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Detection aflatoxin production by local isolates of *Aspergillus* spp. and molecular characterization

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Abstract

Five isolates of *Aspergillus* spp. were isolated from stored wheat cereal and peanuts (groundnuts) in the El-Monofiya governorate in Egypt. The isolates were identified using morphological and microscopic properties. The ITS (internally transcribed spacer) region was found in the location of 600 bp. Results compared with data in GenBank confirmed that three isolates belonged to *Aspergillus flavus* and two isolates belonged to *Aspergillus parasiticus*. Three strains of *Aspergillus flavus* (Af1, Af2 and Af3) and two isolates of *Aspergillus parasiticus* (Ap4 and Ap5) were tested to produce aflatoxin on Czapek's agar medium. To distinguish between aflatoxigenic and non-aflatoxigenic isolates, TLC (thin layer chromatography) and PCR (polymerase chain reaction) were used. The TLC technique was previously used to quantify aflatoxin (B1, B2, G1 and G2) of five strains of *Aspergillus*. Three strains Af1, Af2 and Af3 gave B2 (50 µg/kg). The other strains Ap4 and Ap5 gave B1 (50 µg/kg) and G1 (75 µg/kg) for Ap4 and G2 (100 µg/kg) for Ap5. The DNA from all isolates has been extracted and amplified by PCR to encode target genes for the development of toxins (omt-A). It was observed in 3 (60%) of isolates Af1, Af2 & Af3 that the genes O-methyltransferase gene (omt-A) 300 bp. In addition, four SCoT primers and six ISSR primers were used to genetically link five *Aspergillus* spp. All five strains were divided into two groups for ISSR and SCoT primers. ISSR and SCOT analysis yielded similar results with TLC and aflatoxin-specific genes.

Keywords: *Aspergillus* spp., molecular, aflatoxin, identification, TLC.

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1. Introduction

In crops, *Aspergillus parasiticus* and *Aspergillus flavus* produce aflatoxin. The amount of aflatoxins created by culture, as well as the capacity to create mycotoxin or morphology of mycelia and conidiospores differ across these species (Linz *et al*., 2014). Investigate the genetic basis for biochemical and morphological distinctions. *Aspergillus flavus* is a major aflatoxin producer that can tarnish a diverse a variety of agricultural products in the field or in a warehouse. Aspergillus belongs to the phylum Ascomycetes and contains over 185 recognized species. Approximately 20 of them have been linked to harmful infections in humans and animals. Foods contaminated by Aspergillus species include many types of vegetables such as beans, wheat, etc. (Pitt and Hocking, 2009). *Aspergillus flavus* section comprises six economically significant species which are morphologically and phylogenetically closely related and sometimes divided into two categories based on their influence on foodstuffs or in terms of human health. The parasites are *A. flavus*, *A. parasiticus*, and *A. nomius* non-aflatoxigenic species are wreaking havoc on agricultural commodities all over the world. *A. Oryzae*, *A. Sojae* and *A. Tamarii* were used historically in Asia for fermented food production (Godet and Munaut, 2010; Kumeda and Asao, 2001). The distinction inter alia non-aflatoxigenic and aflatoxigenic of *Aspergillus flavus* has been a matter of dispute (Jamali *et al*., 2013). Microbiological recognition high performance thin layer chromatography (TLC) testing is a traditional method used to detect aflatoxins. These procedures are time consuming, so effective detection techniques for aflatoxins are considered (Hussain *et al*., 2015). PCR is easy to use, rapid and accurate (Luque *et al*., 2012). ISSR, like RAPD, is a dominant marker with increased repeatability and extreme variability. ISSR outperforms other readily available marker systems when it comes to investigating genetic variation among very closely related individuals. The technique is useful in a wide range of plant species for purposes such as genetic diversity, phylogenetic studies, and gene mapping (Dje *et al*., 2006; Fang and Roose, 1997; Nagaoka and Ogihara, 1997). Genomic sequencing is a new method of analysing a person's genome. Scientists are developing a plethora of novel and promising approaches (Gupta and Rustgi, 2004). SCoT polymorphism markers were used by the researchers to study genetic diversity in Aspergillus genotypes. They discovered genetic variations based on molecular markers between and within species for the first time (Saeed *et al*., 2011; Tanya *et al*., 2011). Because of the high degree of genetic variation in the ITS1-5.8S-ITS2 region, comparisons among Aspergillus species are extremely useful for strain classification and phylogenetic studies (Kumeda and Asao, 2001; Henry *et al*., 2000). The use of ITS markers to identify species may be more dependable than examining D1-D2 domain sequences of large ribosomal subunits (Hinrikson *et al*., 2005). Due to the diffusion of contamination in *A.* spp. food and the difficulty of obtaining a diagnostic method to differentiate aflatoxin gene encoding, molecular screening, and detection isolates. Thus, our

research aims at: (1) Isolation and identification of *A.* spp. from wheat cereal and peanut (2) determine molecular detection for aflatoxin-encoding genes (omt-A) of *A.* spp. (3) distinguish between aflatoxigenic and non-aflatoxigenic according to PCR by ISSR and SCoT and (4) to analyze the role of TLC in the analysis and identification of the level of aflatoxin from *A.* spp.

