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# Genetic Diversity of Aflatoxin Producing *Aspergillus flavus* Contamination of Rice Grains from Saudi Arabia

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**Twenty *Aspergillus flavus* isolates were isolated from 72 rice grain samples collected from three main regions at Saudi Arabia. Potential ability to produce aflatoxins (AFs) B1, B2, G1 and G2 was studied by isolate culture followed by HPLC analysis of these AFS in the culture extracts. Results indicated that 65% of the *A. flavus* strains produced AFs. Most of the *A. flavus* strains presented moderate toxigenicity with mean levels of AFs ranging from 0.2 µg/Kg to 2.5 µg/kg. The isolates were classified into five chemotypes based on the ability to produce AF that majority of them (30%) belonged to chemotype V comprising non-aflatoxigenic isolates. Inter-simple sequence repeats (ISSR), and random amplified polymorphic DNA (RAPD) molecular markers were used, with the aim of genetic diversity and discriminate aflatoxigenic from non-aflatoxigenic isolates. RAPD and ISSR analysis revealed a high level of genetic diversity in the *A. flavus* population and useful for genetic characterization. RAPD and ISSR were successes to discriminate aflatoxigenic from non-aflatoxigenic isolates with different percentage, but ISSR was more sensitive to discriminate aflatoxigenic from non-aflatoxigenic isolates.**

**Key words**: Genetic diversity, *Aspergillus flavus*, rice, RAPD, ISSR.

Rice (*Oryza sativa* L.) is a very important foodstuff for millions of people in the world. It is the dominant grain for half of the world population and provides 20% of the world's dietary energy supply, with wheat and maize supplying 19 and 5%, respectively (FAO, 2004). Saudi Arabia is second of the ten major rice-importing countries. Others include Philippines, Malaysia, Côte d'Ivoire, Iran, Iraq, United Arab Emirates, South Africa, United States of America and Cameroon. Saudi Arabia imported 1.25 million tones on the average annually (IRRI, 2010). The worldwide contamination of foods and feeds with mycotoxins poses a significant health problem (Moss, 1996). The Food and Agriculture Organization (FAO) estimates that at least 25% of the world cereal production is contaminated with mycotoxins (Dowling, 1997). The established presence of these major mycotoxins and the fungi that produce them in rice in several parts of the world (Reddy *et al*. 2008). Fungi are a normal part of the microflora of stored crops, but the contamination by mycotoxins depends upon the fungi present, agronomic practices, the composition of the commodity and the conditions of harvesting, handling, and storage and shipment (Bryden, 2009). There are hundreds of mycotoxins found in foods, but those that pose the greatest risk to human and animal health are aflatoxins (AFs), trichothecenes [e.g. deoxynivalenol (DON), T-2 toxin], fumonisins (FBs), zearalenone (ZEA), patulin (PAT) and ochratoxin A (OTA) (CAST 2003). AFs are toxic secondary metabolites produced by many species of

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*Aspergilli*. Among 22 closely related species in *Aspergillus* section *Flavi* which are grouped in seven clades, *Aspergillus flavus* and *Aspergillus parasiticus* are the most important AF-producers frequently encountered in agricultural products (Godet and Munaut 2010; Varga *et al*. 2011). The four major naturally occurring AFs are known as type B (AFB1 and AFB2) and types G (AFG1 and AFG2), AFB1 which is listed as a group I carcinogen by the International Agency for Research on Cancer (IARC, 1982). *Aspergillus* spp. are common contaminants of stored rice (Oh *et al*. 2010; Park *et al*. 2005; Sales and Yoshizawa 2005). RAPD markers have found a wide range of applications in genetic diversity, characterization, genetic structure of populations and genome mapping. This is mainly due to the speed, cost and efficiency of the RAPD technique to generate large numbers of markers in a short period compared with other methods (Bardakci, 2001). Besides, with RAPD, the appearance of new ligation sites only creates new annealing sites without impairing pre-existing ones, which would continue to be amplified. RAPD markers were detecting genetic variability between the isolates of *A. flavus* and related species (Batista *et al*., 2008; Reddy *et al*., 2009a; Irshad and Nawab, 2012). RAPD markers were used to discriminate between aflatoxigenic from non-aflatoxigenic isolates of *A. flavus* (Lourenço *et al*., 2007; Gashgari *et al*., 2010). ISSR markers are highly polymorphic and are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology (Reddy *et al*., 2002). Inter simple sequence repeat (ISSR) technique is a PCR based method, which involves amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions. Different isolates can be distinguished from each Other based on differences in repeat numbers. All repeat numbers of the analyzed markers form a genotype for each individual isolate. These genotypes are easily compared to each other (Hadrich *et al*., 2011). ISSR markers were very useful for investigating the diversity and population structure of *A. flavus* and related species (Tran-Dinh and Carter, 2000; Batista *et al*., 2008; Hadrich *et al*., 2010; Neal *et al*. 2011). ISSR markers were used to determine similarity and dissimilarity between aflatoxigenic from non-aflatoxigenic isolates of *A. flavus* (Hatti *et al*., 2011).

## MATERIALS AND METHOD

### Sampling of rice

The market survey of rice grain samples was conducted from 15 April 18 May to 2011. A total of 72 rice samples were randomly collected from market in three regions consider cardinal direction for every region (Table 1). The market survey of rice samples were divided Saudi Arabia into the eastern region (Dammam), where the weather was hot (30–45°C) and high humid, middle region (Riyadh) where it was hot (30-45°C) and dried and south western region (Abha) 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. To avoid sampling error due to the highly heterogeneous nature of fungal distribution, each 1 kg composite sample collected from one market was a mixture of 4 sub-samples (250 g each).

