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Survey of *Aspergillus flavus* in Some Nuts

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In this study conducted a survey of nuts crops (peanuts, almond and hazelnuts) available in different market of Riyadh, Saudi Arabia for the presence of *Aspergillus flavus* and used RAPD and ISSR markers to investigate the genetic variability of *A. flavus* isolates. From a total of 72 samples comprising (24 each crop), 20 isolates were isolated. Identification of strains by colony morphology. Potential ability to produce aflatoxins (AFs) B1, B2, G1 and G2 was studied by HPLC analysis of these AFs in the culture extracts. Eleven (55%) *A. flavus* isolates produced detectable levels of AFs at concentrations ranging from 0.2 to 1.2 µg/kg. Fifty-five percent of the isolates produced AFs. The isolates were classified into five chemotypes based on the ability to produce AFs and sclerotia. RAPD and ISSR analysis revealed a high level of genetic variability in the *A. flavus* population. Clustering, based on RAPD and ISSR genotype, were unrelated to aflatoxin production or substrate origin.

Key words: *Aspergillus flavus*, Nuts, HPLC, RAPD, ISSR.

Grains (cereals and oilseeds) and nuts in general are subject to mold attack, in preharvest and postharvest. Among molds that can attack these foods *A. flavus*, and *A. parasiticus* are important because they can produce aflatoxins that are considered a potent natural toxin (Wild and Gong, 2010). Aflatoxin can be produced mainly by different *Aspergillus* species, but *Emiricella* and *Petromyces* have been reported as aflatoxin producers (Frisvald *et al*., 2005). Aflatoxin contamination has been reported for grains as corn, soya, wheat, rice, and cottonseed, and nuts such as peanuts, almonds, Brazil nuts, hazelnuts, walnuts, cashew nuts, pecans, and pistachio nuts (Gürses, 2006). Despite aflatoxin contamination having been observed in several foodstuffs, the contamination of maize, peanuts, and oilseeds can be considered, in terms of diet exposure, the most important worldwide (Benford *et al*. 2010). 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 (Anonymous, 2007). 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). Random amplified polymorphic DNA (RAPD) markers have found a wide range of

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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 were (1) survey for the presence of *A. flavus* in nuts crops potentially at risk of aflatoxin contamination, (2) assess whether isolated strains could produce aflatoxins; and (3) investigate their genetic variability using RAPD and ISSR marker.

MATERIALS AND METHODS

Sampling of peanut, almond and hazelnut

The market survey of peanut, almond and hazelnut samples was conducted from 15 November to 15 December 2012. A total of 72 samples (50-100 grams) were randomly collected from market in Riyadh region, in the Saudi Arabia, based on cardinal direction, 24 samples for every kind.

Isolation of *A. flavus*

Seventy two samples were examined for the presence of *A. flavus* using standard techniques (Pitt and Hocking, 1997). Nuts grains were surface disinfected in 10% household chlorine bleach (i.e. 0.5% active chlorine) for 2 min, then rinsed twice with water. Twenty grains from each nut 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 3°C 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_2HPO_4, 3% sucrose, 0.5% yeast extract, 0.3% $NaNO_3$, 0.05% KCl, 0.05% $MgSO_4 7H_2O$, 0.001% $FeSO_4 7H_2O$, 0.005% $CuSO_4 5H_2O$, 0.01% $ZnSO_4$7H2O, 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 formation 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 in sterilized SMKY liquid medium (20 g sucrose, 0.5 g magnesium sulfate, 3 g potassium nitrate and 7 g yeast extract in 1,000 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 d (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 aflatoxin extraction, the filtrates from each flask were treated three times with 50 ml of chloroform using a separating funnel. The chloroform extracts were then separated dehydrated with anhydrous sodium sulfate and evaporated to dryness in a water bath at 50°C under vacuum. The residues were dissolved in 10 ml of methanol and stored in dark vials, and the extracts were passed through a 0.45 µm micro-filter. Analysis of the compounds present in the specimens was performed using an HPLC apparatus (PerkinElmer series 200 UV/VIS) with a C18 column with an internal diameter of 300 mm x

3.9 mm, 4 µm. The HPLC instrument was equipped with a UV detector, and fluorescence was measured using 365 nm excitation and 430 emission wavelengths. The mobile phase consisted of methanol: acetic acid: water (20:20:60 v/v/v). The total run time for the separation was approximately 25 min at a flow rate of 1 ml/min (Christian, 1990).

