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Characterization of *Aspergilli* from dried red chilies (*Capsicum* spp.): Insights into the etiology of aflatoxin contamination

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ABSTRACT

Aflatoxins are toxic carcinogens produced by several species of *Aspergillus* section *Flavi*, with some aflatoxin producers associated with specific crops. Red chilies (*Capsicum* spp.) are grown in warm regions that also favor aflatoxin-producers. Aflatoxins in red chilies may result in serious health concerns and severe economic losses. The current study sought to gain insight on causal agents of aflatoxin contamination in red chilies. Naturally contaminated chilies from markets in Nigeria (n = 55) and the United States (US) (n = 169) were examined. The *A. flavus* L strain was the predominant member of *Aspergillus* section *Flavi* (84%) in chilies. Highly toxigenic fungi with S strain morphology were also detected in chilies from both countries (11%), followed by *A. tamarii* (4.6%) and *A. parasiticus* (0.4%). Fungi with L morphology produced significantly lower quantities of aflatoxins (mean = 43 μg g^{-1}) compared to S morphology fungi (mean = 667 μg g^{-1}; $p < 0.01$) in liquid fermentation. Eighty-one percent of S morphology fungi from chilies in US markets produced only B aflatoxins, whereas 20%, all imported from Nigeria, produced both B and G aflatoxins; all S morphology fungi from Nigerian chilies produced both B and G aflatoxins. Multi-gene phylogenetic analyses of partial gene sequences for nitrate reductase (*niaD*, 2.1 kb) and the aflatoxin pathway transcription factor (*aflR*, 1.9 kb) resolved *Aspergilli* recovered from chilies into five highly supported distinct clades: 1) *A. parasiticus*; 2) *A. flavus* with either L or S morphology; 3) *A. minisclerotigenes*; 4) *A. aflatoxiformans*, and 5) a new lineage. *Aspergillus aflatoxiformans* and the new lineage produced the highest concentrations of total aflatoxins in chilies, whereas *A. flavus* L strains produced the least. The results suggest etiology of aflatoxin contamination of chili is complex and may vary with region. Knowledge of causal agents of aflatoxin contamination of chilies will be helpful in developing mitigation strategies to prevent human exposure.

1. Introduction

Aflatoxins are hepatocarcinogenic metabolites produced by several *Aspergilli*, which frequently contaminate food and feed crops including maize, groundnut, cottonseed, spices, and tree nuts (Doster et al., 2014; Kachapulula et al., 2017). Of the four major aflatoxins, B_1, B_2, G_1 and G_2, aflatoxin B_1 is the most toxic, and is carcinogenic to both humans and animals (IARC, 2002). Most developed nations stringently enforce aflatoxin regulatory limits within food and feed (e.g. US regulates aflatoxins at 20 μg/kg total aflatoxins in human food) resulting in significant economic losses to growers (Robens and Cardwell, 2003; van Egmond et al., 2007). Across regions of the globe where regulations are either lacking or are not strictly enforced, aflatoxin contamination exposes humans and animals to severe health risks. Sub-lethal concentrations are associated with stunted development (Khlangwiset et al., 2011), immune suppression (Turner et al., 2003), and liver cancer (Liu and Wu, 2010), whereas high levels can cause liver cirrhosis followed by rapid death (CDC, 2004).

Aspergillus flavus is the most frequently implicated causal agent of aflatoxin contamination of crops (Klich, 2007). The species can be divided into two major morphotypes known as the L and S strains. The S strain isolates produce copious amounts of small sclerotia (< 400 μm) and fewer conidia, whereas the L strain isolates produce sparse amounts of large sclerotia (> 400 μm) but abundant conidia (Cotty, 1989). The L strain produces variable quantities of aflatoxins, and isolates can either be atoxigenic or produce moderate to high levels of aflatoxins; however, the S strain of *A. flavus* and other members of section *Flavi* with S morphology are known to consistently produce high concentrations of aflatoxins (Cotty, 1989; Cotty and Cardwell, 1999; Probst et al., 2010). Aflatoxin contamination can start before harvest when crops are

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infected by resident *Aspergilli* dispersed by various mechanisms. Crop infection is facilitated by plant stress (physical damage, by insects, drought) and warm temperatures (above 28 °C) (Cotty et al., 2008). Contamination continues after crop maturation in the presence of conducive conditions, both in the field, and post-harvest during storage, processing or transportation (Cotty et al., 2008; Marín et al., 2009). Since conidia of *Aspergilli* are air-borne and ubiquitous, new infections may occur during post-harvest stages, exacerbating contamination levels (Cotty et al., 1994).

Red chili (*Capsicum* spp.), a member of the nightshade family of Solanaceae, is a globally consumed spice. *Capsicum* was domesticated in America about 6000 years ago and has now spread into Asia, Africa and Europe (Perry et al., 2007). Chilies are mainly cultivated in warm regions that provide suitable conditions for crop infection by *Aspergillus* propagules and subsequent contamination with aflatoxins. India, China and Thailand produced most of the world's dried red chilies during the past decade (FAOSTAT, 2017). Post-harvest practices and conditions during growth render the spice susceptible to aflatoxin contamination. Contaminated chilies can result in loss of lucrative European and US markets where aflatoxins are stringently regulated (European Spice Association, 2004; Yu, 2012). Consumption of aflatoxin contaminated chilies has recently been associated with gall bladder cancer in Bolivia, Chile and Peru (Asai et al., 2014; Nogueira et al., 2015). Aflatoxin contamination is a problem in major chili producing regions (Reddy et al., 2001; Shamsuddin et al., 1995; Singh and Cotty, 2017), yet *Aspergillus* section *Flavi* associated with chilies and the etiology of chili contamination with aflatoxins have not been examined in detail. Structure of fungal communities associated with crops is an important determinant of the severity of aflatoxin contamination (Cotty et al., 2008; Probst et al., 2010), since higher incidences of aflatoxin-producers result in increased average aflatoxin-producing potentials of fungal communities, leading to overall high contamination (Cotty et al., 2008).

