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# Genetic Characterization of *Aspergillus flavus* Contamination of Sorghum Grains using DNA Marker

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**Twelve species from six fungal genera were found to be associated with Sorghum (*Sorghum bicolor* [L.] Moench) grain samples collected from different markets at three main regions of Saudi Arabia. The average frequencies of the most common genera were *Aspergillus* (10.3%), *Penicillium* (9.8%), *Fusarium* (9.5%) and *Alternaria* (7.7%). Thirteen isolates of *Aspergillus flavus* were screened by HPLC for their ability to produce aflatoxins (AFs). The percentage of aflatoxigenic *A. flavus* isolates was 46.2%. Six isolates produced AFs, at concentrations ranging 0.0-6.1 $\mu$g/ml. Inter simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) molecular markers were used to genetically characterize strains 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 dendograms 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*; Sorghum, Random amplified polymorphic DNA; Inter-simple sequence repeats; Aflatoxin; HPLC.

Sorghum (*Sorghum bicolor* [L.] Moench) is the fifth most important cereal crop grown in the world after rice, wheat, maize and barley. Grains sorghum is used for human food and livestock feeds. Total world production of sorghum reached about 65.5 million tons in 2008 (FAO, 2009). Grains sorghum is the principal source of energy, protein, vitamins and minerals for millions of poor people in 30 countries of the semi-arid tropics. Saudi Arabia product about 272.000 tonnes and imported about 2.2 million tonnes sorghum in 2007 ( FAO, 2008).

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In Saudi Arabia, there is a lack of accurate information on diseases and seed-borne fungi of sorghum. Previous studies of grain sorghum samples in Saudi Arabia had shown the presence of several seed-borne fungi genera such as *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria* (Yassin *et al*., 2010; Mahmoud *et al*., 2013). In addition to the effects of seed-borne fungi on seed health, such fungi may also produce harmful mycotoxins that can affect both human and animal health (Palanee *et al*., 2001; David *et al*., 2005). Several mycotoxigenic fungi recovered from both freshly harvested and stored Indian sorghum (Sreenivasa *et al*., 2010; Abdulsalaam and Shenge, 2011). AFs are toxic secondary metabolites produced by species of *Aspergilli*, especially *Aspergillus flavus* and *Aspergillus parasiticus* (Pildain *et al*., 2008).

AFs can enter the food chain in the field, during storage, or later, under favorable conditions of temperature and humidity. The most potent of the four naturally occurring AFs (B1, B2, G1 and G2) is B1, which is listed as a group I carcinogen by the International Agency for Research on Cancer (IARC, 1982). The genetic techniques provide a useful tool for detection, analysis and assessment of genetic information of *Aspergilli* (Batista *et al*., 2008). Many genetic markers include randomly amplified polymorphic DNA (RAPD) (Gashgari *et al*., 2010; Irshad and Nawab, 2012; Al-Wadai *et al*., 2013). Inter-simple sequence repeat (ISSR) (Batista *et al*., 2008; Tran-Dinh *et al*., 2009; Hatti *et al*., 2010) restriction fragment length polymorphisms (RFLPs) (Mohankumar, *et al*., 2010) and amplified fragment length polymorphisms (AFLP) Martins Reis *et al*., 2012) which has been used widely for estimating of genetic characterization for *A. flavus*. 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.

## METHODS

**Collection of samples**

Fifteen samples (250 g each) of grain sorghum were collected from different markets located in Riyadh, Dammam, and Abha regions (Figure 1) in the Kingdom of Saudi Arabia during March 2011. The market survey of grain sorghum samples were divided Saudi Arabia into three regions, the first region Dammam, (eastern) where the weather was hot (30–45°C) and high humid, the second region Riyadh, (middle) where it was very hot (40-50°C) and dried and the third region Abha, (south western) where it was cool (15-30°C) and wet. Collected samples were transported immediately to laboratory for labelling, like date and place of collection and kept in cool place for further mycological analysis ( Czerwiecki *et al*., 2002).

**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 and identified up to the species level using keys and manuals (Raper and Fennell 19650. The isolation frequencies of fungal species were calculated according to the method of (Gonzalez *et al*., 1999). 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.

**Detection of aflatoxins based on fluorescence**

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 in the center of a Petri dish containing test medium (PDA, Czapek agar (CZ) or malt extract agar (Davis *et al*., 1987).The plates were then incubated at 25°C for 4 days in the dark, and tested for 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 ( 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 the toxigenic Aspergillus spp. 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, filtration and separation using HPLC (PerkinElmer series 200 UV/VIS) were carried out according to (Christian, 1990).

**DNA extraction of *A. flavus* isolates**

*A. flavus* isolates were cultured in double layer media, consisting of a solid and a liquid layer, in 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. DNA was extracted from 50 mg

fresh mats according to ( Amer *et al*., 2011).

**RAPD PCR**

We selected 3 primers from standard RAPD primer; sequences of primers (e-oligos, Gene link, USA) are listed in Table 1. Total DNA of *A. flavus* isolates were using a template. PCR amplifications were run using mixtures with a final volume of 25 ¼L containing reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 3.4 mM $MgCl_2$, 0.25 mM dNTPs, 0.4 mM each primer, 2 U Taq DNA polymerase (BioLabs) and 25 ng genomic DNA.

**ISSR PCR**

PCR amplification of ISSRs was performed with the primers $(GTG)_5$, $(GACA)_4$ and $(AGAG)_4G$. Sequences of primers are listed in Table 1. The reaction mixtures had a final volume of 25 ¼L and contained reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 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.

