GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Molecular characterization of aflatoxigenic and non-aflatoxigenic *Aspergillus flavus* isolates collected from corn grains

M.A. Mahmoud[1], H.M. Ali[2], A.R.M. El-Aziz[2], M.R. Al-Othman[2] and A.S. Al-Wadai[2]

[1]Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt
[2]Botany and Microbiology Department, College of Science, King Saud University, Riyadh, Kingdom of Saudi Arabia

Corresponding author: A.R.M. El-Aziz
E-mail: aabdelaziz@ksu.edu.sa

Genet. Mol. Res. 13 (4): 9352-9370 (2014)
Received September 24, 2013
Accepted May 8, 2014
Published November 11, 2014
DOI http://dx.doi.org/10.4238/2014.November.11.2

ABSTRACT. Twelve species from six fungal genera were found to be associated with corn (*Zea mays* L.) grain samples collected from three main regions of Saudi Arabia. The average frequencies of the most common genera were *Aspergillus* (11.4%), *Fusarium* (9.5%), *Penicillium* (5.1%), and *Alternaria* (5.8%). Fifteen isolates of *Aspergillus flavus* were screened by HPLC for their ability to produce aflatoxins (AF). The percentage of aflatoxigenic *A. flavus* isolates was 53%. Eight isolates produced AF, at concentrations ranging 0.7-2.9 ppb. Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) molecular markers were used to genetically characterize isolates of *A. flavus* and to discriminate between the aflatoxigenic and non-aflatoxigenic isolates. RAPD and ISSR analysis revealed a high level of genetic diversity in the *A. flavus* population, which was useful for genetic characterization. The clustering in the RAPD and ISSR dendrograms obtained was unrelated to geographic

origin. The RAPD and ISSR markers could not discriminate between aflatoxigenic and non-aflatoxigenic isolates, but the ISSR primers were somewhat better.

Key words: *Aspergillus flavus*; Random amplified polymorphic DNA; Corn; Inter-simple sequence repeats

INTRODUCTION

Corn (*Zea mays* L.) is one of the main cereals that is used as a source of food, forage, and processed products for industry. World corn production is approximately 790 million tons, and as a staple food, it provides more than one-third of the calories and protein in some countries (Chulze, 2010). Mycotoxins contaminate approximately 25-50% of the total crops harvested, and because molds thrive in tropical environments, they damage approximately 80% of the crops in tropical regions. Mycotoxins, such as aflatoxins (AF), ochratoxins, zearalenone, trichothecenes, and fumonisins, are the major mycotoxins that influence public health and agricultural crops.

In Saudi Arabia, there is a lack of accurate data on *Aspergillus flavus* contamination of corn. Few studies concerning *A. flavus* and AF production have been conducted (Abd El-Aziz Abeer et al., 2012; Yassin et al., 2012; Mahmoud et al., 2013) and no major survey has addressed this topic. AF are produced by a large number of *Aspergillus* species. The main producers of AF are *A. flavus*, *A. parasiticus*, *A. nomius*, *A. pseudotamarii*, *A. parvisclerotigenus* and *A. bombycis* (Frisvad et al., 2005). AF occur in several different chemical forms, designated AF B1, B2, G1, and G2 (Murphy et al., 2006). AF production depends on various factors such as the presence of toxigenic fungi, the chemical composition of the substrate, moisture content, relative humidity, temperature, and the time course of fungal growth. However, high incidences of AF contamination in cereal seeds and animal feed have been reported worldwide (Placinta et al., 1999). When AF are present in foods at sufficiently high levels, these fungal metabolites can have toxic effects that range from acute (liver or kidney deterioration) to chronic (e.g., liver cancer) toxicity and can be mutagenic and teratogenic. A promising method for controlling these toxigenic fungi is to use competitive biological control through colonizing soils with nontoxigenic *A. flavus* isolates, which can suppress their toxigenic counterparts (Yin et al., 2008). In experiments involving plants artificially infected with nontoxigenic fungal strains, a reduction in AF contamination of up to 90% was observed (Tran-Dinh et al., 1999). However, before biological control strategies can be implemented, the genetic diversity within this fungal group and the critical factors leading to the retention or loss of characteristics such as toxigenic capacity and virulence to agricultural crops must be investigated.

The genetic techniques for performing such analyses include the examination of random amplified polymorphic DNA (RAPD) markers and inter-simple sequence repeats (ISSR). The RAPD technique has been used to detect genetic variability between isolates of *A. flavus* and related species (Batista et al., 2008; Gehlot et al., 2011; Irshad and Nawab, 2012) and to discriminate between aflatoxigenic and non-aflatoxigenic isolates of *A. flavus* (Lourenço et al., 2007; Gashgari et al., 2010; Sepahvand et al., 2011). The ISSR technique has been employed to investigate the diversity and population structure of *A. flavus* (Tran-Dinh and Carter, 2000;

Batista et al., 2008; Tran-Dinh et al., 2009; Hadrich et al., 2010; Wang et al., 2012) and to determine the similarities and dissimilarities between aflatoxigenic and non-aflatoxigenic isolates of this species (Tran-Dinh et al., 2009; Hatti et al., 2010).

The aims of the present study were therefore 1) to survey the presence of *A. flavus* in corn in three main regions in Saudi Arabia; 2) to test methods for the detection and determination of AF involving HPLC; and 3) to characterize and discriminate aflatoxigenic and non-aflatoxigenic isolates genetically using RAPD and ISSR markers.

MATERIAL AND METHODS

Collection of samples

Fifteen samples (250 g each) of corn grain were collected from different markets located in Riyadh, Dammam, and Abha regions (Figure 1) in the Kingdom of Saudi Arabia during March 2011. The samples were stored at 2°C until use (Czerwiecki et al., 2002).

Figure 1. Map of Saudi Arabia indicating the regions which corn grain samples were collected during March 2012.

Isolation, purification, and identification of pathogens

Samples were surface-sterilized with 5% sodium hypochlorite for 1 min, before they were rinsed three times with sterile distilled water. Five grains were placed randomly

on potato dextrose agar (PDA) on three 9-cm Petri dishes. The Petri dishes were incubated at 25°C and observed daily for emergence of colonies for 5 days, after which the colonies were counted. Isolates were purified by single spore method and then transferred to PDA slants. All the isolates of fungal species were identified up to the species level using keys and manuals (Raper and Fennell, 1965; Domsch et al., 1993). The isolation frequencies of fungal species were calculated according to the method of González et al. (1995). Identification of fungal isolates was carried out on the basis of morphological and microscopic characteristics at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt.