A previous study evaluated aflatoxin contamination of dried red chilies between the US and Nigeria, two markets differing in regulation enforcement (Singh and Cotty, 2017). Aflatoxin concentrations and fungal load were significantly higher in Nigerian chilies compared to those from US markets. The current study sought to understand communities of *Aspergillus* section *Flavi* associated with dried red chilies and to obtain insight into the etiology of contamination of dried red chilies. Our objectives were to (i) relate *Aspergillus* section *Flavi* in chilies from markets in the US and Nigeria to previously described members of section *Flavi*, and (ii) to determine the relative importance of each as etiologic agents of contamination. The acquired knowledge on aflatoxin-producing fungi associated with red chilies may be helpful in devising aflatoxin management strategies for the spice.

2. Materials and methods

2.1. Sampling

Dried red chili was collected from markets in the US and Nigeria, as reported previously (Singh and Cotty, 2017). Briefly, 169 chili samples, sealed in airtight packets were purchased in the US from retail markets including supermarkets and ethnic groceries, where chilies were kept at ambient room temperature throughout storage. The retail stores were sampled in Arizona (n = 64), California (n = 68), Minnesota, (n = 3), and New York (n = 34) during 2014–15 as representative locations for the US. US samples consisted of whole (n = 60, mean = 250 g), ground (n = 78, mean = 200 g) and crushed chili (n = 12, mean = 150 g), and paprika (n = 19, mean = 180 g). Fifty-eight percent of chili samples collected from US markets were imported (Singh and Cotty, 2017). Nations of origin for the imported chili samples are listed in Table 1S. Only whole red chilies were collected in Nigeria (n = 55, mean = 70 g), primarily from rural, small-scale markets in Kaduna (n = 50) and Lagos (n = 5) states during 2015–16. Nigerian samples were imported to the USDA-ARS laboratory in the School of Plant Sciences at the University of Arizona, Tucson, under permits issued by the USDA Animal and Plant Health Inspection Service (APHIS) within a week of purchase.

2.2. Fungal isolation and characterization

Whole chili samples were dried (forced air oven, (40 °C)) to below 8% moisture content and sealed in plastic bags; ground chili, crushed chili and paprika were sealed in plastic bags immediately after receipt. After bagging, samples were stored at room temperature. After drying, whole and crushed chili samples were finely ground in a laboratory mill (Retsch Grindomix GM200, Newtown, PA) for 30 s at 10,000 rpm prior to fungal isolation.

In a previous study (Singh and Cotty, 2017), fungi belonging to *Aspergillus* section *Flavi* were recovered from the above samples by dilution plate technique on modified rose Bengal agar (Cotty, 1994a). Fungi isolated in Singh and Cotty, 2017 were assigned to morphological groups (S morphology, *A. flavus* L morphotype, and *A. parasiticus*) using colony characteristics, sclerotia and spore morphology (Cotty, 1989; Klich and Pitt, 1988). Fungal isolations were performed at least twice from each chili sample. All fungal isolates were subjected to dilution plating on malt agar (1% malt, 2% agar, 1000 ml of water) followed by incubation at 31 °C for 48 h. At dilutions providing < 10 colonies per plate, discrete colonies were transferred to 5–2 agar (5% V-8 juice; 2% agar; pH 6.0) and incubated at 31 °C for 5–7 days in dark. Fungal isolates were stored as plugs of sporulating culture in sterile distilled water (2 ml) and used as working cultures for conducting aflatoxin and phylogenetic analyses.

2.3. Screen for aflatoxin producers

Aflatoxin production was evaluated for both L and S morphology isolates recovered from chilies. The L morphology fungi (n = 130) were randomly selected with at least one isolate from each chili sample positive for *Aspergillus* section *Flavi*. Fungi with S morphology (n = 75) were randomly selected from 30 Nigerian and 5 US chili samples from which S morphology fungi were recovered. At least 1 isolate from each of the 35 samples positive for S morphology fungi was included. Fungi were evaluated in a chemically-defined aflatoxin production liquid medium (Mateles and Adye, 1965) supplemented with 22.5 mM urea as the sole nitrogen source (Cotty and Cardwell, 1999; Probst et al., 2012). Fungal inoculum for each isolate was prepared as described previously (Probst et al., 2012). Erlenmeyer flasks containing 70 ml of the liquid medium were seeded with conidial suspensions (10^6 conidia ml^{-1}), covered with stoppers that allow gas exchange, and incubated with agitation in dark for 5 days (31 °C, 160 rpm). Fermentations were terminated by addition of acetone (70 ml acetone per 70 ml fermentation) and swirled to allow mixing. Cultures were allowed to sit for at least one hour to allow for lysis of fungal cells and release of aflatoxins contained in the mycelia. Acetone extracts were directly spotted onto thin-layer chromatography (TLC) plates (Silica gel 60; EMD, Darmstadt, Germany) and separated adjacent to aflatoxin standards (Aflatoxin Mix Kit-M; Supelco, Bellefonte, PA). Plates were developed in a solution of ethyl ether-methanol-water (96:3:1), air-dried, and aflatoxins were visualized under 365-nm UV light. Total aflatoxins were quantified directly on TLC plates using a scanning densitometer (TLC Scanner 3, Camag Scientific Inc., Wilmington, N.C.). Filtrates initially negative for aflatoxins were partitioned twice with dichloromethane and concentrated prior to quantification (limit of detection 104 μg/kg mycelia) as previously described (Cardwell and Cotty, 2002). Mycelial mass from the fermentation was captured during vacuum filtration on Whatman No. 1 filter paper and dried (40 °C, 48 h) in a forced air oven. Aflatoxin concentrations were expressed as μg total aflatoxin per g mycelium.

