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Genetic Diversity of *Aspergillus flavus* Contamination of Sorghum Grains

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Seventeen *Aspergillus flavus* isolates were isolated from 48 sorghum grain samples from different localities of Riyadh, in the Saudi Arabia. Potential ability to produce aflatoxins (AFs) B1, B2, G1 and G2 was studied by HPLC analysis of these AFs in the culture extracts. Ten (59%) *A. flavus* isolates produced detectable levels of AFs at concentrations ranging from 0.4 to 1.6 µg/kg. The isolates were classified into four chemotypes based on the ability to produce AFs and sclerotia. Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) markers were used, with the aim of study 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 markers were not suitable to discriminate aflatoxigenic and non-aflatoxigenic isolates, but ISSR primers were better compared to RAPD.

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

Sorghum (*Sorghum bicolor* L.) is the fifth most important cereal after rice, wheat, maize and barley. It is the staple food grain for over 750 million people who live in the semi-arid tropics of Africa, Asia, and Latin America (Sreenivasa *et al*. 2010). Besides being an important food, feed and forage crop, sorghum also provides raw material for the production of starch, fiber, dextrose syrup, biofuels, alcohol, and other products (Jeya Prakash *et al*. 2010). Saudi Arabia product about 272.000 tonnes and imported about 152850 tonnes sorghum in 2009 (FAO, 2010). Recovery of several mycotoxigenic fungi (*Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria* genera) from sorghum grains have been frequently documented (Hussaini *et al*., 2009, Yassin *et al*., 2010, Abdulsalaam and Shenge, 2011; Abd El-Aziz *et al*., 2013). Mycotoxins can cause acute or chronic intoxication and damage to humans and animals after ingestion of contaminated food and feed (Moss, 1996). Among the mycotoxins, aflatoxins (AFs) and ochratoxin A (OTA) are of special interest, given their high occurrence and toxicity. All AFs are regulated in different products in most countries worldwide (Commission of the European Communities, 2001). Aflatoxins (AFs) are toxic secondary metabolites produced by species of *Aspergilli*, especially *Aspergillus flavus* and *Aspergillus parasiticus*. 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) because of its demonstrated carcinogenicity in humans (Castegnaro and Wild, 1995). Aflatoxigenic seed-borne *Aspergillus* strains have been isolated from

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sorghum grains (Da Silva *et al*. 2004, Yassin *et al*., 2010). Random amplified polymorphic DNA (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). RAPD markers have detected genetic variability between the isolates of *A. flavus* and related species (Batista *et al*., 2008; Reddy *et al*., 2009; Irshad and Nawab, 2012). RAPD markers have been used to discriminate between aflatoxigenic from non-aflatoxigenic isolates of *A. flavus* (Lourenço *et al*., 2007; Gashgari *et al*. 2010, Al-Wadai *et al*. 2013, Mahmoud *et al*. 2013). The inter-simple sequence repeat (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). ISSR markers are 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 have been used to determine similarity and dissimilarity between aflatoxigenic and non-aflatoxigenic isolates of *A. flavus* (Hatti *et al*. 2010, Al-Wadai *et al*. 2013).

The objective of the present study was 1) to detection the AFs in *A. flavus* isolates, 2) to genetically characterize *A. flavus* isolates, 3) to compare the genomic profile of aflatoxigenic and non- aflatoxigenic *A. flavus* isolates using RAPD and ISSR markers.

MATERIALS AND METHODS

Sampling of sorghum grains

The market survey of sorghum grain samples was conducted from 5 September to 25 September 2012. A total of 48 sorghum grain samples were randomly collected from market in Riyadh region, in the Kingdom of Saudi Arabia, based on cardinal direction, 12 samples for every direction.

Isolation of *A. flavus*

Forty eight samples were examined for the presence of *A. flavus* and *A. parasiticus* using standard techniques (Pitt and Hocking, 1997). Sorghum grains were surface disinfected in 10% household chlorine bleach (i.e. 0.5% active chlorine) for 2 min, then rinsed twice with water. Twenty kernels from each peanut and corn sample were randomly selected and transferred onto two *Aspergillus flavus* and *parasiticus agar* (AFPA: 1% peptone, 2% yeast extract, 0.05% ferric ammonium citrate, 0.01% chloramphenicol, 9.7 lM dichloran, 1.5% agar) plates (ten per plate) using sterile forceps. Plates were incubated at 3C for 3 days (Pitt *et al*, 1983).

