Food Additives and Contaminants
Vol. 27, No. 5, May 2010, 576–590

Taylor & Francis
Taylor & Francis Group

Molecular characterization of atoxigenic strains for biological control of aflatoxins in Nigeria

M. Donner[a], J. Atehnkeng[b], R.A. Sikora[a], R. Bandyopadhyay[b] and P.J. Cotty[c]*

[a]*Department of Plant Pathology, INRES Institute of Crop Science and Resource Conservation, University of Bonn, Bonn, Germany;* [b]*International Institute of Tropical Agriculture (IITA), PMB 5320, Ibadan, Nigeria;* [c]*USDA, ARS, Division of Plant Pathology and Microbiology, Department of Plant Sciences, University of Arizona, Tucson, AZ 85721, USA*

(*Received 11 October 2009; final version received 10 December 2009*)

Aflatoxins are highly toxic carcinogens produced by several species in *Aspergillus* section *Flavi*. Strains of *A. flavus* that do not produce aflatoxins, called atoxigenic strains, have been used commercially in North America as tools for limiting aflatoxin contamination. A similar aflatoxin management strategy is being pursued in Nigeria. In the current study, loci across the 68 kb aflatoxin biosynthesis gene cluster were compared among 18 atoxigenic and two aflatoxin-producing vegetative compatibility groups (VCGs) from Nigeria and an atoxigenic VCG used commercially in North America. Five of the atoxigenic VCGs had large deletions (37–65 kb) extending from the teleomeric side of the aflatoxin biosynthesis cluster. In one VCG (AV0222) the deletion extended through the cluster to the adjacent sugar cluster. The remaining twelve atoxigenic VCGs, including the VCG used for aflatoxin management in North America, contained all the aflatoxin pathway genes, but with defects. Two observations support the long-term persistence of atoxigenicity within *A. flavus*: first, a comparison of pathway genes revealed more changes in atoxigenic than in aflatoxin-producing isolates relative to the aflatoxin-producing strain NRRL 3357; and second, several non-synonymous changes are unique to atoxigenics. Atoxigenic VCG diversity was assessed with phylogenetic analyses. Although some atoxigenics share relatively recent ancestry, several are more closely related to aflatoxin producers than to other atoxigenics. The current study demonstrates VCGs of *A. flavus* in West Africa with diverse mechanisms of atoxigenicity and potential value in aflatoxin management programmes.

Keywords: polymerase chain reaction (PCR); mycology; health significance; aflatoxins; cereals

Introduction

Aflatoxins are a group of toxic secondary metabolites produced by several *Aspergillus* species (Payne and Brown 1998). They are highly carcinogenic and can contaminate food and feeds resulting in serious human and domestic animal health problems (Williams et al. 2004). Regulations limiting the concentrations of aflatoxins allowed in foods and feeds exist in most developed countries (van Egmond et al. 2007). Nevertheless, in Africa products of small-scale farms may move from field to mouth without the opportunity for practical monitoring. This can result in severe human health effects as exemplified by the hundreds of deaths associated with acute aflatoxicosis in Kenya (Lewis et al. 2005). In Nigeria, mycotoxin monitoring is rare, but high concentrations of aflatoxin in pre- and post-harvest maize are known (Adebajo et al. 1994; Udoh et al. 2000; Bankole and Adebanjo 2003; Atehnkeng, Ojiambo, Donner et al. 2008).

Aspergillus flavus is the most common causal agent of aflatoxin contamination of crops (Klich 2007). *A. flavus* isolates produce only B-aflatoxins, while three other aflatoxin producers known in West Africa, *A. parasiticus*, *A. nomius*, and the unnamed taxon S_{BG}, produce both B- and G-aflatoxins (Diener et al. 1987; Kurtzman et al. 1987; Cotty and Cardwell 1999). The molecular basis for aflatoxin biosynthesis is known and the more than 20 genes involved are clustered in a 65–70 kb DNA region (Yu et al. 1995; Yu, Bhatnagar et al. 2004; Yu, Chang et al. 2004).

A. flavus is widely distributed with the greatest quantities of the fungus in warm climates (Cotty et al. 1994; Boyd and Cotty 2001). Gene flow within *A. flavus* is limited by a vegetative compatibility system (Papa 1986; Bayman and Cotty 1991) that delineates numerous genetic groups called vegetative compatibility groups (VCGs), which are clonal lineages (Ehrlich et al. 2004) that vary in many characteristics, including aflatoxin-producing ability.

Populations of *A. flavus* in individual agricultural fields are complex communities that contain many VCGs (Cotty et al. 1994; Ehrlich et al. 2007). Aflatoxin-producing potential is known to vary less among isolates within a VCG than among isolates

*Corresponding author. Email: pjcotty@email.arizona.edu

ISSN 0265–203X print/ISSN 1464–5122 online

DOI: 10.1080/19440040903551954
http://www.informaworld.com

from different VCGs (Ehrlich and Cotty 2004). Communities of *A. flavus* VCGs resident in different fields, areas, and regions may vary widely in average aflatoxin-producing ability (Schroeder and Boller 1973; Lisker et al. 1993; Cotty et al. 1997). Isolates that do not produce aflatoxin, called atoxigenic, are common within *A. flavus* communities (Joffe and Lisker 1969; Schroeder and Boller 1973; Lisker et al. 1993; Cotty et al. 1997). Surveys of *A. flavus* isolates from various geographic regions have revealed differences in the proportions of isolates that produce low, medium, and high amounts of aflatoxins (Cotty 1997; Cotty and Cardwell 1999; Horn and Dorner 1999). In Argentina (Vaamonde et al. 2003) and Iran (Razzaghi-Abyaneh et al. 2006), fewer than 30% of *A. flavus* isolates are capable of producing aflatoxin, whereas in Nigeria more than 50% produce aflatoxins (Donner et al. 2009). In the south-western United States, most *A. flavus* isolates produce aflatoxin due to the high incidences of the aflatoxin-producing *A. flavus* S-strain (Cotty 1997; Horn and Dorner 1999).

There are two atoxigenic strains of *A. flavus* used to reduce aflatoxin contamination of commercial crops in the United States (Dorner 2004; Cotty 2006; Cotty et al. 2008). Each strain is in a distinct VCG and affects aflatoxin levels by competitively excluding aflatoxin producers (Cotty 2006). One of these strains, AF36, has successfully suppressed aflatoxin producers on cottonseed in Arizona and Texas, where it occurs naturally, since 1999 (Cotty et al. 2007). Recently, efforts were initiated to develop similar technology for use in Africa where several indigenous atoxigenic strains have been shown to exclude aflatoxin producers and thereby reduce aflatoxin concentrations in maize (Atehnkeng, Ojiambo, Ikotun et al. 2008; Hell et al. 2008).

The loss of aflatoxin production by members of *Aspergillus* section *Flavi* is not well understood. For example, neither *A. sojae* nor *A. oryzae* produce aflatoxins (Wei and Jong 1986), even though homologues of aflatoxin biosynthetic genes are present in each (Chang et al. 1995; Klich et al. 1995; Yu et al. 2000). Both species have been used for centuries in the food fermentation industry and are generally considered safe (Machida et al. 2005; Chang et al. 2007). Although *A. oryzae* strains have the aflatoxin biosynthesis gene cluster, it is not functional (Tominaga et al. 2006). *A. oryzae* is a domesticated atoxigenic strain of *A. flavus* (Wicklow 1984; Kurtzman et al. 1986; Chang et al. 2006). The aflatoxin biosynthesis genes in *A. oryzae* contain deletions, frame-shift mutations, and base pair substitutions that explain the lack of aflatoxin production (Tominaga et al. 2006). Deletion of portions of the aflatoxin biosynthetic gene cluster within atoxigenic *A flavus* isolates is not rare (Chang et al. 2005) and strains of *A. flavus* with large deletions in the aflatoxin gene cluster have been used to study the genetics of aflatoxin biosynthesis for over a decade (Prieto et al. 1996). A single nucleotide polymorphism (SNP) in a polyketide synthase gene results in atoxigenicity in the biocontrol strain AF36 (Ehrlich and Cotty 2004; Ehrlich et al. 2007). Nevertheless, molecular mechanisms responsible for the loss of aflatoxin production are diverse and for most atoxigenic *A. flavus* strains the specific genetic changes resulting in atoxigenicity are unknown.

In the current study, in order to provide a basis for understanding atoxigenicity in Nigeria, aflatoxin biosynthetic gene clusters of 18 atoxigenic *A. flavus* VCGs were compared with two aflatoxin-producing *A. flavus* isolates from Nigeria and an isolate from North America belonging to the atoxigenic VCG to which AF36 belongs using two approaches. First, sequences from 14 genes from the aflatoxin gene cluster were generated to assess nucleotide polymorphism. Second, eight additional cluster genes were polymerase chain reaction (PCR)-amplified to determine the presence/absence of the genes (Chang et al. 2005). To assess the diversity of atoxigenic strains available for biocontrol in Nigeria, relationships among the examined isolates were assessed with phylogenetic analyses that included two additional protein coding loci outside the aflatoxin gene cluster, *pecA* and *taka amylase*. Molecular characteristics were discovered that will be useful both for monitoring the stability of atoxigenic phenotypes and for specifically identifying each of these candidate biocontrol agents.

Materials and methods

Isolates, vegetative compatibility and aflatoxin production

Isolates belonging to the L strain of *A. flavus* (Table 1) were recovered by a dilution plate technique based on modified rose Bengal agar (MRBA; Cotty 1994) from maize collected in several districts of Nigeria (Atehnkeng, Ojiambo, Donner et al. 2008) and placed into vegetative compatibility groups (VCGs) by complementation of nitrate non-utilizing mutants as previously described (Bayman and Cotty 1991, 1993; Cotty 1994, 1997). The commercial biocontrol agent from the United States, *A. flavus* AF36 (ATCC96045) was used as a reference culture throughout the study.