### Isolation of *A. flavus* from rice sample

Fungal isolation from collected rice samples was carried by using direct plating method. 10 grains were inoculated randomly in each of petri plates containing potato dextrose agar (PDA) medium and Rose Bengal at 50 ppm (Cotty, 1994). The plates were incubated at room temperature and the presence of *A. flavus* was observed after 6 days. The *A. flavus* isolated from samples were further purified individually by sub culturing PDA slants. They were then identified according to Raper and Fennell (1965) and Klich (2002).

### Detection of Aflatoxin Production

Aflatoxin production was assessed by growing twenty isolates of *A. flavus* on sterilized different agar media (potato dextrose agar (PDA), Czapek agar (CZ) and Malt extract agar (MEA) at 25 °C for 4 days (Davis *et al*; 1987). Three replications were maintained for each isolate for each media. After that observed colonies under long wavelength (365 nm) ultraviolet light. The appearance of intense fluorescence around fungal colonies was presumptive evidence that a strain could produce aflatoxin.

### HPLC determination of AF

Isolates were grown on sterilized SMKY liquid medium (sucrose, 20 g, magnesium sulfate 0.5 g, potassium nitrate 3 g, yeast extract 7 g and distilled water, 1000 ml) (Davis *et al*., 1987). The

flasks were inoculated with discs of 6 mm diameter of the toxigenic *Aspergillus* spp. at 25 ± 2°C for 7 days (Paranagama *et al*., 2003) three replicates were performed. After incubation, content of each flask was filtered (Whatman, No. 1). For aflatoxins extractions, the filtrates of each flask were treated three times with 50 ml of chloroform in a separating funnel. The chloroform extract was separated and dehydrated with anhydrous sodium sulfate and evaporated till dryness on water bath at 50°C under vacuum. The residues were dissolved in 10 ml methanol and stored in dark vials.

The extract was passed through a 0.45 µm micro-filter. Analysis of compounds was performed on HPLC model (model PerkinElmer series 200 UV/VIS) with a C18 column with an internal diameter of 300 mm x 3.9 mm, 4 micron. The HPLC was equipped with an UV detector and fluorescence with 365 nm excitation and 430 emission wavelengths. The mobile phase consisting 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 on double layer media in 50 mm Petri dishes, one solid and the other liquid. Base media solid, was potato dextrose agar as a film, and the top media, liquid, was peptone yeast glucose (PYG, 1200 µl). Fungi were incubated at 25°C for two days. Fungi mycelia (50 mg) were scraped using slides covers and transferred to sterile Eppendorf tubes (1.5 ml) for DNA isolation. DNA was extracted from 50 mg fresh mat according to Amer *et al*. (2011).

**RAPD by PCR**

In order to identify the best primers to establish the RAPD profile, we tested 6 primers from the standard RAPD primer Kits (Amersham Pharmacia), using total DNA from *A. flavus* isolates as template. The three primers with high reproducibility and clear banding profiles including RAPD primer 1, RAPD primer 3 and RAPD primer 5 were selected. The PCR mixtures were made to a final volume of 25 µL, containing reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 3.4 mM $MgCl_2$, 0.25 mM dNTP, 0.4 mM of each primer, 2 U *Taq* DNA polymerase (BioLabs)) and 25 ng genomic DNA. Amplification 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) as described in Mahmoud *et al*. (2011). The primer tested is shown in Table 2.

**ISSR by PCR**

PCR amplification of ISSR was performed with $(GTG)_5$, $(GACA)_4$ and $(AGAG)_4G$ primers. The reaction mixture were made to a final volume of 25 µL, containing reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.25 mM dNTP, 0.25 mM of the primer, 1.25 U *Taq* DNA polymerase (BioLabs) and 25 ng genomic DNA (Williams *et al*., 1990). Amplification 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 as described in Batista *et al*. (2008). The primer tested is shown in Table 2.

**DNA electrophoresis**

For all samples, the amplified DNA (15 µL) was electrophoresed using electrophoresis unit (wide mini-sub-cell GT Bio-RAD) on 2% agarose containing ethedium bromide (0.5 µg/mL), at a constant 75 volt and 60 mA, and visualized with UV trans-illuminator.

**Gel analysis for DNA**

DNA gel was scanned for band Rf using gel documentation system (AAB Advanced American Biotechnology 1166 E. Valencia Dr. Unit 6 C, Fullerton, CA 92631). The different molecular weights of bands were determined against a DNA standard (kb DNA ladder, Stratagene, Canada) with molecular weights 250, 500, 750, 1.000, 1.500 and 2.000 bp. The similarity level was determined by un-weighted pair group method based on arithmetic mean (UPGMA).

## RESULTS

**Detection of AFs Production**

Three culture media included Cazpek's agar, Potato dextrose agar (PDA) and yeast extract sucrose agar (YES agar) were used to screen examined for aflatoxin production. The appearance of intense fluorescence around fungal colonies was presumptive evidence that a strain could produce aflatoxin. Blue/violet fluorescence indicated that a strain was able to produce B aflatoxin only, while a

blue/white fluorescence indicated that a strain produced both B and G aflatoxins. Data in (Table 3) show that fourteen isolates of *A. flavus* expressed as positive for aflatoxin production while six isolates *A. flavus* expressed as negative.