Isolates of *A. flavus* was recognised by bright orange colouration of the reverse colonies and were subcultured onto new AFPA plates for verification.

Identification of *A. flavus* isolates

Isolates were identified following subculturing on Czapek Yeast Agar (CYA: 0.1% K_2HPO4, 3% sucrose, 0.5% yeast extract, 0.3% $NaNO_3$, 0.05% KCl, 0.05% $MgSO_47H_2O$, 0.001% $FeSO_47H_2O$, 0.005% $CuSO45H_2O$, 0.01% $ZnSO_47H2O$, 1.5% agar) media and incubation at 25 °C for 7 days Klich and Pitt, 1998). Isolates were initially identified macroscopically and confirmed microscopically by conidiophore structure and conidial roughening. sclerotia production were determined by culturing the isolates on Czapek–Dox agar (CZ), Czapek yeast extract agar (CYA), and malt extract agar (MEA) plates for 7 days at 25°C and at 42°C on CYA.

Detection of aflatoxin production

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 (Williams *et al*., 1990). 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*. (2013). Sequences of primers are listed in Table 1.

ISSR by PCR

PCR amplification of ISSR was performed with $(GTG)_5$, $(GACA)_4$ and $(AGAG)_4G$ primers. The reaction mixture;po s 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). Am-plification 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). Sequences of primers are listed in Table 1.

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, and 1.500 bp. The similarity level was determined by un-weighted pair group method based on arithmetic mean (UPGMA).

RESULTS

AFs production

Ten isolates were capable of producing detectable levels of both B and G aflatoxins, although seven isolates failed to produce any detectable amount (Table 2).The highest level (1.5 and 1.6 µg/kg) of B1 and B2 were obtained from isolates AF15 and AF03. Isolate AF11 was the highest producer (0.8 and 0.6 µg/kg) for both G1 and G2.

Chemotype patterns

A. flavus isolates were classified into three chemotypes (I to III) based on their ability to produce B1, B2, G1 and G2 (Table 3). Chemotype I produced all AFs comprised 17.6% of the isolates (three 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 III) were comprised 41.2% of the isolates (seven isolates).

Genetic characterization of *A. flavus* isolates

Genetic characterization was evaluated by PCR amplification using a set of 6 primers (three RAPD and three ISSR). The amplification products

were analyzed for polymorphisms by gel electrophoresis to determine whether pathotypes could be distinguished at the molecular level.

Phenetic analysis by three RAPD primers

Phenetic analysis by RAPD primer 1 of 17 *A. flavus* isolates

Dendrogram analysis grouped the isolates into two main clusters at a genetic similarity level (GSL) of 20% based on the banding pattern (Figure 1). GSL between the tested isolates ranged from 20%-98%. The first main cluster included two groups at 24% GSL. The second main cluster included two groups at 26.5% GLS.

Five sub-clusters included eleven isolates, seven isolates producing AFB and AFG and four isolates non-producing AFB or/and AFG isolated from Riyadh region. Four sub-clusters included nine isolates combination of aflatoxigenic and non-aflatoxigenic isolates, for example, sub-cluster included three isolates AF06 (non-producing AFB and AFG), AF02 and AF09 (producing AFB) and had 86% GLS. Only one sub-cluster included two aflatoxigenic isolates AF01 and AF03 had 98% GLS. AFB and AFG producing isolates were resided in both clusters with a random distribution and, Based on the dendrogram data; an exact association was not established between RAPD genotypes and the ability to produce AFB and AFG.

Phenetic analysis by RAPD primer 3 of 17 *A. flavus* isolates

Dendrogram analysis grouped the isolates into two main clusters at 20% GSL based on the banding pattern (Figure 2). GSL between the tested isolates ranged from 20%-97%. The first main cluster included only two isolates (AF17 and AF15). The second main cluster included two groups at 22.5% GLS.

Five sub-clusters included thirteen isolates, eight isolates producing AFB and AFG and five isolates non-producing AFB or/and AFG isolated from Riyadh region. Four sub-clusters included eleven isolates combination of aflatoxigenic and non-aflatoxigenic isolates, for example, sub-cluster included three isolates AF07, AF03, AF02 (producing AFB and AFG), AF05 (non-producing AFB) and had 81% GLS. Only one sub-cluster included two aflatoxigenic isolates AF01 and AF04 had 98% GLS.