Aflatoxin content was quantified as previously described (Cotty, 1997; Cotty and Cardwell 1999). Flasks (250 ml) containing 70 ml of a chemically defined medium with ammonium as the sole nitrogen source were inoculated with an isolate belonging to *Aspergillus* section *Flavi*. After 5 days of incubation, 50 ml acetone were added to lyse the mycelium and solubilize the aflatoxins. The cultures were filtered and the aflatoxins partitioned into methylene chloride and spotted on thin-layer chromatography (TLC) plates to

determine the presence or absence of aflatoxins. Extracts negative for aflatoxin content were evaporated to dryness, dissolved in 60% methanol, and loaded onto a column with immunoaffinity to aflatoxins (Aflatest P column, VICAM, Watertown, MA, USA). Aflatoxins were eluted from the column with methanol. The eluate was concentrated, spotted onto TLC plates, developed, and quantified as previously described (Cotty and Cardwell 1999). The limit of detection was 0.5 ng aflatoxin g^{-1} mycelium.

Table 1. *Aspergillus flavus* strains from Nigeria and one strain from the United States used in the study.

Isolate	VCG	Geographical origin	Aflatoxin production
AV3279[a]	B	Lafia	No
AV3304[a]	D	Lafia	No
AV2216[a]	E	Abuja	No
AV0106	F	Ogbomosho	Yes
AV0222[a]	G	Ogbomosho	No
AV0173	H	Ogbomosho	No
AV0165	I	Ogbomosho	No
AV0452	L	Ogbomosho	No
AV3108[a]	M	Lafia	No
AV3150	N	Lafia	No
AV3228[a]	O	Lafia	Yes
AV3224	Q	Lafia	No
AV3303[a]	R	Lafia	No
AV3306	S	Lafia	No
AV0205	T	Ogbomosho	No
AV2757[a]	U	Akwanga	No
AV3058[a]	V	Akwanga	No
AV4216[a]	W	Lokoja	No
AV16127[a]	X	Kaduna	No
AV3020[a]	Y	Akwanga	No
AV0216	n.a.[c]	Ogbomosho	No
AV0230	n.a.	Ogbomosho	No
AV3193	n.a.	Lafia	No
AF36[b]	YV36	Arizona	No

Notes: [a]Isolates from Nigeria used in a previous study by Atehnkeng, Ojiambo, Ikotun et al. (2008).
[b]AF36 (ATCC 96045) is a strain that produces no aflatoxin and which is used commercially for aflatoxin biocontrol in the United States.
[c]n.a., Not available. They could not be assigned to any VCG because of incompatibility.

DNA isolation

Fungi were cultured in 70 ml Czapek-Dox Broth (Difco Laboratories, Detroit, MI, USA) in 250 ml flasks agitated at 150 rpm at 32°C for 48–72 h. DNA from most cultures was isolated using the FastDNA SPIN Kit and the FastPrep Instrument according to manufacturer's instructions (Qbiogene, Inc., Carlsbad, CA, USA). If a PCR was unsuccessful, it was repeated with DNA isolated with a second method using a chloroform–phenol protocol as follows.

Mycelia were collected by vacuum filtration, ground to a fine powder in liquid nitrogen, and stored at −80°C. In 1.5 ml tubes, 200 mg of mycelial powder were resuspended in 750 µl spermidine-sodium dodecylsulphate (SDS) buffer (4 mM spermidine, 10 mM ethylenediamine tetra-acetic acid (EDTA), 0.1 M NaCl, 0.5% SDS, 10 mM β-mercaptoethanol, 40 mM Tris-HCl pH 8.0). After adding an equal volume of phenol, the suspension was centrifuged at 14,000 rpm for 15 min. Mycelial lysates were recovered, mixed with an equal volume of phenol–chloroform (1 : 1), and recentrifuged for 15 min at 14,000 rpm. The supernatant was mixed with an equal volume of chloroform–isoamyl alcohol (24 : 1) and centrifuged. DNA was recovered from the aqueous phase by mixing a 0.1 vol. of 3 M sodium acetate pH 5.5 and adding 2 vols of 100% ethanol. The DNA was pelleted by centrifugation, washed with 70% ethanol, air dried, and redissolved in TE buffer (20 mM Tris-HCl pH 7.5, 0.1 mM EDTA). The DNA concentration was measured with a spectrophotometer (model ND-1000, NanoDrop).

PCR conditions

Oligonucleotide primer sets (Table 2) targeting PCR products of 0.3–1.2 kb were derived from known sequences of aflatoxin biosynthetic pathway genes of

Table 2. Primers designed during this study and annealing temperatures (T_a) for PCR amplification.

Designation	Forward primer	Reverse primer	T_a (°C)
norB[a]	GTGAGGGATAGCAATAAGTGT	TCCTGGATTTCCGCATAC	54
pksA[a]	GCTGGGATTCTGCATGGGTT	CCATCTGAGGCATCGCACA	58
afIR[a]	GGAAACAAGTCTTTTCTGG	CAGAGCGTGTGGTGGTTGAT	59
norA[a]	GGAGCACCTCAAGGAGAACA	GGAACCTTGCGTCGATTCTA	60
ver-1[a]	AGCCAAAGTCGTGGTGAACT	CCATCCACCCCAATGATCT	60
verB[a]	CCCAATACAGTTCCGCAGTC	AGTGAAGAGTGCCGACGATAA	59
omtB[a]	TTTACTCGGATTGGGATGTGGT	CGCAGTCCTTGTTAGAGGTGAT	56
pecA	GCTTAGCCTAGACTCAAG	AAGAGGAGTCCAGCTTGTG	49
taka amylase	TATCCAGGGAATGGGCTT	TTAGAGGTCGTCCATGCTGCC	56

Note: [a]Derived from aflatoxin biosynthetic genes and flanking regions.

A. flavus AF36 (AY 510455), AF13 (AY 510451), and AF70 (AY 510453). Some primers sets were based on Chang et al. (2005) and Ehrlich et al. (2005). Primers for the genes *norB*, *pksA*, *afIR*, *norA*, *ver-1*, *verB*, *omtB*, *pecA*, and *taka amylase* were designed using DNAMAN version 6 (Lynnon Biosoft, Vandereuil, Canada). Primers used to PCR-amplify the genes *C2*, *aflT*, *fasA(hexA)*, *aflJ*, *estA*, *omtA*, *verA*, *avnA*, *avfA*, *vbs*, *cypX*, *ordB*, *hypA*, and *glcA* were previously described by Chang et al. (2005) and *norB-cypA* by Ehrlich et al. (2004).

PCR used 5 ng genomic DNA, 50 pmol of forward and reverse oligonucleotides, and the HotMaster PCR kit (Eppendorf, Westbury, NY, USA) in a 50 µl final volume. Annealing temperatures (Table 2) were optimized for each primer set (Chang et al. 2005; Ehrlich et al. 2005). PCR reactions were performed with a MyCycler thermocycler (Bio-Rad Laboratories, Richmond, CA, USA) under the following conditions: 5 min at 95°C followed by 38 cycles of 95°C for 30 s, locus-specific annealing temperature for 20 s, 72°C for 30 s, and 10 min at 72°C. Amplicons were visualized with SYBR Gold after 1.2% agarose gel electrophoresis. Amplicons of the genes *norB*, *aflT*, *hexA*, *aflR*, *alfJ*, *estA*, *ver-1*, *avnA*, *avfA*, *omtB*, *omtA*, *vbs*, *hypA*, and *glcA* were sequenced by The Genomic Analysis and Technology Core Facility (GATC) at the University of Arizona, Tucson.

Analysis of synonymous and non-synonymous substitutions

Sequences were aligned to codon-aligned nucleotide sequences of *A. flavus* AF13 from Genbank. Synonymous (silent) and non-synonymous (amino acid-altering) nucleotide substitutions were calculated with reference to *A. flavus* NRRL3357. Minor manual modifications were made to the automatic DNA alignments to optimize alignments and ensure indels did not erroneously split codons. Estimates of synonymous and non-synonymous substitution rates were made with Synonymous Non-synonymous Program (SNAP) (Korber 2000) based on Nei and Gojobori (1986) and incorporating the statistic of Ota and Nei (1994). The ratio dN/dS (equivalent to K_a/K_s) was calculated with SNAP.

Pyrosequencing (Biotage, Uppsala, Sweden) was used as previously described (Das et al. 2008) to assess distribution of the single nucleotide polymorphism in *pksA* responsible for atoxigenicity in *A. flavus* AF36 (Ehrlich and Cotty 2004).

Statistical analyses

Analyses were performed with SAS (version 9.1.3, SAS Institute, Inc., Cary, NC, USA). Analysis of variance (ANOVA) was performed on all data with the general linear model (GLM) suitable for unbalanced data. The GLM of SAS uses the least-squares method to fit data to a general linear model. Tukey's honest significant difference (HSD) test was used to separate means at the 5% level.

Phylogenetic analysis

Phylogenetic analyses included sequences from Genbank for *A. flavus* L strain AF13 and NRRL3357, *A. flavus* S strain AF70, and *A. parasiticus*. DNA sequences were aligned with DNAMAN version 6 (Lynnon Biosoft, Vandereuil). A concatenated data set of 13 genes within and three genes outside of the aflatoxin biosynthesis pathway was subjected to phylogenetic analyses using PAUP* Version 4.0b10 (Swofford 2002). Parsimony trees were obtained using heuristic search methods with stepwise sequence addition and the tree–bisection–reconnection (TBR) branch-swapping algorithm. All sites were equally weighted and gaps treated as missing characters. Bootstrap analyses were based on 1000 replicates.