Extraction of DNA from *A. flavus* isolates

A. flavus isolates were cultured in double layer media, consisting of one solid and one liquid layer, in 50-mm Petri dishes. The solid base medium was potato dextrose agar, and the top liquid medium was peptone yeast glucose (PYG, 1,200 µL). The fungi were incubated at 25°C for 2 days, after which the fungal mycelia (50 mg) were scraped using slide covers and transferred to sterile Eppendorf tubes (1.5 ml) for DNA isolation. DNA was extracted from 50 mg of fresh mats according to Amer *et al*. (2011).

RAPD PCR

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

ISSR PCR

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

DNA electrophoresis

For all samples, the amplified DNA (15 µL) was electrophoresed using an electrophoresis unit (wide mini-sub-cell GT Bio-RAD) in 2% agarose containing ethidium bromide (0.5 µg/mL) at a 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 (100 DNA ladder, Stratagene, Canada) with molecular weights 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

AFs production

Eleven isolates were capable of producing detectable levels of both B and G aflatoxins, although nine isolates failed to produce any detectable amount (Table 2). The highest level (1.2 and 0.7 µg/kg) of B1 and B2 were obtained from isolates AF10 and AF03. Isolate AF15 was the highest producer (0.2 and 0.3 µg/kg) for both G1 and G2.

Chemotype patterns

A. flavus isolates were classified into five chemotypes (I to V) based on their ability to produce AFs and sclerotia (Table 3). Chemotype I produced all AFs comprised 5% of the isolates (one isolate). Chemotype II produced B1 and B2 and sclerotia was comprised 25% of the isolates (five isolates). Five isolates produced B1 and B2 only was comprised 25% of the isolates (Chemotype III). Nine isolates failed to produce any detectable amount of AFs (Chemotype V) were comprised 45% of the 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 20 *A. flavus* isolates

Dendrogram analysis grouped the isolates into two main clusters at a genetic similarity (GS) of 20.5% based on the banding pattern (Figure 1). GS between the tested isolates ranged from 20.5%-100%. The first main cluster included two isolates AF14 (non-aflatoxigenic) and AF17 (aflatoxigenic) at 42% GS. The second main cluster included two groups at 27% GS. First group included two isolates AF8 (aflatoxigenic) and AF13 (non-aflatoxigenic) with 30% GS. Second group

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. AFs and sclerotia formation by *A. flavus* isolated from peanut, almond and hazelnut collected from Riyadh region

A. flavus code	AFs (µg/kg)				Sclerotia
	B1	B2	G1	G2	
Peanut	0.6	0.2	0.0	0.0	+
AF01	0.8	0.5	0.0	0.0	-
AF02	1.0	0.6	0.0	0.0	-
AF03	0.0	0.0	0.0	0.0	-
AF04	0.0	0.0	0.0	0.0	-
AF05	0.9	0.4	0.0	0.0	+
AF06	0.7	0.5	0.0	0.0	+
AF07	0.0	0.0	0.0	0.0	+
AF08	1.0	0.6	0.0	0.0	-
Almond					
AF09	0.0	0.0	0.0	0.0	-
AF10	1.2	0.7	0.0	0.0	+
AF11	0.8	0.5	0.0	0.0	-
AF12	0.0	0.0	0.0	0.0	-
AF13	0.0	0.0	0.0	0.0	-
AF14	0.0	0.0	0.0	0.0	-
AF15	0.9	0.6	0.2	0.3	+
Hazelnut					
AF16	0.0	0.0	0.0	0.0	-
AF17	0.8	0.3	0.0	0.0	+
AF18	0.0	0.0	0.0	0.0	-
AF19	0.0	0.0	0.0	0.0	+
AF20	0.6	0.5	0.0	0.0	-

Table 3. Chemotype patterns of *A. flavus* isolated peanut, almond and hazelnut collected from Riyadh region based on the ability for producing AFs and sclerotia

Chemotype	AFs		Sclerotia			No. (%)
	B1	B2	G1	G2		ofisolates
I	+	+	+	+	+	1 (5%)
II	+	+	-	-	+	5 (25%)
III	+	+	-	-	-	5 (25%)
IV	-	-	-	-	+	1 (5%)
V	-	-	-	-	-	8 (40%)

included eighteen isolates appeared in overlapping many sub clusters. Only one sub cluster included six aflatoxigenic (1, 6, 5, 10, 15, 11) and three non-aflatoxigenic (3, 19, 7) with 52% GS.