2.4. DNA isolation and gene amplification

DNA extraction, PCR amplification and sequencing for fungi

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

Primer pair	Sequence	T_a (°C)	Product size (bp)	Reference
aflR1F-1R	F-AGAGAGCCAACTGTCGGACCAA R-GGGTGACCAGAGAACTGCGTGAT	57	737	Probst et al., 2012
aflR2F-2R	F-GACTTCCGGCGCATAACACGTA R-ACGGTGGCGGGACTGTTGCTACA	57	745	Probst et al., 2012
aflR4F-4R	F-CGCCCATGACGGACTACGTT R-TGGTGGTTGATTCGATTGAGG	57	735	Current Study
niaDF-AR	F-CGGACGATAAGCAACAACAC R-GGATGAACACCCGTTAATCTGA	52	795	Probst et al., 2012
niaDBF-BR	F-ACGGCCGACAGAAGTGCTGA R-TGGGCGAAGAGACTCCCCGT	57	799	Probst et al., 2012
niaDCF-CR	F-GCAGCCCAATGGTCACTACGGC R-GGCTGCACGCCCAATGCTTC	55	792	Current study

recovered from chilies and all reference isolates were done in the current study. Fungal cultures were grown and DNA was extracted as described previously (Callicott and Cotty, 2015). The reference isolates used in the current study were obtained from the ARS Culture Collection, Peoria, IL, USA (indicated with NRRL in Table 4), the American Type Culture Collection, Manassas, USA (indicated with ATCC in Table 4), or were present in the USDA, ARS, Tucson Laboratory Culture Collection. Sequence analyses for chili and reference isolates were performed using two genomic regions; nitrate reductase (*niaD*) and aflatoxin pathway transcription factor (*aflR*). Partial gene sequences of both *niaD* (chromosome 4) and *aflR* (chromosome 3) were amplified and sequenced in both directions, each with three sets of primers (Table 1) that covered approximately 2.1 kb of *niaD* and 1.9 kb of *aflR* genes. The primers aflR4F-4R and niaDCF-CR were designed in the current study based on genome sequence of *A. flavus* NRRL 3357 (GenBank accession no. AAIH02000041 and AAIH02000071) using Primer3 version 0.4.0 (Koressaar and Remm, 2007; Untergasser et al., 2012). PCR reactions were performed under the following conditions: 5 min at 94 °C followed by 38 cycles of 94 °C for 30 s, locus-specific annealing temperature for 30 s, 72 °C for 30 s, and 5 min at 72 °C. Amplicons were visualized on 1.0% agarose gels and sequenced by the University of Arizona Genetics core sequencing facility (UAGC, Tucson, AZ).

2.5. DNA sequence data and phylogenetics

Phylogenetic reconstruction was performed using bidirectional sequences of the genes *aflR* (1.9 kb) and *niaD* (2.1 kb) from 62 fungal isolates of *Aspergillus* section *Flavi* recovered from chilies and 17 reference isolates. Fungi were selected such that for each morphology (L, S or *A. parasiticus*) and aflatoxin producing ability (B or B and G), fungi from as many samples as possible were examined. All sequences utilized in phylogenetics were obtained during the current study. Consensus sequences were created for each isolate by assembly of 6 reads per gene with visual inspection and alignment using the MUSCLE algorithm with the default settings in Geneious Pro Version 7.1.9. (Biomatters Ltd., Auckland, New Zealand). DNA sequence alignments were refined manually. Phylogenetic analysis of sequence data for the two loci were performed for concatenated and individual sequences using Bayesian inference with 10 million generations (MrBayes version 3.2.0; Huelsenbeck and Ronquist, 2001). Trees were drawn mid-point rooted using FigTree v.1.4.3 (Rambaut, 2012). Maximum likelihood analysis for individual and concatenated sequences was performed in parallel to confirm tree topologies using PhyML (Phylogeny.fr (Dereeper et al., 2008; Dereeper et al., 2010)). Data sets were bootstrapped with 500 replicates.

2.6. Crop inoculations

Results based on phylogenetic analyses were used to select fungi for inoculation onto sterile piquin chili (*Capsicum annuum*), maize (*Zea mays*) and groundnut (*Arachis hypogaea*). Four fungal isolates were randomly selected from each phylogenetically distinct clade and assessed for aflatoxin production on the three crops. In cases where clades contained fungi from both US and Nigerian chilies, isolates representative of both nations were randomly selected for aflatoxin analyses. For L morphology isolates, only aflatoxin producers, as determined from preliminary liquid culture aflatoxin assays, were included. Both maize and groundnut are hosts to members of section *Flavi* (Kachapulula et al., 2017). Healthy, undamaged chili pods were autoclaved for 20 min at 121 °C in sealed paper bags. The intact, autoclaved paper bags containing the chili were dried in a forced air oven (40 °C) for 48 h. Each chili bag was opened under asceptic conditions in a biological safety cabinet and allowed to sit for another 24 h to let the volatiles formed during autoclaving to escape. Chilies were then weighed out into previously sterilized Erlenmeyer flasks (5 g per flask).

Healthy, undamaged kernels of maize and groundnut, were autoclaved in Erlenmeyer flasks (5 g per flask) for 20 min at 121 °C. Each crop was inoculated with conidial suspensions adjusted to 10^6 conidia ml^{-1}. Inoculated crops adjusted to 30% moisture were incubated for 10 d at 31 °C in the dark. Each crop was incubated in a different experiment. Treatments were replicated four times and each experiment was performed twice.

2.7. Aflatoxin quantification in crops

Crop cultures were ground in 85% acetone (50 ml) in a laboratory grade Waring Blender (seven-speed laboratory blender, Waring Laboratory, Torrington, CT) at full speed for 30 s. The ground crop-acetone slurry was allowed to sit for an hour in the dark, and the culture filtrate was spotted directly onto TLC plates (Silica gel 60, EMD, Darmstadt, Germany) adjacent to an aflatoxin standard (Aflatoxin Mix Kit-M, Supelco, Bellefonte, PA) containing a mixture of known concentrations 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 examined for aflatoxins under 365-nm UV light. Total aflatoxins were quantified directly on TLC plates with a scanning densitometer (TLC Scanner 3, Camag Scientific Inc., Wilmington, NC, USA). Chili cultures initially negative for aflatoxins were concentrated with a previously reported modification to the method of the Association of Official Analytical Chemists (Cotty and Lee, 1989; Mckinney, 1975; Stoloff and Scott, 1984). Briefly, a 20 ml solution of 1.1 M $(CH_3COO)_2Zn$ and 0.04 M $AlCl_3$ diluted with 80 ml of deionized water was added to the negative samples. After 5 min, 5 g of diatomaceous earth was added and the resulting mixture was shaken to allow mixing. These mixtures were allowed to sit for 1–2 h in order to minimize interfering pigments, fatty acids, and trace lipids, and to obtain cleaner extracts for TLC (Mckinney, 1975). Extracts were filtered through number 4 Whatman paper. Aflatoxins were extracted from 100 ml filtrate by partitioning the filtrate twice with 25 ml of dichloromethane. The extracts were passed through a bed of

anhydrous Na_2SO_4 and air-dried. Residues were dissolved in dichloromethane, spotted on TLC plates, developed and quantified as above. Both maize and groundnut cultures were positive for aflatoxins in the initial round of extraction and did not require concentration.