Results

Aflatoxin production of aflatoxin-producing VCGs

The 21 atoxigenic strains previously selected for use in biocontrol of aflatoxin contamination of maize (Atehnkeng, Ojiambo, Donner et al. 2008) were confirmed as atoxigenic (limit of detection = 0.5 ng aflatoxin B_1 g^{-1} mycelium) (Table 1). The two aflatoxin-producing isolates from West Africa produced widely varying aflatoxin concentrations from 560 ng g^{-1} aflatoxin B_1 for AV0106 to 23,000 ng g^{-1} for AV3228. Out of the initial 21 atoxigenic isolates tested, 18 were assigned to a VCG. Complementary mutants could not be generated for three isolates and these were not assigned to a VCG (Table 1).

Deletions in aflatoxin biosynthetic gene cluster of atoxigenic A. flavus *isolates*

All 21 gene fragments attempted were successfully PCR-amplified for both aflatoxin-producing isolates from West Africa. In addition, all fragments were successfully amplified for *A. flavus* AF36, the atoxigenic strain used commercially for aflatoxin biological control in the United States, and for 13 of the 21 atoxigenic strains belonging to different VCGs from Nigeria. PCR results for seven of the atoxigenic *A. flavus* isolates consistently indicated large deletions in the aflatoxin biosynthetic pathway (Figure 1). Deletions ranged from the entire cluster for AV0222 to deletion of all genes from *norB* through *norA*, over 35 kb. The seven isolates

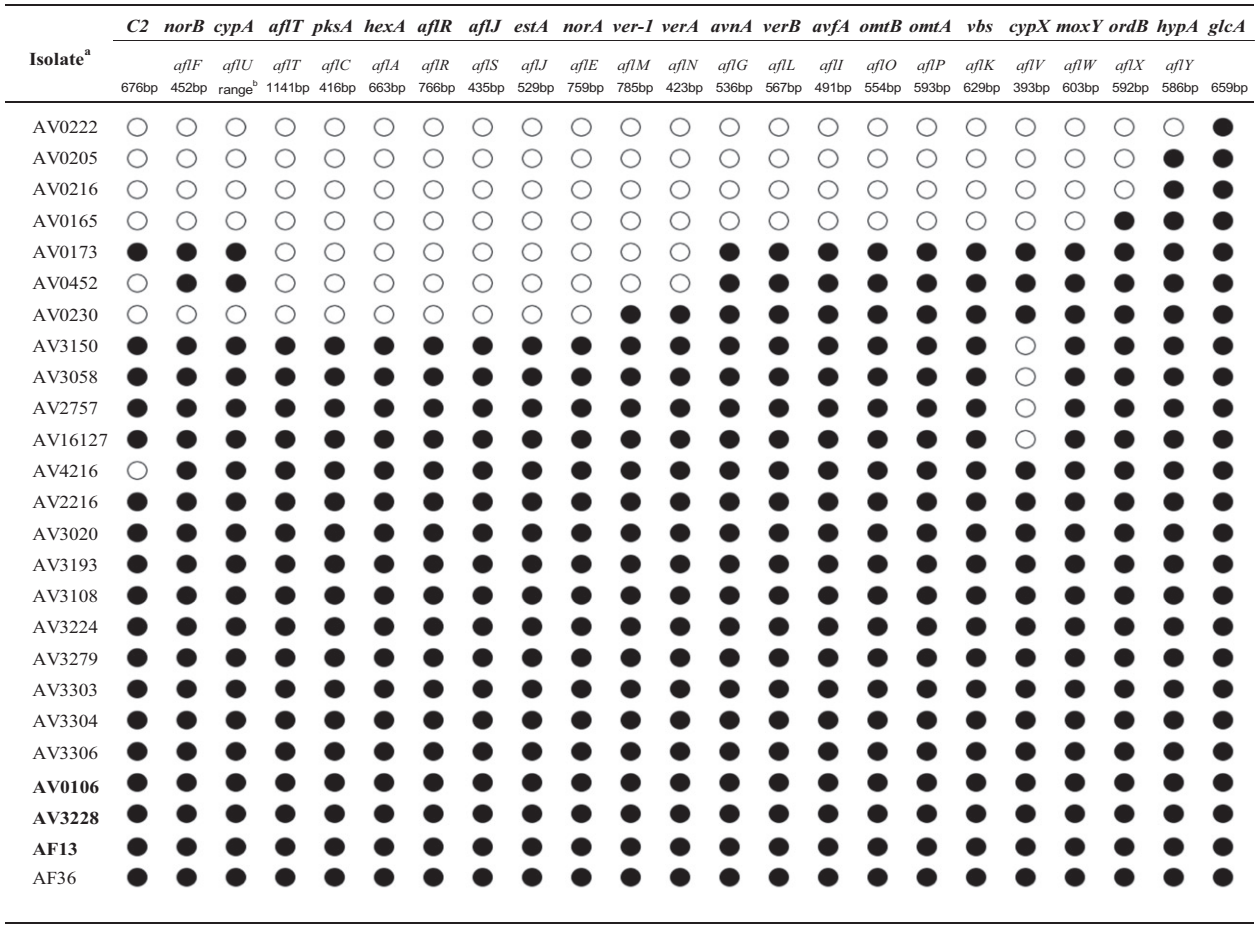

Figure 1. Segments of the *Aspergillus flavus* aflatoxin gene cluster present (filled circles) or absent (empty circles) in Nigerian and US isolates. Original gene names are above and new names below (Yu, Chang et al. 2004). Amplicon sizes are indicated. The *glcA* is a gene in the sugar utilization cluster adjacent to the 3′ end of the aflatoxin cluster; C2 is a gene-flanking region of the aflatoxin gene cluster at the 5′ end. [a]Isolates names in bold indicate the aflatoxin producers. [b]Two amplicon sizes were detected. Amplicons of all isolates except AV3228 were 0.3 kb; the amplicon of AV3228 was 0.8 kb.

belonged to five different VCGs and all originated from the same district: Ogbomosho. The large deletions included the 5′ end (proximal to the teleomere) of the aflatoxin gene cluster except for AV0173 and AV0452 which retained remnants of the *norB-cypA* region. For four VCGs (AV3150, AV3058, AV2757 and AV16127), the PCR protocol failed to amplify the 603 bp target within the gene *cypX* (aflV) while protocols for the adjacent genes *vbs* (aflK) and *moxY* (aflW) produced the expected amplicons. These four VCGs originated from three districts (Figure 1 and Table 1). The protocols for the three genes not involved in aflatoxin biosynthesis, *pecA*, *taka amylase*, and *glcA*, produced the predicted amplicons for each of the 24 *A. flavus* isolates included in the current study.

All *A. flavus* isolates had deletions in the *norB–cypA* region when compared with the *norB-cypA* region of *A. parasiticus* (AY371490). In all isolates except AV3228, deletions included coding regions for amino acids 1–280 of *norB* and 1–112 of *cypA*, and the *norB-cypA* intergenic region. In AV3228, coding regions for amino acids 1–181 and 300–310 of *norB* and 1–29 of *cypA* were deleted along with the *norB-cypA* intergenic region.

The pyrosequencing assay used to detect the SNP responsible for atoxigenicity in *A. flavus* AF36 (Ehrlich and Cotty 2004; Das et al. 2008) successfully identified the target SNP in AF36 but not in any other *A. flavus* isolate.

Distribution of nucleotide polymorphisms

In *A. flavus*, the aflatoxin-biosynthesis gene cluster is 66.1–66.5 kb in length. In the current study, 0.4–8.1 kb of the cluster was sequenced for the 18 atoxigenic and two aflatoxin-producing *A. flavus* VCGs. Of the sequenced cluster regions, 5 kb were coding sequence and 3.1 kb were non-coding. In addition, three gene pieces (2.2 kb) outside of the cluster were sequenced, including 649 bp of *glcA*, a component of the sugar cluster adjacent to the aflatoxin-biosynthesis cluster. All polymorphisms were measured in relation to

NRRL3357, the fully sequenced *A. flavus* isolate and a producer of high aflatoxin concentrations. Gene segments differed significantly ($p = 0.05$) in percentages of both polymorphic bases and non-synonymous substitutions. The greatest rates of polymorphism occurred in *ver-1* among atoxigenic VCGs and in *omtB* among all VCGs in both coding and non-coding segments. Among all VCGs, significantly ($p = 0.05$) more polymorphisms were detected in non-coding (3.4%) than in coding (2.8%) gene segments. This was true both when the toxigenic strains were included and when they were removed from the analysis. Among atoxigenic VCGs, gene segments outside the aflatoxin cluster were less polymorphic (total = 0.3%, synonymous = 0.2%, non-synonymous = 0.1%) than segments within the cluster.

Polymorphisms in *hypA*, a gene present in all but one of the isolates from Nigeria, ranged from 0.0% to 2.7% in atoxigenics exhibiting large deletions in the remainder of the cluster (six isolates) and from 0.0% to 1.1% in atoxigenics with no deletion. The two aflatoxin producers had 0.4–0.9% polymorphism. For the isolates without large deletions from the cluster, there were significant ($p = 0.05$) differences in per cent polymorphic bases between aflatoxin producers and atoxigenic isolates in the coding segments of *aflT*, *hexA*, *aflR*, *aflJ*, *ver-1*, and *omtB*. Only the segments of *aflT*, *ver-1*, and *omtB* had significantly ($p = 0.05$) more non-synonymous substitutions in atoxigenic strains than in the aflatoxin producers. Neither of the aflatoxin producers contained polymorphisms in the aflatoxin regulatory genes *aflR* and *aflJ*. All but one of the atoxigenics had polymorphisms in *aflR* and several atoxigenics had polymorphisms in the *aflJ* segment in which several changes were detected in protein sequences. Although frequencies of polymorphisms varied among atoxigenic VCGs for several genes (i.e. *avfA* and *omtA*), polymorphism frequency was consistent among atoxigenics for many other genes. Atoxigenic strains had significantly greater polymorphism frequencies than aflatoxin producers in both coding and non-coding segments of the aflatoxin-biosynthesis cluster.