Phenetic analysis by RAPD primer 3 of 20 *A. flavus* isolates

Dendrogram analysis grouped the isolates into two main clusters at a genetic similarity (GS) of 19.5% based on the banding pattern (Figure 2). GS between the tested isolates ranged from 19.5%-87%. The first main cluster included two isolates AF12 and AF16 (non-aflatoxigenic) at 27% GS. The second main cluster included three clusters. First one included four aflatoxigenic isolates (18, 6, 11, 15) and one non-aflatoxigenic isolate (19) with 25% GS. Second cluster included two non-aflatoxigenic isolates (13, 7) and one aflatoxigenic isolate (17) with 41% GS. Third cluster included four aflatoxigenic isolates (1, 5, 9, 2, 10) and two non-aflatoxigenic isolates (4, 9) with 93% GS.

Phenetic analysis by RAPD primer 5 of 20 *A. flavus* isolates

Dendrogram analysis grouped the isolates into two main clusters at a genetic similarity (GS) of 12% based on the banding pattern (Figure 3). GS between the tested isolates ranged from 12%-100%. The first main cluster included one isolate AF127 (aflatoxigenic). The second main cluster included nineteen isolates distributing in many overlapping clusters (four clusters). First one included two aflatoxigenic isolates (20, 6) and two non-aflatoxigenic isolate (19, 3) with 29% GS. Second cluster included two non-aflatoxigenic isolates (7, 9) and one aflatoxigenic isolate (18) with 35% GS. Third cluster included four aflatoxigenic isolates (1, 2, 10, 11) and two non-aflatoxigenic isolates (12, 16) with 43% GS.

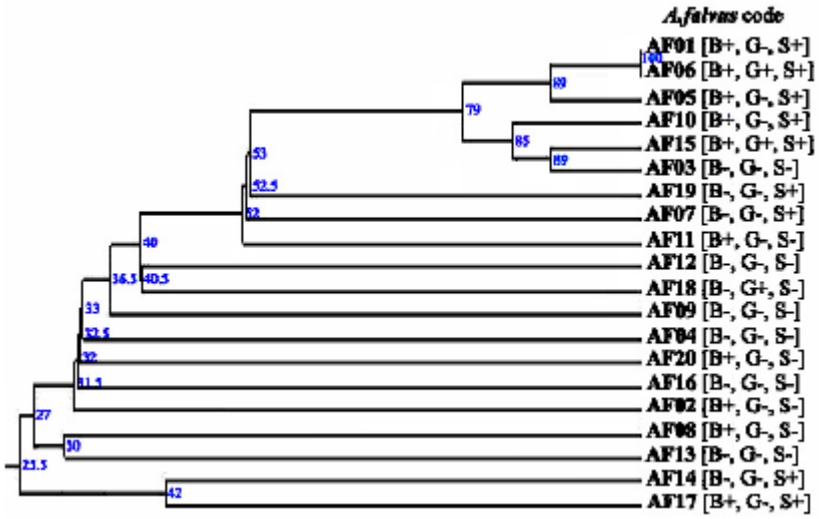

Fig. 1. Dendrogram obtained by UPGMA method derived from PCR amplification banding of RAPD with RAPD primer 1 of 20 *A. flavus* isolates

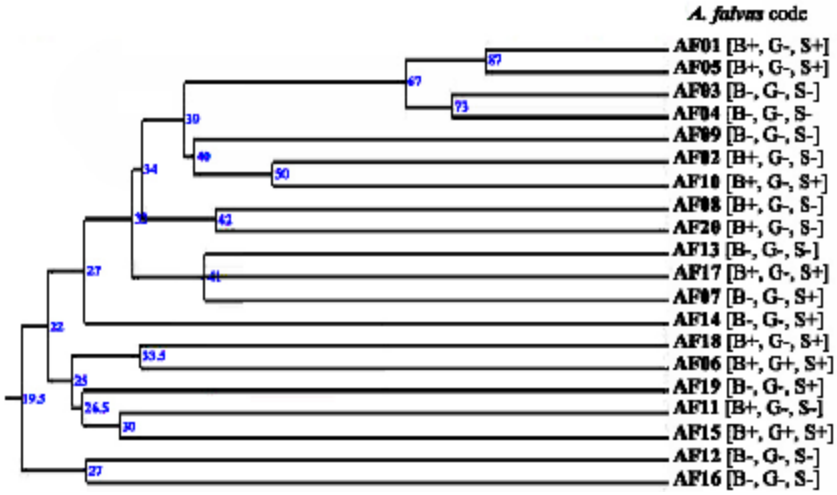

Fig. 2. Dendrogram obtained by UPGMA method derived from PCR amplification banding of RAPD with RAPD primer 3 of 20 *A. flavus* isolates