2.8. Data analysis

Total aflatoxin was measured in $\mu g\, g^{-1}$. Aflatoxin concentrations were log transformed and subjected to Analysis of Variance (JMP 11.1.1, SAS Institute, Cary, NC). In experiments with significant differences by ANOVA, mean separations were performed using Tukey's HSD test ($p = 0.05$). True means are presented for clarity.

3. Results

3.1. Incidence of Aspergillus section Flavi in dried red chilies

A total of 530 *Aspergillus* section *Flavi* isolates were recovered from 169 samples purchased from US markets (Table 2). *Aspergillus flavus* L strain had the highest incidence (92.1%), followed by *A. tamarii* (4.3%), and fungi with S morphology (3.0%). *Aspergillus parasiticus* occurred in low numbers (0.6%). Aflatoxin assays and phylogenetic analyses further characterized the S morphology fungi into *A. flavus* S strain (2.4%) and *A. aflatoxiformans* (0.6%).

Fungal isolation from Nigerian samples resulted in a total of 565 *Aspergillus* section *Flavi* isolates from 55 samples (Table 2). Majority of these fungi were assigned to *A. flavus* L strain (76.7%). Incidences of fungi with S morphology were higher in Nigerian chilies than in chilies from US markets. Phylogenetics resolved the S morphology fungi into *A. aflatoxiformans* (8.3%), *A. minisclerotigenes* (8.0%), and a new lineage (2.8%) discovered in the current study. *Aspergillus tamarii* occurred at 4% whereas incidences of *A. parasiticus* were low (0.2%).

3.2. Aflatoxin producers from chilies: occurrence of non-native aflatoxin producers in chilies from US markets

Fungi recovered from chilies were initially assayed for aflatoxin production in liquid fermentation. Out of the 205 isolates tested, over 70% of isolates produced aflatoxins (LOD = 104 μg/kg mycelia). All L morphology aflatoxin-producers recovered from chilies produced only B aflatoxins, with 71% from US (n = 65; mean = $27\ \mu g\, g^{-1}$ mycelium) and 40% from Nigerian (n = 65; mean = $58\ \mu g\, g^{-1}$ mycelium) markets producing detectable quantities of aflatoxins (Table 3). The S morphology fungi produced higher concentrations ($p < 0.01$) of aflatoxins in A&M medium with urea than the Ls (Table 3). Three isolates from imported chili purchased at US markets had S morphology and produced both B and G aflatoxins. The chilies were imported from Nigeria. All S morphology fungi from Nigerian chilies produced both B and G aflatoxins.

3.3. Phylogenetic analyses

Phylogenetic analyses of the concatenated *niaD* and *aflR* sequences (2.1 kb for *niaD* and 1.9 kb for *aflR*) resolved fungi from red chilies into five highly supported clades representing five taxa (Fig. 1). The first taxon, *A. parasiticus* (e.g. reference isolates 2999 and BN009-E), was recovered in low numbers from both US and Nigerian chilies. The second taxon, consisting of B aflatoxin-producers, included previously described L and S strain *A. flavus* isolates. *Aspergillus flavus* S strain isolates were only detected in US chilies, whereas L strain isolates occurred in both US and Nigerian chilies. The third taxon, *A. minisclerotigenes* (e.g. reference isolates A-11611, 4-2 and TAR3N43) was recovered only from Nigerian chilies. The fourth taxon, *A. aflatoxiformans*, included fungi that grouped with reference isolates A-11612 and BN038-G, and consisted solely of fungi from chilies produced in Nigeria. The fifth and final clade was occupied by a novel lineage discovered in the current study. This new lineage with S morphology was detected only in Nigerian chilies. No reference isolates grouped with this lineage.

The new lineage was sister to *A. korhogoensis* (Fig. 1). In addition to the results from liquid fermentation, phylogenetic reconstruction validated the occurrence of *A. aflatoxiformans* isolates in chilies from US markets (Fig. 1, Table 4). Based on DNA sequence analysis and phylogenies, 44% of S morphology isolates from Nigeria were identified as *A. aflatoxiformans*, 42% as *A. minisclerotigenes* and 15% as the new lineage. *Aspergillus korhogoensis* was not detected in the current study.

3.4. Aflatoxin production in crops

Individual isolates of *A. flavus* S strain, *A. parasiticus*, the new lineage and *A. aflatoxiformans* varied in aflatoxin producing ability on each of the hosts ($p < 0.05$) (Table 5). Both *A. aflatoxiformans* and the new lineage produced the highest concentrations of aflatoxins in chilies, maize and groundnuts. On chilies, these two species produced over 10 fold more total aflatoxins than the other S morphology taxa. The S strain of *A. flavus* and *A. parasiticus* produced similar concentrations of aflatoxins in chilies (Table 5). Among the S morphology fungi, *A. minisclerotigenes* produced the lowest concentrations of aflatoxins in all the three crops. The L strain of *A. flavus* was the least toxic in chilies but produced quantities of aflatoxins comparable to *A. minisclerotigenes* in groundnuts and maize.

4. Discussion

Most aflatoxin-producers belong to *Aspergillus* section *Flavi*. Since aflatoxin-producing ability can be highly variable among species, strains and isolates, it is difficult to attribute specific etiologies to aflatoxin contamination of crops. To identify the most important causal agents of aflatoxin contamination of chilies, both, frequency of crop infection and aflatoxin-producing ability must be considered. Characterization of causal agents is critical for development of management procedures. Although occurrence of *Aspergillus* spp. in chilies has been reported in previous studies (Flannigan and Hui, 1976; Kiran et al., 2005; Reddy et al., 2011), this study provides the first comprehensive report of identity and toxigenicity of species within section *Flavi* associated with dried red chilies. The *A. flavus* L strain was the predominant member of section *Flavi* in chilies (Table 2). Similar dominance of the L strain has been reported in other important hosts

Table 2
Incidence of *Aspergillus* section *Flavi* in chilies from US and Nigerian markets.