The majority of the nucleotide changes in gene segments of the aflatoxin biosynthesis gene cluster resulted in synonymous substitutions (Table 3). The ratio dN/dS, as calculated with SNAP (Korber 2000), resulted in a ratio far less than 1 for most gene segments including genes with very high polymorphism rates (i.e. *omtB* and *ver-1*). However, one gene (*avnA*) had dN/d_s values greater than 1 both for twelve of 15 atoxigenics and for one aflatoxin producer. In that segment the highest simple ratio of synonymous to non-synonymous substitutions was also measured. In the *hypA* gene segment, twelve of 15 atoxigenics and one aflatoxin producer contained two non-synonymous substitutions and no synonymous (Table 3). The ratio of non-synonymous to synonymous changes of all isolates was almost balanced in the *aflT* gene segment. Values for several genes could not be calculated because the values for dS or Sd were zero.

Although only 9.5% of the coding sequence of the aflatoxin biosynthesis pathway was sequenced, several DNA polymorphisms which lead to predicted amino acid changes were detected that did not occur in aflatoxin-producing isolates (Table 4). Several of these cause changes in amino acid type (i.e., serine to proline or proline to threonine) and reactivity (i.e., arginine to cysteine).

Phylogenetic analysis

Phylogenetic analyses on the 13 concatenated gene segments (*norB-hypA*) were performed on those atoxigenic strains without large deletions in the cluster (Figure 2). Twelve of 14 atoxigenic isolates formed a highly supported clade separated from the aflatoxin producers from Nigeria and the United States. Nevertheless, two atoxigenic isolates, AV3108 and AV2216, were closely related to *A. flavus* aflatoxin producers from the United States. The aflatoxin-producing isolates from Nigeria, AV3228 and AV0106, which have low polymorphism frequencies in the analysed gene segments, belonged to well-supported clades shared by the North American aflatoxin producers AF70 (AV0106) and NRRL3357 (AV3228).

The phylogenetic analysis from genes outside the cluster (*taka amylase-pecA-glcA*) resulted in most parsimonious trees with only a single highly supported clade, that shared by two VCGs (AV0452 and AV0173) with the same deletion pattern in the aflatoxin gene cluster (Figure 3). The majority of Nigerian atoxigenic isolates shared a clade separated from both atoxigenic and aflatoxin-producing isolates from the United States.

Discussion

Aspergillus flavus isolates that do not produce aflatoxin have been found worldwide and are not rare (Cotty et al. 1994). Some of these isolates are used successfully to reduce aflatoxin contamination of susceptible crops in fields by competitively excluding native aflatoxin-producing *A. flavus* strains (Antilla and Cotty 2002; Dorner 2004). The safety and stability of atoxigenic strains must be demonstrated before atoxigenic strains can be registered as biopesticides (Bacchus 2007; Cotty et al. 2007). The atoxigenicity of *A. flavus* isolate AF36, a registered biopesticide, is the result of a single nucleotide polymorphism which inserts a stop codon near the beginning of the coding sequence (*pksA*) for polyketide synthase (Ehrlich and Cotty 2004). The AF36 *pksA* polymorphism was not found among the

Table 3. Frequencies of single nucleotide polymorphisms (SNP) in sequenced segments of the aflatoxin biosynthesis gene cluster compared with the toxin producer NRRL3357 for *Aspergillus flavus* isolates in the current study lacking a large deletion in the cluster. Aflatoxin producers are shown in bold. Nigerian VCGs are with prefix AV while AF36 is an atoxigenic strain of North American origin.

		Isolates																
Genes[a]		AV3193	AV3224	AV3108	AV3279	AV3304	AV3303	AV3150	AV3306	AF36	AV3058	AV2757	AV4216	AV3020	AV2216	AV16127	**AV3228**	**AV0106**
norB 120bp	Sd	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0	0.0	1.0	1.0	0.0	1.0	0.0	1.0	0.0	1.0
	Sn	1.0	1.0	2.0	1.0	1.0	1.0	2.0	1.0	2.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.0
	dN/dS	0.284	0.284	NA	0.284	0.284	0.284	0.574	0.284	NA	0.284	0.284	NA	0.284	NA	0.284	NA	0.574
	P (%)	1.7	1.7	1.7	1.7	1.7	1.7	2.5	1.7	1.7	1.7	1.7	0.8	1.7	0.8	1.7	0.8	2.5
	Sn/Sd	1.0	1.0	NA	1.0	1.0	1.0	2.0	1.0	NA	1.0	1.0	NA	1.0	NA	1.0	NA	2.0
aflT 612bp	Sd	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	3.0	2.0	3.0	2.0	0.0	2.0
	Sn	2.0	2.0	1.0	2.0	2.0	2.0	2.0	2.0	1.0	2.0	2.0	1.0	2.0	1.0	2.0	0.0	1.0
	dN/dS	0.346	0.346	0.173	0.346	0.346	0.346	0.346	0.346	0.173	0.346	0.346	0.115	0.346	0.115	0.346	NA	0.173
	P (%)	0.7	0.7	0.5	0.7	0.7	0.7	0.7	0.7	0.5	0.7	0.7	0.7	0.7	0.7	0.7	0.0	0.5
	Sn/Sd	1.0	1.0	0.5	1.0	1.0	1.0	1.0	1.0	0.5	1.0	1.0	0.3	1.0	0.3	1.0	NA	0.5
hexA 582bp	Sd	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	1.0	2.0	2.0	2.0	2.0	2.0	2.0	0.0	0.0
	Sn	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	dN/dS	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	P (%)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.0	0.0
	Sn/Sd	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	NA	NA
aflR 354bp	Sd	1.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.0	0.0
	Sn	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0
	dN/dS	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.339	NA	NA	NA
	P (%)	0.3	0.3	0.0	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.6	0.3	0.0	0.0
	Sn/Sd	0.0	0.0	NA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	NA	NA
aflJ 190bp	Sd	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	1.0	1.0	0.0	0.0
	Sn	0.0	1.0	0.0	1.0	1.0	1.0	1.0	1.0	0.0	1.0	1.0	0.0	1.0	0.0	0.0	0.0	0.0
	dN/dS	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	P (%)	0.5	0.5	0.0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.0	0.0
	Sn/Sd	0.0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
estA 426bp	Sd	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Sn	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	dN/dS	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	P (%)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Sn/Sd	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
ver1 483bp	Sd	19.0	19.0	19.0	18.0	19.0	19.0	18.0	19.0	20.0	19.0	19.0	19.0	19.0	20.0	18.0	0.0	1.0
	Sn	1.0	2.0	0.0	1.0	2.0	2.0	1.0	2.0	0.0	2.0	2.0	1.0	2.0	0.0	1.0	0.0	0.0
	dN/dS	0.015	0.030	NA	0.015	0.030	0.030	0.015	0.030	NA	0.030	0.030	0.015	0.030	NA	0.015	NA	NA
	P (%)	4.1	4.3	3.9	3.9	4.3	4.3	3.9	4.3	4.1	4.3	4.3	4.1	4.3	0.4	3.9	0.0	0.2
	Sn/Sd	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.0	0.1	NA	0.0
avnA 387bp	Sd	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.0	1.0	1.0	1.0	1.0	2.0	1.0	1.0	1.0

(*continued*)

Table 3. Continued.

Genes[a]		Isolates																
		AV3193	AV3224	AV3108	AV3279	AV3304	AV3303	AV3150	AV3306	AF36	AV3058	AV2757	AV4216	AV3020	AV2216	AV16127	**AV3228**	**AV0106**
	Sn	4.0	4.0	3.0	4.0	4.0	4.0	4.0	4.0	3.0	4.0	4.0	4.0	4.0	2.0	4.0	2.0	4.0
	dN/dS	1.290	1.290	0.963	1.290	1.290	1.290	1.290	1.290	0.477	1.290	1.290	1.290	1.290	0.319	1.290	0.645	1.290
	P (%)	1.3	1.3	1.0	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.0	1.3	0.8	1.3
	Sn/Sd	4.0	4.0	3.0	4.0	4.0	4.0	4.0	4.0	1.5	4.0	4.0	4.0	4.0	1.0	4.0	2.0	4.0
avfA 429bp	Sd	5.0	5.0	18.0	5.0	5.0	5.0	5.0	5.0	18.0	5.0	5.0	5.0	5.0	19.0	5.0	1.0	5.0
	Sn	3.0	3.0	12.0	3.0	3.0	3.0	3.0	3.0	12.0	3.0	3.0	3.0	3.0	03.0	3.0	1.0	3.0
	dN/dS	0.170	0.170	0.174	0.170	0.170	0.170	0.170	0.170	0.174	0.170	0.170	0.170	0.170	0.17	0.170	0.288	0.170
	P (%)	1.9	1.9	7.0	1.9	1.9	1.9	1.9	1.9	7.0	1.9	1.9	1.9	1.9	7.5	1.9	0.5	1.9
	Sn/Sd	0.6	0.6	0.7	0.6	0.6	0.6	0.6	0.6	0.7	0.6	0.6	0.6	0.6	0.7	0.6	1.0	0.6
omtB 249bp	Sd	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	16.0	17.0	16.0	0.0	16.0
	Sn	5.0	5.0	3.0	5.0	5.0	5.0	5.0	5.0	4.0	5.0	5.0	5.0	5.0	4.0	5.0	0.0	5.0
	dN/dS	0.094	0.094	0.056	0.094	0.094	0.094	0.094	0.094	0.075	0.094	0.094	0.094	0.094	0.09	0.094	NA	0.094
	P (%)	8.4	8.4	7.6	8.4	8.4	8.4	8.4	8.4	8.0	8.4	8.4	8.4	8.4	8.4	8.4	0.0	8.4
	Sn/Sd	0.3	0.3	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.3	NA	0.3
omtA 122bp	Sd	2.0	2.0	2.0	7.0	2.0	2.0	7.0	2.0	7.0	2.0	2.0	7.0	2.0	6.0	2.0	0.0	7.0
	Sn	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	dN/dS	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	P (%)	1.6	1.6	1.6	5.7	1.6	1.6	5.7	1.6	5.7	1.6	1.6	5.7	1.6	4.9	1.6	0.0	5.7
	Sn/Sd	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	Na	0.0
vbs 540bp	Sd	7.0	5.0	8.0	5.0	5.0	5.0	7.0	5.0	05.0	5.0	5.0	5.0	5.0	5.0	7.0	5.0	5.0
	Sn	3.0	2.0	02.0	1.0	2.0	2.0	2.0	2.0	02.0	2.0	2.0	1.0	2.0	1.0	3.0	1.0	1.0
	dN/dS	0.123	0.116	NA	0.058	0.116	0.116	0.082	0.116	NA	0.116	0.116	0.058	0.116	0.058	0.123	0.058	0.058
	P (%)	1.9	1.3	1.5	1.1	1.3	1.3	1.7	1.3	0.0	1.3	1.3	1.1	1.3	1.1	1.9	1.1	1.1
	Sn/Sd	0.4	0.4	0.40	0.2	0.4	0.4	0.3	0.4	NA	0.4	0.4	0.2	0.4	0.2	0.4	0.2	0.2
hyper 525bp	Sd	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Sn	2.0	2.0	0.0	0.0	2.0	2.0	2.0	2.0	1.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	0.0
	dN/dS	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	P (%)	0.4	0.4	0.2	0.0	0.4	0.4	0.4	0.4	0.2	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.0
	Sn/Sd	NA	NA	0.0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
glcA 462bp	Sd	1.0	1.0	4.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.0	1.0
	Sn	1.0	0.0	1.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	1.0	1.0	1.0
	dN/dS	0.027	0.0	0.066	0.027	0.0	0.0	0.027	0.0	0.0	0.027	0.0	0.0	0.027	0.0	0.027	0.089	0.027
	P (%)	0.4	0.2	1.1	0.4	0.2	0.2	0.4	0.2	0.2	0.4	0.2	0.2	0.4	0.2	0.4	0.9	0.4
	Sn/Sd	1.0	0.0	0.3	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	1.0	0.3	1.0
Total	Sd	58.0	55.0	73.0	59.0	55.0	55.0	61.0	55.0	69.0	55.0	55.0	61.0	55.0	59.0	57.0	10.0	39.0
	Sn	22.0	22.0	23.0	19.0	22.0	22.0	23.0	22.0	23.0	23.0	22.0	18.0	23.0	25.0	22.0	8.0	17.0