**AFs production**

Fourteen isolates were capable of producing detectable levels of both B and G aflatoxins, although six isolates failed to produce any detectable amount (Table 4). The highest level (1.8 and 2.1 ppb) of B1 and B2 were obtained from isolate R9. Isolate R17 was the highest producer (10.7 ppb) of G1 while isolate R18 was the highest producer (0.3 ppb) of G2.

**Chemotype patterns**

*A. flavus* isolates were classified into eight chemotypes (I to V) based on their ability to produce B1, B2, G1 and G2 (Table 5). Chemotype II (produced B1 and B2) and Chemotype V (non produced B1, B2, G1 and G2) were the prominent groups comprising 60% of the isolates. Four isolates only produced all AFs (chemotype I) was the second prominent group comprising 20% of the isolates. Three isolates produced B1 only (chemotype I) was the third prominent group comprising 15% of the isolates. Finally chemotype III (produced B1and G2) comprising 5% of the isolates.

**RAPD profiles**

A total of 20 *A. flavus* isolates were subjected to RAPD analysis based on their source (Riyadh, Dammam and Abha regions) and aflatoxin-producing ability (AFB and AFG). The initial tests to select the best RAPD primers were performed on a small number of *A. flavus* isolates. Three of the six tested primers that produced clear and reproducible banding patterns were selected for the RAPD amplification of twenty *A. flavus* isolates. The isolates produced between seven and ten clear and reproducible bands. A total of 189 bands of between 300 and 2,030 bp were scored and used in the analysis. The RAPD profiles displayed significant differences within some *A. flavus* isolates.

**Phenetic analysis using three RAPD primers**

**Phenetic analysis of the 20 *A. flavus* isolates based on RAPD primer 1**

The dendrogram generated using the similarity matrix produced from the banding patterns obtained with RAPD primer 1 exhibited genetic similarity level (GSL) between the tested isolates ranged from 11%-87% (Fig. 2). The dendrogram generated the two major clusters, with an 11% (GSL). The first major cluster comprised

**Table 1.** Governorates, locations, sources of rice samples collected in this study the number of varieties covered

| Governorate | Location | No. of sample |
|---|---|---|
| Riyadh | North | 6 |
| | South | 6 |
| | East | 6 |
| | west | 6 |
| Dammam | North | 6 |
| | South | 6 |
| | East | 6 |
| | west | 6 |
| Abha | North | 6 |
| | South | 6 |
| | East | 6 |
| | west | 6 |
| Total | | 72 |

**Table 2.** Primers used to amplify *A. flavus* DNA.

| Primer code | RAPD primers | | |
|---|---|---|---|
| | Sequence | Amplified region | References |
| RAPD primer 1 | 52 -GGT GCG GGA A-32 | fragments from 1-10 genomic sites simultaneously | Mahmoud et al, 2012 |
| RAPD primer 2 | 52 -GTT TCG CTC C-32 | | |
| RAPD primer 5 | 52 -AAC GCG CAA C-32 | | |
| Primer code | ISSR primers | | |
| | Sequence | Amplified region | References |
| $(GTG)_5$ | 52 -GTG GTG GTG GTG GTG-32 | Minisatellite-region DNA | Batista et al., 2008 |
| $(GACA)_4$ | 52 -GAC AGA CAG ACA GAC A-32 | | |
| $(AGAG)_4G$ | 52 -AGA GAG AGA GAG AGA GG-32 | | |

only one isolate R10 from the Dammam region. The second major was divided into two minor cluster with a 13% GLS. The first minor cluster included two isolates R4 and R7 from the Riyadh region, which displayed a 20% GSL. The second minor was divided into two cluster with an 18% GLS. The first cluster contained isolates R15, R11 and R14 form the Dammam region and R5 from the Riyadh region with a 22% GLS. The second cluster contained thirteen isolates form the Riyadh, the Dammam and the Abha regions ranged from 19-87% GLS. These data indicate that this primer was not able to differentiate between isolates according to geographic regions.

**Table 3.** Detection of Aflatoxigenic *A. flavus* isolates under long wavelength (365 nm) ultraviolet light

| *A. flavus* code | PDA+NaCl | Czapacks | YES |
|---|---|---|---|
| Riyadh region | | | |
| R1 | - | + | + |
| R2 | + | + | + |
| R3 | - | - | + |
| R4 | - | - | - |
| R5 | - | - | + |
| R6 | - | - | + |
| R7 | - | - | + |
| R8 | - | - | - |
| Dammam region | | | |
| R9 | - | - | + |
| R10 | - | - | + |
| R11 | - | + | - |
| R12 | - | - | + |
| R13 | - | - | + |
| R14 | - | - | - |
| R15 | - | - | - |
| Abha region | | | |
| R16 | - | - | + |
| R17 | - | - | + |
| R18 | + | + | + |
| R19 | - | - | - |
| R20 | - | - | - |

Czapeks: Czapeks Dox agar, YES: yeast extract sucrose agar, PDA: potato dextrose agar