Country	Year[a]	Chili samples	No. of isolates	Species/Taxon (%)[b]						
				A. flavus L strain	*A. flavus* S strain	*A. minisclerotigenes*	*A. aflatoxiformans*	New Lineage	*A. tamarii*	*A. parasiticus*
USA[#]	2014–15	169	530	92.1	2.4	0.0	0.6	0.0	4.3	0.6
Nigeria	2015–16	55	565	76.7	0.0	8.0	8.3	2.8	4.0	0.2

[a] Year samples were collected.
[b] Assignment of fungal isolates recovered from red chilies to species/taxa based on morphological, physiological and DNA sequence data.
[#] Some chilies (98 samples) purchased in the US were produced in other countries and imported.

Table 3
Aflatoxin production by members of *Aspergillus* section *Flavi* from red chilies in liquid fermentation.

Country	Fungal morphology	# Isolates tested	Aflatoxin (AF) production				
			AFB producers (%)	AFG producers (%)	Mean AFB (μg g^{-1})	Mean AFG (μg g^{-1})	Mean total AF (μg g^{-1})
USA[ρ]	L	65	71	0	27 (ND-761)	NA	27[B]
	S	15	100	20	247 (33–614)	103 (25.5–255)	268[A]
Nigeria	L	65	40	0	58 (ND-2,450)	NA	58[B]
	S	60	100	100	535 (3–4,960)	232 (2–2,765)	767[A]

Total aflatoxin concentrations followed by different letters differed significantly (Tukey's HSD, $p < 0.01$). Values in the parenthesis indicate range of aflatoxin concentrations measured.
LOD = 104 μg/kg mycelium.
ND- Not Detected.
NA- Not applicable. These fungi do not produce G aflatoxins.
[ρ] Chili samples were purchased in USA but all did not originate from USA.

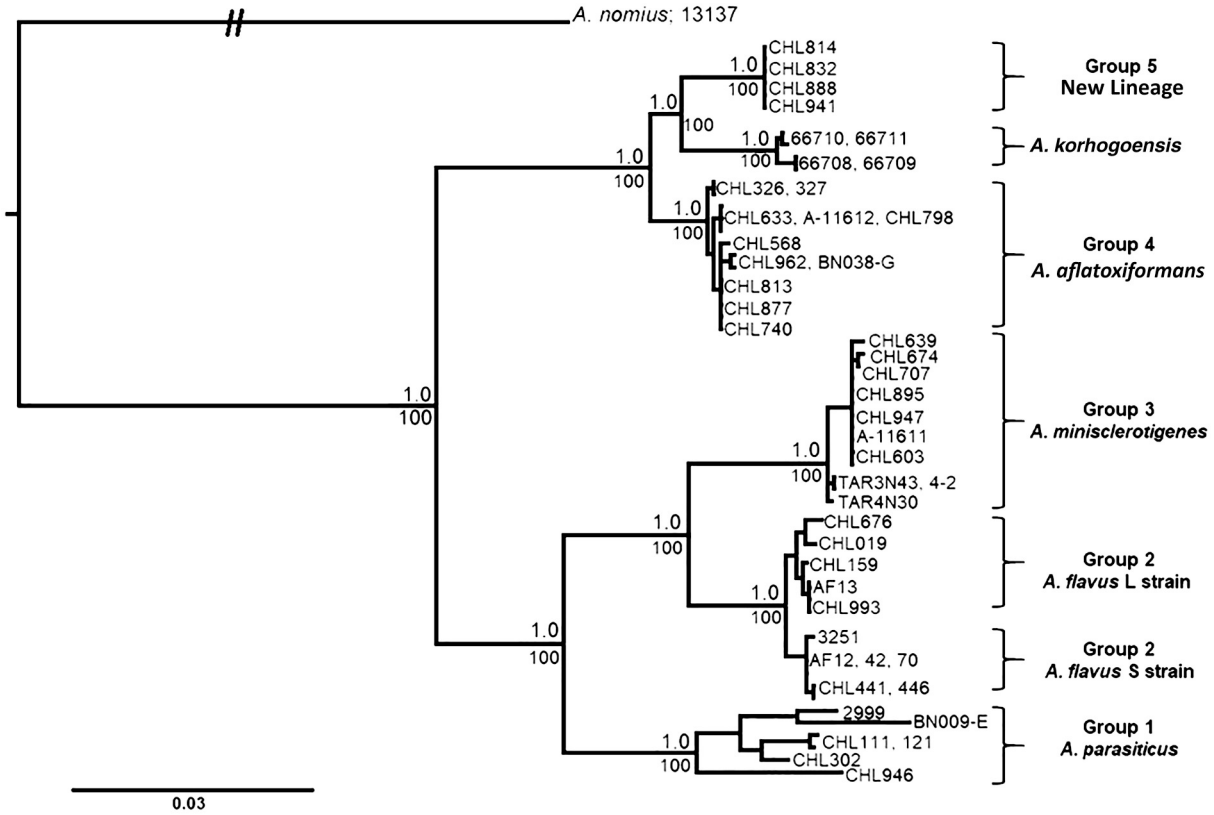

Fig. 1. Mid-point rooted Bayesian phylogeny of 62 isolates of *Aspergillus* section *Flavi* based on concatenated partial gene sequences of *aflR* (1.9 kb) and *niaD* (2.1 kb) genes. Values above nodes are Bayesian posterior probabilities and values below nodes are bootstrap values from 500 replicates.

such as maize, cottonseed, legumes and tree nuts (Boyd and Cotty, 2001; Doster et al., 2014; Bayman and Cotty, 1991; Probst et al., 2014). Fungi with S morphology were recovered from chilies purchased in both the US and Nigeria, with higher incidence in Nigerian chilies (Table 2). *Aspergillus parasiticus*, a highly toxigenic B and G aflatoxin-producing species, was rare in chilies, comprising of only 0.6% and 0.2% of *Aspergillus* section *Flavi* in chilies from US and Nigerian markets, respectively. Lower recoveries of fungal isolates from US samples were due to failure to recover section *Flavi* fungi from 102 (60%) samples; however, fungi were recovered from all Nigerian samples (Singh and Cotty, 2017).