Aflatoxin production in different culture media

For the determination of AF production based on fluorescence in culture media, all of the *A. flavus* isolates obtained were cultivated on PDA at 25°C for 7 days. A mycelium plug from each strain was placed on the center of a Petri dish containing test medium [Czapek agar (CZ) and yeast extract sucrose (YES)] (Davis et al., 1987). The plates were then incubated at 25°C for 4 days in the dark, and the presence or absence of fluorescence in the agar surrounding the growing *Aspergillus* colonies was determined by exposing the Petri dishes to UV light (365 nm), which was expressed as positive or negative.

Detection of aflatoxins based on fluorescence

The culture media used in these assays were PDA with sodium chloride, CZ and YES. Detection was performed at 25°C after 4 days in darkness. The presence or absence of fluorescence in the agar surrounding the colonies was determined under UV irradiation (365 nm) and was expressed as positive or negative according to Franco et al. (1998).

Determination of the toxigenic potential of *A. flavus* isolates

Isolates were grown in sterilized SMKY liquid medium (20 g sucrose, 0.5 g magnesium sulfate, 3 g potassium nitrate and 7 g yeast extract in 1000 mL distilled water) (Davis et al., 1987). Flasks were inoculated with 6-mm diameter discs of *A. flavus* isolates at 25 ± 2°C for 7 days (Paranagama et al., 2003). The experiments were performed in triplicate. After incubation, the contents of each flask were filtered through Whatman No. 1 filter paper. For AF extraction, the filtrates from each flask were treated three times with 50 mL chloroform using a separatory funnel. The chloroform extracts were then separately dehydrated with anhydrous sodium sulfate and evaporated to dryness in a water bath at 50°C under vacuum. The residues were dissolved in 10 mL methanol and stored in dark vials, and the extracts were passed through a 0.45-µm micro-filter. Analysis of the compounds present in the specimens was performed using an HPLC apparatus (PerkinElmer series 200 UV/VIS) with a C18 column, 300 mm x 3.9 mm ID, 4-µm particle size. The HPLC instrument was equipped with a UV detector, and fluorescence was measured using 365 nm excitation and 430 nm emission wavelengths. The mobile phase consisted of methanol: acetic acid: water (20:20:60 v/v/v). The total run time for the separation was approximately 25 min at a flow rate of 1 mL/min (Christian, 1990).

Extraction of DNA from *A. flavus* isolates

A. flavus isolates were cultured in double layer media, consisting of a solid and a liquid layer, on 50-mm Petri dishes. The solid base medium was PDA, and the top liquid medium was peptone yeast glucose (PYG, 1200 µL). The fungi were incubated at 25°C for 2 days, after which the fungal mycelia (50 mg) were scraped using slide covers and transferred to sterile Eppendorf tubes (1.5 mL) for DNA isolation. DNA was extracted from 50 mg fresh mats according to Amer et al. (2011).

RAPD PCR

To identify the best primers for establishing the RAPD profile, we tested 6 primers from standard RAPD primer kits (Amersham Pharmacia) suppliers need locations using total DNA from *A. flavus* isolates as a template. The three primers with the highest reproducibility and clearest banding profiles, RAPD primers 1, 3, and 5 were selected. PCR amplifications were run using mixtures with a final volume of 25 µL containing reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl) (Biolabs), 3.4 mM $MgCl_2$, 0.25 mM dNTPs, 0.4 mM of each primer, 2 U *Taq* DNA polymerase (BioLabs), and 25 ng genomic DNA (Williams et al., 1990). The amplification program consisted of an initial denaturation step at 95°C for 5 min (one cycle), followed by 45 cycles of denaturation at 95°C for 1 min, annealing at 36°C for 1 min and amplification at 72°C for 2 min, with a final extension at 72°C for 5 min (one cycle). Sequences of primers are listed in Table 1.

Table 1. Sequences of the nucleotide primers used in this study.

Primer code		RAPD primers	
	Sequence	Amplified region	References
RAPD primer 1 RAPD primer 2 RAPD primer 5	5'-GGT GCG GGA A-3' 5'-GTT TCG CTC C-3' 5'-AAC GCG CAA C-3'	Fragments from 1-10 genomic sites are amplified simultaneously	Mahmoud et al., 2012
Primer code		ISSR primers	
$(GTG)_5$ $(GACA)_4$ $(AGAG)_4G$	5'-GTG GTG GTG GTG GTG-3' 5'-GAC AGA CAG ACA GAC A-3' 5'-AGA GAG AGA GAG AGA GG-3'	Minisatellite-region DNA	Batista et al., 2008

ISSR PCR

PCR amplification of ISSRs was performed with the primers $(GTG)_5$, $(GACA)_4$ and $(AGAG)_4G$. The reaction mixtures had a final volume of 25 µL and contained reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl) (Biolabs), 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.25 mM primers, 1.25 U *Taq* DNA polymerase (BioLabs), and 25 ng genomic DNA. The amplification program consisted of an initial denaturation step at 93°C for 5 min, followed by 40 cycles of denaturation at 93°C for 20 s, annealing at 55°C for 45 s and amplification at 72°C for 90 s, with a final extension at 72°C for 6 min. Sequences of primers are listed in Table 1.

DNA electrophoresis

For all samples, the amplified DNA (15 µL) was electrophoresed using an electrophoresis unit (Wide Mini-Sub-Cell GT, Bio-RAD) in 2% agarose containing ethidium bromide

(0.5 µg/mL) at constant 75 V and 60 mA and was visualized with a UV trans-illuminator.

Gel analysis of DNA

The DNA gel was scanned for band Rf using a gel documentation system (AAB Advanced American Biotechnology, Fullerton, CA, USA). The different molecular weights of the bands were determined against a DNA standard (kb DNA ladder, Stratagene, Canada) with molecular weights of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, and 1500 bp. The similarity level was determined by unweighted pair group method with arithmetic mean (UPGMA).