Note: [a]Only amplicons including coding regions with protein information are included; gene names and length of the portion of coding region used; Sd, synonymous substitutions; Sn, non-synonymous substitutions; non-synonymous (dN) and synonymous (dS) as calculated by applying the Jukes–Cantor correction for back-mutations; dN/dS, ratio of non-synonymous to synonymous substitutions; P, percentage polymorphic sites for each gene/sequence; Sn/Sd, simple ratio of non-synonymous to synonymous substitutions; n.a., not applicable because either Sn/Sd or dN/dS could not be calculated; isolates names are shown in bold (AV3228 and AV0106) indicate the aflatoxin producers.

Table 4. Non-synonymous nucleotide (Nt) substitutions in gene sequences of atoxigenic *Aspergillus flavus* isolates that were not found in the toxigenic isolates NRRL3357, AF13, AF70, AV3228, and AV0106, and the impact of substitutions on amino acid changes.

Genes	Nt change	Nt position respect to the translation with start site	Amino acid change	Amino acid position with respect to the translation start	Isolates
norB	G → A	1057	G → S	353	AV0452,AV0173
	T → C	1075	V → A	359	AF36,AV3108
aflT	G → T	710	M → I	237	AV3193, AV3303, AV3150, AV3303, AV3224, AV3279, AV3304, AV3058, AV16127, AV2757, AV3020
aflR	G → C	191	M → I	64	AV2216
aflJ	T → C	768	S → P	257	AV3303, AV3150, AV3306, AV3224, AV3279, AV3304, AV3058, AV2757, AV3020
ver1	G → A	198	D → N	67	AV3303, AV3306, AV3224, AV3304, AV3058, AV2757, AV3020
	G → A	213	E → K	72	AV3193, AV3303, AV3150, AV3306, AV3279, AV3304, AV3058, AV16127, AV2757, AV4216, AV3020
	C → T	390	R → C	131	AV0230
avnA	C → T	159	L → F	54	AV0230
	T → A	191	D → E	64	AF36
	C → A	294	P → T	99	AV3108
	A → C	415	D → G	139	AV0230
omtB	A → G	234	T → A	79	AF36
	A → G	297	T → A	100	AV0452, AV0173
vbs	G → A	1065	G → R	551	AV3193, AV16127
	G → A	1785	V → I	596	AV0230
	T → C	1791	S → P	598	AV3193, AV3150, AV16127, AV3279, AV2216
	G → T	1809	G → C	604	AV0452
hypA	G → C	975	P → A	326	AV0173, AV0216, AV0452
	A → G	1087	Y → C	363	AF36
	G → T	1118	K → N	373	AV3193, AV3224, AV3303, AV3306, AV3150, AV3058, AV3020, AV2757, AV2216, AV16127, AV0425, AV4216
	G → C	1149	E → Q	384	AV0173, AV0216, AV0452
	T → A	1220	N → K	407	AV0173, AV0216, AV0452
	T → C	1251	F → L	418	AV0173, AV0216, AV0452

atoxigenic isolates from Nigeria. Nevertheless, other molecular lesions within aflatoxin pathway genes were observed in atoxigenic *A. flavus* isolates from Nigeria.

In the present study, atoxigenic *A. flavus* isolates from Nigeria were found that did not generate PCR products for several regions within the aflatoxin gene cluster, indicating the presence of large deletions. Deletions within the aflatoxin gene cluster were found previously in isolates of both *A. flavus* and the closely related *A. oryzae* (Kusumoto et al. 2000; Chang et al. 2005). Kusumoto et al. (2000) classified strains of *A. oryzae* into three groups based on the deletion pattern. Strains belonging to groups 2 and 3 contained large deletions, while group 1 had a nearly intact aflatoxin gene cluster. Lee et al. (2006) confirmed that *A. oryzae* isolates belonging to group 2 had deletions on the left side of the aflatoxin gene cluster extending to a chromosome breakpoint in the gene *ver-1*. The deletion type of group 2 is apparently identical to that of isolate AV0230 of the current study. Interestingly, several deletion patterns observed by Chang et al. (2005) were also found within Nigerian atoxigenic *A. flavus* VCGs. Nevertheless, in the current study new types of deletions were also found. Isolates AV0205, AV0216, and AV0165 contained large lesions extending to *ordB* and *hypA*, at the distal end of the aflatoxin gene cluster. A lack of PCR products also suggested deletion of *cypX* in four *A. flavus* isolates; deletion of *cypX* is known to result in atoxigenicity (Wen et al. 2005). Lesions of the isolates AV0173 and AV0452