**Table 4.** Aflatoxin production by *A. flavus* isolated from rice sample collected from Riyadh, Dammam and Abha regions

| *A. flavus* code | Aflatoxin (µg/kg) | | | |
|---|---|---|---|---|
| | B1 | B2 | G1 | G2 |
| Riyadh region | | | | |
| R1 | 0.5 | 1.3 | 0.0 | 0.0 |
| R2 | 1.2 | 0.7 | 0.0 | 0.0 |
| R3 | 0.4 | 0.3 | 0.2 | 0.4 |
| R4 | 0.0 | 0.0 | 0.0 | 0.0 |
| R5 | 1.8 | 1.6 | 0.0 | 0.0 |
| R6 | 1.3 | 0.0 | 0.0 | 0.0 |
| R7 | 1.5 | 0.9 | 0.0 | 0.0 |
| R8 | 0.0 | 0.0 | 0.0 | 0.0 |
| Dammam region | | | | |
| R9 | 1.8 | 2.1 | 0.0 | 0.0 |
| R10 | 1.2 | 0.8 | 0.5 | 0.6 |
| R11 | 2.5 | 0.6 | 0.0 | 0.0 |
| R12 | 1.8 | 0.0 | 0.0 | 0.0 |
| R13 | 1.4 | 0.0 | 0.5 | 0.0 |
| R14 | 0.0 | 0.0 | 0.0 | 0.0 |
| R15 | 0.0 | 0.0 | 0.0 | 0.0 |
| Abha region | | | | |
| R16 | 1.3 | 0.0 | 0.0 | 0.0 |
| R17 | 0.6 | 0.3 | 0.7 | 0.2 |
| R18 | 1.4 | 0.7 | 0.5 | 0.3 |
| R19 | 0.0 | 0.0 | 0.0 | 0.0 |
| R20 | 0.0 | 0.0 | 0.0 | 0.0 |

**Table 5.** Chemotype patterns of *A. flavus* isolated from Riyadh, Dammam and Abha regions based on the ability for producing aflatoxins (AF) B1, B2, G1 and G2

| Chemotype | Aflatoxin | | | | No. (%) |
|---|---|---|---|---|---|
| | B1 | B2 | G1 | G2 | of isolates |
| I | + | + | + | + | 4 (20.0) |
| II | + | + | - | - | 6 (30.0) |
| III | + | - | + | - | 1 (05.0) |
| IV | + | - | - | - | 3 (15.0) |
| V | - | - | - | - | 6 (30.0) |

The five subclusters included ten isolates. These subclusters were divided into two aflatoxigenic (B and/or G producing isolates) subclusters and three combination of aflatoxigenic and non-aflatoxigenic (B and/or G non producing isolates) clusters (mixed cluster). Two aflatoxigenic subclusters contained four isolates. For example one subcluster included isolates R1 and R2 which displayed an 87% GLS. Three mixed subclusters comprised six isolates. For example one subcluster included isolates R19 and R15 which displayed a 37% GLS. These findings indicated that this primer was succeeding to discriminate aflatoxigenic from non-aflatoxigenic isolates with 40%.

**Phenetic analysis of the 20 *A. flavus* isolates based on RAPD primer 3**

The dendrogram generated using the similarity matrix produced from the banding patterns obtained with RAPD primer 3 exhibited GSLs between the tested isolates ranged from 54%-83% (Figure 3). The dendrogram generated the two major clusters, with a 54% (GSL). The first major cluster comprised only one isolate R15 from the Dammam region. The second major was divided into two minor cluster with a 58% GLS. The first minor cluster included isolates R3, R4 and R5 from the Riyadh region, with a 79% GLS. The second minor cluster was divided into two clusters which


**Fig. 1.** Map of Saudi Arabia indicating the governorates which rice grain samples were collected between 15 April 18 May 2011

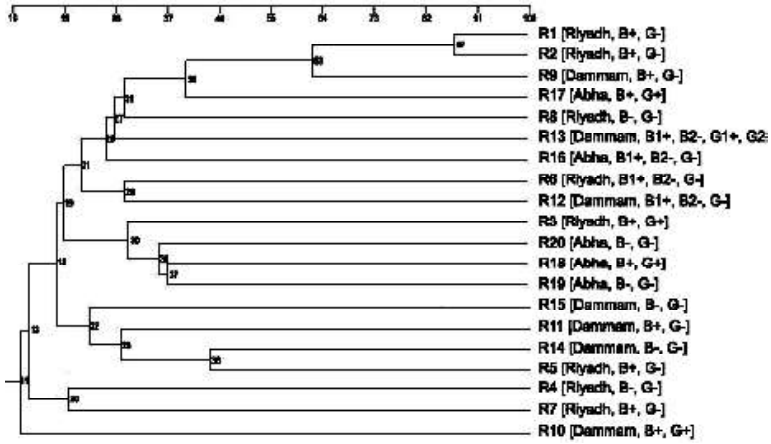


**Fig. 2.** Dendrogram obtained by the UPGMA method derived from PCR-amplified RAPD banding using RAPD primer 1 for 20 *A. flavus* isolates

showed a 60% GLS. The first cluster comprised isolates R18, R16 and R17 from Abha region with a 70% GLS. The second cluster comprised thirteen isolates from the Riyadh, the Dammam and the Abha regions ranged 62-83% GLS. These data indicate that this primer was able to differentiate between isolates according to geographic regions.

The four subclusters included nine isolates. These subclusters were divided into two aflatoxigenic subclusters and three mixed cluster. One aflatoxigenic subclusters contained two isolates. For example one subcluster included isolates R16 and R17 which showed a 73% GLS. Three mixed subclusters comprised seven isolates. For example one subcluster included isolates R1 and R8 which showed an 83% GLS. These findings indicated that this primer was succeeding to discriminate aflatoxigenic from non-aflatoxigenic isolates with 25%.