RESULTS

Frequency percentage of isolated fungi in corn grains from Riyadh, Dammam, and Abha regions

Frequency of the fungi isolated from wheat grains from the Riyadh, Dammam, and Abha regions of Saudi Arabia. Twelve species from six fungal genera were identified from the samples tested (Table 2). In the Riyadh region, *A. flavus*, *Fusarium moniliforme*, and *F. oxysporum* were the dominant fungi isolated from wheat grains, showing frequencies of 20.00, 12.00, and 12.00%, respectively. The other isolated fungi occurred at frequencies ranging from 0.00 to 8.00%. In the Dammam region, *A. flavus*, *A. niger*, and *F. moniliforme* were the dominant fungi isolated from the wheat grains, displaying frequencies of 17.90, 14.30, and 14.30%, respectively. The other isolated fungi were present at frequencies ranging from 3.6 to 10.70%. In the Abha region, *A. flavus*, *F. moniliforme* and *F. oxysporum* were the dominant fungi isolated from the wheat grains, exhibiting frequencies of 29.20, 11.80 and 11.80%, respectively. The other isolated fungi occurred at frequencies ranging from 0.00 to 5.90%. *A. flavus* isolates represented the dominant fungi in these three regions, with five *A. flavus* isolates being obtained from each region.

Table 2. Frequency of fungi isolated from corn kernels from Riyadh, Dammam, and Abha regions in Saudi Arabia.

Isolated fungi	Riyadh	Dammam	Abha
Aspergillus flavus	20.00	17.90	29.20
A. niger	8.00	14.30	5.90
A. parasiticus	4.00	3.60	0.00
Alternaria longipes	8.00	7.10	5.90
A. chlamydospora	4.00	3.60	5.90
Fusarium moniliforme	12.00	14.30	11.80
F. oxysporum	12.00	10.70	11.80
F. solani	8.00	7.10	0.00
Penicillium funiculosum	8.00	7.10	5.90
P. implicatum	0.00	3.60	5.90
Eupenicillium alutaceum	8.00	7.10	5.90
Rhizopus stolonifer	8.00	3.60	11.80
Total	100.00	100.00	100.00

Detection of aflatoxigenic *A. flavus* isolates under UV irradiation (365 nm)

Three different culture media, CZ, PDA, and YES agar, were used to screen for AF

production. The production of AF was readily detectable by direct visualization of a beige ring surrounding the colonies after 4 days of incubation. The presence or absence of fluorescence in the agar surrounding the colonies was determined under UV irradiation (365 nm) and was expressed as positive or negative. The data presented in Table 3 show that fifteen isolates of *A. flavus* were positive for AF production, and that four *A. flavus* isolates were negative. Example of non-aflatoxigenic and aflatoxigenic isolates are shown in Figure 2.

Table 3. Detection of aflatoxigenic *Aspergillus flavus* isolates under UV radiation (365 nm).

A. flavus code	Medium		
	PDA+NaCl	Czapek	YES
Riyadh region			
C1	+	+	+
C2	-	+	+
C3	-	+	-
C4	-	+	-
C5	-	+	+
Dammam region			
C6	-	+	+
C7	-	-	-
C8	+	+	+
C9	-	+	+
C10	-	-	-
Abha region			
C11	-	-	-
C12	+	+	+
C13	-	-	+
C14	-	-	-
C15	-	-	-

Czapeks = Czapeks Dox agar; YES = yeast extract sucrose agar; PDA = potato dextrose agar.

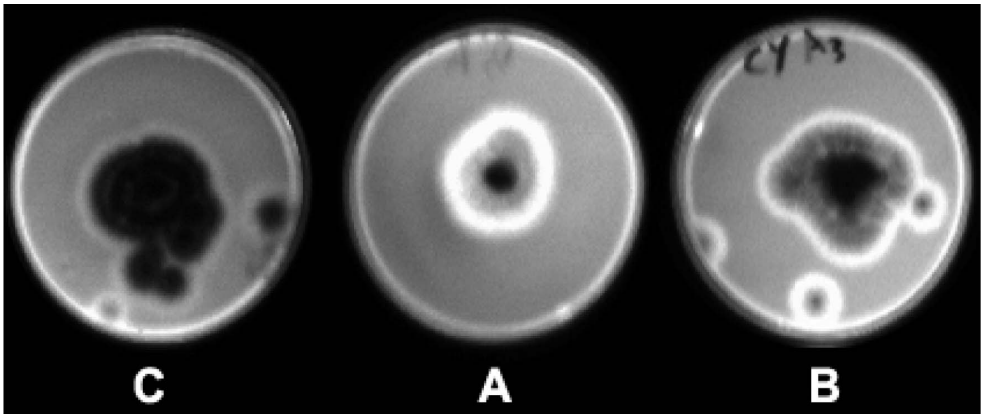

Figure 2. Non-aflatoxigenic isolate of *Aspergillus flavus* (**C**), two aflatoxigenic isolates (**A**) and (**B**) visualized under 365-nm UV light. The white ring around colonies of aflatoxigenic isolates displays faint blue or green fluorescence.

Aflatoxin production

Fifteen isolates were capable of producing detectable levels of both B and G AF,

whereas six isolates failed to produce a detectable amount of the toxins. The highest levels of B1 and B2 (2.9 and 2.3 ppb) were obtained from isolate C8. Isolate C3 showed the highest production of G1 (1.9 ppb), whereas isolate C1 exhibited the highest production of G2 (1.6 ppb) (Table 4).

Table 4. Aflatoxin production by *Aspergillus flavus* isolated from corn grains collected from the Riyadh, Dammam, and Abha regions.

A. flavus code	Aflatoxin (ppb)			
	B1	B2	G1	G2
Riyadh region				
C1	2.6	2.1	1.7	1.6
C2	1.8	1.5	0.9	1.1
C3	2.8	2.2	1.9	1.2
C4	0.0	0.0	0.0	0.0
C5	2.1	1.8	1.5	1.2
Dammam region				
C6	2.3	2.1	0.9	0.7
C7	0.0	0.0	0.0	0.0
C8	2.9	2.3	1.6	1.3
C9	2.5	2.1	1.7	1.5
C10	0.0	0.0	0.0	0.0
Abha region				
C11	0.0	0.0	0.0	0.0
C12	1.4	1.1	0.9	0.7
C13	1.8	1.3	1.0	0.8
C14	0.0	0.0	0.0	0.0
C15	0.0	0.0	0.0	0.0

Chemotype patterns

The *A. flavus* isolates were classified into two chemotypes on the basis of their ability to produce B1, B2, G1, and G2 (Table 5). Chemotype I was the prominent group and included eight isolates (aflatoxigenic), comprising 53.3% of the isolates obtained. Chemotype II included seven isolates (non-aflatoxigenic), corresponding to 46.7% of the isolates.

