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Molecular Identification and Inter-Simple Sequence Repeat (ISSR) Differentiation of Toxigenic *Aspergillus* Strains

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Abstract

The *Aspergillus* genera are one of the most abundant and widely distributed fungi. Among the *Aspergillus* genera are *A. parasiticus*, *A. flavus*, which produces aflatoxins, *A. ochraceus*, and *A. niger* that can produce ochratoxin A. Therefore, the aim of this study is to perform molecular identification and differentiation using the ISSR technique to the *Aspergillus* species, and study the ability of fungal isolates to produce mycotoxins. The ISSR markers are used in the analysis of the genetic similarity of *Aspergillus* isolated from peanuts. Data show the ability of the isolated *A. flavus*, and *A. parasiticus* to produce aflatoxins, whereas *A. ochraceus* produced ochratoxin A. To analyze the genetic similarity among the different fungal isolates, a total of 165 full bands were scored from the amplified products with the ten ISSR primers; 116 bands were polymorphic, and forty-one were unique bands with an average range size of (143-1939) bp. The similarity matrix between the isolated fungi revealed that the isolates *A. parasiticus* and *A. niger* showed the highest similarity (59 %). The ISSR molecular markers are an extremely valuable means to characterize the genetic similarity of the *Aspergillus* genera.

Keywords: *Aspergillus* fungi, Mycotoxins, PCR, ISSR , Internal transcribed spacer

1. Introduction

The growth of several fungi in different agricultural crops leads to a decrease in the yield and quality with major economic losses (Richard, 2007; Adejumo and Adejoro, 2014). Among these fungi is *Aspergillus*, which is one of the most economically important genera, as foods, plants, and soils are commonly inhabited by the majority of these species (Yu *et al*., 2005). The excessive occurrence of *Aspergillus* isolates is considered significant since these species are well-known to produce several mycotoxins (Sahab *et al*., 2014).

Aflatoxins (AFs) are among the most potent mycotoxins produced by the toxigenic strains of *Aspergillus flavus* and *A. parasiticus* (Ashiq, 2015). Meanwhile, ochratoxin A (OTA) is produced by several genera of the *Penicillium* and *Aspergillus* species including *A. ochraceus* (Aly *et al*., 2012). Aflatoxins have been classified as group one human carcinogen (IARC, 2012). Moreover, OTA is classified as group 2B, that is possible human carcinogens (IARC, 2002). Territrems produced by *A. terreus*, induce acute toxicities such as tremor, as well as hepatocellular and nephrotoxic damage in rats and mice (Abdalla *et al*., 1998).

Morphological parameters including colony diameter, color and texture, size, and texture of conidia and hypha structure are traditional strategies for fungal species identification. Nevertheless, due to the intensive divergence of the morphological characters created by a high level of genetic variability, each inter-and intraspecific fungal species classification may be difficult (Kumeda and Asao, 2001). In spite of numerous investigations, the taxonomy of fungi is still highly complex. To minimize the problem, the genetic methods help determine the level of polymorphism and similarity amongst fungal strains.

From their DNA sequences, taxonomy, population structure, and the epidemiology associated with fungi, molecular biology have presented numerous insights into the detection and genetic relationships of fungal isolates (Paplomatas, 2004). The 18S rRNA gene, mitochondrial DNA, the intergenic spacer region, and the internal transcribed spacer (ITS) regions are targets for the genus level detection of *Aspergillus*. For the appropriate detection of pathogens at the species level, the ribosomal RNA (rRNA) genes in ribosomal DNA possess the characteristics (O´Donnell, 1992). These rDNA sequences show a variety of conserved and different regions within the genome that are highly stable (Bruns *et al*., 1991). They each consist of the 18S small subunit (SSU), the 5.8S, and the 28S large subunit (LSU) genes, and occur in multiple copies with up to 200 copies per haploid genome arranged in tandem repeats (Paplomatas, 2004). To amplify the entire 5.8S rRNA gene, ITS primers 1 and 4, ITS regions I and II, and a portion of the 18S small-subunit rRNA gene have been used.

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Recently, the development of molecular biology tools and technologies has enormously progressed (Frankham *et al*., 2002). Consisting of several simple sequence repeats, the inter-simple sequence repeat (ISSR) is established on closely-spaced microsatellites by primers (25-30 bp) and the amplification of regions (100-3000 bp) between inversely-oriented microsatellites (Reischl and Lohmann, 1997). These primers that are abundant throughout the eukaryotic genome and grow fast are annealed to simple-sequence repeats (microsatellites) (Morgante *et al*., 2002). However, primers that work for one may not work for another, since there is a lot of diversity among fungi. Therefore, the ISSR primers need to be enhanced for each fungal species (Klaassen, 2009).

