-producing *Fusarium* species. In general, deoxynivalenol levels were not a concern with less than 10% exceeding the proposed action level (0.75 μg/g) for the European Union (Agriculture and Consumer Protection, 2004).

In recent years, increased attention has been devoted to strategies directed at decreasing human exposure to aflatoxins (Atehnkeng et al., 2010; Cotty and Bandyopadhyay, 2010; Wu and Khlangwiset, 2010; Probst et al., 2011). Native, well adapted atoxigenic strains of *A. flavus* have been successfully used to competitively displace aflatoxin-producing fungi and reduce aflatoxins (Cotty et al., 2007; Atehnkeng et al., 2010; Probst et al., 2011). The current study provides insights into both distributions of aflatoxin-producing fungi across the continent and causal agents of aflatoxin contamination of maize of potential regional importance. Fungal communities on maize in Africa were dominated by the L strain of *A. flavus*, a morphotype in which all the atoxigenic isolates currently used for biocontrol are found. Isolates collected in the current study may be valuable for identification of atoxigenic *A. flavus* L strain isolates native across much of sub-Saharan Africa.

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