Table 5. Chemotype patterns of *Aspergillus flavus* isolated from corn kernels based on the ability to produce AFs B1, B2, G1, and G2.

Chemotype	Aflatoxin				No. of isolates (%)
	B1	B2	G1	G2	
I	+	+	+	+	8 (53.3)
II	-	-	-	-	7 (46.7)

Genetic characterization of *A. flavus* isolates

Genetic characterization was evaluated by PCR amplification using a set of 6 primers (3 RAPD and 3 ISSR). The amplification products were analyzed for polymorphisms by gel electrophoresis to determine whether pathotypes could be distinguished at the molecular level.

RAPD profiles

Phenetic analysis using three RAPD primers

Phenetic analysis of the 15 A. flavus isolates based on RAPD primer 1

The dendrogram generated using the similarity matrix produced from the banding patterns obtained with primer 1 exhibited two main clusters, with 63.82% genetic similarity (GS) (Figure 3). The GSs between the isolates tested ranged from 63.82-98.42%. The first main cluster included two groups with 74.13% GS. The first group contained Abha region isolates C12, C15, C13, and C14, which showed 85.03% GS. The second group consisted of only one isolate, C8 from the Dammam region. The second main cluster included two groups with 83.44% GS. The first group comprised isolates C3 from the Riyadh region and C7 from the Dammam region, which displayed 91.61% GS. The second group contained two subgroups and displayed 89.10% GS. The first subgroup included only one isolate, C11 from the Dammam region, and the second subgroup was divided into two subclusters, with 90.16% GS. The first subcluster consisted of isolates C2, C4, and C5 from the Riyadh region and C6 and C9 from the Dammam region, with 93.60% GS being detected between isolates. The second subcluster included isolate C1 from the Riyadh region and isolate C10 from the Dammam region, which showed 94.51% GS.

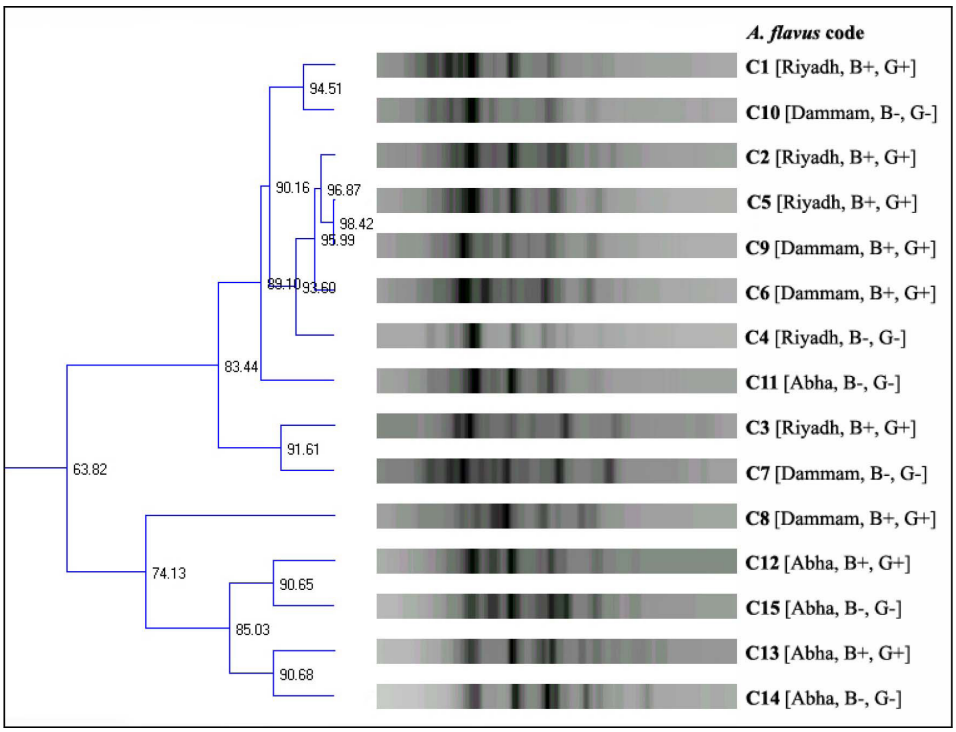

Figure 3. Dendrogram obtained by UPGMA method derived from PCR-amplification banding of RAPD with RAPD primer 1 of 15 *Aspergillus flavus* isolates.

These data indicated that this primer was not able to differentiate between isolates according to geographic regions, except for the Abha region, *i.e*., isolates C12, C13, C14, and C15, which exhibited 85.03% GS and were located in a single subcluster.

The four subclusters included eight isolates. The four AFB and AFG-producing isolates (C1, C3, C12, and C13) and the four isolates that did not produce AFB or AFG (C7, C10, C14, and C15) from different regions resided in two main clusters, with a random distribution. All of the subclusters included a combination of aflatoxigenic and non-aflatoxigenic isolates. For example, one subcluster included isolates C10 (non-AFBand AFG-non-producing) and C1 (AFB and AFG-producing) and showed 94.51% GS. No correlation was detected between the DNA banding patterns obtained and AF-producing ability.

Phenetic analysis of the 15 A. flavus isolates based on RAPD primer 3

The dendrogram generated using the similarity matrix produced from the banding patterns obtained with primer 3 exhibited two main clusters, with 50.35% GS (Figure 4). The GS between the isolates tested ranged 50.35-96.07%. The first main cluster included two isolates from the Abha region, C11 and C12, which showed 74.31% GS. The second main cluster contained two groups with 50.87% GS. The first group included only one isolate, C10, from the Dammam region, while the second group was divided into two subgroups, which showed 74.81% GS. The first subcluster included isolates C2, C3, and C4 from the Riyadh region, C6, C7, C8, and C9 from the Dammam region, and C14 from the Abha region, with 75.88% GS being detected between these isolates. The second subcluster included isolates C1 and C5 from the Riyadh region and C13 and C15 from the Abha region, which showed 84.52% GS. The isolates representing the Dammam region, i.e., isolates C6, C7, and C9, showed GS greater than 92%. The isolate pairs C13 + C15 and C11 + C12 from the Abha region showed GS of 91.63 and 79.31%, respectively.