**DNA electrophoresis**

For all samples, the amplified DNA (10 ¼L) was electrophoresed using an electrophoresis unit (Wide Mini-Sub-Cell GT, Bio-RAD) in 1.5% 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

**Isolation frequencies of fungi recovered from sorghum grains from Riyadh, Dammam, and Abha regions**

Twelve fungi belonging to six genera were isolated from tested samples shown in Table 2. Nine fungi belonging to five genera were isolated from tested sorghum grains. Isolation frequencies of isolated fungi were *Aspergillus* spp. (42.8%), *Alternaria* spp. (14.3%), *Fusarium* spp. (23.8%), *Penicillium* spp. (14.3%) and Rhizoups stolonifer (4.8%) were isolated from Riyadh region. Eight fungi belonging to four genera were isolated from tested sorghum grains. Isolation frequencies of isolated fungi were *Aspergillus* spp. (33.2%), *Fusarium* spp. (44.5%), *Penicillium* (11.2%) and *Eupenicillum alutaceum* (11.1%) were isolated from Dammam region.

Nine fungi belonging to six genera were isolated from tested wheat grains. Isolation frequencies of isolated fungi were *Aspergillus* spp. (16.7%), *Alternaria* spp. (16.6) *Fusarium* spp. (16.6%), Penicillium (33.4%), *Eupenicillum alutaceum* (8.3%) and Rhizoups stolonifer (8.3%) were isolated from Abha region. *A. flavus* was the dominant fungi isolated from Riyadh, Dammam and Abha with isolation frequencies 33.30%, 22.10% and 16.70%, respectively.

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

| Primer | RAPD primers | | |
|---|---|---|---|
| code | Sequence | Amplified region | References |
| RAPD primer 1 | 5' -GGTGCGGGAA-3' | fragments from 1-10 genomic sites simultaneously | Mahmoud *et al*., 2012 |
| RAPD primer 2 | 5' -GTTTCG CTCC-3' | | |
| RAPD primer 5 | 5' -AACGCG CAAC-3' | | |
| Primer code | ISSR primers | | |
| | Sequence | Amplified region | References |
| $(GTG)_5$ | 5' -GTGGTGGTGGTGGTG-3' | Minisatellite-region DNA | Batista *et al*., 2008 |
| $(GACA)_4$ | 5' -GACAGACAGACAGACA-3' | | |
| $(AGAG)_4G$ | 5 -AGAGAGAGAGAGAGAGG-3' | | Hatti *et al*., 2010 |

**Detection of aflatoxins produced by *A. flavus* under UV irradiation (365 nm)**

Thirteen *A. flavus* isolates were isolated from tested samples in all regions and detected of aflatoxins under 365 nm UV irradiation using three different culture media, CZ, PDA and YES agar (Table 3). The production of AF was readily detectable by direct visualization of a beige ring surrounding the colonies after 4 days of incubation. For example, Isolates S2, S4 and S5 expressed as positive for aflatoxin production whereas, isolates S1, S3, S6 and S7 were expressed as negative for aflatoxin production, these isolates from Riyadh region.

**Determination of aflatoxins for *A. flavus* isolates from sorghum grains using HPLC**

Seven isolates were capable of producing detectable levels of both B and G aflatoxins,

**Table 2.** Isolation frequencies of fungi recovered from sorghum grains sorghum grains collected from different markets located in Saudi Arabia

| Isolated fungi | Riyadh | Dammam | Abha |
|---|---|---|---|
| *Aspergillus flavus* | 33.30 | 22.10 | 16.70 |
| *A. niger* | 00.00 | 11.10 | 00.00 |
| *A. parasiticus* | 09.50 | 00.00 | 00.00 |
| *Alternaria longipes* | 04.80 | 00.00 | 08.30 |
| *A. Chlamydospora* | 09.50 | 00.00 | 08.30 |
| *Fusarium monilinforme* | 09.50 | 16.70 | 08.30 |
| *F. oxysporum* | 14.30 | 16.70 | 00.00 |
| *F. solani* | 00.00 | 11.10 | 08.30 |
| *Penicillum funiculosum* | 09.50 | 05.60 | 16.70 |
| *P. implicatum* | 04.80 | 05.60 | 16.70 |
| *Eupenicillum alutaceum* | 00.00 | 11.10 | 08.30 |
| *Rhizoups stolonifer* | 04.80 | 00.00 | 08.30 |
| Total | 100.00 | 100.00 | 100.00 |

**Table 3.** Detection of AFs produced by *A. flavus* isolated from sorghum grains under UV irradiation (365 nm)

| *A. flavus* code | PDA+NaCl | Czapack | YES |
|---|---|---|---|
| | Riyadh region | | |
| S1 | - | - | - |
| S2 | - | - | + |
| S3 | - | - | - |
| S4 | - | + | + |
| S5 | - | + | + |
| S6 | - | - | - |
| S7 | - | - | - |
| | Dammam region | | |
| S8 | - | + | + |
| S9 | + | + | - |
| S10 | - | - | - |
| S11 | + | - | - |
| | Abha region | | |
| S12 | - | + | - |
| S13 | - | - | - |

Czapek: Czapeks Dox agar, YES: yeast extract sucrose agar, PDA: potato dextrose agar (+) positive for aflatoxin production, (-) negative for aflatoxin production