Recently, several fungi with S morphology that are phylogenetically distinct but morphologically indistinguishable have been reported (Cotty, 1989; Pildain et al., 2008; Probst et al., 2012). The B aflatoxin-producing *A.flavus* S strain is only known to be common in crops and soil in the US (Cotty, 1989; Horn, 2003). This contrasts with *A. flavus* L strain, which has been reported in high frequencies across warm regions of the globe (Bayman and Cotty, 1991; Ehrlich et al., 2007; Probst et al., 2014). The S strain of *A. flavus* has never been reported in West Africa. However, a distinct species with S morphology and ability to produce both B and G aflatoxins has been frequently isolated from maize and soil in Nigeria and Benin, and has been referred to as the unnamed taxon S_{BG} (Atehnkeng et al., 2008; Cardwell and Cotty, 2002; Donner et al., 2009). This taxon was recently described as *A. aflatoxiformans* (Frisvad et al., 2019). In the current study, all S morphology fungi from Nigerian chilies produced high concentrations of B and G aflatoxins in liquid fermentation. This is in agreement with findings from previous studies on aflatoxin-producing fungi in West Africa (Atehnkeng et al., 2008; Cardwell and Cotty, 2002; Donner et al., 2009). These distinct S morphology fungi that produce both B and G aflatoxins are resident in agroecosystems of Nigeria. In contrast, the only S morphology fungus known in the US is the *A. flavus* S strain,

Table 4
Aspergillus section *Flavi* isolates used for phylogenetic reconstruction in the current study.

Substrate	Country of purchase[a]	Country of origin[b]	Isolate[c]	Morphology[d]	Aflatoxins[e]	Group[f]	Species[g]
Chili	USA	Myanmar	CHL019	L	B	2	AF
	USA	Unknown	CHL111	N/A	BG	1	AP
	USA	Unknown	CHL121	N/A	BG	1	AP
	USA	Unknown	CHL133	L	B	2	AF
	USA	Unknown	CHL159	L	B	2	AF
	USA	Pakistan	CHL187	L	B	2	AF
	USA	India	CHL302	N/A	BG	1	AP
	USA	India	CHL303	N/A	B	1	AP
	USA	Nigeria	CHL326	S	BG	4	AA
	USA	Nigeria	CHL327	S	BG	4	AA
	USA	Unknown	CHL358	S	B	2	AF
	USA	India	CHL441	S	B	2	AF
	USA	India	CHL446	S	B	2	AF
Soil		USA	AF12 = ATCC® MYA382*	S	B	2	AF
		USA	AF12 = ATCC® MYA383*	S	B	2	AF
		USA	AF12 = ATCC® MYA384*	S	B	2	AF
		USA	AF13 = ATCC® 96044*	L	B	2	AF
Walnuts		USA	NRRL 3251*	S	B	2	AF
Chili	Nigeria	Nigeria	CHL568	S	BG	4	AA
	Nigeria	Nigeria	CHL583	S	BG	3	AM
	Nigeria	Nigeria	CHL603	S	BG	3	AM
	Nigeria	Nigeria	CHL627	S	BG	3	AM
	Nigeria	Nigeria	CHL633	S	BG	4	AA
	Nigeria	Nigeria	CHL639	S	BG	3	AM
	Nigeria	Nigeria	CHL650	S	BG	3	AM
	Nigeria	Nigeria	CHL674	S	BG	3	AM
	Nigeria	Nigeria	CHL676	L	B	2	AF
	Nigeria	Nigeria	CHL707	S	BG	3	AM
	Nigeria	Nigeria	CHL723	L	B	2	AF
	Nigeria	Nigeria	CHL740	S	BG	4	AA
	Nigeria	Nigeria	CHL755	L	B	2	AF
	Nigeria	Nigeria	CHL798	S	BG	4	AA
	Nigeria	Nigeria	CHL801 = NRRL 66829	S	BG	5	New Lineage
	Nigeria	Nigeria	CHL813	S	BG	4	AA
	Nigeria	Nigeria	CHL814	S	BG	5	New Lineage
	Nigeria	Nigeria	CHL816	S	BG	5	New Lineage
	Nigeria	Nigeria	CHL819	S	BG	4	AA
	Nigeria	Nigeria	CHL820	S	BG	5	New Lineage
	Nigeria	Nigeria	CHL832 = NRRL 66830	S	BG	5	New Lineage
	Nigeria	Nigeria	CHL845	S	BG	3	AM
	Nigeria	Nigeria	CHL877	S	BG	4	AA
	Nigeria	Nigeria	CHL878	S	BG	4	AA
	Nigeria	Nigeria	CHL884 = NRRL 66831	S	BG	5	New Lineage
	Nigeria	Nigeria	CHL888	S	BG	5	New Lineage
	Nigeria	Nigeria	CHL895	S	BG	3	AM
	Nigeria	Nigeria	CHL941	S	BG	5	New Lineage
	Nigeria	Nigeria	CHL946	N/A	BG	1	AP
	Nigeria	Nigeria	CHL947	S	BG	3	AM
	Nigeria	Nigeria	CHL962	S	BG	4	AA
	Nigeria	Nigeria	CHL993	L	B	2	AF
Groundnut		Nigeria	NRRL A-11612*	S	BG	4	AA
		Nigeria	NRRL A-11611*	S	BG	3	AM
Soil		Benin	BNO38G = ATCC®MYA380*	S	BG	4	AA
		Benin	BN009-E*	N/A	BG	1	AP
Soil		Uganda	NRRL 2999*	N/A	BG	1	AP
Groundnut		Argentina	TAR3N43*	S	BG	3	AM
Groundnut		Argentina	TAR4N30*	S	BG	3	AM
Soil		Australia	4-2*	S	BG	3	AM
Groundnut		Côte d'Ivoire	NRRL 66708*	S	BG	N/A	AK
Groundnut		Côte d'Ivoire	NRRL 66709*	S	BG	N/A	AK
Groundnut		Côte d'Ivoire	NRRL 66710*	S	BG	N/A	AK
Groundnut		Côte d'Ivoire	NRRL 66711*	S	BG	N/A	AK

[a] Country of purchase- Country from where chilies were purchased.
[b] Country of origin- Country from where the samples actually originated.
[c] Fungal isolates recovered from chilies and reference isolates (indicated by *). Isolates with NRRL deposited at USDA ARS Culture Collection; with ATCC deposited at American Type Culture Collection. Isolates without NRRL or ATCC at the USDA, ARS Tucson laboratory where this work was performed.
[d] N/A; classification into morphology or group is not applicable.
[e] B refers to B aflatoxin production by fungal isolates; BG refers to production of both B and G aflatoxins. Aflatoxin profiles of fungi recovered from chilies were determined in the current study; aflatoxin profiles of reference cultures were previously reported (Probst et al., 2012; Carvajal-Campos et al., 2017).
[f] Group indicates phylogenetic groups in Fig. 1.
[g] Species assignment; AM- AP- *A. parasiticus*, AF- *A. flavus*, *A. minisclerotigenes*, AA- *A. aflatoxiformans*, and AK- *A. korhogoensis*.