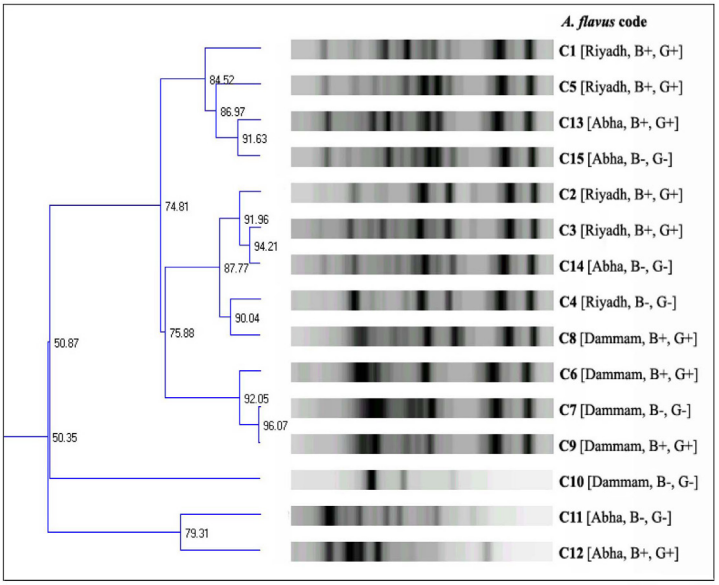

Figure 4. Dendrogram obtained by UPGMA method derived from PCR-amplification banding of RAPD with RAPD primer 3 of 15 *Aspergillus flavus* isolates.

These findings indicated that that this primer was, to some extent, able to differentiate between regions. With reference to this primer, there was a partial relationship detected between the clustering observed in the RAPD dendrogram and the geographic origin of the isolates tested.

The five subclusters obtained included ten isolates. The five AFB and AFG-producing isolates (C13, C3, C8, C9, and C12) and the five non-AFB- or non-AFG-producing isolates (C15, C14, C4, C7, and C11) came from different regions and resided in the two main clusters with a random distribution.

All subclusters included a combination of aflatoxigenic and non-aflatoxigenic isolates. For example, one subcluster included isolates C14 (non-AFB and AFG non-producing) and C3 (AFB and AFG producing), which showed 94.21% GS. No correlation was detected between the DNA banding patterns obtained and the AF-producing ability.

Phenetic analysis of the 15 A. flavus isolates based on RAPD primer 5

The dendrogram generated using the similarity matrix produced with the banding patterns obtained with primer 5 showed the formation of two main clusters, with 49.75% GS (Figure 5). The GS between the isolates tested ranged 49.75-98.65%. The first main cluster included two isolates, C11 and C12 from the Abha region, which displayed 79.19% GS. The second main cluster included two groups, with 71.69% GS. The first group was divided into two subclusters, which showed 84.51% GS. The first subcluster included only one isolate, C6, from the Dammam region, and the second included isolates from both the Riyadh region (C5) and the Abha region (10.13 and 15), with 85.42% GS. The second group was divided into two subclusters, with 88.45% GS. The first subcluster included isolates C8 and C9 from the Dammam region and isolate C14 from the Abha region, which showed 92.41% GS, while the second included isolates C1, C2, C3, and C4 from the Riyadh region and C7 from the Dammam region, which displayed 92.93% GS. The isolates representing the Riyadh region, i.e., isolates C1, C7, C3, and C4, exhibited GS greater than 96%, while isolates C8 and 9 from the Dammam region and isolates C13 and C15 from the Abha region showed GS of 93.80 and 91.69%, respectively.

These findings indicated that this primer was, to some extent, able to differentiate between regions. With reference to this primer, there was a partial relationship found between the clustering observed in the RAPD dendrogram and the geographic origin of the isolates tested.

The three subclusters included six isolates. The three AFB and AFG-producing isolates (C1, C4, and C12) and the three non-AFB or non-AFG-producing isolates (C7, C3, and C10) came from different regions and resided within the two main clusters, with a random distribution.

All subclusters included a combination of aflatoxigenic and non-aflatoxigenic isolates. For example, one subcluster included isolate C4 (non-AFB- and AFG-non-producing) and C3 (AFB- and AFG-producing), which showed 99.40% GS. No correlation was detected between the DNA banding patterns obtained and the AF-producing abilities of the isolates.

Phenetic analysis based on three ISSR primers

Phenetic analysis of 15 A. flavus isolates based on ISSR primer (*GTG*)$_5$

The dendrogram generated using the similarity matrix produced with the banding patterns obtained from the $(GTG)_5$ primer showed the formation of two main clusters, with 65.19% GS (Figure 6). The GS between the isolates tested ranged 65.19-99.22%. The first

main cluster included one isolate, C2, from the Riyadh region. The second main cluster included two groups, with 75.27% GS. The first group included isolates C11 and C12 from the Abha region, which displayed 97.31% GS. The second group was divided into two subclusters, exhibiting 81.22% GS. The first subcluster included isolates C3 and C5 from the Riyadh region, with 97.17% GS. The second subcluster comprised ten isolates: C1 and C4 from the Riyadh region, C6, C7, C8, C9, and C10 from the Dammam region and C13, C14, and C15 from the Abha region, which showed 81.56% GS.