**Table 4.** AFs production by *A. flavus* isolated from sorghum grains using HPLC

| *A. flavus* code | AFs (µg/ml) | | | |
|---|---|---|---|---|
| | B1 | B2 | G1 | G2 |
| | Riyadh region | | | |
| S1 | 0.0 | 0.0 | 0.0 | 0.0 |
| S2 | 0.8 | 1.1 | 4.4 | 5.2 |
| S3 | 0.0 | 0.0 | 0.0 | 0.0 |
| S4 | 0.4 | 0.0 | 6.1 | 3.2 |
| S5 | 0.6 | 3.1 | 2.8 | 1.3 |
| S6 | 0.0 | 0.0 | 0.0 | 0.0 |
| S7 | 0.0 | 0.0 | 0.0 | 0.0 |
| | Dammam region | | | |
| S8 | 0.0 | 2.9 | 4.3 | 1.6 |
| S9 | 0.5 | 1.7 | 0.0 | 1.9 |
| S10 | 0.0 | 0.0 | 0.0 | 0.0 |
| S11 | 0.0 | 0.0 | 0.0 | 0.0 |
| | Abha region | | | |
| S12 | 0.3 | 0.0 | 3.3 | 1.7 |
| S13 | 0.0 | 0.0 | 0.0 | 0.0 |

although six isolates failed to produce any detectable amount (Table 4). The highest level (0.8 and 3.1 µg/ml) of B1 and B2 were obtained from isolates S2 and S5. Isolate S4 was the highest producer (6.1 µg/ml) of G1 while isolate S2 was the highest producer (5.2 µg/ml) of G2.

**Chemotype patterns**

*A. flavus* isolates were classified into three chemotypes (I to IV) based on their ability to produce B1, B2, G1 and G2 (Table 5). Chemotype I produced all AFs comprised 15.4% of the isolates (two isolates). Chemotype II produced B1, B2 and G2 was comprised 7.7% of the isolates (one isolate). Seven isolates failed to produce any detectable amount of AFs (Chemotype V) were comprised 53.8% of the isolates (seven isolates).

**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.

**Phenetic analysis using three RAPD primers**

Phenetic analysis of 13 *A. flavus* isolates obtained with RAPD primer 1

Dendrogram analysis grouped the isolates into two main clusters at 67.28% genetic similarity (GS) based on the banding pattern (Fig. 2). The GSs between the tested isolates ranged from 67.28% to 99.01%. The first main cluster included two isolates S2 and S4 from Riyadh region showed GS of 98.87%. The second main cluster included two groups at 85.70% GS. First group included two subclusters. First one included one isolate S11 from Dammam region, second subcluster included overlapping for four isolates (S12 and S13, from Abha region, S6 and S7 from Riyadh region. The second group included two subclusters at 89.96% GS. The first one included only one isolate S9 from Dammam region. The second subcluster included overlapping for five isolates (S1, S5 and S3, from Riyadh region, S8 and S10, from Dammam region). In this subcluster S1 and isolate S5 had more than 99% GS. This fact indicating that this primer was able, for somewhat, to differentiated that regions. With reference to this primer, there was partial relationship between clustering in the RAPD dendrogram and geographic origin of the tested isolates, especially with Riyadh region.

**Table 5.** Chemotype patterns of *A. flavus* isolates based on the ability for producing AFs

| Chemotype | Aflatoxin | | | | No. (%) |
|---|---|---|---|---|---|
| | B1 | B2 | G1 | G2 | of isolates |
| I | + | + | + | + | 2 (15.4) |
| II | + | + | - | + | 1 (7.7) |
| III | + | - | + | + | 2 (15.4) |
| IV | - | + | + | + | 1 (7.7) |
| V | - | - | - | - | 7(53.8) |

Three subclusters included six isolates; one subcluster included isolate S2 and S4 (producing AFB and AFG) with 98.87% GS. Two subclusters, first one included S6 (non- producing AFB or AFG) and S10 (producing AFB and AFG) with 98.41% GS, second included S1 and S5 (non-producing AFB and AFG) with 99.01% GS. There no was clear-cut relationship between clustering in the RAPD dendrogram and the ability of aflatoxin production or non-production of the tested isolates.

**Phenetic analysis of 13 *A. flavus* isolates obtained with RAPD primer 3**

Dendrogram analysis grouped the isolates into two main clusters at GS of 68.73% based on the banding pattern (Figure 3). The GSs between the tested isolates ranged from 68.73%-99.85%. The first main cluster included only one isolate S1 from Riyadh region. The second main cluster included two groups at 92.93% GS. First group included two isolates S11 and S13 from Riyadh region with 98.34% GS. Second group divided into two sub-group at 98.14% GS. First

one included two isolates S6 and S7 from Riyadh region with 99.18% GS. Second sub-group included overlapping for many subclusters with eight isolates. No correlation was found between RAPD data and geographic origin of the *A. flavus* isolates tested by using this primer.


**Fig. 1.** Map of Saudi Arabia indicating the governorates which sorghum grain samples were collected during March 2011

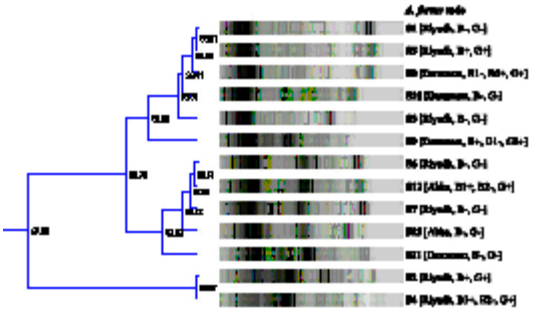

**Fig. 2.** Dendrogram obtained by UPGMA method derived from PCR amplification banding of RAPD with RAPD primer 1 of 13 *A. flavus* isolates.