2. Materials and methods

2.1 Isolation and purification of fungal isolates

The five fungal isolates of *Aspergillus* were isolated from wheat cereal and peanuts (groundnuts) were stored at room temperature from El-Monofiya governorate in Egypt. The seeds were surface sterilized before being transferred to Petri dishes containing PDA (potato dextrose agar) medium and incubated at 30^{o}C for 7 days. All fungal isolates were cleansed using the solitary spore or hyphal tip method and kept on PDA medium slants at 4^{o}C until identified or needed by Alexopoulos *et al*. (1996) and Hogg (2005).

2.2 Identification of the aflatoxin-producing fungal isolate

The fungal isolates chosen for aflatoxin production were identified by morphological and cultural characteristics on malt extract agar (MEA) and Czapeks media by examining colony color, form, reverse, size, appearance and cell morphology according to the handbook in order to identify fungi (Alexopoulos, *et al*., 1996; Hogg, 2005). The microscopic observation of the fungal morphology in the case of each of the isolates was done using a binocular microscope. The aflatoxin producing fungal isolates were described in fungal identification manuals (Domsch and Gams, 1972; McClenny, 2005).

2.3 Physiological and biochemical characteristics

The criteria of physiological properties such as the ability to degrade starch, cellulose, gelatin liquefaction and lipid hydrolysis as substrates were studied for genus confirmation. The different carbon utilization was examined to reach a possible classification of species. The carbon utilization test was carried out in the carbon utilizing medium as described in Alexopoulos *et al*. (1996) and Hogg (2005) with the addition of one of the following sugars: glucose (control), sucrose, lactose, maltose and fructose. In addition, other compounds such as sole carbon were examined.

2.4 Fluorescence cultivation and observation of aflatoxin production

Cultures were grown on Czapeks dox medium was used as a basal medium for aflatoxin production plates and inoculated by mass conidial transfer to the center of the plates, which were then incubated at 28°C. An uninoculated plate was observed for comparison. Each plate

contained a central colony, the reverse side of which was regularly tested for blue fluorescence under long wave (365 nm) UV light (Gurav and Medhe, 2018).

2.5 Preparation of aflatoxin standard solutions

Sigma Chemical Company supplied the aflatoxins B1, B2, G1 and G2 were dissolved separately in benzene-acetonitril mixture (98:2, v/v) to give a concentration of 1µg toxin/ml. The standards were checked for purity and concentration as recommended by AOAC (1990) where the authentic toxins were measured spectrophotometrically (CE-Cil instrument) at wave lengths of 233, 265 and 360 nm. The optical densities of each toxin were recorded, and the concentrations were calculated according to AOAC (1990).

2.6 Quantitative determination of aflatoxin

To scan the positive spots identified under UV light, a photo documentation system (SIM Gel Documentatio System, Bio–Best, 140 A) was used. The concentration of aflatoxins in peanut samples was calculated in µg/L (ppb) Using the BIO-ID V.6.10 computer programme, follow the manufacturer's instructions which, is set up to analyze the intensity of the spots created by TLC and compare them to those of the standards to quantify the aflatoxin content in the samples.

2.7 DNA Isolation

The DNeasy Plant Tissue Mini Kit (QIAGEN) were used to obtain genomic DNA from all strains. The concentration of DNA was then determined by comparing the DNA samples to standard lambda DNA on a 1% (w/v) agarose gel, and it was adjusted to 5 ng/l.

2.8 Amplification of the rDNA ITS region

The PCR mixtures were prepared to a firm volume of 50 L and inclusive of reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM MgCl2, 0.2 mM dNTP, 12.5 mM of all primers, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATG C-3′), 1.25 U Taq DNA polymerase and 50 ng genomic DNA (White *et al*., 1990). Amplification depended on an initial denaturation step at 95°C for 4 minutes directed by 40 cycles of denaturation at 92°C for 1 minute, annealing at 51.3°C for 1 minute and amplification at 72°C for 2 min, with an end extension step at 72°C for 5 minutes. The products were insular on a 0.1% agarose gel using a 100-bp ladder DNA marker. Ethidium bromide is added to the gel before being photographed under UV light.