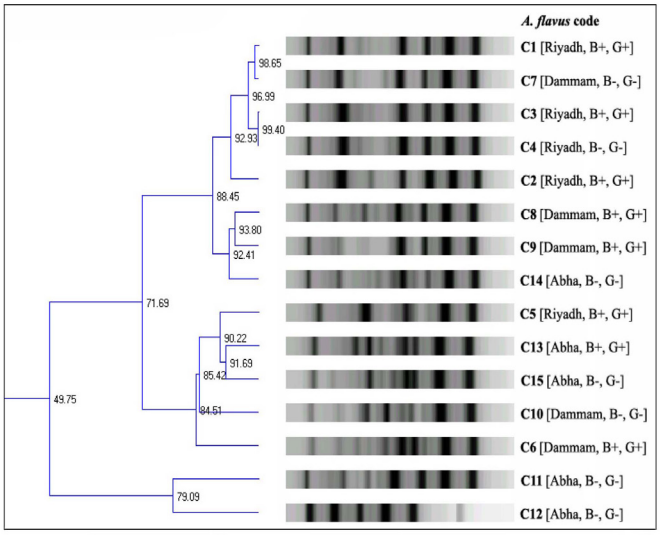

Figure 5. Dendrogram obtained by UPGMA method derived from PCR-amplification banding of RAPD with RAPD primer 5 of 15 *Aspergillus flavus* isolates.

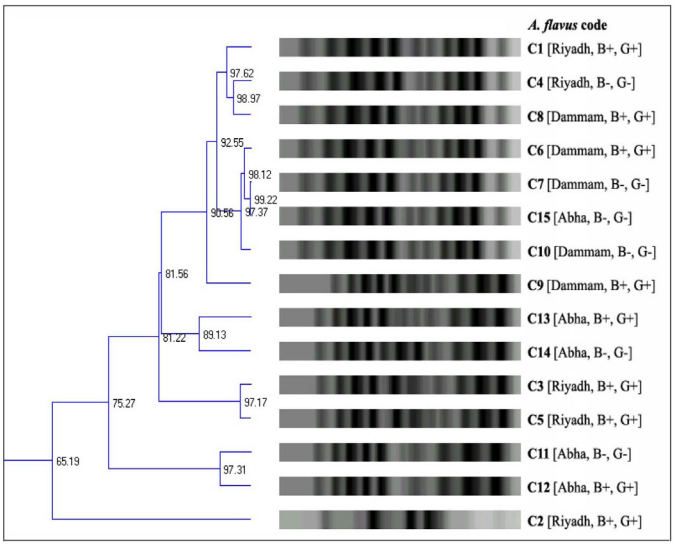

Figure 6. Dendrogram obtained by UPGMA method derived from PCR-amplification banding of ISSR with primer $(GTG)_5$ of 15 *Aspergillus flavus* isolates.

With regard to this primer, there was no relationship found between the clustering observed in the ISSR dendrogram and the geographic origin of the isolates tested.

The three subclusters included six isolates. The three AFB- and AFG-producing isolates (C8, C12, and C13) and the three non-AFB- or non-AFG-producing isolates (C4, C10, and C14) from different regions resided in the two main clusters with a random distribution.

All subclusters included a combination of aflatoxigenic and non-aflatoxigenic isolates. For example, one subcluster included isolates C4 (non-AFB- and non-AFG-producing) and C8 (AFB- and AFG-producing), showing 98.97% GS. No correlation was found between the DNA banding patterns obtained and the AF-producing ability of the isolates. One subcluster included isolates C7 and C15, which were AFB and AFG non-producers and showed 99.22% GS.

Phenetic analysis of the 15 A. flavus isolates based on ISSR primer (*GACA*)$_4$

The dendrogram generated using the similarity matrix produced with the banding patterns obtained with the $(GAGA)_4$ primer exhibited two main clusters, with 80.37% GS (Figure 7). The GS between the isolates tested ranged 80.37-99.52%. The first main cluster included only one isolate, C2, from the Riyadh region, and the second main cluster included two groups, showing 81.99% GS. The first group was divided into two subclusters, displaying 92.83% GS. The first subcluster included isolates C11, C12, C13, C14, and C15 from the Abha region, with 97.53% GS, and the second subcluster comprised only one isolate, C5, from the Riyadh region. The second group was divided into two subclusters, exhibiting 92.48% GS. The first subcluster contained only one isolate, C4, from the Riyadh region, and the second subcluster contained isolates C1 and C3 from the Riyadh region and C7, C8, C9, and C10 from the Dammam region.

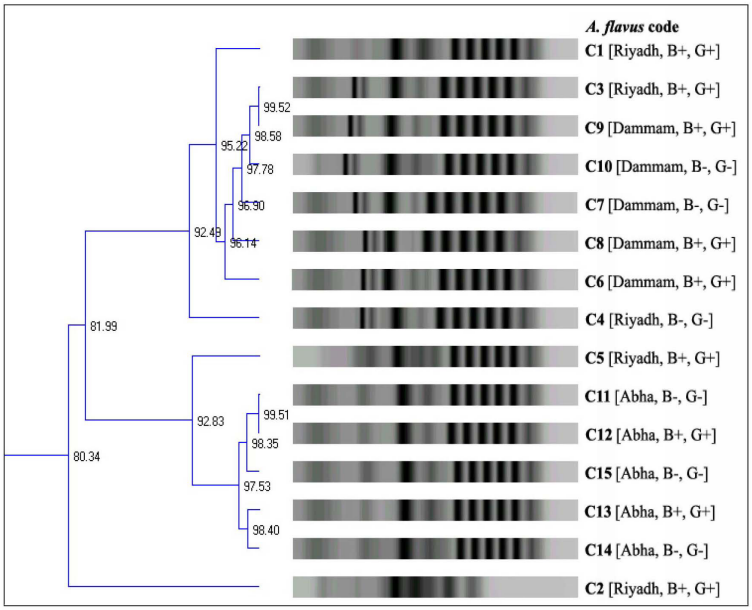

Figure 7. Dendrogram obtained by UPGMA method derived from PCR-amplification banding of ISSR with primer $(GACA)_4$ of 15 *Aspergillus flavus* isolates.

In reference to this primer, there was a strong relationship found between the clustering observed in the ISSR dendrogram and the geographic origin of the isolates tested, especially with the Abha region isolates and the Dammam region isolates.

The three subclusters included seven isolates. The four AFB and AFG-producing isolates (C3, C9, C12, and C13) and three non-AFB or AFG-producing isolates (C10, C11, and C14) came from different regions and resided in the two main clusters with a random distribution. All subclusters included a combination of aflatoxigenic and non-aflatoxigenic isolates. For example, one subcluster contained isolate C11 (non-AFB and AFGnon-producing) and C12 (AFB and AFG producing), which showed 99.51% GS. No correlation was detected between the DNA banding patterns obtained and the AF-producing ability of the isolates.