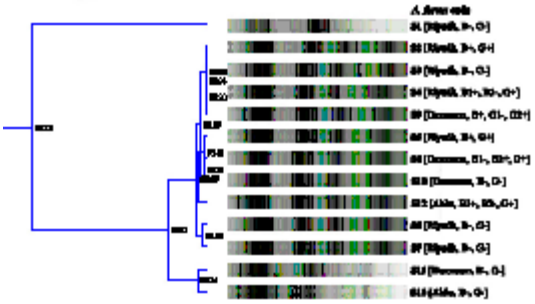

**Fig. 3.** Dendrogram obtained by UPGMA method derived from PCR amplification banding of RAPD with RAPD primer 3 of 13 *A. flavus* isolates.

Four subclusters included eight isolates; two subclusters included four isolates non-producing AFB and AFG, first one included isolate S6 and S7 (non-producing AFB and AFG) with 99.18% GSL. Second cluster S11 and S13 (non-producing AFB and AFG) with 98.34% GS. One subcluster included two isolates producing AFB and AFG, S8 and S10 with 99.39% GS. One subcluster included S3 (non-producing AFB or AFG) and S4 (producing AFB and AFG) with 99.94% GSL. There was clear-cut relationship between clustering in the RAPD dendrogram and the ability of aflatoxin production or non-production of the tested isolates.

**Phenetic analysis of 13 *A. flavus* isolates obtained with RAPD primer 5**

Dendrogram analysis grouped the isolates into two main clusters at 84.14% GS based on the banding pattern (Figure 4). The GSs between the tested isolates ranged from 84.14%-99.65% GS. The first main cluster included only one isolate S9 from Dammam region. Second main cluster included two groups at 92.02% GS. The first group included two groups at 85.70% GS. First group included one isolate S1 from Riyadh region, second group included two sub-groups at 94.1% GS. First sub-group included S8 and S10 from Dammam region with 95.90% GS. Second sub-group divided into two subclusters at 97.98% GS. First subcluster included isolates S6 and S7 from Riyadh region with 99.05% GS. Second sub-group included overlapping for many subclusters, contain seven isolates. In these subclusters, isolates S2, S3 and S5 from Riyadh region had more 99% GS. This fact indicating that this primer was able, for somewhat, to differentiated that regions. With reference to this primer, there was partial relationship between clustering in the RAPD dendrogram and geographic origin of the tested isolates, especially with Riyadh region.

Five subclusters included ten isolates; four subclusters included combination of aflatoxigenic and non-aflatoxigenic isolates for example, subcluster included two isolate S11 (non-producing AFB or AFG) and S12 (producing AFB and AFG) had 99.65% GS. One subcluster included two isolates (S6 and S7) non-producing AFB or AFG) with 99.05% GS. No correlation between RAPD banding patterns and the ability of aflatoxin production or non-production of the tested isolates.

The genetic relationships among the 13 *A. flavus* genotypes were also visualized by principle coordinate analysis of the RAPD data (Figure 5). The two-dimensional plot generated from PCA supports the clustering pattern of the UPGMA dendrogram.

**Phenetic analysis of 13 *A. flavus* isolates obtained with ISSR primer $(GTG)_5$**

Dendrogram analysis grouped the isolates into two main clusters at 75.28% GS based on the banding pattern (Figure 6). The GSs between the tested isolates ranged from 75.28%-99.88%. The first main cluster included two isolates S2 and S5 from Riyadh region with 78.62% GS. Second main cluster included two groups at 80.57% GS. First group included two subclusters at 89.36% GS. First one included only one isolates S9 from Dammam, second subcluster included overlapping for many subclusters. In these subcluster isolates S12 and S13 had more 98% GS. Second group included two subclusters at 83.44% GS. First subcluster included one isolate S4 from Riyadh, second subcluster included overlapping for many subclusters. In these subcluster isolates S1 and S3 had more 95% GS. This fact indicating this primer was able, for somewhat, to differentiated that regions. Also, with reference to this primer, there was partial relationship between clustering in the ISSR dendrogram and geographic origin of the tested isolates, especially with Riyadh and Abha regions. Three subclusters included six isolates; two subclusters included combination of aflatoxigenic and non-aflatoxigenic isolates for example, subcluster included two isolate S13 (non-producing AFB and AFG) and S12 (producing AFB or AFG) had 99.65% GS. One subcluster included two isolates S2 and S5 (producing AFB or AFG) with 78.62% GS. No correlation between RAPD banding patterns and the ability of aflatoxin production or non-production of the tested isolates.

**Phenetic analysis of ISSR profile of 13 *A. flavus* isolates obtained with ISSR primer $(GACA)_4$**

Dendrogram analysis grouped the isolates into two main clusters at 75.58% GS based on the banding pattern (Figure 7). The GSs between the tested isolates ranged from 75.58%-96.19%. The first main cluster included one isolate S6 from Riyadh region. Second main cluster included two groups at 81.06% GS. First group included three isolates (S2 and S4, from Riyadh region, S12 from Abha region. Isolate S2 and S4 had more 90% GS. Second group included two subclusters at 87.75% GS. First subcluster included three isolates (S10 and S11, from Dammam region, S3 from Riyadh region). Isolates S10 and S11 had more 90% GS.