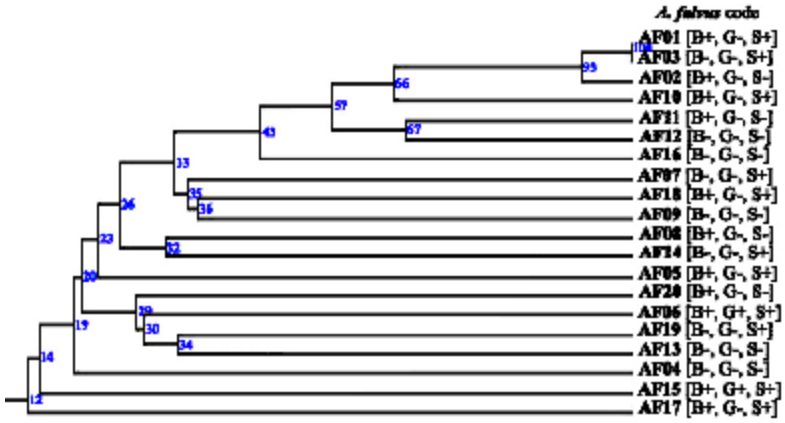

Fig. 3. Dendrogram obtained by UPGMA method derived from PCR amplification banding of RAPD with RAPD primer 5 of 20 *A. flavus* isolates

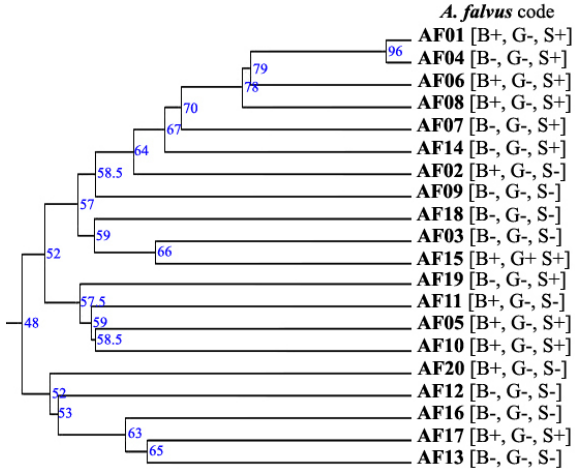

Fig. 4. Dendrogram obtained by UPGMA method derived from PCR amplification banding of ISSR with primer $(GTG)_5$ of 20 *A. flavus* isolates

RAPD markers show the genetic relationship between the 20 isolates of *A. flavus* isolated from different substrate. A high level of genetic variability was seen in the 20 isolates with no evident correlation between both isolate toxigenicity and sclerotia formation with RAPD genotype. No correlation between substrate origin of isolates and genotype was evident either.

Phenetic analysis by three ISSR primers

Phenetic analysis by $(GTG)_5$ primer of 20 *A. flavus* isolates

Dendrogram analysis grouped the isolates into two main clusters at a genetic similarity (GS) of 48% based on the banding pattern (Figure 4). GS between the tested isolates ranged from 48%-96%. The first main cluster included three non-aflatoxigenic isolates (12, 16, 13) and two

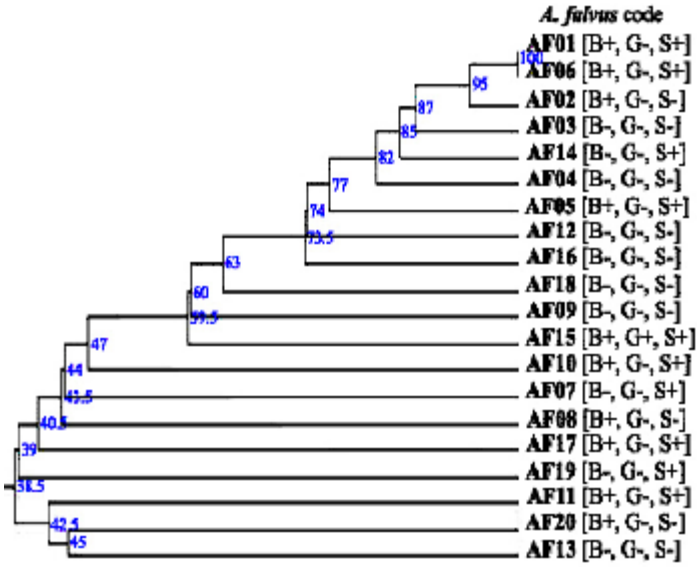

Fig. 5. Dendrogram obtained by UPGMA method derived from PCR amplification banding of ISSR with primer $(GACA)_4$ of 20 *A. flavus* isolates