Phenetic analysis of 15 A. flavus isolates based on ISSR primer $(AGAG)_4G$

The dendrogram generated using the similarity matrix produced from the banding patterns obtained with primer $(AGAG)_4G$ showed two main clusters, with 67.27% GS (Figure 8). The GS between the isolates tested ranged 67.27-99.49%. The first main cluster included only isolate C9, from the Dammam region, and the second main cluster included two groups, with 78.75% GS. The first group was divided into two subclusters, which showed 82.92% GS. The first subcluster comprised isolates C6, C7, and C8 from the Dammam region and isolates C11, C12, C13, C14, and C15 from the Abha region, and the second subcluster included two isolates, C4, and C10, from the Riyadh and Dammam regions, with 92.25% GS. The second group included isolates C1, C2, C3, and C5 from the Riyadh region, which displayed 95.40% GS.

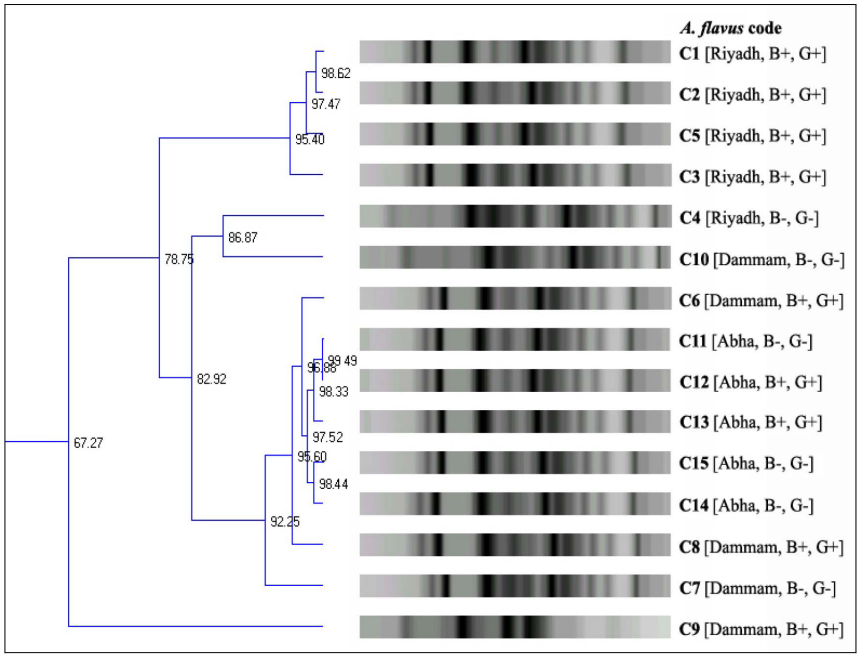

Figure 8. Dendrogram obtained by UPGMA method derived from PCR amplification banding of ISSR with primer $(AGAG)_4G$ of 15 *Aspergillus flavus* isolates.

With regard to this primer, there was a strong relationship detected between the clustering observed in the ISSR dendrogram and the geographic origin of the isolates tested, especially for the Riyadh region isolates and the Abha region isolates.

The four subclusters included eight isolates. One subcluster included isolates C1 and C2 (AFB or AFG-producing), with 98.62% GS. The first subcluster included C4 and C10 (non-AFB and AFG non-producing), which showed 86.87% GS, and the second subcluster included C15 and C14 (non-AFB and AFG-non-producing), displaying 98.44% GS. Another subcluster included isolates C12 (AFB and AFG-producing) and C11 (non-AFB and AFG non-producing), with 99.49% GS. There was a strong relationship detected between the clustering observed in the ISSR dendrogram and the AF-producing ability of the isolates.

DISCUSSION

In the corn samples examined, the isolation frequency among the fungal genera was the highest for *Aspergillus*, followed by *Penicillium* and *Fusarium*, and the lowest for *Alternaria*. *P. verrucosum* was the most frequently isolated species, followed by *A. niger* aggregates, *A. ochraceus, A. flavus, P. chrysogenum*, *A. parasiticus*, *A. carbonarius*, and *Fusarium* and *Alternaria* spp. Abd El-Aziz Abeer et al. (2012) and Mahmoud et al. (2013) reported *A. flavus* as the predominant species isolated from maize grains in Saudi Arabia. Gao et al. (2007) isolated *Aspergilli* as the predominant species in China, followed by *Penicillium*, *Fusarium*, and other fungi. A predominance of *Aspergillus* species, followed by *Fusarium* species, has also been reported from maize silage in Argentina (González et al., 2008).

The prevalence of AF production, determined by the fluorescence of fungal colonies and recorded as the percentage of positive isolates observed in the PDA+NaCl, CZ, and YES agar media, was 20, 60, and 53.33%, respectively. AF are produced by some isolates of *A. flavus,* but not all isolates were able to produce AF, which has encouraged screening for AF production abilities based on the fact that aflatoxigenic isolates produce blue fluorescence on the reverse side of colonies under UV light, whereas non-aflatoxigenic strains do not fluoresce (Davis et al., 1987). A new, reliable, fast, simple method for the detection of aflatoxigenic *Aspergillus* strains involves the addition of a cyclodextrin to the common media used for testing isolates for AF production to enhance the natural fluorescence of AF. Colonies of *Aspergillus* produce a bright yellow-orange pigment, and blue (B1, B2) or green (G1, G2) fluorescent halos appear around aflatoxigenic colonies upon exposition to UV light (Fente et al., 2001).