Phenetic analysis by RAPD primer 5 of 17 *A. flavus* isolates

Dendrogram analysis grouped the isolates into two main clusters at 33% GSL based on the banding pattern (Figure 3). GSL between the tested isolates ranged from 33%-98%. The first main cluster included only one isolate (AF17). The second main cluster included two groups at 35% GLS.

Six sub-clusters included eleven isolates combination of aflatoxigenic and non-aflatoxigenic isolates, for example, sub-cluster included two isolates AF01 (producing AFB and AFG), AF06 (non-producing AFB and AFG) and had 98% GLS.

AFB and AFG producing and non-producing isolates were resided in RAPD clusters with a random distribution, Based on the dendrograms data; an exact association was not established between RAPD genotypes and the ability to produce AFB and AFG.

Sclerotia producing isolates were also scattered in both clusters. There was no association between both sclerotia formation and RAPD

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

Primer code	RAPD primers		
	Sequence	Amplified region	References
RAPD primer 1	5' -GGT GCG GGA A-3'	Fragments from 1-10 genomic sites simultaneously	Mahmoud et al, 2013
RAPD primer 2	5' -GTT TCG CTC C-3'		
RAPD primer 5	5' -AAC GCG CAA C-3'		
Primer code	ISSR primers		
	Sequence	Amplified region	References
$(GTG)_5$	5' -GTG GTG GTG GTG GTG-3'	Minisatellite-region DNA	Batista et al., 2008
$(GACA)_4$	5' -GAC AGA CAG ACA GAC A-3'		
$(AGAG)_4G$	5' -AGA GAG AGA GAG AGA GG-3'		

Table 2. Aflatoxin and sclerotia production by *A. flavus* isolated from sorghum grains collected from Riyadh region

A. flavus code	Aflatoxin (µg/kg)				Sclerotia
	B1	B2	G1	G2	
Northern region					
AF01	0.8	1.5	0.0	0.0	+
AF02	0.9	1.1	0.0	0.0	+
AF03	1.2	1.6	0.0	0.0	+
AF04	0.5	1.4	0.4	0.3	+
Southern region					
AF05	0.0	0.0	0.0	0.0	-
AF06	0.0	0.0	0.0	0.0	-
AF07	1.1	0.4	0.6	0.5	+
AF08	0.0	0.0	0.0	0.0	+
Eastern region					
AF09	0.9	1.5	0.0	0.0	+
AF10	0.0	0.0	0.0	0.0	+
AF11	1.3	0.7	0.8	0.6	+
AF12	0.0	0.0	0.0	0.0	+
Western region					
AF13	1.1	1.3	0.0	0.0	+
AF14	1.3	0.8	0.0	0.0	+
AF15	1.5	0.9	0.0	0.0	+
AF16	0.0	0.0	0.0	0.0	-
AF17	0.0	0.0	0.0	0.0	-

genotypes in both aflatoxigenic and non-aflatoxigenic isolates.

In general, the RAPD primers could not be useful in discriminate between aflatoxigenic and non- aflatoxigenic isolates.

Phenetic analysis by three ISSR primers

Phenetic analysis by $(GTG)_5$ primer of 17 *A. flavus* isolates

Dendrogram analysis grouped the isolates into two main clusters at 40% GSL based on the banding pattern (Figure 4). GSL between the tested isolates ranged from 40%-99%. The first main cluster included only two isolates (AF16 and AF17). The second main cluster included two groups at 45% GLS.

Three sub-clusters included six isolates combination of aflatoxigenic and non-aflatoxigenic isolates, for example, sub-cluster included two isolates AF02 (producing AFB), AF05 (non-producing AFB and AFG) and had 92.5% GLS. Three sub-cluster included six aflatoxigenic isolates, for example AF01 and AF04 had 99% GLS. Only one sub-cluster included two non-aflatoxigenic (AF16 and AF17) had 53 % GLS.

Phenetic analysis by $(GACA)_4$ primer of 17 *A. flavus* isolates

Dendrogram analysis grouped the isolates into two main clusters at 34% GSL based on the banding pattern (Figure 5). GSL between the tested isolates ranged from 34%-96%. The first main cluster included only one isolate (AF17). The second main cluster included two groups at 35% GLS.

One sub-cluster included two aflatoxigenic isolates (AF01 and AF03) had 96 % GLS. One sub-clusters included two non-aflatoxigenic isolates (AF08 and AF06) had 79 % GLS. One sub-cluster included four isolates combination of aflatoxigenic and non-aflatoxigenic isolates, for example, sub-cluster included four isolates AF12 (non-producing AFB and AFG) and AF11, AF14, AF15 (producing AFB or/and AFG) and had 59.5% GLS.