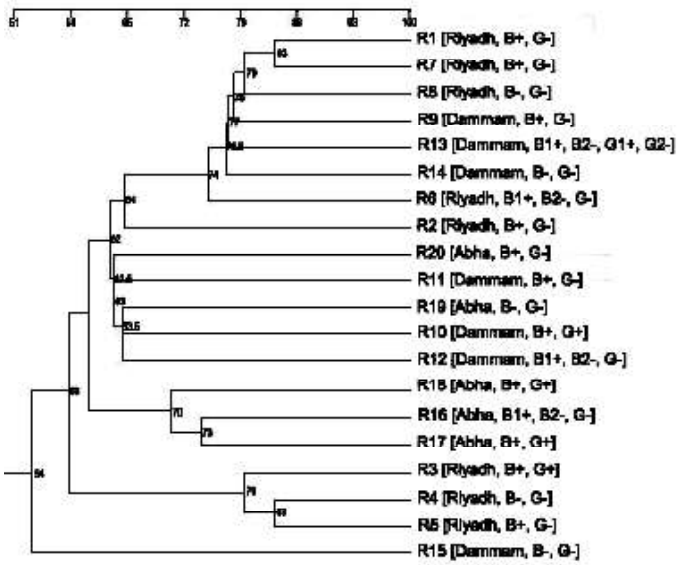


**Fig. 3.** Dendrogram obtained by the UPGMA method derived from PCR-amplified RAPD banding using RAPD primer 3 for 20 *A. flavus* isolates

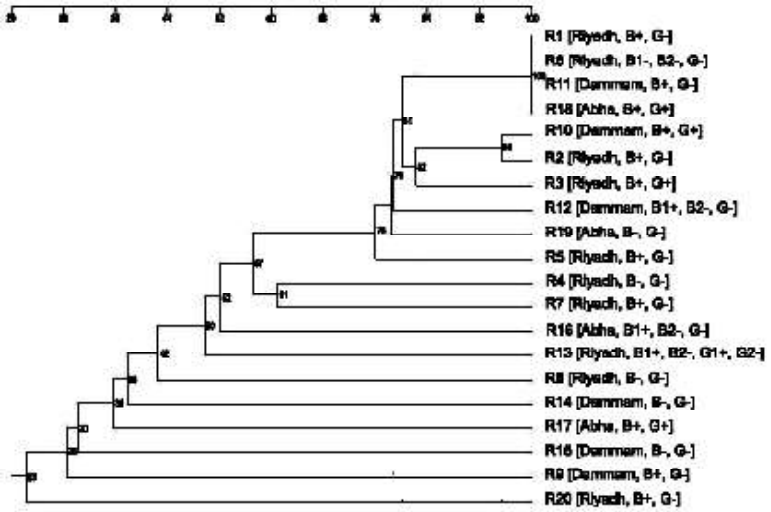


**Fig. 4.** Dendrogram obtained by the UPGMA method derived from PCR-amplified RAPD banding using RAPD primer 5 for 20 *A. flavus* isolates.

**Phenetic analysis of the 20 *A. flavus* isolates based on RAPD primer 5**

The dendrogram generated using the similarity matrix produced from the banding patterns obtained with RAPD primer 5 exhibited GSL between the tested isolates ranged from 23-100% (Figure 4). The dendrogram generated the two major clusters, with a 23% GSL. The first major cluster included only one isolate R20 from the Abha region. The second major included overlapping of many clusters. These data indicate that this primer was not able to differentiate between isolates according to geographic regions.

The three mixed subclusters included eight isolates. For example one subcluster included isolates R1, R14, R8 and R18 with a 100% GLS. These findings indicated that this primer was failure to discriminate aflatoxigenic from non-aflatoxigenic isolates.

**ISSR profiles**

A total of 20 *A. flavus* isolates were subjected to ISSR analysis based on their source (Riyadh, Dammam and Abha regions) and aflatoxin-producing ability (AFB and AFG). The initial tests to select the best ISSR primers were performed on a small number of *A. flavus* isolates. Three of the six

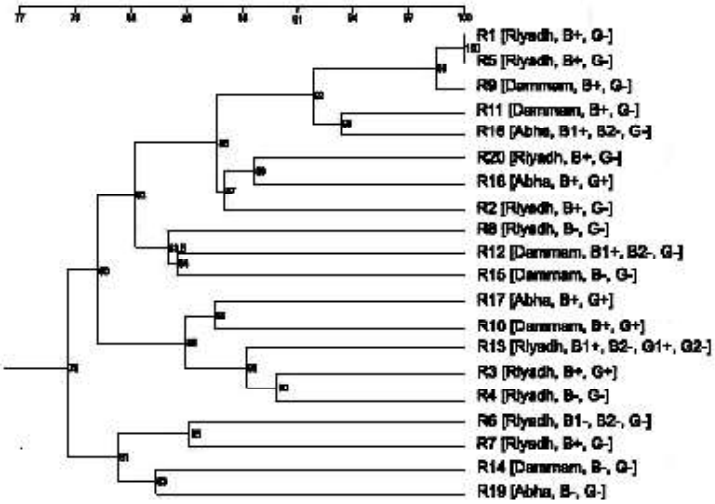


**Fig. 5.** Dendrogram obtained by the UPGMA method derived from PCR-amplified ISSR banding using primer (GTG)5 for 19 *A. flavus* isolates