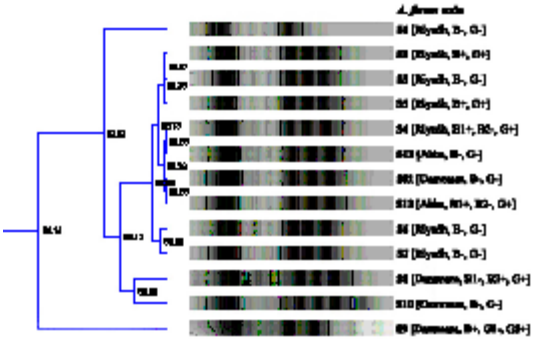

**Fig. 4.** Dendrogram obtained by UPGMA method derived from PCR amplification banding of RAPD with RAPD primer 5 of 13 *A. flavus* isolates

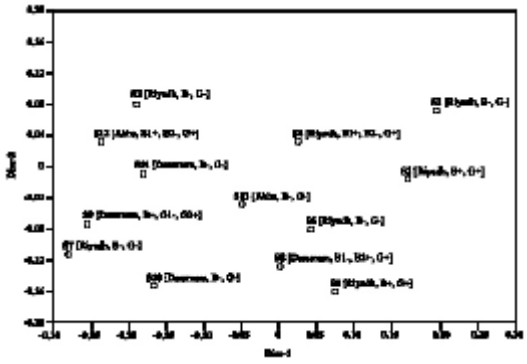

**Fig. 5.** Principle coordinate analysis for 3 RAPD primers used to profile 13 *A. flavus* genotype. The 3 RAPD primers used were RAPD primer 1, 3 and 5

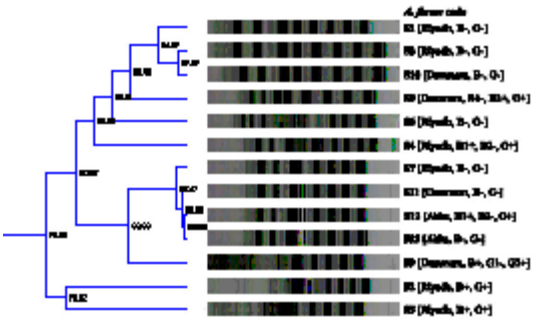

**Fig. 6.** Dendrogram obtained by UPGMA method derived from PCR amplification banding of ISSR with primer $(GTG)_5$ of 13 *A. flavus* isolates.

Second subcluster included many subclusters at 91.05% GS. Isolates S1, S7 and S5 from Riyadh region had more 95% GS. This fact indicating that this primer was able, for somewhat, to differentiated that regions. With reference to this primer, there was partial relationship between clustering in the RAPD dendrogram and geographic origin of the tested isolates, especially with Riyadh and Abha regions.

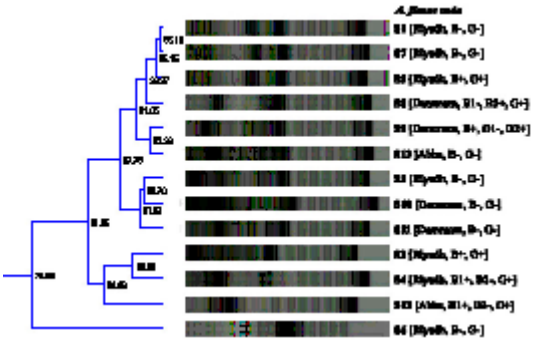

**Fig. 7.** Dendrogram obtained by UPGMA method derived from PCR amplification banding of ISSR with primer $(GACA)_4$ of 13 *A. flavus* isolates.

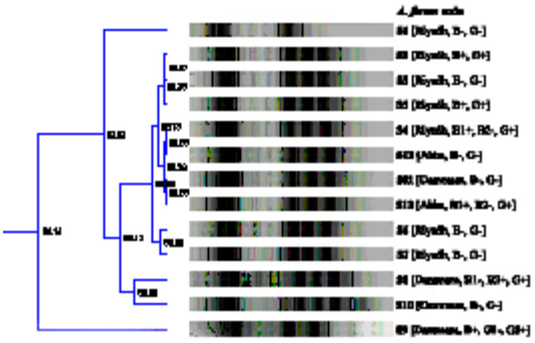

**Fig. 8.** Dendrogram obtained by UPGMA method derived from PCR amplification banding of ISSR with ISSR primer $(AGAG)_4G$ of 13 *A. flavus* isolates

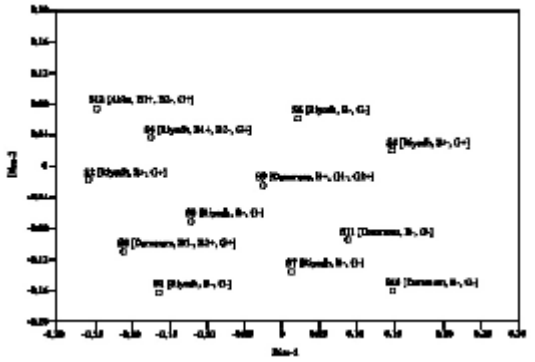

**Fig. 9.** Principle coordinate analysis for 3 ISSR primers used to profile 13 *A. flavus* genotype. The 3 ISSR primers used were ISSR primers $(GTG)_5$ , $(GACA)_4$ and $(AGAG)_4G$

Four subclusters included eight isolates; three subclusters included combination of aflatoxigenic and non-aflatoxigenic isolates for example, subcluster included two isolate S13 (non-producing AFB and AFG) and S12 (producing AFB or AFG) had 93.99% GS. One subcluster included two isolates (S1 and S7) non-producing AFB or AFG) with 78.62% GS. No correlation between ISSR banding patterns and the ability of aflatoxin production or non-production of the tested isolates.