2.9 Phylogenetic identification

Phylogenetic identification was performed using the fungal kingdom's highly variable region 5.8S rDNA gene described by Fakruddin *et al.* (2013). The homology of the genes was checked against those of other organisms that had already been submitted to the GenBank database using the BLASTN algorithm.

2.10 Target genes and PCR reagents

Total DNA was used for detection of genes which encode aflatoxin produced by *A.* spp. isolates. PCR was conducted to amplify the gene O-methyltransferase gene (omt-A) for aflatoxin B1 biosynthesis. These genes and specific primer sequences were chosen from previously published data (Deabes *et al*., 2018; Shweta *et al.*, 2013). The primer sequence, the name of the gene and the output size are offered in Table (1). The PCR reagents were provided and synthesized by (Bioneer-Korea).

2.11 Optimization of PCR for target genes

PCR reaction was conducted in 25 µl included (5 µl) premix (Taq DNA polymerase, 250 µM (each) dATP, dGTP, dCTP, dTTP and 1.5 Mm $MgCl_2$, reaction buffer (PH 9) and loading dye buffer, 2 µl of primers, 4 µl target DNA and 12 µl water. Thermal cycle conditions were carried out for these gene initial denaturation 95˚C for 4 minutes annealing at 58-62˚C for 1 minute, followed by 30 seconds of extension at 72˚C, followed by 10 minutes of final extension at 72˚C. Gel electrophoresis was used to verify PCR products on 1% gel and make them visible by using the gel documentation system (Abdel-Hadi *et al*., 2011).

2.12 ISSR-PCR

In this study, six ISSR primers were used. Amplification was completed in 35 cycles, with denaturation at 94˚C for 1 minute, annealing at 45˚C for 1 minute, and extension at 72˚C for 2 minutes.

Table (1): Primer was used to amplify the (omt-A) gene in this study.

Primer	Sequence (´5-´3)	Product size	References
omt-F	GACCAATACGCCCACACAG	300 bp	Deabes *et al*. (2018)
omt-R	CTTTGGTAGCTGTTTCTCGC		

Denaturation of 5 minutes at 94˚C was after that a final extension of 5 minutes at 72˚C. 20 ng of DNA template, 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1 % Triton X-100, 1.5 mM MgCl2, 0.1 mM dNTP, 2 mM primer, and 0.5 unit Taq DNA polymerase were included in the reaction mixture (25 µl). To prevent evaporation, master mixes of each reaction were overlaid with 10 l of mineral oil. The products amplification was separated on a 1.2 % agarose gel. Table (2) shows the primers that were tested.

2.13 SCoT-PCR

In this study, there were four SCoT primers used. The reaction volume was 25 µL and it contained 1.5µL of template DNA (25ng/µL), 1.0 µL primer at 10 M, 2.0 µL dNTPs at 10 M, 0.125 µL, Taq DNA polymerase at 5U/µL, 2.5µL 10X PCR buffer and 17.875 µL ddH_2O. The initial denaturation was carried out at

94°C for 5min. followed by 35 cycles of 94°C for 1 minute, 50°C for 1 minute, 72°C for 2min. and final extension at 72°C for 5 minutes. The products were separated in 1.2% agarose gels containing ethidium bromide by electrophoresis and photographed. Table (2) shows the primers that were tested.

Table (2): ISSR and SCoT primer sequences were used in the analysis.

ISSR Primers		SCoT Primers	
P. Name	P. sequence 5'-3'	P. Name	P. sequence 5'-3'
ISSR-10	$(GAGA)_3CC$ *	SCoT 1	CAACAATGGCTACCACCA
ISSR-11	$(GT)_6CC$ *	SCoT 2	CAACAATGGCTACCACCC
ISSR-12	(CAC)3GC *	SCoT 3	CAACAATGGCTACCACCG
ISSR-13	(AG)8YT *	SCoT 4	CAACAATGGCTACCACCT
ISSR-14	(AC)8CG		
ISSR-15	$(AC)_8YG$ *		

2.14 Statistical analysis

Polymorphism was calculated as a percentage by dividing the number of polymorphic bands by the total number of bands. SCoT and ISSR bands were manually scored as present (1) or absent (1) to estimate the similarity among all tested samples (0) Jaccard's coefficient was used to compute pairwise comparisons.

3. Results and Discussion

3.1 Isolation of Aspergillus isolates

Five isolates of *Aspergillus* were isolated from wheat cereal and peanut (groundnut) was stored at room temperature from El-Monofiya Governorate in Egypt. The isolation was done by sterilized surface seeds and transferred into petri dishes containing PDA medium, the fungal colony appearance was streaked on PDA medium. Subsequently, the fungal isolates were subculture into their respective selective growth media until pure cultures were isolated (Kaur and Kaur, 2014; Okonji, *et al*., 2019). These isolates were named Af1, Af2, Af3, Ap4 and Ap5.