Table 5
Aflatoxin production by members of *Aspergillus* section *Flavi* from the current study in various hosts.

Species[a]	Isolates	Aflatoxin (AF) (μg g^{-1})								
		Chili			Maize			Groundnut		
		AFB	AFG	Total AF	AFB	AFG	Total AF	AFB	AFG	Total AF
AM	CHL674	1.0	0.8	1.8	11.3^{C}	37.4^{B}	48.7^{B}	9.8^{B}	31.6^{B}	41.4^{B}
	CHL707	0.5	0.7	1.2	31.0^{B}	95.0^{A}	126.0^{A}	10.6^{B}	33.7^{B}	44.3^{B}
	CHL845	0.7	0.5	1.2	68.0^{A}	133.0^{A}	201.0^{A}	34.0^{A}	70.0^{A}	104.0^{A}
	CHL947	2.1	1.2	3.3	50.0^{AB}	111.0^{A}	161.0^{A}	30.0^{A}	71.0^{A}	101.0^{A}
	Average	**1.1^{z}**	**0.8^{z}**	**1.9^{y}**	**39.9^{z}**	**94.0^{z}**	**134.0^{z}**	**21.3^{y}**	**51.5^{y}**	**72.8^{y}**
AF S	CHL178	1.0^{B}	NA	1.0^{B}	55.7^{C}	NA	55.7^{C}	31.5^{B}	NA	31.5^{B}
	CHL303	13.8^{A}	NA	13.8^{A}	184.0^{B}	NA	184.0^{B}	37.7^{B}	NA	37.7^{B}
	CHL358	30.4^{A}	NA	30.4^{A}	512.0^{A}	NA	512.0^{A}	287.0^{A}	NA	287.0^{A}
	CHL441	22.3^{A}	NA	22.3^{A}	171.0^{B}	NA	171.0^{B}	49.1^{B}	NA	49.1^{B}
	Average	**16.7^{y}**	**NA^{Φ}**	**16.7^{x}**	**231.0^{x}**	**NA^{Φ}**	**212.0^{yz}**	**101.0^{x}**	**NA^{Φ}**	**101.0^{y}**
AF L	CHL280	0.8	NA	0.8	336.0^{A}	NA	336.0^{A}	139.0^{A}	NA	139.0^{A}
	CHL404	0.2	NA	0.2	131.0^{BC}	NA	131.0^{BC}	55.1^{B}	NA	55.1^{B}
	CHL537	0.2	NA	0.2	182.0^{B}	NA	182.0^{B}	80.7^{AB}	NA	80.7^{AB}
	CHL755	0.3	NA	0.3	93.5^{C}	NA	93.5^{C}	23.5^{C}	NA	23.5^{C}
	Average	**0.4^{z}**	**NA^{Φ}**	**0.4^{z}**	**185.0^{x}**	**NA^{Φ}**	**166.0^{yz}**	**74.6^{x}**	**NA^{Φ}**	**74.6^{y}**
AP	CHL111	1.1^{C}	0.6^{C}	1.7^{C}	27.0^{D}	73.0^{C}	100.0^{C}	35.0^{B}	75.0^{B}	111.0^{B}
	CHL121	4.2^{B}	4.2^{B}	8.4^{B}	59.0^{C}	195.0^{AB}	254.0^{B}	92.0^{A}	263.0^{A}	355.0^{A}
	CHL302	25.2^{A}	30.6^{A}	55.8^{A}	106.0^{B}	268.0^{A}	374.0^{A}	63.0^{AB}	158.0^{A}	221.0^{A}
	2999	5.1^{B}	2.6^{B}	7.7^{B}	154.0^{A}	192.0^{B}	345.0^{A}	123.0^{A}	161.0^{A}	284.0^{A}
	Average	**8.9^{y}**	**9.5^{y}**	**18.4^{x}**	**86.5^{y}**	**182.0^{y}**	**239.0^{y}**	**78.2^{x}**	**164.0^{x}**	**243.0^{x}**
NL	CHL801	101.0^{AB}	101.0^{AB}	202.0^{AB}	128.0^{C}	334.0^{B}	462.0^{B}	82.0	137.0	219.0
	CHL832	61.8^{B}	72.5^{B}	134.0^{B}	98.0^{D}	257.0^{C}	354.0^{C}	85.0	137.0	222.0
	CHL888	86.2^{AB}	89.8^{AB}	176.0^{AB}	178.0^{B}	440.0^{A}	618.0^{A}	112.0	235.0	347.0
	CHL941	221.0^{A}	199.0^{A}	420.0^{A}	212.0^{A}	496.0^{A}	708.0^{A}	131.0	264.0	395.0
	Average	**118.0^{x}**	**116.0^{x}**	**233.0^{w}**	**154.0^{x}**	**382.0^{x}**	**519.0^{x}**	**103.0^{x}**	**193.0^{x}**	**296.0^{x}**
AA	CHL326	76.7^{A}	124.0^{A}	201.0^{A}	166.0^{C}	399.0^{B}	565.0^{B}	136.0^{B}	292.0^{A}	428.0^{A}
	CHL633	8.5^{B}	10.9^{B}	19.4^{B}	70.0^{D}	134.0^{D}	204.0^{C}	30.8^{C}	59.0^{B}	89.8^{B}
	CHL819	159.0^{A}	149.0^{A}	308.0^{A}	303.0^{A}	480.0^{A}	782.0^{A}	412.0^{A}	523.0^{A}	935.0^{A}
	CHL962	64.7^{A}	67.3^{A}	132.0^{A}	212.0^{B}	275.0^{C}	487.0^{B}	167.0^{AB}	232.0^{A}	398.0^{A}
	Average	**77.0^{x}**	**88.0^{x}**	**165.0^{w}**	**188.0^{x}**	**322.0^{x}**	**510.0^{x}**	**186.0^{x}**	**276.0^{x}**	**463.0^{x}**