**Phenetic analysis of 13 A. flavus isolates obtained with ISSR primer $(AGAG)_4G$**

Dendrogram analysis grouped the isolates into two main clusters at 76.74% GS based on the banding pattern (Figure 8). The GSs between the tested isolates ranged from 76.74%-99.52%. The first main cluster included one isolate S6 from Riyadh region. Second main cluster included two groups at 80.86% GS. First group included one isolate S4 from Riyadh region. Second group included two subgroups at 83.78% GS. First sub-group divided into two subclusters at 85.89% GS. First subcluster included S11 from Dammam region; second subcluster included overlapping for many subclusters. Isolates S8, S9 and S10 had more 98% GS. Second sub-group included two subclusters at 86.91% GS. Isolates S1, S3, S2, S5 and S7 from Riyadh had more 86.91% GS. With reference to this primer, there was clear-cut relationship between clustering in the ISSR dendrogram and geographic origin of the tested isolates.

Three subclusters included six isolates; two subclusters included combination of aflatoxigenic and non-aflatoxigenic isolates for example, subcluster included two isolate S5 (producing AFB and AFG) and S12 (non-producing AFB or AFG) had 99.52% GS. One subcluster included two isolates (S1 and S3) non-producing AFB or AFG) with 96.33% GS. No correlation between ISSR banding patterns and the ability of aflatoxin production or non-production of the tested isolates.

The genetic relationships among the 13 *A. flavus* genotypes were also visualized by

principle coordinate analysis of the ISSR data (Figure 9). The two-dimensional plot generated from PCA supports the clustering pattern of the UPGMA dendrogram.

## DISCUSSION

Our results indicated that the type of fungal contamination of the sorghum grains, at Saudi Arabia, was qualitatively similar to that found in other sorghum producing countries such as India (Sreenivasa *et al*., 2010), Nigeria (Abdulsalaam and Shenge, 2011), Saudi Arabia (Yassin *et al*., 2010; Mahmoud *et al*., 2013). Different of Recovery fungal genera from sorghum grains in many countries, could be attributed to prevailing climatic conditions, agronomic practices, the conditions of harvesting, handling and storage and transport (Setamou *et al*., 1997). In our study, the average frequencies of the most common genera were *Aspergillus* (10.3%), *Penicillium* (9.8%), *Fusarium* (9.5%) and *Alternaria* (7.7%). Most grain molding fungi are known to produce diverse toxic secondary metabolites generally called mycotoxins. The genera of most concern globally are *Aspergillus*, *Fusarium* and *Penicillium*. The major toxins produced by these three genera include: aflatoxins, ochratoxins, trichothecenes, fumonisins and zearalenone (Miller, 1998). The amount of toxin produced will depend on physical factors (temperature and mechanical damage, relative humidity and moisture), chemical factors (oxygen, carbon dioxide, pesticide and fungicides, composition of substrate,), and biological factors (stress, spore load, plant variety, insects) (Bryden, 2012).

The prevalence of AFs 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 15.3%, 38.5% and 30.8%, respectively. AF are produced by some isolates of *A. flavus*, but not all isolates were able to produce AFs, aflatoxigenic isolates produce blue fluorescence (B1 and B2) or green fluorescence (G and G2) on the reverse side of colonies under UV light, whereas non-aflatoxigenic isolates do not fluoresce (Davis *et al*., 1987). Our results indicated the 13 *A. flavus* isolates described in this study were classified into five major chemotypes, where chemotypes I, II, III, IV included AF producers (46.2%) and chemotype V included AF non-producers (53.8%). 17 *A. flavus* isolates were classified into four chemotypes (I to IV) based on their ability to produce B1, B2, G1 and G2. Chemotype I, II, II produced AFs comprised 76.4% of the isolates (three teen isolates). Chemotype II produced B1 and B2 was comprised 41.2% of the isolates (seven isolates). Seven isolates failed to produce any detectable amount of AFs (Chemotype IV) were comprised 41.2% (Abd El-Aziz *et al*., 2013). In this study, thirteen isolates of *A. flavus* were screened by HPLC for their ability to produce AFs. The percentage of aflatoxigenic *A. flavus* isolates was 46.2%. Six isolates produced AFs, at concentrations ranging 0.0-6.1 µg/ml. 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 sorghum grains (Yassin *et al*., 2010; Mahmoud *et al*., 2013).

Three primers were used to generate an RAPD dendrogram using the similarity matrix produced with the banding patterns obtained with RAPD. GS for RAPD primers 1, 3, and 5 were 67.28-99.01%, 68.73-99.85%, and 84.14-99.65%, respectively, for all isolates. 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 high level of genetic diversity. Regarding the geographic sources of the isolates, two relationships were found: first, there was no apparent correlation observed for primer RAPD 3 dendrogram; second, there was a partial relationship observed for RAPD primers 1 and 5 with the RAPD dendrograms, especially with isolates of Riyadh region, but no correlation was detected between the RAPD dendrograms and the AF-producing ability of the isolates. RAPD molecular markers used 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 (Batista *et al*., 2008). 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 used to characterization 19 *A. flavus* isolates form three different regions in Saudi Arabia.