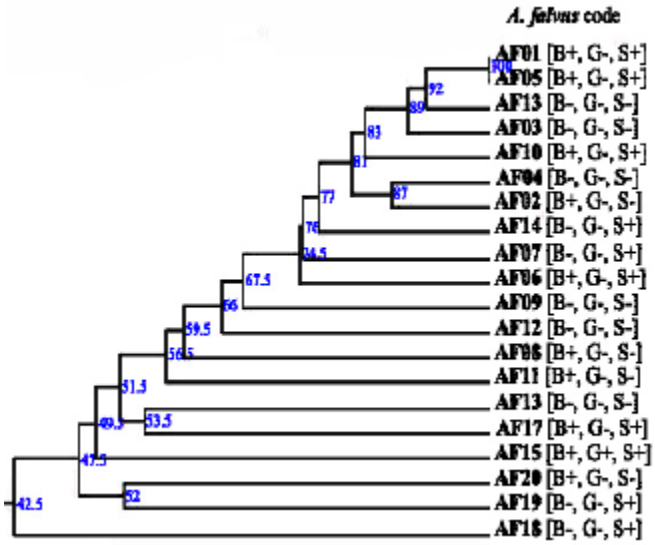

Fig. 6. Dendrogram obtained by UPGMA method derived from PCR amplification banding of ISSR with primer $(AGAG)_4G$ of 20 *A. flavus* isolates

aflatoxigenic isolates (12, 16) at 52% GS. The second main cluster included three groups at 52% GS. The second main clusters included three clusters. The first cluster included three aflatoxigenic isolates (11, 5, 10) and one non-aflatoxigenic isolate (19) at 57.5% GS. The second cluster included two non-aflatoxigenic isolates (18, 3) and one aflatoxigenic isolate (15) at 59% GS. The third cluster included four aflatoxigenic isolates (1, 6, 8, 2) and four non-aflatoxigenic isolate (4, 7, 14, 9) at 58.5% GS.

Phenetic analysis by $(GACA)_4$ primer of 20 *A. flavus* isolates

Dendrogram analysis grouped the isolates into two main clusters at a genetic similarity (GS) of 38.5% based on the banding pattern (Figure 5). GS between the tested isolates ranged from 38.5%-100%. The first main cluster included two aflatoxigenic isolates (11, 20) and one non-aflatoxigenic isolate (13) at 42.5% GS. The second main clusters included seventeen isolates. No separate cluster was appears because overlapping of seventeen isolates.

Phenetic analysis by $(AGAG)_4G$ primer of 20 *A. flavus* isolates

Dendrogram analysis grouped the isolates into two main clusters at a genetic similarity (GS) of 42.5% based on the banding pattern (Figure 6). GS between the tested isolates ranged from 42.5%-100%. The first main cluster included one non-aflatoxigenic isolates (18). The second main clusters included seventeen isolates. No separate cluster was appears because overlapping of seventeen isolates.

ISSR analysis revealed a high level of genetic variability in 20 *A. flavus* population. No correlation was found between ISSR genotype and the ability to produce aflatoxin and sclerotia also substrate origin.