3.2 Identification of fungal isolates

Following the incubation period, colonies were subculture to the appropriate media and incubated further to achieve pure cultures of each fungal species. Fungal isolates were identified using morphological and microscopic features. The classification considered several macroscopic characteristics (Table 3). On malt extract agar (MEA) medium, distinguishing traits included colony coloration: white and brown shades, orangish brown, dark brown and green shades, whereas on Czapek's medium, colony coloration was observed as green and white, yellowish green, color and floccose or flat colonies. Sclerotia and exudates were rarely observed in this medium. The colony diameter for five isolates of *Aspergillus* ranged from 30 to

70 mm. The colony reverse, on the other hand, three strains penetrated the agar, and reverse coloration was in goldish to red brown, or orange shades, whereas the Aspergillus strains Ap4 and Ap5 were colorless for colony reverse on Cezapike medium. These findings corroborate those of Alexopoulos *et al.* (1996), who stated that *Aspergillus flavus* was identified by deep yellow-green, light yellow-green, olive brown, or brown spore heads that were compact, spherical, or columnar. Microscopic analysis of five *Aspergillus* isolates was performed. These isolates were observed to have conidial heads typical of *Aspergillus* with a radial or apical head. Conidial heads were mostly biseriate, but occasionally uniseriate; conidia were usually round, and hyphae were septate.

Table (3): Five isolates of *Aspergillus* spp. were studied for their morphological, cultural, and biochemical properties.

Fungal isolates / Characteristics	Af1	Af2	Af3	Ap4	Ap5
Morphological colony					
Form	Circular	Circular	Circular	Circular	Circular
Margin	Entire	Entire	Entire	Entire	Entire
Colour	White to brownish	Yellow to green	Orangish to green	Yellowish green	Yellowish green
Reverse	Goldish to red brown	Goldish to red brown	Goldish to red brown	Colourless	Colourless
Growth at temperature					
25 ^{O}C	++	++	++	+++	+++
30 ^{O}C	+++	+++	+++	+++	+++
35 ^{O}C	+++	+++	+++	+++	+++
40 ^{O}C	++	++	++	++	++
45 ^{O}C	+	+	+	+	+
Microscopic characteristics					
Shape of mycelium	Separate	Separate	Separate	Separate	Separate
Spore formation	Positive	Positive	Positive	Positive	Positive
Biochemical characteristics					
Gelatin liquefaction	+++	+++	+++	+++	+++
Starch hydrolysis	++	++	++	+++	+++
cellulose hydrolysis	++	++	++	++	++
Lipid hydrolysis	+++	+++	+++	+++	+++
Growth at carbon sources					
Glucose	+++	+++	+++	++	++
Sucrose	+++	+++	+++	+++	+++
Lactose	++	++	++	++	++
Maltose	+++	++	++	++	++
Fructose	+++	+++	+++	++	+++

+ means weak growth, ++ means moderate growth, +++ means highly growth.

Microscopic examination confirmed that the isolated isolates belonged to the genus Aspergillus. The biochemical and physiological characteristics were derived from the study described by Alexopoulos *et al.* (1996) and Hogg (2005). All tests were done at 30^{o}C. The observed data of physiological and biochemical tests of the isolates are recorded in Table (3).

3.3 Molecular identification for the fungal isolates

Molecular techniques have become increasingly popular used to identify *Aspergillus* species. Amplification of

DNA directed by DNA sequence is a powerful tool in research. *Aspergillus* is in fact, one of the most genetically fungi research. In July 2005, the entire genome of *A. flavus* NRRL 3357 was sequenced and liberal to the NCBI (National Centre for Biotechnology Information). By using the ITS1 and ITS4 primers, a unique band at 600 bp was show for each of the five *Aspergillus* strains (Figure 1). DNA sequencing of the five strains shows extra than 98% similarity with sequences of *Aspergillus flavus* for isolates (Af1, Af2 and Af3 and *Aspergillus parasiticus* for isolates (Ap4 and Ap5) (Figure 2). These results agree with Schoch *et al.* (2012).

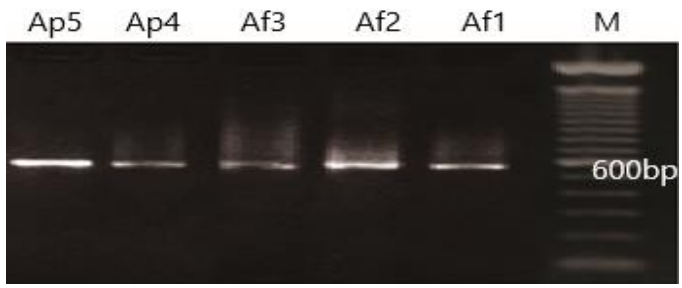

Figure (1): Profile of the rDNA ITS region of five strains of *Aspergillus* (lanes Af1, Af2, Af3, Ap4 and Ap5) at 600-bp unique band of a 100-bp DNA ladder marker (M).