The 15 *A. flavus* isolates described in this study were classified into two major chemotypes, where chemotype I included AF producers (53.3%) and chemotype II included AF non-producers (46.7%). HPLC determination of AF is sensitive and accurate in unprocessed cereals and cereal-based products (Pascale, 2009). Several authors have applied HPLC methods for low-ppb detection of AF (B1, B2, G1, and G2) and AF residues in cereals. Ahsan et al. (2009) found that of the 40 maize samples examined, 34 were contaminated with AF in Pakistan. The presence of AF (B1, B2, G1, and G2) was determined by RP-HPLC, and the mean values of B1and B2 were (10-46 µg/kg) and G1 and G2 were (0.2-12 µg/kg). Yassin et al. (2012) found that most of the *Aspergilli* isolated from corn grains produced in Saudi Arabia were toxigenic and varied in the type and quantity of AF. B1 was generally the most dominant toxin, especially from *A. flavus*. Three isolates of *A. flavus* produced B1 in concentrations ranging from 2 to 8 ppb, whereas two isolates produced B2 at concentrations ranging from 1 to 2 ppb. Three isolates produced G1 in concentrations ranging from 2 to 4 ppb, and two

isolates produced G2 in concentrations ranging from 1 to 3 ppb. Martins et al. (2008) detected and quantified AF using HPLC in Portugal. Five isolates of *A. flavus* produced B1, B2, G1 and G2. The concentrations detected for B1, B2, G1, and G2 ranged from 18-24, 6-18, 9-12, and 6-8 mg/kg, respectively. Rocha et al. (2009) reported that among 200 corn samples analyzed, 21 (10.5%) were contaminated with B1, seven (3.5%) with B2 and only one (0.5%) with G1 and G2. Sixteen (76.2%) of the 21 positive samples showed a total AF (B1+B2+G1+G2) concentration that was higher than the limit established by Brazilian regulations (20 µg/kg).

All of the RAPD primers employed in the present study were able to reveal information contributing to genetic characterization for the isolates as well as determination of their genetic diversity. Regarding the geographic sources of the isolates, two relationships were found: first, there was no apparent correlation observed for primer RAPD 1; second, there was a partial relationship observed for RAPD primers 3 and 5 with the RAPD dendrogram, but no correlation was detected between the RAPD dendrogram and the AF-producing ability of the isolates. Batista et al. (2008) used RAPD molecular markers with the aim of genetically characterizing the diversity of *A. flavus* strains. High genetic diversity was revealed by their RAPD analysis, and a high efficiency of strain characterization was achieved. The characterization of seven different *Aspergillus* species by RAPD was useful for estimating the distances between and within species and may contribute to future management and conservation programs. Their results indicated that the genetic differences between *Aspergillus* species maintained genetic diversity within this population (Irshad and Nawab, 2012). RAPD was also used for the analysis of 14 isolates of *A. flavus* from two geographically distinct sites in Brazil. The distribution of the isolates showed a non-random pattern, though it cannot be assumed that this is a fully discriminatory result, and thus, the utility of RAPD analysis is debatable (Lourenço et al., 2007). Similar findings have been reported by Tran-Dinh et al. (1999). Twenty-one isolates of *Rhizoctonia solani* were characterized by RAPD-PCR. There was a partial relationship found between the genetic similarity of the isolates and the recorded AF, level of virulence or geographic origin based on the RAPD dendrogram obtained (Mahmoud et al., 2012). Other authors have reported detecting no correlation between DNA banding profiles and the production or nonproduction of AF (Tran-Dinh et al., 1999; Lourenço et al., 2007; Sepahvand et al., 2011). However, in one investigation involving isolates of *Aspergillus* section *flavi* based on RAPD analysis, Egel et al. (1994) grouped strains with similar toxigenic capacities in a manner that allowed more subtle differentiation compared to the simple classification of toxin producers and non-producers. Because the RAPD-PCR technique amplifies random fragments of the fungal genome, the fragments that contain the genes regulating toxin production may not have been amplified using this technique with the primers selected. Similar results have been described in other studies with *Aspergillus* spp that have shown no correlation between DNA banding profiles and the production or non-production of mycotoxins (Gashgari et al., 2010).

All ISSR primers used in the present study contributed to the genetic characterization of the diversity of the isolates. With respect to the geographic sources of the isolates, two relationships were found: first, there was no apparent correlation with primer $(GTG)_5$, and second, there was a strong correlation observed for primers $(GACA)_4$ and $(AGAG)_4G$ with the ISSR dendrogram, whereas there was no correlation between the ISSR dendrogram and AF-producing ability of the isolates found when using the primers $(GTG)_5$ and $(GACA)_4$. There was a strong correlation for primer $(AGAG)_4G$. No apparent correlation was found between the ISSR dendrogram and AF-producing ability based on primers $(GTG)_5$ and $(GACA)_4$, whereas a strong correlation was found using primer $(AGAG)_4$. ISSR microsatellite markers have been

employed to characterize *A. flavus* isolates in a wheat crop. ISSR analysis can be useful in population genetic analyses, epidemiological surveys and ecological studies of *A. flavus*. Additionally, the $(GTG)_5$ primer can be applied to generate unique products from different *Aspergillus* species that can then be converted to sequences, and the characterized amplified regions can aid in taxonomic identification (Batista et al., 2008). Microsatellite analysis of Vietnamese *A. flavus* strains (isolated from corn and peanut) revealed high genetic diversity (Tran-Dinh et al., 2009). The microsatellite markers presented here will be useful for investigating the diversity and population structure of *A. flavus* and *A. parasiticus* (Tran-Dinh and Carter, 2000). The dendrogram produced using the ISSR markers showed no relationship between the clustering of the isolates and their geographic origin. The dendrogram produced using the ISSR data showed high genetic similarity according to the geographical origin (Batista et al., 2008). Genetic relationships were found between 84 strains of *A. flavus* isolated from Vietnam. No correlation was found between the geographic origin of the strains, and no genotype was evident. For example, the strains collected from both northern and southern regions were interspersed throughout the dendrogram (Tran-Dinh et al., 2009). ISSR markers can differentiate between aflatoxigenic and non-aflatoxigenic strains, and the ISSR primers showed a relationship between the ISSR dendrogram and the AF-producing ability of the isolates, especially when using primers $(GTG)_5$ and $(GACA)_4$. High genetic diversity was observed in the 84 strains, with no evident correlation detected between strain toxigenicity and genotype (Tran-Dinh et al., 2009). *A. flavus* strains were isolated from different oil seeds (groundnut, sunflower, and soybean), and four of these isolates were found to be nontoxic, while eight were toxic. No correlation was found between AF production and an ISSR dendrogram (Hatti et al., 2010).

ACKNOWLEDGMENTS

Research supported by the King Saud University, Deanship of Scientific Research, College of Science Research Center.

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