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

Dendrogram analysis grouped the isolates into two main clusters at 32% GSL based

Table 3. Chemotype patterns of *A. flavus* isolated from Riyadh region based on the ability for producing aflatoxins (AF) B1, B2, G1 and G2 and sclerotia

Chemotype	Aflatoxin				Sclerotia	No. (%)
	B1	B2	G1	G2		ofisolates
I	+	+	+	+	+	3 (17.6)
II	+	+	-	-	+	7 (41.2)
III	-	-	-	-	+	3 (17.6)
IIII	-	-	-	-	-	4 (23.6)

on the banding pattern (Figure 6). GSL between the tested isolates ranged from 32%-94%. The first main cluster included only one isolate (AF17). The second main cluster included two groups at 34% GLS.

Three sub-clusters included six isolates combination of aflatoxigenic and non-aflatoxigenic isolates, for example, sub-cluster included four isolates AF04 (producing AFB and AFG) and AF05 (non-producing AFB and AFG) and had 90% GLS. Two sub-clusters included four aflatoxigenic isolates, for example (AF01 and AF02) had 94 % GLS. One sub-clusters included two non-aflatoxigenic isolates (AF08 and AF10) had 89 % GLS.

Sclerotia producing isolates were also scattered in both clusters. There was no association between both sclerotia formation and RAPD genotypes in both aflatoxigenic and non-aflatoxigenic isolates.

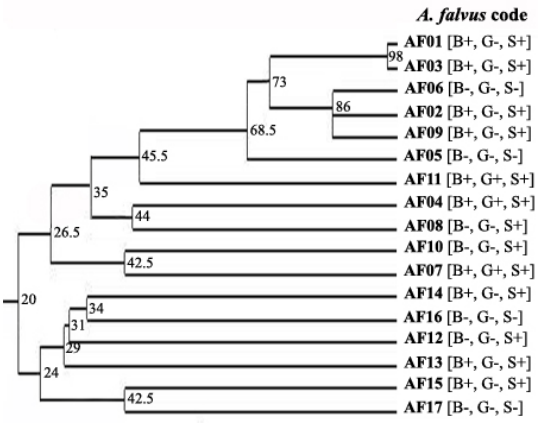

Fig. 1. Dendrogram obtained by UPGMA method derived from PCR amplification banding of RAPD with RAPD primer 1 of 17 *A. flavus* isolates

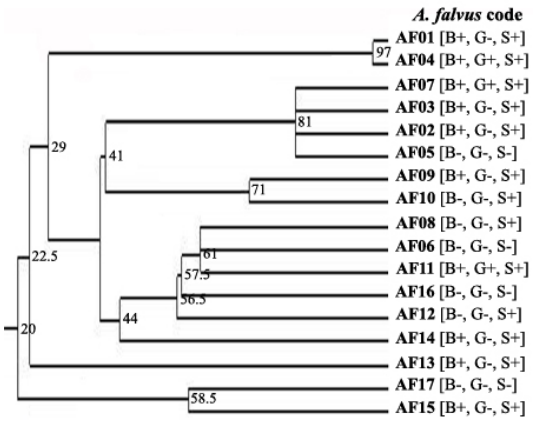

Fig. 2. Dendrogram obtained by UPGMA method derived from PCR amplification banding of RAPD with RAPD primer 3 of 17 *A. flavus* isolates

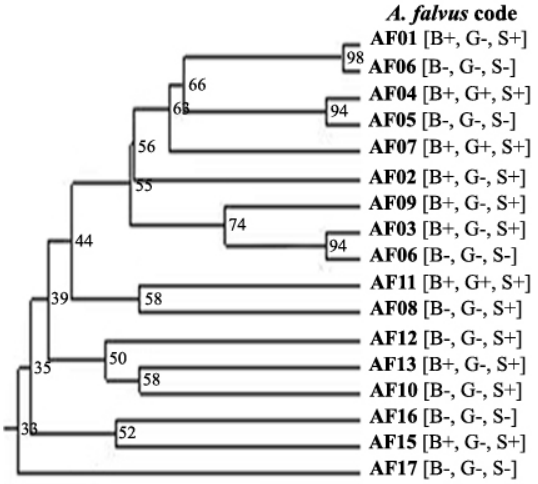

Fig. 3. Dendrogram obtained by UPGMA method derived from PCR amplification banding of RAPD with RAPD primer 5 of 17 *A. flavus* isolates.