DISCUSSION

In our work, detection of aflatoxin showed that eleven (55%) of twenty tested *A. flavus* isolates produced detectable levels of AFs at concentrations ranging from 0.2 to 1.2 µg/kg. Ten isolates produced AFB1 and AFB2, while one isolate produced all AFs. Nine isolates failed to produce any detectable amount. *Aspergillus* was consistently the most frequent genus in seeds and in shell peanuts and was the dominant mycotoxigenic component of the mycobiota. The most common species were from *Aspergillus* section *Flavi* (4.7-78.3%), *Aspergillus* section *Nigri* (9.4-52.6%) and *Aspergillus* section *Circumdati* (5.1-30.9%). Of a total of 88 Aspergillus section Flavi strains examined, 95% were *A. flavus* based on production of aflatoxin B1 (Sultan and Magan, 2010). A total of 18 strains of *Aspergillus* section *Flavi* isolated from Egyptian peanuts were assessed for aflatoxin production using HPLC. The AFB1 was in the range 1.27- 213.35 µg/g one strain was a very high producer (213.35 µg/g), nine strains were high producers (15.92-50.63 µg/g) and five strains were low producers (0.15-6.59 µg/g). Five of the strains showed no detectable levels of aflatoxin (Abdel-Hadi *et al*, 2011). Thirty-one isolates of *Aspergillus* Section *Flavi*, isolated from almonds from the Northeast of Portugal (region of Trás-os-Montes), Morphological characterization of the isolates is classified as *A. parasiticus* (18 isolates, 58%) and *A. flavus* (13 isolates, 42%). 13 isolates of *A. flavus* were isolated from almonds. These isolates were classified into three chemotype first one included 10 isolates (77%) were atoxigenic, whereas second included 2 isolates (15%) were CPA and AFB producers and third had one isolate (8%) produced AFB only (Rodrigues *et al*, 2009). 352 fungi belonging to *Aspergillus* section *Flavi* were isolated from Portuguese almonds: 127 were identified as *A. flavus* (of which 28% produced aflatoxins B), 196 as typical or atypical *A. parasiticus* (all producing aflatoxins B and G), and 29 as *A. tamarii* (all nonaflatoxigenic) (Rodrigues *et al*, 2012). *A. flavus* was recovered from a total of 20 random samples of almond collected from different locations in Riyadh, Kingdom of Saudi Arabia (Yassin *et al*, 2013).

One hundred samples of hazelnut were collected randomly from supermarkets in Isfahan. 78% of the samples were contaminated and 9 genera of fungi. The most predominant *Aspergillus* isolated fungus was were *A. flavus*, *A. niger*, *A. fumigatus* and *A. terreus* (Saffari *et al*, 2013).

RAPD markers show the genetic relationship between the 20 isolates of *A. flavus* isolated from different substrate. A high level of genetic variability was seen in the 20 isolates with no evident correlation between RAPD genotype and toxigenicity of isolates, sclerotia formation and

substrate origin. RAPD fingerprints analysis appeared genetic variability between *A. niger*, *A. flavus*, *A. parasiticus*. The similarity percent was 37% in *A. niger*, 58% in *A. flavus* and 51.5% in *A. parasiticus* (Nahid, 2006). RAPD was used to detectable genomic difference between toxigenic and non toxigenic *A. flavus*. RAPD phenetic and cladistic analysis failure to discrimination but RAPD was useful for isolates characterization, especially for preliminary evaluation over extensive collections. (Lourenço *et al*., 2007). 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 (Batista *et al*, 2008). RAPD showed no correlation between DNA banding profiles and the production or non-production of aflatoxins (Gashgari *et al*., 2010). RAPD profiles appeared no obvious correlation between RAPD genotypes and the ability to produce aflatoxin B1 (AFB1) and cyclopiazonic acid also sclerotia formation (Sepahvand *et al*., 2011). RAPD indicated that genetic differences between seven *Aspergillus* species (*A. flavus*, *A. niger*, *A. parasiticus*, *A. japonicas*, *A. nidulans*, *A. oryzae* and *A. fumigatus*) of the same genus maintain genetic diversity within this population. Results showed that useful in estimating distances between and within same species and might help future programs of management and conservation (Irshad and Nawab, 2012). RAPD-PCR could not be useful in discriminate between aflatoxigenic and non-aflatoxigenic *A. flavus* isolates. There was no association between both sclerotia formation and RAPD genotypes in both aflatoxigenic and non-aflatoxigenic isolates. (Abd El-Aziz *et al*, 2013). However, in two investigations involving isolates of *Aspergillus* section *flavi* based on RAPD analysis, Egel *et al*. (1994) grouped strains with similar toxigenic capacities in a manner that allowed more subtle differentiation compared to the simple classification of toxin producers and non-producers. RAPD primers were successes to discriminate aflatoxigenic *A. flavus* from non-aflatoxigenic isolates with different percentage ranged from 20% to 40% (Mohamoud *et al*, 2013). RAPD was not suitable to discriminate aflatoxigenic *A. flavus* from non-aflatoxigenic isolates, this is due to RAPD-PCR 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).

ISSR

ISSR analysis revealed a high level of genetic variability in 20 *A. flavus* population

There was no correlation between the ISSR dendrogram and the aflatoxin production ability of the isolates using these primers. No correlation was found between ISSR genotype and sclerotia formation also substrate origin.

The microsatellite markers presented here will be useful for investigating the diversity and population structure of *A. flavus* and *A. parasiticus* (Tran-Dinh and Carter, 2000).