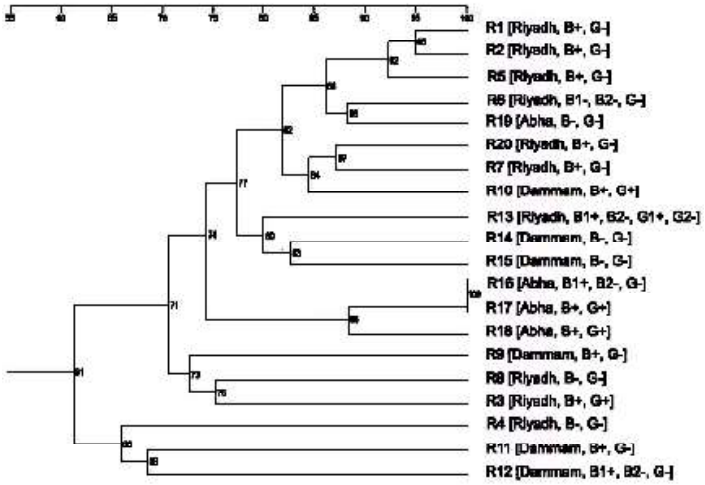


**Fig. 6.** Dendrogram obtained by the UPGMA method derived from PCR-amplified ISSR banding using primer $(GACA)_4$ for 19 *A. flavus* isolates

tested primers produced clear and reproducible banding patterns and were selected for the ISSR amplification of twenty *A. flavus* isolates. The isolates produced between ten and seventeen clear and reproducible bands. A total of 985 bands ranging from 200 to 2,070 bp were scored and used in the analysis. The ISSR profiles displayed significant differences within some *A. flavus* isolates.

**Phenetic analysis based on three ISSR primers**

**Phenetic analysis of 19 *A. flavus* isolates using 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 major clusters, with a 78% GSL (Figure 5). The GSL between the tested isolates ranged from 78-100%. The first major cluster comprised two subclusters with exhibiting an 81% GLS. The first subcluster comprised isolates R14 from the Dammam region and R19 from Abha region which displayed 83% GLS. The second subcluster included isolates R6 and R7 from the Riyadh region which displayed 85% GLS. The second major was divided into two minor clusters with an 80%. The First minor was divided into one cluster and one subcluster with an 85% GLS. The second minor was divided into two clusters with an 82% GLS. In reference to this primer, there was a strong relationship found between the ISSR dendrogram and the geographic origin of the tested isolates, especially with the Riyadh region.

The eight subclusters included sixteen isolates. These subclusters were divided into five aflatoxigenic subclusters, one non-aflatoxigenic subcluster and two mixed clusters.

The five aflatoxigenic subclusters for example, subcluster included isolates R1 and R5 which displayed a 100% GLS. The one non-aflatoxigenic subcluster for example, subcluster contained R14 and R19 which showed an 83% GLS. The two mixed subclusters for example, subcluster comprised R12 and R15 which showed an 84% GLS. These findings indicated that this primer was succeeding to discriminate aflatoxigenic from non-aflatoxigenic isolates with 75%.

**Phenetic analysis of 19 *A. flavus* isolates using ISSR primer $(GACA)_4$**

The dendrogram generated using the similarity matrix produced with the banding patterns obtained with the $(GAGA)_4$ primer exhibited two major clusters, with an 61% GSL (Figure 6). The GSLs between the tested isolates ranged from 61%-100%. The first major cluster included isolates R4 from the Riyadh region, R11 and R12 from the Dammam region showing a 66% GSL. The second major cluster was divided into two minor clusters, displaying a 71% GSL. The first minor cluster included isolates R9 from the Dammam region and R8 and R3 from the Riyadh region with a 73% GSL.

The second minor cluster was divided into two clusters, exhibiting a 74% GSL. The first cluster comprised R16, R17 and R18 from the Abha region, which presented an 88% GLS. The second cluster comprised eleven isolates from the Riyadh, the Dammam and the Abha region, ranged from a 77-95% GLS. In reference to this primer, there was a strong relationship found between the ISSR

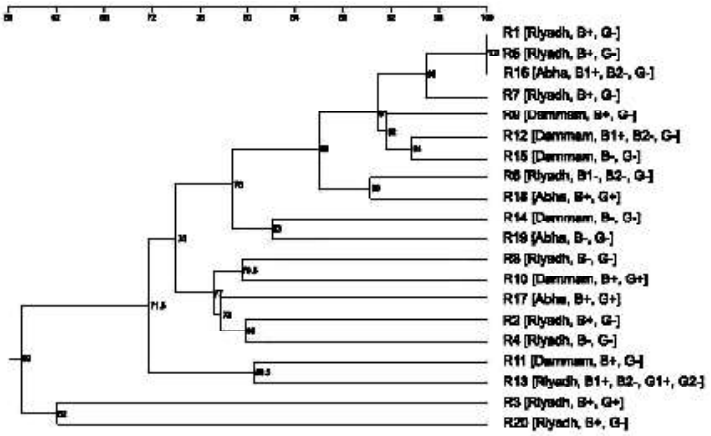


**Fig. 7.** Dendrogram obtained by the UPGMA method derived from PCR-amplified ISSR banding using primer $AGAG)_4G$ for 19 *A. flavus* isolates

dendrogram and the geographic origin of the tested isolates, especially with the Riyadh and Abha regions.

The seven subclusters included fourteen isolates. These subclusters were divided into four aflatoxigenic subclusters, one non- aflatoxigenic subcluster and two mixed clusters. The four aflatoxigenic subclusters for example, subcluster included isolates R16 and R17 which showed a 100% GLS. The one non- aflatoxigenic subcluster for example, subcluster comprised isolates R14 and R15 which presented an 83% GLS. The two mixed subclusters for example, subcluster contained isolates R8 and R3 which showed a 75% GLS. These findings indicated that this primer was succeeding to discriminate aflatoxigenic from non-aflatoxigenic isolates with 71.5%.