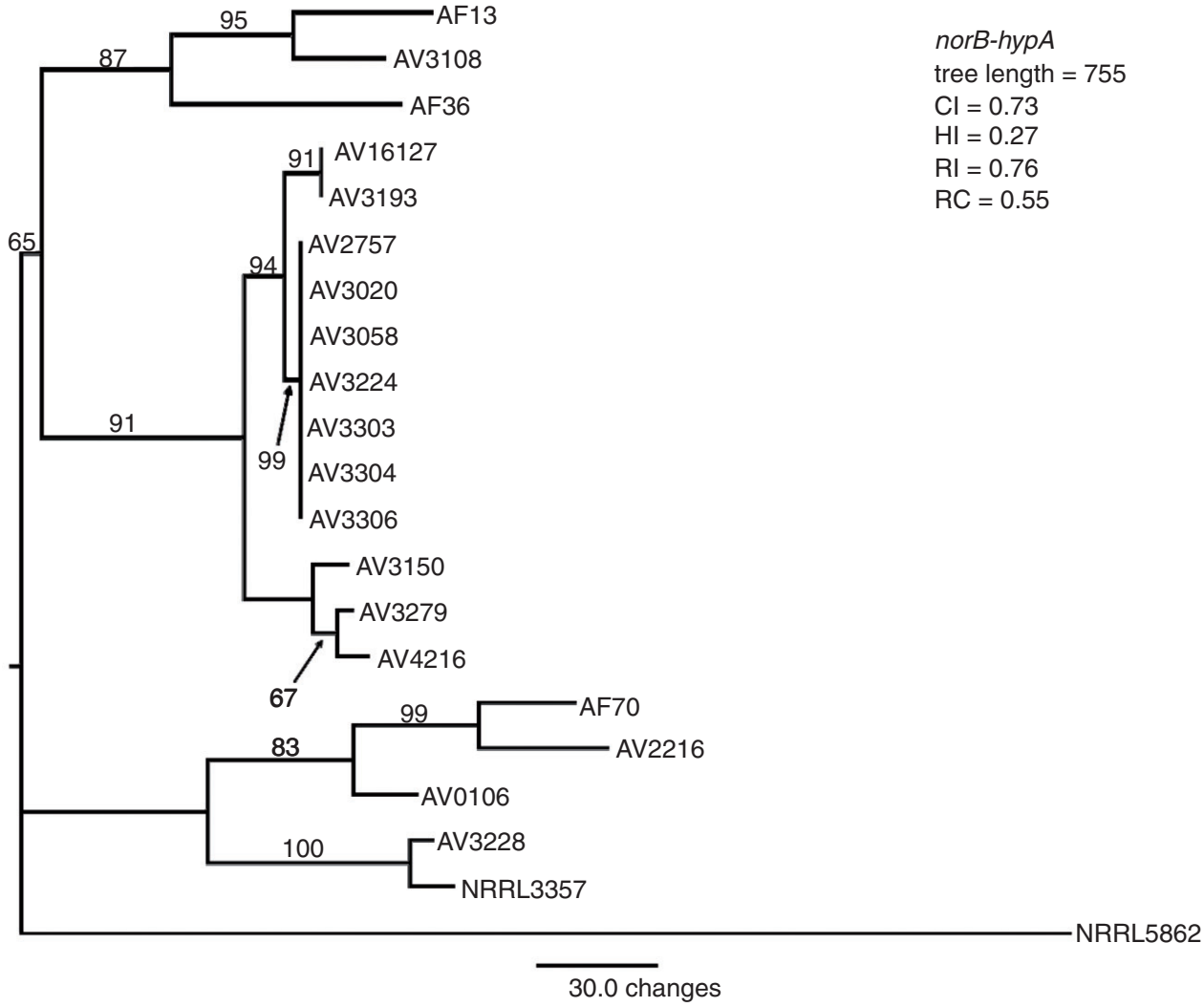

Figure 2. One of five most parsimonious trees for selected Nigerian *Aspergillus flavus* VCGs (with prefix AV and selected on the basis of lack of large deletions in aflatoxin gene cluster) based on the combined aflatoxin biosynthesis gene data set (*norB*, *aflT*, *hexA*, *aflR*, *aflJ*, *estA*, *ver1*, *avnA*, *avfA*, *omtB*, *omtA*, *vbs*, and *hypA*) with 7825 total characters, 238 parsimony informative. Tree was rooted with *A. parasiticus* (NRRL5862). Bootstrap values based on 1000 replicates are shown above the line. Aflatoxin producers are shown in bold. NRRL 3357, AF13, AF36, and AF70 are of North American origin. CI, consistency index; HI, homoplasy index; RI, retention index; RC, rescaled consistency index.

(Figure 1) were similar to deletion pattern C identified by Chang et al. (2005). The loss of the entire aflatoxin gene cluster of isolate AV0222 is comparable with the deletion pattern H of Chang et al. (2005).

Atoxigenic isolates from the district of Ogbomosho formed a unique group among the isolates from Nigeria in that they alone contained large deletions in the aflatoxin gene cluster. All the Ogbomosho strains belong to different VCGs and were found within a relatively small geographic area and may be derived from a common ancestor. This is supported by the phylogenetic analysis for the concatenated gene pieces *taka amylase–pecA-glcA*, which resolved these isolates into a single clade. Both adaptations and selective forces that may cause such isolates to become established in Ogbomosho are unknown.

Genes controlling secondary metabolites are generally organized in clusters, many of which are species specific (Nierman et al. 2005). As has been observed for mammals, nematodes and yeasts, repeats and subtelomeric sequences are associated with rearranged regions (Eichler and Sankoff 2003; Galagan et al. 2005). The aflatoxin gene cluster of the toxigenic *A. flavus* isolate NRRL3357 genome is biased toward the telomere (Chang et al. 2007). Similarly, the left side of the aflatoxin gene cluster of *A. oryzae* isolate RIB40 and that of RIB62 are close to the telomere (Lee et al. 2006) with the distance from the beginning of the aflatoxin gene cluster to the telomere being only 18 kb in *A. oryzae* RIB40. The side of the aflatoxin gene cluster toward the sugar utilization cluster is relatively conserved, whereas, at the opposite end, the regions

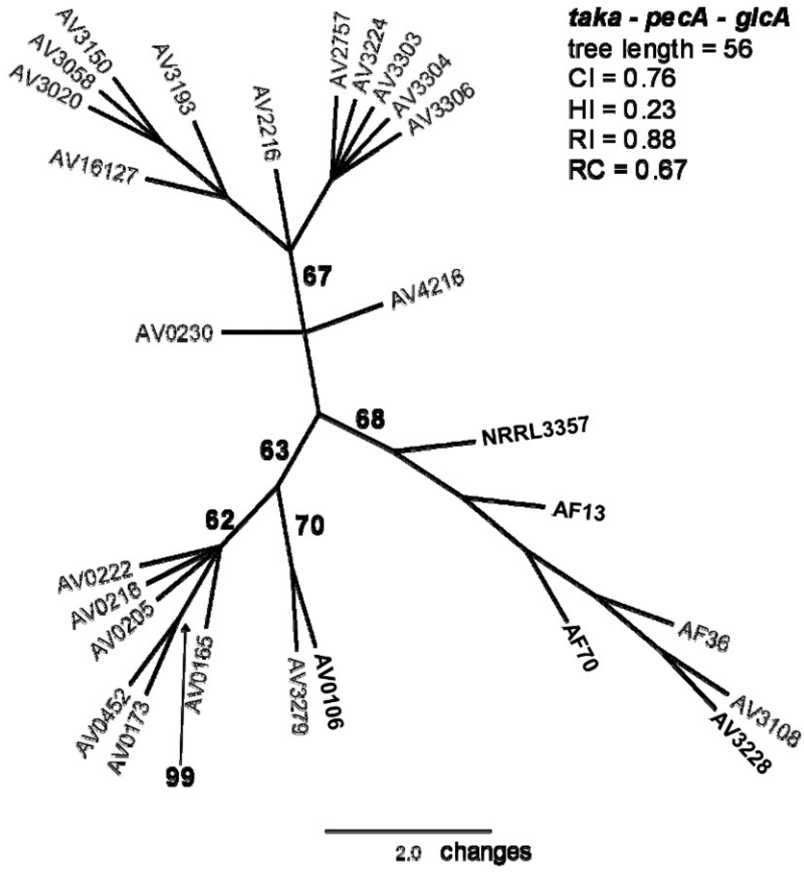

Figure 3. One of 86 most parsimonious trees of *Aspergillus flavus* isolates based on concatenated gene segments of taka-amylase, *pecA*, and *glcA*. Of 2035 total characters, 24 were parsimony informative. Tree is unrooted. Bootstrap values based on 1000 replicates are indicated. Nigerian VCGs are with prefix AV, while NRRL 3357, AF13, AF36, and AF70 are of North American origin. Aflatoxin producers are shown in bold. CI, consistency index; HI, homoplasy index; RI, retention index; RC, rescaled consistency index.

toward the telomere are highly variable (Ehrlich et al. 2005). Preliminary, genome analyses reveal large non-syntenous regions resulting from insertions or deletions in subtelomeric sequences, intra-molecular recombination, and variation in the number of gene duplications (Nierman et al. 2005). For example, a deletion in the *cypA* gene is the reason why *A. flavus* is incapable of producing G-aflatoxins (Ehrlich et al. 2007). The *cypA* gene is located toward the telomeric region at the beginning of the aflatoxin gene cluster. The location of the aflatoxin gene cluster in the telomeric region of *A. nidulans*, *A. oryzae* and *A. flavus* facilitates gene loss as well as recombination, DNA inversions, partial deletions, translocations and other genomic rearrangements (Kusumoto et al. 2000; Chang et al. 2005; Ehrlich et al. 2005; Wong and Kenneth 2005; Carbone et al. 2007). The large deletions found in the current and previous studies are consistent with these suggestions.

Eleven atoxigenic VCGs were tested previously to evaluate *A. flavus* strains as potential biocontrol agents for maize in Nigeria (Atehnkeng, Ojiambo, Ikotun et al. 2008). These isolates varied in efficacy reducing aflatoxin in maize from 70.1% to 99.9%. AV3279 was the most effective atoxigenic isolate (greater than 99.3%) followed by the isolates AV3303, AV0222, and AV4216 with an average aflatoxin reduction of greater than 92% (Atehnkeng, Ojiambo, Ikotun et al. 2008). Among these highly effective atoxigenic strains, several lacked deletions in the portions of the aflatoxin biosynthesis gene cluster examined, as does the biocontrol strain AF36 (Ehrlich and Cotty 2004) and one (AV0222) had a deletion covering the entire gene cluster. These results suggest that remnants of the aflatoxin gene cluster are not necessary for isolates effectively to exclude aflatoxin producers during host infection.

Differences between atoxigenic and aflatoxin-producing strains were observed based on the combined aflatoxin biosynthesis gene data set. Phylogenetic analysis of gene segments both within aflatoxin gene clusters and outside indicate most atoxigenic isolates are more closely related to each other than to aflatoxin producers. Atoxigenic isolates exhibited significantly more polymorphisms, in relation to the NRRL3357 sequence, in coding gene

segments than aflatoxin producers. *Ver1* averaged 4.0% polymorphism for atoxigenic strains, but only 0.1% for toxigenic isolates. Most nucleotide changes in *avnA* and *hypA* resulted in non-synonymous amino acid substitutions suggesting a response to selective pressure. One amino acid change occurred in the *aflJ* gene in nine atoxigenic isolates, but not in high aflatoxin producers. This gene is involved in the regulation of aflatoxin biosynthesis (Meyers et al. 1998; Ehrlich et al. 1999; Chang et al. 2000). Similar to the current study, the majority of atoxigenic *A. flavus* and *A. oryzae* had aflatoxin gene clusters without significant deletions (Kusumoto et al. 2000; Chang et al. 2005; Lee et al. 2006). In these cases, atoxigenicity probably results from SNPs (Ehrlich and Cotty 2004) or small deletions in aflatoxin-cluster genes (Calvo et al. 2004). Molecular mechanisms responsible for atoxigenicity in *A. flavus* appear to be diverse, and the mechanism of atoxigenicity in most isolates lacking large deletions in the gene cluster is still not firmly established. Differences in frequencies of non-synonymous substitutions among isolates may reflect the initial stages of differential adaptation.