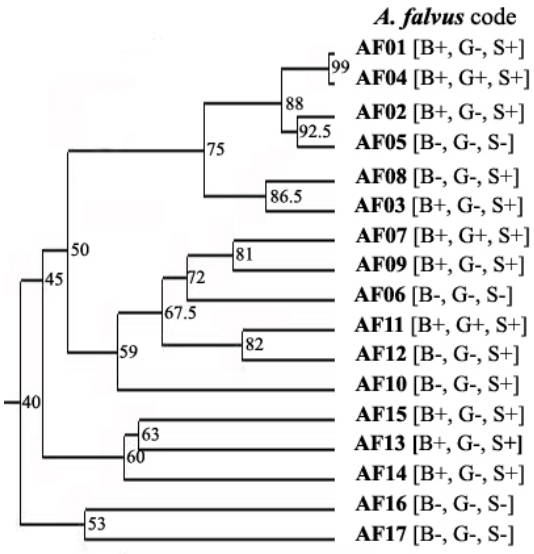

Fig. 4. Dendrogram obtained by UPGMA method derived from PCR amplification banding of ISSR with primer $(GTG)_5$ of 17 *A. flavus* isolates

Fig. 5. Dendrogram obtained by UPGMA method derived from PCR amplification banding of ISSR with primer $(GACA)_4$ of 17 *A. flavus* isolates

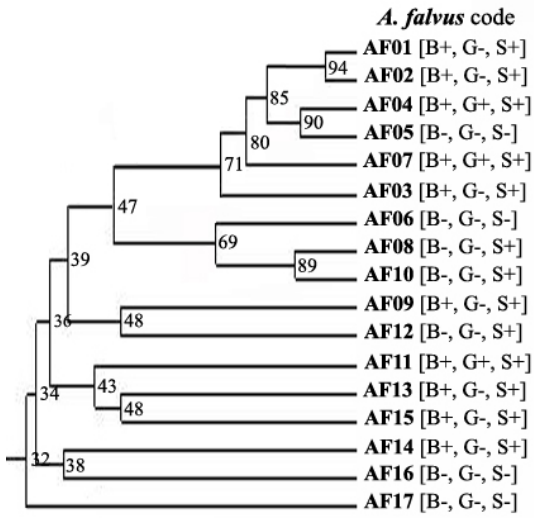

Fig. 6. Dendrogram obtained by UPGMA method derived from PCR amplification banding of ISSR with primer $(AGAG)_4G$ of 17 *A. flavus* isolates

In general, the ISSR primers could be useful in discriminate between aflatoxigenic and non- aflatoxigenic isolates in some cases.

DISCUSSION

In our work, detection of aflatoxin showed that ten (58.8%) of seventeen tested *A. flavus* isolates produced detectable levels of aflatoxins at concentrations ranging from 0.3 to 1.6 µg/kg. seven isolates produced AFB1 and AFB2, while three isolates produced all AFs. In Brazil aflatoxin analysis showed that 38 (64.4%) of 59 tested *A. flavus* strains produced detectable levels of aflatoxins at concentrations ranging from 12.00 to 3282.50 µg/kg (AFB1+AFB2) (Da Silva *et al*. 2004). Determination of AFs found 23% of *A. flavus* strains isolated from sorghum in Morocco produced AFB1 and AFB2 (Kichou and Walser 1993). The proportion of nontoxigenic to toxigenic strains in elsewhere in the world ~ (50:50) (Klich and Pitt, 1988, Viquez *et al*. 1994).

These differences may be due to differences in the fungi present, prevailing climatic conditions, agronomic practices, the composition of the commodity and the conditions of harvesting, handling and storage (Bryden, 2009). The amount of toxin produced will depend on physical factors (moisture, relative humidity, temperature and mechanical damage), chemical factors (carbon dioxide, oxygen, composition of substrate, pesticide and fungicides), and biological factors (plant variety, stress, insects, spore load) (Wicklow, 1995). Moisture and temperature have a major influence on mould growth and mycotoxin production. Although water activity is the most useful expression of the availability of water for microorganism growth (Pitt and Hocking, 1997). Our results differ from those of Yassin *et al*. (2010) who found that *A. niger* and isolates were the aflatoxigenic pathogens in sorghum grain samples in Saudi Arabia.