RAPD analysis revealed a high level of genetic diversity in the *A. flavus* population, useful for genetic characterization. Clustering based on RAPD dendograms was unrelated to geographic origin (Al-Wadai *et al*., 2013). Many authors have reported detecting no correlation between DNA banding profiles and the production or nonproduction of AFs (Gashgari *et al*., 2010; Al-Wadai *et al*., 2013). We think RAPD primers failure to found clear-cut relationship between clustering in the RAPD dendrogram and the ability of AFs production or non-production of the tested isolates because the RAPD technique amplifies anonymous regions of the *A. flavus* genome, the regions that contain aflatoxin biosynthesis gene (regulatory and structural) may not have been amplified using this primers.

Three primers were used to generate an ISSR dendrogram using the similarity matrix produced with the banding patterns obtained with ISSR. GSs for ISSR primers $(GTG)_5$, $(GACA)_4$, and $(AGAG)_4G$ were 75.82-99.88%, 75.58-96.19%, and 76.74-99.52%, respectively, for all isolates. Considering the results obtained about the geographic sources of the *A. flavus* isolates, two relationships were found: first, there was no apparent correlation observed for primer $(AGAG)_4G$ cluster analysis; second, there was a partial relationship observed for ISSR primers $(GTG)_5$ and $(GACA)_4$ with the ISSR cluster analysis, especially with isolates of Abha and Riyadh region, but no correlation was detected between the ISSR cluster analysis and the AF-producing ability of the *A. flavus* isolates. 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. No correlation was found between 84 strains of *A. flavus* isolated from Vietnam and 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).

Clustering of 19 *A. flavus* isolates based on 3 RAPD dendrograms were appeared unrelated to geographic origin (Al-Wadai *et al*., 2013). High genetic diversity was observed in the 84 *A. flavus* strains, with no evident correlation being 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). 22 ISSR primers used to characterize the genetic diversity of 24 *A. flavus* isolates from peanut-cropped soils in China. ISSR technology is effective molecular approaches for studying characterize the genetic diversity. The 24 *A. flavus* isolates were not clustered based on their produces aflatoxin. Some toxigenic and atoxigenic isolates appeared in one cluster. For example, eight atoxigenic isolates and eight toxigenic isolates existed in the same cluster, indicated that atoxigenic and toxigenic *A. flavus* isolates were genetically similar based on their ISSR profiles (Zhang *et al*., 2013). The 19 *A. flavus* isolated from wheat amplified by 3 ISSR primers to discriminate between aflatoxigenic and non-aflatoxigenic isolates. The results indicated no correlation between ISSR banding patterns and the ability of aflatoxin production or non-production of the tested isolates (Al-Wadai *et al*., 2013).

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## REFERENCES

1. Abd El-Aziz, A.R.M., Mahmoud M.A., Al-Othman M.R. Genetic diversity of *Aspergillus flavus* contamination of sorghum grains. *J. Pure Appl. Microbiol.* 2013; **7**(Spl. Edn.), 369–378.
2. Abdulsalaam, S., Shenge, K.C. Seed borne pathogens on farmer-saved sorghum (*Sorghum bicolor* L.) seeds. *J. Stored Prod. Postharvest Res.* 2011; **2**: 24–28.