The production of aflatoxins by the species *Aspergillus* involves approximately 25 genes within a complex biosynthetic pathway which requires a considerable expenditure of energy (Yu, Chang et al. 2004). Ehrlich et al. (2005) estimated that the aflatoxin gene cluster of *A. flavus* was maintained for at least 25 million years and that the loss of genes for production of G-aflatoxins must have occurred less than 17 million years ago. Conservation of aflatoxin biosynthesis among certain *A. flavus* strains suggests important adaptive values for aflatoxins in character-shaping niches (Ehrlich et al. 2005). Around half of the *A. flavus* L-strain isolates do not produce aflatoxin in Nigeria (Atehnkeng, Ojiambo, Donner et al. 2008; Donner et al. 2009). In contrast, *A. parasiticus*, *A. nomius*, and the unnamed taxon S_{BG} of West Africa uniformly produce large quantities of aflatoxins (Kurtzman et al. 1987; Ehrlich et al. 2003; Donner et al. 2009). The precise function of aflatoxins in fungal life cycles is a subject of debate, but for these three species aflatoxin production clearly has adaptive value. Within the L strain, this value is apparently less. Ecological roles for aflatoxins are not well characterized, but aflatoxins may protect against competing microbes and/or insects (Matsumura and Knight 1967; Drummond and Pinnock 1990; Dowd 1992). Aflatoxins have low phytotoxicity (McLean et al. 1995; Hasan 2001) and are not suspected to be involved in plant virulence (Cotty 1989). Aflatoxin production is not essential for fungal growth and is not required for successful competition with other aflatoxin-producing strains during crop infection (Horn et al. 2000; Bhatnagar et al. 2003). In nature, atoxigenic and toxigenic *A. flavus* successfully coexist, and atoxigenic *A. flavus* strains are equally capable of infecting susceptible crops (Cotty 1989, 1997). Ehrlich et al. (2005) suggested that over the last several million years the average adaptive value of aflatoxins may have decreased due to movement into new niches or loss of formative niches in the current environment. Aflatoxin production does not appear to be advantageous to the L strain of *A. flavus* in crop-associated niches; thus, loss of aflatoxin-producing ability may make the fungus more energy efficient, and more competitive in crops.

In nature, VCGs largely behave as clonal lineages (Ehrlich et al. 2007) and, in the present study, some isolates belonging to different VCGs had identical deletion patterns and were closely related. Thus, in some cases, atoxigenicity may predate divergence of new VCGs and, as such, atoxigenicity may be a relatively stable trait that does not impair long-term competitiveness and survival in certain niches.

Most of the atoxigenic VCGs examined yielded amplicons for each of the aflatoxin biosynthesis genes examined. Further studies will be needed to determine the precise mutations causing atoxigenicity. The current study demonstrates that for several West African atoxigenic VCGs being developed as biocontrol agents, atoxigenicity is derived from deletions in portions of the aflatoxin biosynthesis gene cluster and that these deletions have long-term stability.

Acknowledgements

This study was funded by the Bundesministerium für wirtschaftliche Zusammenarbeit und Entwicklung (BMZ; German Federal Ministry for Economic Cooperation and Development) (Project Number 2002.7860.6 – 001.00). The authors thank Peter J. Cotty's laboratory staff members for excellent technical assistance.

References

Adebajo LO, Idowu AA, Adesanya OO. 1994. Mycoflora, and mycotoxins production in Nigerian corn and corn-based snacks. Mycopathologia. 126(3):183–192.

Antilla L, Cotty PJ. 2002. The ARS-ACRPC partnership to control aflatoxin in Arizona cotton: current status. Mycopathologia. 155:64.

Atehnkeng J, Ojiambo PS, Donner M, Ikotun T, Sikora RA, Bandyopadhyay R. 2008. Distribution and toxigenicity of *Aspergillus* species isolated from maize kernels from three agro-ecological zones in Nigeria. Int J Food Microbiol. 122:74–84.

Atehnkeng J, Ojiambo PS, Ikotun T, Sikora RA, Cotty PJ, Bandyopadhyay R. 2008. Evaluation of atoxigenic strains of *Aspergillus flavus* as potential biocontrol agents for aflatoxin in maize. Food Addit Contam. 25:1264–1271.

Bacchus S. 2007. Aflatoxin control in cotton and groundnuts. In: Vincent C, Goettel MS, Lazarovits G, editors.

Biological control: A global perspective. Wallingford (UK): CABI. p. 254–262.
Bankole SA, Adebanjo A. 2003. Mycotoxins in food in West Africa: current situation and possibilities of controlling it. Afr J Biotechnol. 2:254–263.
Bayman P, Cotty PJ. 1991. Vegetative compatibility and genetic diversity in the *Aspergillus flavus* population of a single field. Can J Bot. 69:1707–1711.
Bayman P, Cotty PJ. 1993. Genetic diversity in *Aspergillus flavus*: association with aflatoxin production and morphology. Can J Bot. 71:23–31.
Bhatnagar D, Ehrlich KC, Cleveland TE. 2003. Molecular genetic analysis and regulation of aflatoxin biosynthesis. Appl Microbiol Biotechnol. 61:83–93.
Boyd ML, Cotty PJ. 2001. *Aspergillus flavus* and aflatoxin contamination of leguminous trees of the Sonoran desert in Arizona. Phytopathology. 91:913–919.
Calvo AM, Bok J, Brooks W, Keller NP. 2004. veA is required for toxin and sclerotial production in *Aspergillus parasiticus*. Appl Environ Microbiol. 70:4733–4739.
Carbone I, Jakobek JL, Ramirez-Prado JH, Horn BW. 2007. Recombination, balancing selection and adaptive evolution in the aflatoxin gene cluster of *Aspergillus parasiticus*. Mol Ecol. 6:4401–4417.
Chang P-K, Bhatnagar D, Cleveland TE, Bennett JW. 1995. Sequence variability in homologs of the aflatoxin pathway gene aflR distinguishes species in *Aspergillus* section Flavi. Appl Environ Microbiol. 61:40–43.
Chang P-K, Ehrlich KC, Hua SS. 2006. Cladal relatedness among *Aspergillus oryzae* isolates and *Aspergillus flavus* S and L morphotype isolates. Int J Food Microbiol. 108:172–177.
Chang P-K, Horn BW, Dorner JW. 2005. Sequence breakpoints in the aflatoxin biosynthesis gene cluster and flanking regions in nonaflatoxigenic *Aspergillus flavus* isolates. Fungal Genet Biol. 42:914–923.
Chang P-K, Matsushima K, Takahashi T, Yu J, Abe K, Bhatnagar D, Yuan GF, Koyama Y, Cleveland TE. 2007. Understanding nonaflatoxigenicity of *Aspergillus sojae*: a windfall of aflatoxin biosynthesis research. Appl Microbiol Biotechnol. 76:977–984.
Chang P-K, Yu J, Bhatnagar D, Cleveland TE. 2000. Characterization of the *Aspergillus parasiticus* major nitrogen regulatory gene, areA. BBA-Gene Struct Expr. 1491:263–266.
Cotty PJ. 1989. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology. 79:808–814.
Cotty PJ. 1994. Comparison of four media for the isolation of *Aspergillus flavus* group fungi. Mycopathologia. 125:157–162.
Cotty PJ. 1997. Aflatoxin-producing potential of communities of *Aspergillus* section Flavi from cotton producing areas in the United States. Mycol Res. 101:698–704.
Cotty PJ. 2006. Biocompetitive exclusion of toxigenic fungi. In: Barug D, Bhatnagar D, van Egmond HP, van der Kamp JW, van Osenbruggen WA, Visconti A, editors. The mycotoxin factbook. Wageningen (the Netherlands): Wageningen Academic Publishers. p. 179–197.
Cotty PJ, Antilla L, Wakelyn PJ. 2007. Competitive exclusion of aflatoxin producers: Farmer-driven research and development. In: Vincent C, Goettel MS, Lazarovits G, editors. Biological control. A global perspective: Wallingford (UK): CABI. p. 241–254.
Cotty PJ, Bayman DS, Egel DS, Elias KS. 1994. Agriculture, aflatoxins and *Aspergillus*. In: Powell K, editor. The genus *Aspergillus*. New York (NY): Plenum. p. 1–27.
Cotty PJ, Cardwell KF. 1999. Divergence of West African and North American communities of *Aspergillus* section Flavi. Appl Environ Microbiol. 65:2264–2266.
Cotty PJ, Howell DR, Bock C, Tellez A. 1997. Aflatoxin contamination of commercially grown transgenic BT cottonseed. In: Proceedings of the Beltwide Cotton Production Research Conference; National Cotton Council of America; Memphis, TN, USA. p. 108–110.
Cotty PJ, Probst C, Jaime-Garcia R. 2008. Etiology and management of aflatoxin contamination. In: Leslie JF, Bandyopadhyay R, Visconti A, editors. Mycotoxins: Detection methods, management, public health and agricultural trade. Wallingford (UK): CABI. p. 287–299.
Das MK, Ehrlich KC, Cotty PJ. 2008. Use of pyrosequencing to quantify incidence of a specific *Aspergillus flavus* strain within complex fungal communities associated with commercial cotton crops. Phytopathology. 98:282–288.
Diener UL, Cole RJ, Sanders TH, Payne GA, Lee LS, Klich MA. 1987. Epidemiology of aflatoxin formation by *Aspergillus flavus*. Annu Rev Phytopathol. 25:249–270.
Donner M, Atehnkeng J, Sikora RA, Bandyopadhyay R, Cotty PJ. 2009. Distribution of *Aspergillus* section Flavi in soils of maize fields in three agroecological zones of Nigeria. Soil Biol Biochem. 41:37–44.
Dorner JW. 2004. Biological control of aflatoxin contamination of crops. J Toxicol-Toxin Rev. 23:425–450.
Dowd PF. 1992. Insect interaction with mycotoxin-producing fungi and their hosts. In: Bhatnagar D, Lillehoj EB, Arora DK, editors. Mycotoxins in ecological systems. New York (NY): Marcel Dekker. p. 137–155.
Drummond J, Pinnock DE. 1990. Aflatoxin production by entomopathogenic isolates of *Aspergillus parasiticus* and *Aspergillus flavus*. J Invertebr Pathol. 55:332–336.
Ehrlich KC, Cary JW, Montalbano BG. 1999. Characterization of the promoter for the gene encoding the aflatoxin biosynthetic pathway regulatory protein AFLR. BBA-Gene Struct and Expr. 1444:412–417.
Ehrlich KC, Chang P-K, Yu J, Cotty PJ. 2004. Aflatoxin biosynthesis cluster gene cypA is required for G aflatoxin formation. Appl Environ Microbiol. 70(11):6518–6524.
Ehrlich KC, Cotty PJ. 2004. An isolate of *Aspergillus flavus* used to reduce aflatoxin contamination in cottonseed has a defective polyketide synthase gene. Appl Microbiol Biotechnol. 65:473–478.
Ehrlich KC, Montalbano BG, Cotty PJ. 2003. Sequence comparison of aflR from different *Aspergillus* species provides evidence for variability in regulation of aflatoxin production. Fungal Genet Biol. 38:63–74.
Ehrlich KC, Montalbano BG, Cotty PJ. 2007. Analysis of single nucleotide polymorphisms in three genes shows evidence for genetic isolation of certain *Aspergillus flavus* vegetative compatibility groups. FEMS Microbiol Lett. 268:231–236.
Ehrlich KC, Yu J, Cotty PJ. 2005. Aflatoxin biosynthesis gene clusters and flanking regions. J Appl Microbiol. 99:518–527.