The $(GTG)_5$ and $(GACA)_4$ primers produced differential amplification products, varying both in size and band intensity. Although $(GACA)_4$ revealed higher genetic variability, the number and size of $(GTG)_5$ bands were in a characteristic pattern in several strains of *A. flavus*, even though high interspecific variation was observed (Batista *et al*, 2008). Genetic relationships were found between 84 strains of *A. flavus* isolated from Vietnam. Microsatellite analysis revealed a high level of genetic diversity in the Vietnamese *A. flavus* population. Clustering, based on microsatellite genotype, was unrelated to aflatoxin production, geographic origin or substrate origin. For example, the strains collected from both northern and southern regions were interspersed throughout the dendrogram (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). RAPD-PCR could not be useful in discriminate between aflatoxigenic and non-aflatoxigenic *A. flavus* isolates. There was no association between both sclerotia formation and RAPD genotypes in both aflatoxigenic and non-aflatoxigenic isolates. (Abd El-Aziz *et al*, 2013). However, in one investigation involving isolates of *A. flavus* based on ISSR analysis, ISSR primers were successes to discriminate aflatoxigenic *A. flavus* from non-aflatoxigenic isolates with different percentage ranged from 60% to 75% (Mohamoud *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 and Appl. Microbiol.*, 2013; **7**: (Spl. Edi): 369-78.
2. Abdel-Hadi, A., Carter, D. Magan, N. Discrimination between aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* section *Flavi* group contaminating Egyptian peanuts using molecular and analytical techniques. *World Mycotoxin J.*, 2011; **4**: 69-77.
3. Al-Wadai, A.S., Al-Othman, M.R., Mahmoud, M.A. 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**: 3335-52.
4. Anonymous. Discussion paper on ochratoxin A in cocoa. Codex Alimentarius Commission, Codex Committee on Food Additives and Contaminants, First session. 2007; Beijing, China (16-20 April 2007, CX/CF 07/1/19).
5. Amer, O.E., Mahmoud, M.A., El-Samawaty, A.M.A., Sayed, R.M. Non liquid nitrogen-based-method for isolation of DNA from filamentous fungi. *Afr. J. Biotechnol.*, 2011; **10**: 14337-41.
6. Bardakci, F. Random amplified polymorphic DNA (RAPD) markers. *Turk. J. Biol.*, 2001; **25**: 185-96.
7. Batista P.P., Santos J.F., Oliveira N.T., Pires A.P., Motta C.M., Luna-Alves L.E.A. Genetic characterization of Brazilian strains of *Aspergillus flavus* using DNA markers. *Genet. Mol. Res*., 2008; **7**: 706-17.
8. Benford, D., Leblanc, J.C., Setzer, R.W. Application of the margen of exposure (MoE) approach to substances in food that are genotoxic and carcinogenic. Example: Aflatoxin B1 (AFB1). *Food Chem. Toxicol*., 2010; **48**: 534-41
9. Castegnaro, M., and Wild, C.P. IARC activities in mycotoxin research. *Nat. Toxins*, 1995; **3**: 327-31.
10. Christian G (ed). HPLC Tips and Tricks. Great Britain at the Iden Press, Oxford. 1990.
11. 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**: 1593-95.
12. Egel, D.S., Cotty, P.J., Elias, K.S. Relationships among isolates of *Aspergillus* sect. *Flavi* that vary in aflatoxin production. *Phytopathology*, 1994; **84**: 906-12.
13. Frisvald, J.C., Skoube, P., Samson, R.A. Taxonomic comparison of three different groups of aflatoxin producers and a new efficient producer of aflatoxin B1, sterigmatocystin and 3-O-methylsterigmatocystin, *Aspergillus rambellii* sp. nov. *Sys. Appl. Microbiol.*, 2005; **28**:442-53.
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**: 1047-54.
15. Gürses, M. Mycoflora and Aflatoxin Content of Hazelnuts, Walnuts, Peanuts, Almonds and Roasted Chickpeas (LEBLEBI) Sold in Turkey. *Int. J. Food Prop.*, 2006; **9**(3): 395-9.
16. Hadrich, I., Makni F., Ayadi, A., Ranque, S. Microsatellite typing to trace *Aspergillus flavus* infections in a hematology unit. *J. Clin. Microbiol.*, 2010; **48**: 2396-401.
17. 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**: 61-6.
18. IARC. The Evaluation of the Carcinogenic Risk of Chemical to Humans. In: IARC Monograph Supplement, 1982; 4, International Agency for Research on Cancer, Lyon.
19. 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.
20. Klich, M.A., Pitt, J.I. A laboratory guide to the common *Aspergillus* species and their teleomorphs. Sydney: Commonwealth Scientific and Industrial Research Organisation, Division of Food Processing; 1988.
21. Lourenço, A., Durigon, E.L., Zanotto, P., Cruz Madeira, J.E., De Almeida A.P., Correa, B. Genetic diversity of environmental *Aspergillus flavus* strains in the state of Sao Paulo, Brazil by random amplified polymorphic DNA. *Mem. Inst. Oswaldo Cruz.*, 2007; **102**: 687-92.
22. Mahmoud, M.A., Al-Othman, M.R., Abd El-Aziz, A.R.M. Genetic diversity of aflatoxin producing *Aspergillus flavus* contamination of rice grains from Saudi Arabia. *J. Pure and Appl.*