**Phenetic analysis of 19 *A. flavus* isolates using ISSR primer $(AGAG)_4G$**

The dendrogram generated using the similarity matrix produced from the banding patterns obtained with primer $(AGAG)_4G$ showed two major clusters, with a 59% GSL (Figure 7). The GSLs between the tested isolates ranged from 59%-100%. The first major cluster included isolates R3 from the Riyadh R20 from the Abha region, which showed a 62% GSL. The second major was divided into two minor clusters, which displayed a 71.5% GSL. The first minor included isolates R11 and R13 from the Dammam region, which presented an 80.5% GSL. The second minor was divided into two clusters, which displayed a 74% GSL. The first cluster contained five isolates from the Riyadh, the Dammam and the Abha with a 77% GLS. The second cluster comprised eleven isolates from the Riyadh, the Dammam and the Abha with a 78% GLS. In reference to this primer, there was no relationship found between the ISSR dendrogram and the geographic origin of the tested isolates.

The eight subclusters included seventeen isolates. These subclusters were divided into four aflatoxigenic subclusters, one non-aflatoxigenic subcluster and three mixed clusters. The four aflatoxigenic subclusters for example, subcluster included isolates, R5 and R16 which showed a 100% GLS. The one non-aflatoxigenic subcluster for example, subcluster comprised isolates R14 and R1 which presented an 83% GLS. The two mixed subclusters for example, subcluster contained isolates R8 and R10 which showed a 79.5% GLS. These findings indicated that this primer was succeeding to discriminate aflatoxigenic from non-aflatoxigenic isolates with 62.5%.

## DISCUSSION

The present investigation revealed the presence of *A. flavus* and contamination of rice grains samples. These results confirm by several studies that reported different contamination levels of *Aspergillus* in rice grain from different countries including Portugal (Magro *et al*., 2006), Sweden (Fredlund *et al*., 2008), Egypt (Aziz *et al*., 2006), India (Reddy *et al*., 2009b), Saudi Arabia (Abd El-Aziz *et al*., 2013), Vietnam (Trung *et al*., 2001), China (Liu *et al*., 2006), United state (Abbas *et al*., 2005), Nigeria (Makun *et al*., 2011), Korea (Oh *et al*., 2010). Storage mycoflora and contamination of *A. flavus* different from country to country depends upon the fungi present and prevailing climatic conditions in the region (Setamou *et al*., 1997) agronomic practices and the conditions of harvesting, handling and storage (Bryden, 2009) transportation and shipment (customs clearance, weather conditions and conditions of storage at the various transit points) (Bhat 1988, Milton & Pawsey 1988).

In this study, the detection of AFs as judged by fluorescence of fungal colonies recorded the percentage of positive samples for PDA+NaCl media, Czapeks Dox agar media and yeast extract sucrose agar media were 10%, 20% and 65%, respectively. AFs produced by some strains of *A. flavus* not all isolates are able to produce aflatoxins, and this has encouraged the use of screening for their aflatoxin production abilities. The methodology commonly used for this survey using coconut agar (CA) medium for detection of aflatoxin. In this method, aflatoxigenic strains produced blue fluorescence on the reverse side of the colony under UV light whereas, Non-aflatoxigenic does not fluorescence (Davis *et al*., 1987). Fente *et al*. (2001) evaluated different agar (Czapeks, YES, coconut agar, aflatoxin producing ability (APA) media, coconut extract agar and coconut cream agar) media for aflatoxin production by *Aspergillus* spp. They reported more aflatoxin production in YES media compared to others. In our investigation we found that YES agar media is the best media for AFB1 production by *A. flavus*. Manisha and Sandip (2003) isolated *A. flavus*

strains from rice mill surrounding air and tested aflatoxin producing capability in Czapeks agar, APA media and CAM agar. They found that the high aflatoxin production in Czapeks media produced by *A. flavus* compared to other media.

In the present study, high percentage of the *A. flavus* isolates (64.1%) was capable of producing AFs using HPLC. Out of 52 strains of *A. flavus* isolated from rice, 22 strains produced AFB1. Twenty eight percent of the isolates produced AFB1 on Czapeks and PDA agar media, 42% of the isolates on YES Media. The production of AFB1 by the strains range of 0.2-40 µg/g (Reddy *et al*., 2009b). The percentage of aflatoxigenic isolates of *A. flavus* has been shown to vary with the nature of substrate, and environmental factors (Klich, 2007). For example, the incidence of aflatoxigenic *A. flavus* strains was higher in peanuts (69%) than in wheat (13%) (Vaamonde *et al*., 2003). In some studies, the majority of isolates were able to produce AFs (Giorni *et al*. 2007; Riba *et al*. 2010), while other studies indicated that less than 30% were able to produce AFs (Tran-Dinh *et al*. 2009; Sepahvand *et al*. 2011).

Our strains were classified into five different chemotypes, based on patterns of AFs production which majority of them belonged to chemotype II (B+ and G+) and V (B- and G-). *A. flavus* populations have been found to be extremely diverse in toxigenicity and divided into groups, depending on their toxigenic profile (Giorni *et al*., 2007, Razzaghi-Abyaneh *et al*., 20 06; Vaamonde *et al*., 2003).

One hundred and fifty isolates belong to *Aspergillus* section *Flavi* isolated from stored wheat in Algeria. The isolates classified into eight different chemotypes, based on patterns of mycotoxin (AFB1, AFG and CPA) and sclerotial production. The results obtained demonstrate a great variability in the AFB and CPA-producing potential of *A. flavus* (Riba *et al*., 2010).