Eichler EE, Sankoff D. 2003. Structural dynamics of eukaryotic chromosome evolution. Science. 301:793–797.

Galagan JE, Calvo SE, Cuomo C, Ma LJ, Wortman JR, Batzoglou S, Lee SI, Bastuerkem M, Spevak CC, Clutterbuck J. 2005. Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. Nature. 438: 1105–1115.

Hasan HA. 2001. Phytotoxicity of pathogenic fungi and their mycotoxins to cereal seedling viability. Acta Microbiol Immunol Hung. 48:27–37.

Hell K, Fandohan P, Bandyopadhyay R, Kiewnick S, Sikora R, Cotty PJ. 2008. Pre- and post-harvest management of aflatoxin in maize: An African perspective. In: Leslie JF, Bandyopadhyay R, Visconti A, editors. Mycotoxins: Detection methods, management, public health and agricultrural trade. Wallingford (UK): CABI. p. 219–229.

Horn BW, Dorner JW. 1999. Regional differences in production of aflatoxin B_1 and cyclopiazonic acid by soil isolates of *Aspergillus flavus* along a transect within the United States. Appl Environ Microbiol. 65:1444–1449.

Horn BW, Greene RL, Dorner JW. 2000. Inhibition of aflatoxin B_1 production by *Aspergillus parasiticus* using nonaflatoxigenic strains: role of vegetative compatibility. Biol Control. 17:147–154.

Joffe AZ, Lisker N. 1969. Effects of light, temperature, and pH value on aflatoxin production in vitro. Appl Microbiol. 18:517–518.

Klich MA. 2007. Environmental and developmental factors influencing aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus*. Mycoscience. 48:71–80.

Klich MA, Yu J, Chang P-K, Mullaney EJ, Bhatnagar D, Cleveland TE. 1995. Hybridization of genes involved in aflatoxin biosynthesis to DNA of aflatoxigenic and non-aflatoxigenic *Aspergilli*. Appl Microbiol Biotechnol. 44:439–443.

Korber B. 2000. HIV signature and sequence variation analysis. In: Rodrigo AG, Learn GH, editors. Computational analysis of HIV molecular sequences. Dordrecht (the Netherlands): Kluwer. p. 55–72.

Kurtzman CP, Horn BW, Hesseltine CW. 1987. *Aspergillus nomius*, a new aflatoxin-producing species related to *Aspergillus flavus* and *Aspergillus tamarii*. Antonie Van Leeuwenhoek. 53:147–158.

Kurtzman CP, Smiley MJ, Robnett CJ, Wicklow DT. 1986. DNA relatedness among wild and domesticated species in the *Aspergillus flavus* group. Mycologia. 78:955–959.

Kusumoto K, Nogata Y, Ohta H. 2000. Directed deletions in the aflatoxin biosynthesis gene homolog cluster of *Aspergillus oryzae*. Curr Genet. 37:104–111.

Lee YH, Tominaga M, Hayashi R, Sakamoto K, Yamada O, Akita O. 2006. *Aspergillus oryzae* strains with a large deletion of the aflatoxin biosynthetic homologous gene cluster differentiated by chromosomal breakage. Appl Microbiol Biotechnol. 72:339–345.

Lewis L, Onsongo M, Njapau H, Schurz-Rogers H, Luber G, Kieszak S, Nyamongo J, Backer L, Dahiye AM, Misore A. 2005. Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in Eastern and Central Kenya. Environ Health Persp. 113:1763–1767.

Lisker N, Michaeli R, Frank ZR. 1993. Mycotoxigenic potential of *Aspergillus flavus* strains isolated from groundnuts growing in Israel. Mycopathologia. 122:177–183.

Machida M, Asai K, Sano M, Tanaka T, Kumagai T, Terai G, Kusumoto KI, Arima T, Akita O, Kashiwagi Y. 2005. Genome sequencing and analysis of *Aspergillus oryzae*. Nature. 438:1157–1161.

Matsumura F, Knight SG. 1967. Toxicity and chemosterilizing activity of aflatoxin against insects. J Econ Entomol. 60:871–872.

McLean M, Watt MP, Berjak P, Dutton MF. 1995. Aflatoxin B_1: its effects on an in vitro plant system. Food Addit Contam. 12:435–443.

Meyers DM, Obrian G, Du WL, Bhatnagar D, Payne GA. 1998. Characterization of aflJ, a gene required for conversion of pathway intermediates to aflatoxin. Appl Environ Microbiol. 64:3713–3717.

Nei M, Gojobori T. 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol Biol Evol. 3:418–426.

Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS, Arrogo J, Berriman M, Abe K, Archer DB, Bermejo C. 2005. Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. Nature. 438:1151–1156.

Ota T, Nei M. 1994. Variance and covariances of the numbers of synonymous and nonsynonymous substitutions per site. Mol Biol Evol. 11:613–619.

Papa KE. 1986. Heterokaryon incompatibility in *Aspergillus flavus*. Mycologia. 78:98–101.

Payne GP, Brown MP. 1998. Genetics and physiology of aflatoxin biosynthesis. Annu Rev Phytopathol. 36:329–362.

Prieto R, Yousibova GL, Woloshuk CP. 1996. Identification of aflatoxin biosynthesis genes by genetic complementation in an *Aspergillus flavus* mutant lacking the aflatoxin gene cluster. Appl Environ Microbiol. 62:3567–3571.

Razzaghi-Abyaneh M, Shams-Ghahfarokhi M, Allameh A, Kazeroon-Shiri A, Ranjbar-Bahadori S, Mizahoseini H, Rezaee M-B. 2006. A survey on distribution of *Aspergillus* Section Flavi in corn field soils in Iran: population patterns based on aflatoxins, cyclopiazonic acid and sclerotia production. Mycopathologia. 161:183–192.

Schroeder HW, Boller RA. 1973. Aflatoxin production of species and strains of the *Aspergillus flavus* group isolated from field crops. Appl Microbiol. 25:885–889.

Swofford DL. 2002. PAUP*: Phylogenetic analysis using parsimony (and other methods) 4.0 Beta. Sunderland (MA): Sinauer Associates.

Tominaga M, Lee YH, Hayashi R, Suzuki Y, Yamada O, Sakamoto K, Gotoh K, Akita O. 2006. Molecular analysis of an inactive aflatoxin biosynthesis gene cluster in *Aspergillus oryzae* RIB Strains. Appl Environ Microbiol. 72:484–490.

Udoh JM, Cardwell KF, Ikotun T. 2000. Storage structures and aflatoxin content of maize in five agroecological zones of Nigeria. J Stored Prod Res. 36:187–201.

Vaamonde G, Patriarca A, Pinto VF, Comerio R, Degrossi C. 2003. Variability of aflatoxin and cyclopiazonic acid production by *Aspergillus* section flavi from different substrates in Argentina. Int J Food Microbiol. 88:79–84.

van Egmond HP, Schothorst RC, Jonker MA. 2007. Regulations relating to mycotoxins in food: perspectives in a global and European context. Anal Bioanal Chem. 389:147–157.

Wei DL, Jong SC. 1986. Production of aflatoxins by strains of the *Aspergillus flavus* group maintained in ATCC. Mycopathologia. 93:19–24.

Wen Y, Hatabayashi H, Arai H, Kitamoto HK, Yabe K. 2005. Function of the cypX and moxY genes in aflatoxin biosynthesis in *Aspergillus parasiticus*. Appl Environ Microbiol. 71:3192–3198.

Wicklow DT. 1984. Conidium germination rate in wild and domesticated yellow-green *Aspergilli*. Appl Environ Microbiol. 47:299–300.

Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D. 2004. Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. Am J Clin Nutr. 80:1106–1122.

Wong S, Kenneth H. 2005. Birth of a metabolic gene cluster in yeast by adaptive gene relocation. Nat Genet. 37:777–782.

Yu J, Bhatnagar D, Cleveland TE. 2004. Completed sequence of aflatoxin pathway gene cluster in *Aspergillus parasiticus*. FEBS Lett. 564:126–130.

Yu J, Chang P-K, Cary JW, Wright M, Bhatnagar D, Payne GA, Linz JE. 1995. Comparative mapping of aflatoxin pathway gene clusters in *Aspergillus parasiticus* and *Aspergillus flavus*. Appl Environ Microbiol. 61:2365–2371.

Yu J, Chang P-K, Ehrlich KC, Cary JW, Bhatnagar D, Cleveland TE, Payne GA, Linz JE, Woloshuk CP, Bennett JW. 2004. Clustered pathway genes in aflatoxin biosynthesis. Appl Environ Microbiol. 70:1253–1262.

Yu J, Woloshuk CP, Bhatnagar D, Cleveland TE. 2000. Cloning and characterization of avfA and omtB genes involved in aflatoxin biosynthesis in three *Aspergillus* species. Gene. 248:157–167.