3. Al-Wadai, A.S., Al-Othman M.R., M.A. Mahmoud Abd El-Aziz, A.R.M. Molecular characterization of *Aspergillus flavus* and aflatoxin contamination of wheat grains from Saudi Arabia. *Genet. Mol. Res.* 2013**; 12**: 3335–3352.
4. Amer, O.E., Mahmoud, M.A., El-Samawaty, A.M., Sayed, R.M. Non liquid nitrogen-based-method for isolation of DNA from filamentous fungi. *Afr. J. Biotechnol*. 2011; **10**: 1437–1441.
5. Batista, P.P., Santos J.F., Oliveira N.T., Pires A.P., Motta C.M.S., Luna-Alves L. E.A. Genetic characterization of Brazilian strains of Aspergillus flavus using DNA markers. *Genet. Mol. Res*. 2008; **7**, 706–717.
6. Bryden, W.L. Mycotoxin contamination of the feed supply chain: Implications for animal productivity and feed security. *Anim. Feed Sci. Technol*. 2012; 173, 134–158.
7. Christian G. HPLC Tips and Tricks. Great Britain at the Iden Press, Oxford, London, UK. 1990.
8. Czerwiecki, L., Czajkowska, D., Witkowska-Gwiazdowska, A. On ochratoxin A and fungal flora in Polish cereals from conventional and ecological farms. Part 2: occurrence of ochratoxin A and fungi in cereals in 1998. *Food Addit. Contam*. 2002; **19**, 1051–1057.
9. David, M.S., Manfred, M., Leane, L. Mutagenicity of the mycotoxin patulin in cultured Chinese hamster V79 cells, and its modulation by intracellular glutathione. *Arch. Toxicol*. 2005; **79**: 110–121.
10. Davis, N.D., Iyer, S.K., Diener, U.L. Improved method of screening for aflatoxin with a coconut agar medium. *Appl. Environ. Microbiol.*1987; **53**: 1593–1595.
11. FAO. FAO Statistical Series, *Production year book*, vol **62**, Rome, 2008.
12. FAO. FAO Statistical Series, *Production year book*, vol **63**, Rome, 2009.
13. Franco, C.M., Fente, C.A., Vazquez, B.I., Cepeda, A. Mahuzier, G., Prognon, P. Interaction between cyclodextrins and aflatoxins Q1, M1 and P1. Fluorescence and chromatographic studies. *J. Chromatogr.* 1998; **A815**: 21–29.
14. Gashgari, R.M., Shebany, Y.M., Gherbawy, Y.A. Molecular characterization of mycobiota and aflatoxin contamination of retail wheat flours from Jeddah markets. *Foodborne Pathog. Dis.* 2010; **7**: 1047–1054.
15. Gonzalez, H.H., Resnik, S.L., Boca, R.T., Marasas, W.F. Mycoflora of Argentinian corn harvested in the main production area in 1990. Mycopathologia, 1995; **130**: 29–36.
16. Hatti, A.D., Taware, S.D., Taware, A.S., Pangrikar, P.P., Chavan, A.M., Mukadam, D.S. Genetic diversity of toxigenic and non-toxigenic *Aspergillus flavus* strains using ISSR markers. *Int. J. Curr. Res.* 2010; **5**: 61–66.
17. IARC. The Evaluation of the Carcinogenic Risk of Chemical to Humans. In: *IARC Monograph Supplement*, vol. 4. International Agency for Research on Cancer, Lyon, 1982.
18. Irshad, S.; Nawab, R. Molecular characterization of seven different species of *Aspergillus* through random amplified polymorphic DNA (RAPD) and enzyme analysis. *J. Microbiol. Res.* 2012; **2**: 47–50.
19. Mahmoud, M.A.; Al-Othman M.R.; Abd El-Aziz A.R.M. Mycotoxigenic fungi contaminating corn and sorghum grains in Saudi Arabia. *Pak. J. Bot*. 2013; **45**: 1831–1839.
20. Mahmoud, M.A., Al-Sohaibani, S.A., Abdelbacki, A.M., Al-Othman, M.R., Abd El-Aziz, A.R.M., Kasem, K.K., Mikhail, M.S., Sabet, K.K., Omar, M.R.; Hussein E.M. Molecular characterization of the pathogenic plant fungus Rhizoctonia solani (Ceratobasidiaceae) isolated from Egypt based on protein and PCR RAPD profiles. *Genet. Mol. Res.* 2012; **11**: 3585–3600.
21. Martins Reis, G., Rocha, L.O., Atayde, D., Batatinha, M.J., Corrêa, B. Molecular characterization by amplified fragment length polymorphism and aflatoxin production of *Aspergillus flavus* isolated from freshly harvested peanut in Brazil. *World Mycotoxin Journal*, **2012**; **5**: 187–194.
22. Miller, J.D. Global significance of mycotoxins. In: Miraglia, M., van Egmond, H., Brera, C., Gilbert, J. (Eds.), *Mycotoxins and Phycotoxins-Developments in Chemistry*, Toxicology and Food Safety.1998, Alaken Inc, Ford Collins, CO, pp. 3–15.
23. Mohankumar, M., Vijayasamundeeswari, A., Karthikeyan, M., Mathiyazhagan, S., Paranidharan, V., Velazhahan, R. Analysis of molecular variability among isolates of *Aspergillus Flavus* by PCR-RFLP of theits regions of rDNA. *J. Plant Prot. Res.* 2010; **50**: 446–451.
24. Palanee, T., Dutton, M.F., Chuturgoon, A. Cytotoxicity of aflatoxin B1 and its chemically synthesized epoxide derivative on the A459 human epithelioid lung cell line. *Mycopathologia*, 2001; **151**: 155–159.
25. Pascale, M. Detection methods for mycotoxins in cereal grains and cereal products. Proc. Nat. Sci. Matica Srpska Novi Sad. 2009; **117**: 15-25.
26. Pildain, M.B., Frisvad, J.C., Vaamonde, G., Cabral, D., Varga, J., Samson, R.A. Two novel

aflatoxin-producing Aspergillus species from Argentinean peanuts. *Int. J. Syst. Evol. Microbiol.* 2008; **58**: 725–735.
27. Raper K.B., Fennell D.I. *The Genus of Aspergillus*, Baltimore: Williams & Wilkins Company, USA.1965.
28. Setamou, M., Cardwell, K.F., Schulthes, F., Hell, K. *Aspergillus flavus* infection and aflatoxin contamination of preharvest maize in Benin. *Plant Dis.* 1997; **81**: 1323–1327.
29. Sreenivasa, M.Y., Dass, R.S., Janardhana, G.R. Survey of postharvest fungi associated with sorghum grains produced in karnataka (India). *J. Plant prot. Res.* 2010; **50**: 335–339.
30. Tran-Dinh, N., Kennedy, I., Bui, T., Carter, D. Survey of Vietnamese peanuts, corn and soil for the presence of *Aspergillus flavus* and *Aspergillus parasiticus*. Mycopathologia, 2009; **168**: 257–268.
31. Yassin, M.A., El-Samawaty, A., Bahkali, A., Moslem, M., Abd-Elsalam, K.A., Hyde K.D. Mycotoxin-producing fungi occurring in sorghum grains from Saudi Arabia. Fungal Divers. 2010; **44**: 45–52.
32. Zhang, C.S., Xing, F.G., Selvaraj, J.N., Yang, Q.L., Zhou, L., Zhao, Y.J., Liu, Y. The effectiveness of ISSR profiling for studying genetic diversity of Aspergillus flavus from peanut-cropped soils in China, *Biochem. Syst. Ecol*. 2013; **50**: 147–153.