A. flavus isolates reported in this study were classified into four major chemotypes of which majority of them belonged to chemotype II comprising producers of both B1 and B2. *A. flavus* isolates were classified into four chemotypes (I to IV) based on their ability to produce AFB1, CPA, and sclerotia. Chemotype IV (non-toxigenic isolates) was the most prominent group comprising 55% of the isolates. Twenty-two isolates (34.9%) only produced CPA (chemotype III) and four isolates (6.3%) produced both AFB1 and CPA (chemotype I). A low percentage (3.2%) of the isolates classified as chemotype II produced only AFB1 (Sepahvand *et al*. 2011). *A. flavus* isolates 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-) (Mahmoud *et al*. 2013). *A. flavus* isolates were classified into eight chemotypes (I to VIII) on the basis of their ability to produce B1, B2, G1, and G2 (Table 5). Chemotype I (aflatoxigenic isolates) was the first prominent group comprising 36.8% of the isolates. Only seven isolates produced all AFs (chemotype I). Chemotype VIII (non-aflatoxigenic) was the second prominent group comprising 31.6% of the isolates (Al-Wadai *et al*. 2013).

Three primers were used to generate an RAPD dendrogram using the similarity matrix produced with the banding patterns obtained with RAPD. GSL for RAPD primers 1, 3, and 5 were 20-98%, 20-97%, and 33-98%, respectively, for all isolates, providing evidence of its high level of genetic diversity similarity. Greater variation was seen within *A. flavus* than in *A. parasiticus*, which was consistent with our previous analysis using RAPD markers (Tran-Dinh *et al*. 1999). Similar results have been described in other studies with *A. flavus*, RAPD profile for *A. flavus* had very different products for each *A. flavus* strain, providing evidence of its high genetic diversity (Batista *et al*. 2008). High genetic diversity was revealed by RAPD markers for *A. flavus* isolated from different agriculture crops. Importance of RAPD depends on the chosen many suitable primers, to get clear results (Lourenço *et al*. 2007, Gashgari *et al*. 2010, Al-Wadai *et al*. 2013).

With regard to RAPD markers and differentiation between aflatoxigenic and non-aflatoxigenic, RAPD primers 1, 3, and 5 showed no relationship between RAPD dendrogram and their ability to produce AFs. Similar results have been described in other studies with *Aspergillus* spp, showing no correlation between DNA band profiles and production or non-production of AF (Lourenço *et al*., 2007, Gashgari *et al*. 2010, Al-Wadai *et al*. 2013). However, in one investigation with isolates of *Aspergillu*s sect. *Flavi* using RAPD, Egel and collaborators (1994) grouped strains with similar toxigenic capacities, in a more subtle differentiation than the simple classification of toxin producers and nonproducers. RAPD-PCR technique amplified random fragments of the fungal genome; the fragment that contained the gene regulating toxin production may not have been amplified using this technique with the used primers (Gashgari *et al*. 2010).

Three primers were used to generate an ISSR dendrogram using the similarity matrix produced with the banding patterns obtained with ISSR primers. GSL for ISSR primers $(GTG)_5$, $(GACA)_4$ and $(AGAG)_4G$ were 40-99%, 34-96%, and 32-94%, respectively, for all isolates, providing evidence of its high level of genetic diversity similarity. The microsatellite markers were very useful for investigating the diversity and population structure of *A. flavus* and *A. parasiticus* (Tran-Dinh and Carter, 2000).

ISSR is a suitable molecular typing technique for the study of genetic diversity of *A. flavus*. Screening to obtain an efficient set of primers is very important when using ISSR typing (Batista *et al*. 2008, Tran-Dinh *et al*. 2009, Al-Wadai *et al*. 2013, Zhang *et al*. 2013).

In our study, the ISSR markers could be useful in discriminate between aflatoxigenic and non- aflatoxigenic isolates in some cases. Clustering, based on microsatellite genotype, was unrelated to aflatoxin production, geographic origin or substrate origin. (Tran-Dinh *et al*. 2009). *A. flavus* strains have been isolated from different oilseeds (groundnut, sunflower, and soybean), where four isolates were found to be nontoxic and eight toxic. No correlation was shown between AF production and ISSR dendrogram in a study by Hatti *et al*. (2010). ISSR markers were not suitable to discriminate aflatoxigenic and nonaflatoxigenic isolates, but ISSR primers were better compared to RAPD (Al-Wadai *et al*. 2013).

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