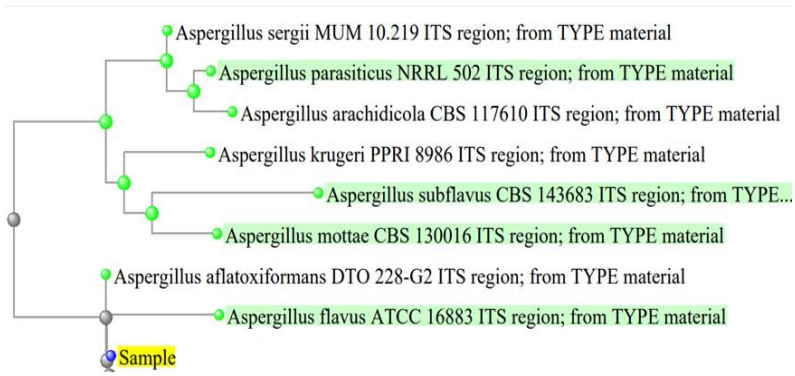

Figure (2): Dendrogram of the ITS region of one strain of *Aspergillus* spp.

3.4 Detection of aflatoxin production by fungal strains

In the current research, a rapid sensitive and precise technique has been created to detect aflatoxigenic and non-aflatoxigenic strains from different sources (Karlovsky *et al*., 2016). The results showed that out of five strains, three strains (Af1, Af2 and Af3) (60%) were positive amplification for the (amt-A) gene targeted by PCR. These stains belonged to *Aspergillus*

flavus, while two strains Ap4 and Ap5 (40%) were negative amplification for the (amt-A) gene targeted by PCR or agarose gel electrophoresis. These strains belong to *Aspergillus parasiticus* as reflected in Figure (3) and Table (4). For these genes, which are perceived to be essential for aflatoxin biosynthesis of aflatoxin B1, the initials were thoroughly selected. This first pair resulted in a single 300 bp DNA fragment. These results agree with Fakruddin *et al*. (2015), Deabes *et al.* (2018) and Shweta *et al.* (2013). TLC (The Thin layer chromatography) technique were formerly quantified aflatoxin B1, B2, G1 and G2 of three *Aspergillus flavus* isolates and two *Aspergillus parasiticus*. TLC is also a standard and excellent method for assessing and measuring highly sensitive aflatoxins. The strains Af1, Af2, and Af3 could produce detectable levels of B1 (50 µg/kg) although strains Ap4 and Ap5 failed to yield any discernible amount. Although Ap4 and Ap5 strains were able to produce detectable levels of B2 (50 µg/kg), three strains Af1, Af2, and Af3 failed to produce any detectable amount. On the other hand, the strains Ap4 could produce detectable levels of G1 (75 µg/kg) although four strains (Af1, Af2, Af3 and Ap5) failed to yield any discernible amount. The most advanced level (100 µg/kg) of G2 was obtained from strain Ap5 although four strains (Af1, Af2, Af3 and Ap4) failed to produce a discernible amount (Table 4). These findings back up those of Al-Wadai *et al.* (2013), who used HPLC to screen 19 strains of *Aspergillus flavus* for aflatoxins.

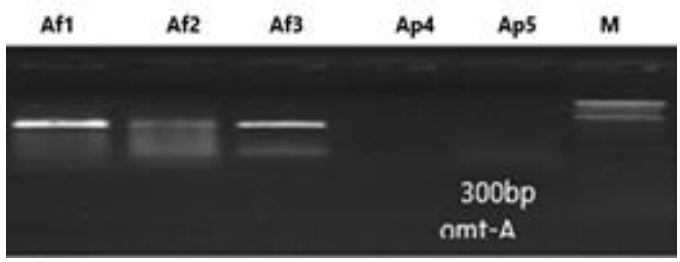

Figure (3): PCR product with (omt-A) primer specific gene by five *Aspergillus* ssp. on 1% agarose gel, M: 100 bp DNA ladder. (Lanes: Af1, Af2, Af3, Ap4 and Ap5).

Table (4): Aflatoxin production by five *Aspergillus* and detection genes were identified using PCR and blue visible fluorescence at 365 nm.