Aflatoxin B, G and total concentrations were compared by column between and within species, separately for chili, maize and groundnut. Each toxin concentration is a mean of 4 replicates.
Differences in aflatoxin concentrations produced by distinct species of *Aspergillus* section *Flavi* within each crop tested are indicated by bold lower case letters (Tukey's HSD, $p < 0.01$).
Differences in aflatoxin concentrations produced within species are indicated by upper case letters (Tukey's HSD, $p < 0.01$).
Values within a column lacking a letter do not differ (ANOVA, $p > 0.05$).
[a] Species recovered from chilies used for crop inoculation; AF- *A. flavus*, AP- *A. parasiticus*, AM- *A. minisclerotigenes*, AA- *A. aflatoxiformans*, NL- New Lineage.
[Φ] Not applicable. These fungi do not produce G aflatoxins. *A. flavus* L and S strain morphotypes were excluded when comparing G aflatoxin between and within species.

which produces only B aflatoxins (Cotty et al., 2008; Jaime-Garcia and Cotty, 2006). In the current study, 3 isolates with S morphology, and B and G aflatoxin producing ability, were detected in chilies from US markets (Table 3). These isolates were traced to chili imported from Nigeria. Crop contamination with B and G aflatoxins in the US has been attributed to *A. parasiticus*, and S morphology fungi with the ability to produce both B and G aflatoxins have not been reported to date, indicating introduction of a highly toxigenic non-native pathogen to the US with chili. Although it is undesirable to allow non-native pathogens in a given region, such cases frequently occur because of trade and human movement. Similarly, both Dutch elm disease and chestnut blight are introduced pathogens that moved through commerce and caused death of billions of trees in the US (Agrios, 2005). If the highly toxigenic non-native aflatoxin producing fungi from Nigeria reach agroecosystems in the US, they may exacerbate aflatoxin contamination.

Phylogenetic reconstruction resolved *Aspergilli* recovered from chilies into five taxa: a) *A. parasiticus*, b) the L and S strain of *A. flavus*, c) *A. minisclerotigenes*, d) *A. aflatoxiformans*, and e) a novel lineage. Fungi with S morphology from Nigerian chilies were indistinguishable based on morphology and types of aflatoxins produced. However, DNA sequence comparisons resolved these fungi into three distinct taxa: *A. minisclerotigenes*, *A. aflatoxiformans*, and a new lineage. *Aspergillus minisclerotigenes* was described from Argentinian groundnuts (Pildain et al., 2008), and has been reported in central and southern Africa, whereas *A. aflatoxiformans* predominates West Africa (Probst et al., 2014). This is the first study to report occurrence of multiple lineages of genetically distinct S morphology fungi with B and G aflatoxin producing ability in West Africa. Furthermore, *A. minisclerotigenes* was detected at a higher frequency in Nigerian chilies (8% of all *Aspergilli*) compared to its sparse occurrence previously reported in maize from Central and Eastern Africa (Probst et al., 2014), maize and almonds from Portugal (Soares et al., 2012), and spices marketed in Morocco (El Mahgubi et al., 2013).

Aspergillus minisclerotigenes is indistinguishable from *A. aflatoxiformans* based on morphology and aflatoxin production. Both *A. aflatoxiformans* and *A. minisclerotigenes* produced similar concentrations of total aflatoxins in liquid fermentation (data not shown). Studies on distribution of *Aspergillus* section *Flavi* in West Africa (Cardwell and Cotty, 2002; Donner et al., 2009) based results on morphological and/or physiological characteristics of S morphology fungi, and as a result, were unable to resolve S morphology fungi with B and G aflatoxin producing ability into multiple taxa.

Members of *A. aflatoxiformans* and the new lineage produced the highest concentrations of aflatoxins in chili, groundnut and maize (Table 5). All of these are important crops in Nigeria based on both production and consumption (FAOSTAT, 2017), and occurrence of these highly toxigenic fungi in Nigeria suggests an unrealized basis for

vulnerability to aflatoxin contamination. More than 90% of Nigerian chilies in the current study were collected from Kaduna state, which falls within the Northern Guinea Savannah (NGS) agroecosystem. Toxigenic S_{BG} fungi have previously been reported in the NGS region of Nigeria in high frequencies (Donner et al., 2009). Incidences within infecting fungal communities and aflatoxin-producing potential must both be considered when assessing the importance of specific taxa to determine etiology of aflatoxin contamination events. Although the most toxic fungi, *A. aflatoxiformans* and the new lineage, had lower incidences than the *A. flavus* L strain, they produced at least 300 times more aflatoxins in chilies. On the other hand, isolates belonging to *A. minisclerotigenes* produced significantly lower concentrations of total aflatoxins in chilies compared to *A. aflatoxiformans* and the new lineage (Table 5); however, increased prevalence of *A. minisclerotigenes* can lead to contamination of the crop with unacceptable concentrations of B and G aflatoxins. Discovery of a novel lineage phylogenetically distinct from *A. aflatoxiformans* but very similar morphologically and physiologically, indicates that diverse communities of genetically distinct S_{BG}-like fungi may be resident in agroecosystems across West Africa.

Aflatoxin management strategies have received increased attention over the past two decades owing to serious health and economic concerns caused by aflatoxin contamination of crops. Biological control of aflatoxins using native, well-adapted atoxigenic L strains of *A. flavus* has been a successful strategy for mitigation of crop contamination (Atehnkeng et al., 2016; Cotty, 1994b; Cotty, 1999; Doster et al., 2014). Atoxigenic isolates are a subset of the L strain of *A. flavus*, which was the predominant member of *Aspergillus* section *Flavi* from chilies in the current study. Of the total L strain isolates tested for aflatoxin production, 45% did not produce detectable concentrations. The L strain isolates of *A. flavus* from the current study are a genetic resource for selection of active ingredients for biological control products directed at reducing aflatoxin contamination of chilies.

Representative isolates of the new lineage (NRRL 66829, NRRL 66830, NRRL 66831) have been deposited at the ARS Culture Collection (NRRL) (Peoria, IL, USA). DNA Sequences of representative isolates from each taxon were deposited at GenBank under accession numbers MH752557 - MH752590 and MH760519 - MH760551.

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Declaration of interest

The authors declare that they have no conflict of interest.

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