Microbiol., 2013; **7**(3): 1679-92.
23. Moss, M.O. Mycotoxins. Centenary review. *Mycol. Res.* 1996; **100**: 513-23.
24. Nahid, A., Genetic variability among three species of *Aspergillus*. Random amplified polymorphic DNA (RAPD) markers for genetic analysis. *J. Appl. Sci. Res.*, 2006; **2**(10): 709-13.
25. Neal, C.O.S., Richardson, A.O., Hurst, S.F., Tortorano, A.M. Global population structure of *Aspergillus terreus* inferred by ISSR typing reveals geographical subclustering. *BMC Microbiol*., 2011; **16**: 203-9.
26. Paranagama, P.A., Abeysekera K.H., Abeywickrama, K. Nugaliyadde, L. Fungicidal and anti-aflatoxigenic effects of the essential oil of *Cymbopogon citratus* (DC.) Stapf. (lemongrass) against *Aspergillus flavus* Link. isolated from stored rice. *Lett. Appl. Microbiol.*, 2003; **37**: 86-90.
27. Reddy, K.R.N., Reddy Ch.S., Nataraj Kumar P., Reddy C.S. Genetic variability of aflatoxin B1 producing *Aspergillus flavus* strains isolated from discolored rice grains. *World J. Microbiol. Biotechnol.* 2009; **25**: 33-9.
28. Pitt, J.I. and Hocking, A.D. Fungi and Food Spoilage Vol. II, ed. Pitt, J.I. and Hocking, A.D. 1997; London: Blackie Academic and Professional.
29. Pitt, J.I., Hocking, A.D., Glenn, D.R. Na improved medium for the detection of *Aspergillus flavus* and *A. parasiticus*. *J. Appl. Bacteriol.*, 1983; **54**: 109-14.
30. Rodrigues, P, Venâncio, A., Kozakiewicz, Z., Lima, N. Polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* Section *Flavi* isolated from Portuguese almonds. *Int. J. Food Microbiol.*, 2009; **129**: 187-93.
31. Rodrigues, P., Venâncio, A., Lima, N. Aflatoxigenic fungi and aflatoxins in Portuguese almonds. *The Scientific World Journal*, **2012**, doi:10.1100/2012/471926.
32. Saffari, E. Madani, M. Karbasizade, V., Toxigenic mycoflora distribution of hazelnut in Isfahan. *Jundishapur J. Microbiol.*, Special Edition, 2013; **2**: p34-34. 1/3p
33. Sepahvand, A., Shams-Ghahfarokhi, M., Allameh, A., Jahanshiri Z., Jamali, M., Razzaghi-Abyaneh M. A survey on distribution and toxigenicity of *Aspergillus flavus* from indoor and outdoor hospital environments. *Folia Microbiol*., 2011; **56**(6): 527-34.
34. Sultan, Y., Magan, N. Mycotoxigenic fungi in peanuts from different geographic regions of Egypt. *Mycotoxin Research*, 2010; **26**: 133-40.
35. Tran-Dinh, N., Carter, D. Characterization of microsatellite loci in the aflatoxigenic fungi *Aspergillus flavus* and *Aspergillus parasiticus*. *Mol. Ecol.*, 2000; **9**: 2170-72.
36. 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.
37. Wild, C.P., Gong, Y.Y. Mycotoxins and human disease: a largely ignored global health issue. *Carcinogenesis*, 2010; **31**(1): 71-82.
38. Yassin, M.A., El-Samawaty, A.M.A., Moslem, M.A. and El-Naggar, M.A. Mycobiota of almond seeds and the toxigenicity of some involved genera. *Life Sci. J.*, 2013; **10**(4):1088-93.