Aspergillus isolates	Blue visible fluorescence at 365 nm	B1(µg/L)	B2 (µg/L)	G1(µg/L)	G2 (µg/L)	Aflatoxin genes (omt-A)
Af1	+	50	0.0	0.0	0.0	+
Af2	+	50	0.0	0.0	0.0	+
Af3	+	50	0.0	0.0	0.0	+
Ap4	+	0.0	50	75	0.0	-
Ap5	+	0.0	50	0.0	100	-

+ positive, - Negative.

Aflatoxin levels in thirteen strains ranged from 0.5 to 2.6. µg/kg. Gurav and Medhe 2018 determine the aflatoxins B1, B2, G1 and G2 in *Aspergillus* by TLCB1 (91.98), B2 (105.2), G1 (87.45) and G2 (90.09) respectively. The same results were published by Linz *et al.* (2014) in *Aspergillus parasiticus* and *Aspergillus flavus*.

3.5 ISSR-PCR analysis

The aim of this experiment was to find out the reason for the reported differences between *A. parasiticus* and *A. flavus*. In this paper, The ISSR-PCR were used to uncover the diversity among five different *A.* spp. strains to search for a polymorphism that could be used as a DNA marker for diagnosis of *A.* spp and study the differences that are induced by environmental conditions. ISSR markers were calculated to detect polymorphic loci between genotypes. ISSR primers were created by combining di-, tri-, tetra-, or penta-nucleotide repeat motifs with a one- to three-nucleotide anchoring sequence (Alexandre *et al.*, 2007; Ruas *et al*., 2003). The six primers that worked with *A. spp* were dinucleotides. Results in Table (5) and Figure (4) showed 48 bands, 25 of which were polymorphic (4.16%) and 23 were monomorphic (3.83%). All primers generated a mean of 4.2 polymorphic fragments on average. The maximum polymorphic primer was ISSR-13, which produced 10 bands, followed by two primers; ISSR-10 and ISSR-12, each of which produced 4 polymorphic bands (Table 2). Figure (4) demonstrates the PCR product of the six ISSR primers and the ability of the six-decamer oligonucleotide primers to resolve variability among *A.* spp. varied greatly. Some primers resulted in multiple bands, while others resulted in only a few bands. The ISSR-13 primer gave the greatest polymorphic band count (10), while ISSR-14 and ISSR-15 primers provided a lower number of polymorphic bands (2). These results agree with Attallah, *et al*. (2014). The polymorphism ranged from 25 to 90.90%. When compared to primer ISSR-14, primer ISSR-13 produced the highest percentage of polymorphism. Each primer's mean band frequency ranged from 0.509 to 0.820. ISSR-13 primer produced the lowest mean frequency of bands (0.509) and the highest polymorphism (90.90%). This primer-13 produced one monomorphic band and the highest polymorphic bands (10), therefore not having higher efficiency. While the ISSR-11 produced the large mean band frequency (0.820) with a polymorphism of 30%. In our study, this primer produced a lot of polymorphic bands in the isolates. Hatti *et al.* (2010) used the same primers and agreed with the present study. (Dheeb, 2013) studied genetic distance between *A. fumigatus* isolates using RAPD technique with the same programming of cluster analysis and genetic distance to make investigation into genetic diversity relationships and attempt to find the DNA fingerprint of isolates, to identify the various isolates. Many studies have

shown that ISSR–PCR is dependent on the reaction condition. To achieve the highest specificity and product yield, the PCR conditions must be optimized (Williams *et al.*, 1990). The analysis of ISSR-PCR and the variation among the five strains, including the absence or presence of bands, differences in molecular weight and intensity of amplified bands (Mayer *et al.*, 2000). Differentiation in the number of bands show by primers, which is influenced by variables such as structure primer and annealing in the genome (Kernodle *et al.*, 1993). In our study, the unique band and the polymorphic bands were generated. The same results obtained by Sayed *et al.* (2017) in *Bacillus* sp.

Table (5): Statistical analysis of ISSR and SCoT primers used for detecting polymorphism in five *Aspergillus* strains.