The three primers used to generate an RAPD dendrogram using the similarity matrix produced with the banding patterns obtained with primers RAPD primers 1, 3 and 5. Genetic similarity level for RAPD primer 1, 3 and 5 were (11%-87%, 54%-83%, 23%-100%, respectively) for all isolates, providing evidence of its high level of genetic diversity. Batista *et al*. (2008) used RAPD molecular markers with the aim of genetically characterizing the diversity of the strains of *A. flavus*. High genetic diversity was revealed by their RAPD analysis, and a high efficiency of strain characterization was achieved.

RAPD results provided by Sepahvand *et al*. (2011) for the *A. flavus* isolates from indoor and outdoor hospital environments. Similarity matrices showed 38% to 100% similarity between the isolates indicating high degree of genetic diversity for *A. flavus*.

Reddy *et al*., (2009a) Twenty-two aflatoxin B1 (AFB1) producing *A. flavus* strains were isolated from discolored rice grain samples. RAPD was used to assess the genetic variability, the isolates showed 17-80% similarity with standard culture of *A. flavus* (MTCC 2799).

Regarding the geographic isolation, two relationships were found: first, there was no apparent correlation observed for primer RAPD 1 and 5 second, there was correlation observed for RAPD primers 3. RAPD was used for fourteen isolates of *A. flavus* from two geographically distinct sites, in Brazil. Isolates distribution were a non-random array, but one cannot assume that this is a fully discriminatory result and, thus, RAPD usefulness is debatable (Lourenço *et al*., 2007). Tran-Dinh and collaborators (1999) have grouped several isolates of *A. flavus* and *A. parasiticus* in a clear association with their origins, prompting them to endorse the use of RAPD as a reliable and reproducible methodology with no significant artifacts.

RAPD was used to discriminate aflatoxigenic from non-aflatoxigenic isolates, RAPD primes 1, 3 and 5 successes with different percentage 40%25% and 0%, respectively.

Jamali *et al*., (2012) The RAPD analysis of 24 selected *A. flavus* isolates demonstrated not established between RAPD genotypes and the ability for producing AF. There was no association between either sclerotium production or the site of isolation (orchards) with RAPD genotypes. Other authors have reported detecting no correlation between RAPD marker and the production or nonproduction of aflatoxins (Jovita and Bainbridge 1996, Tran-Dinh *et al*., 1999, Lourenço *et al*., 2007, Sepahvand *et al*., 2011).

The three primers used to generate an ISSR dendrogram using the similarity matrix produced with the banding patterns obtained with

primers RAPD primers 1, 3 and 5. Genetic similarity level for ISSR primers $(GTG)_5$, $(GACA)_4$ and $(AGAG)_4G$ were (78%-100%, 61%-100%, 59%-100%, respectively) for all isolates, providing evidence of its high level of genetic diversity.

This is the first study to present the use of ISSR microsatellite markers to characterize *A. flavus* isolates with wheat crop. ISSR analysis can be useful in population genetics, epidemiological surveys and ecological studies of *A. flavus*. Additionally, the $(GTG)_5$ primer can be used to generate unique products from different *Aspergillus* species that can be converted to a sequence characterized amplified region to help in taxonomic identification (Batista *et al*., 2008). Microsatellite analysis of the Vietnamese *A. flavus* strains (isolated from corn and peanut) revealed a high genetic diversity, cosmopolitan and genetically connected (Tran-Dinh *et al*., 2009).

The microsatellite markers presented here will be very useful for investigating the diversity and population structure of *A. flavus* and *A. parasiticus* (Tran-Dinh and Carter, 2000). Geographic isolation, the dendrogram produced using the ISSR marker showed no clear-cut relationship between clustering in the ISSR dendrogram and geographic origin. The dendrogram produced using the ISSR data showed a high genetic similarity according to the geographical origin (Batista *et al*., 2008). Genetic relationship between the 84 strains of *A. flavus* isolated from Vietnam. No correlation between geographic origin of strains and genotype was evident either. For example, the strains collected from both northern and southern regions were interspersed throughout the dendrogram (Tran-Dinh *et al*., 2009).

Regarding the geographic isolation, two relationships were found: first, there was no apparent correlation observed for primer ISSR $(AGAG)_4G$ second, there was correlation observed for ISSR primers $(GTG)_5$ and $(GACA)_4$.

The microsatellite markers presented here will be very useful for investigating the diversity and population structure of *A. flavus* and *A. parasiticus* (Tran-Dinh and Carter, 2000). Geographic isolation, the dendrogram produced using the ISSR marker showed no clear-cut relationship between clustering in the ISSR dendrogram and geographic origin. The dendrogram produced using the ISSR data showed a high genetic similarity according to the geographical origin (Batista *et al*., 2008). Genetic relationship between the 84 strains of *A. flavus* isolated from Vietnam. No correlation between geographic origin of strains and genotype was evident either. For example, the strains collected from both northern and southern regions were interspersed throughout the dendrogram (Tran-Dinh *et al*., 2009).

ISSR was used to discriminate aflatoxigenic from non-aflatoxigenic isolates, ISSR primes $(GTG)_5$, $(GACA)_4$ and $(AGAG)_4G$ successes with different percentage 75%, 71.5% and 62.5%, respectively.

High genetic diversity was seen in the 84 *A. flavus* strains with no evident correlation between strain toxigenicity and ISSR genotype (Tran-Dinh *et al*., 2009). *A. flavus* strains were isolated from different oil seed (groundnut, sunflower and soybean), out of which four isolate were found to nontoxic and eight isolates were found to be toxic. No correlation between AFs production and ISSR dendrogram (Hatti *et al*., 2010).

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