Primers Name	Primer sequence 5'-3'	Number of bands (NB)	Mono-morphic bands	Molecular weight bp		Unique bands	Poly-morphic bands (PB)	Polymorphism (PPB) %	Mean of band frequency
				Low M.W	High M.W				
ISSR Primers									
ISSR-10	(GAGA)3CC *	9	5	189	1731	2	4	44.44	0.778
ISSR-11	(GT)6CC *	10	7	195	1696	1	3	30.00	0.820
ISSR-12	(CAC)3GC *	7	3	238	1360	2	4	57.14	0.714
ISSR-13	(AG)8YT *	11	1	184	1307	4	10	90.90	0.509
ISSR-14	(AC)8CG	8	6	362	1377	2	2	25.00	0.800
ISSR-15	(AC)8YG *	3	1	481	1235	1	2	66.66	0.667
Total		48	23			12	25		0.714
Average		8	3.8			2	4.16	52.35	4.288
SCoT Primers									
Primer Name	Primer sequence 5'-3'		NB	PB	Monomorphic bands		PB %	Unique Bands	Mean of Band frequency
SCoT 1	CAACAATGGCTACCACCA		13	5	8		38.46	1	0.785
SCoT 2	CAACAATGGCTACCACCC		22	12	10		54.54	2	0.745
SCoT 3	CAACAATGGCTACCACCG		15	9	6		60.00	1	0.720
SCoT 4	CAACAATGGCTACCACCT		12	9	3		75.00	0	0.667
Total			62	35	27			4	2.917
Average			15.5	8.75	6.75		57	1	0.72925

* Y----C/T.

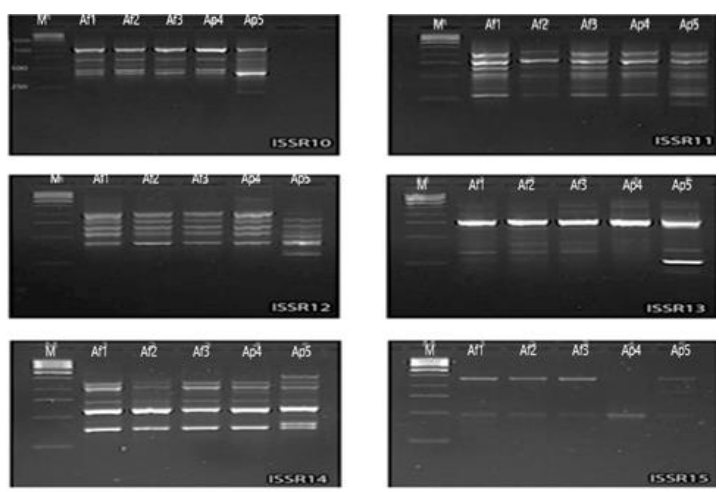

Figure (4): ISSR marker profiles for the five *Aspergillus* strains (Lane Af1, Af2, Af3, Ap4 and Ap5), M Gene Ruler 1Kb DNA (Lane 1).

3.6 SCoT polymorphism

The aim of this experiment was to find out the reason for the reported differences between *A. parasiticus* and *A. flavus*. In this paper, we used marker called start codon targeted polymorphism, which is based on ATG translation start codon (Joshi *et al*., 1997; Sawant *et al*., 1999). PCR is used to create DNA tags, which are designed from the ATG start codon, which is conserved across all genes. As a result, this technique is similar to RAPD or ISSR (Gupta *et al*., 1994; Williams *et al*., 1990). In this study, SCoT primers were used with a flanking region around the ATG start codon derived from previous studies. The distance in base pairs between the template's primer binding sites is critical, and we recommend at least one minute (Joshi *et al*., 1997; Sawant *et al*., 1999). Table (5) shows the primer sequences, codes, number of bands (NB), polymorphic bands (PB), and percent of polymorphic bands (PPB) for SCoT primers. Polymorphism was found in all the primers used in this study. Furthermore, the total number of bands (62) indicates that polymorphism was found in 62 of them. The SCoT2 primer had the most bands (22) while the SCoT4 primer had the fewest (12). Figure (5) depicts the band pattern of all SCoT primers. The average polymorphism percentage was 57%, with the SCoT1 primer producing the least polymorphism (38%) and the SCOT4 primer producing the most polymorphism (75%). The mean band frequency for all primers was 0.729, with SCoT1 producing the highest value and SCoT4 producing the lowest (Table 5).

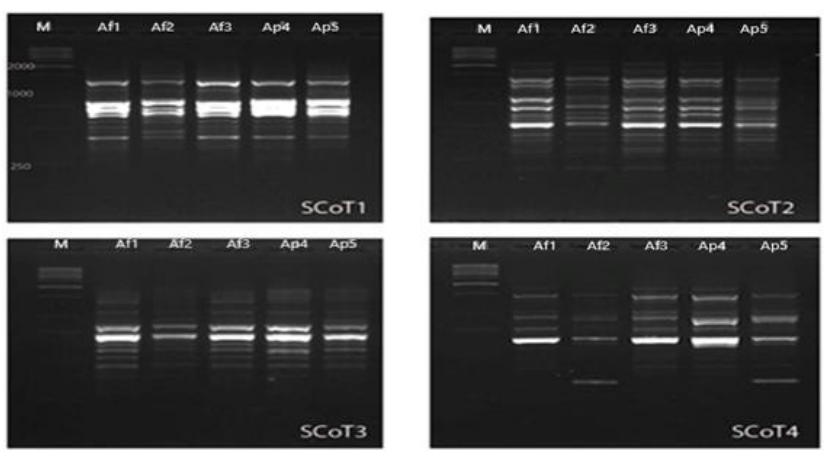

Figure (5): SCoT marker profiles for the five *Aspergillus* strains (Lane 1-5), M Gene Ruler 1Kb DNA (Lane 1).

Table (5) shows that 3 primers (SCoT2, SCoT3 and SCoT1) produced (22, 15 and 13 total bands, respectively). Out of these 62 PCR products generated, (27) were

monomorphic bands, one unique band on average and the remaining (35) bands of the total products scored were polymorphic among the studied isolates. These are like those obtained by Ophel and Kerr (1990), Mackill (1995) and Kaushik *et al.* (2003) in rice and in agrobacterium by EL-Shaer *et al*. (2014). The current study's findings demonstrated that SCoT markers were effective in evaluating genetic diversity among *A.* ssp.

3.7 Cluster analysis by ISSR and SCoT

Cluster analysis works from the bottom up to group all variables (strains) into a single cluster. The most important part of the SPSS output is the dendrogram, which represents the phylogenetic relationships among the variables (strains) under study. Figure (6) shows the cluster analysis for the ISSR and SCoT primers of the five *Aspergillus* strains using average linkage (between groups). Figure (6) shows the two major clusters. The first major group was divided into two subgroups; the first subgroup was divided again into two subgroups; one contains the strain number Af3, and the other contains strain Af1 with the same production of aflatoxin B1 (50 µg/kg). The second subgroup contains strain Af2 with the same production of aflatoxin B1 (50 µg/kg). The second main group included two subgroups; one contains the strain Ap4 and the other contains strain Ap5 with the same production of aflatoxin B2 (50 µg/kg) and differences in G1 and G2 production of aflatoxin. Figure (6) explains the convergence and divergence among the five strains from different sources. Convergence among *Aspergillus* strains resulted for many different reasons like source of isolation, similarity in morphological characteristics, genetic characteristics, aflatoxin production and other characteristics.

3.8 Genetic distance analysis

The genetic distance between *A.* spp. strains was calculated using a genetic program (SPSS Analysis System Version 21 package) based on the number of shared bands between each variety. As the number of shared bands increased, the genetic distance decreased and vice versa. The genetic distance among the five *A.* spp. strains was determined. This occurs through an intersection between each of the two strains. The highest value of genetic distance (94%) occurred between strains (Af1 and Af3) which produced the same amount of B1 (50 µg/kg) of aflatoxin and the lowest value was (74%) between strains (AF2 and Ap5). They are different in aflatoxin production.

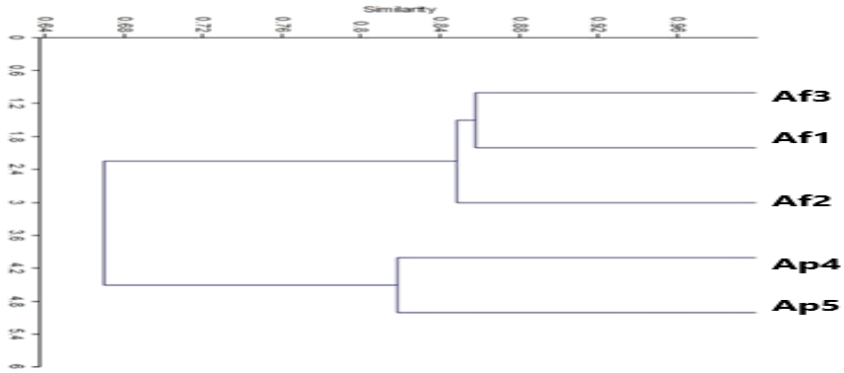

Figure (6): Cluster analysis (UPGMA) dendrogram of five *Aspergillus* ssp. strains based on ISSR and SCoT markers.

4. Conclusion

Contamination with aflatoxin in feed poses and food a serious public health risk, with serious consequences. Most Egyptian crops have been found to be contaminated with aflatoxigenic *Aspergillus* spp. strains. As a result, continuous surveillance is required to detect aflatoxin-contaminated crops. The TLC was used in this study to detect B1, B2, G1, G2 aflatoxins in *Aspergillus* spp. strains isolated from wheat cereal and groundnut samples, as well as to encode target genes for the development of the toxins (Omt-A). Furthermore, the results showed that both the SCoT and ISSR marker systems could be used effectively in the determination of genetic detections of aflatoxin in *Aspergillus* spp. It is possible to conclude that knowledge of genetic similarities and diversity among *Aspergillus* spp. genotypes is required for molecular identification and differentiation methods.

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