The ISSR technology has been used to characterize gene tagging (Ratnaparkhe *et al*., 1998), phylogenetic studies (Dutech *et al*., 2007), population genetic structure (Hadrich *et al*., 2010), genome mapping (Chakravarty, 2011), genetic structure (Rampersad, 2013; Gramaje *et al*., 2014), genetic diversity (Mahmoud *et al*., 2014), and fingerprinting (Priyanka *et al*., 2014) in plant-pathogenic fungi. Therefore, the aim of this study is to perform molecular identification and differentiation using the ISSR technique to *Aspergillus* species, and to study the ability of fungal isolates to produce mycotoxins.

2. Materials and Methods

2.1. Fungal Strains

Aspergillus flavus, *A. parasiticus*, *A. niger*, *A. ochraceus* and *A. terreus* were used in this study. They were isolated from peanut (*Arachis hypogaea*) samples as mentioned previously (Aly *et al*., 2018).

2.2. Ability of Fungal Isolates to Produce Mycotoxins

One mL of each of the fungal spore suspensions were transferred into a 250 mL flask containing 100 mL of yeast extract broth (yeast extract 2 %-Sucrose 20 %). The cultures were incubated for seven days at 28°C. Aflatoxins, ochratoxin A, and territrems were extracted using chloroform. The chloroform extracts were evaporated under nitrogen gas, and the residue was dissolved in methanol and passed at a rate of about 1-2 drops/second through immunoaffinity column (C_{18}). The immunoaffinity column was washed twice with 10 mL of purified water at a rate of about two drops/second. Using 1.0 mL of methanol, elution was performed and analyzed by HPLC.

The HPLC system used for mycotoxin analyses is an Agilent 1200 series system (Agilent, Berks, UK) with a fluorescence detector (FLD G1321A), an autosampler ALS G1329A, FC/ALS thermal G1330B, Degasser G1379B, Bin Bump G1312A,and a C_{18} (Phenomenex, Luna 5 micron, 150 × 4.6 mm) column joined to a pre-column (security guard, 4 × 3-mm cartridge, Phenomenex Luna). The acetonitrile/water/methanol (1/6/3 v/v/v) mobile phase was used for the separation of AFs at a flow rate of 1.0 mL/min, and at ambient temperature. The cetonitrile/water/acetic acid (99/99/2 v/v/v) mobile phase was used for the separation of ochratoxin A(OTA).

Spots were applied on Thin Layer Chromatography plates (Aluminum plate of silica gel $60F_{254}$, Merck No. 5554) for the determination of territrems, and benzene-ethyl acetate (65:35 v/v) was used for the development of plates.

2.3. Molecular Identification of Toxigenic Fungi

2.3.1. Extraction of Genomic DNA

Using the Qiagen Kit (Qiagen Sciences, Maryland, USA), the extraction of genomic DNA was performed by following the manufacturer's instruction manual cat. No 69104.

2.3.2. PCR Amplification of ITS Region

To amplify the ITS gene from the fungal isolates, the PCR reactions were done. Using the primer set; ITS1 (5-CTTGGTCATTTAGAGGAAGTAA-3) and ITS4 (5-GCTGCGTTCTTCATCGATGC-3), the ITS region was amplified with some modification (White *et al*., 1990). The PCR cycles consisted of an initial denaturation step for five minutes at 94°C followed by thirty-five cycles of denaturation for thirty seconds at 94°C, annealing for one minute at 55°C, and amplification for two minutes at 72°C, with a final extension step for five minutes at 72°C. On a 1 % agarose gel by electrophoresis in 1X TBE buffer (Tris-borate EDTA, pH 8.0), the amplification products for the ITS locus were separated using a 100-bp ladder DNA marker (Invitrogen, California, USA). The gel was visualized and photographed using ™XR + Gel Documentation System (Bio-Rad, California 94547, USA).

2.4. Molecular Differentiation of Isolated Fungi Using ISSR Technique

Ten ISSR primers were used in the detection of polymorphism among five isolated fungi. These primers were synthesized by Metabion Corporation, Germany. The primers' code and nucleotide sequences are presented in Table 1. With some modifications, the PCR amplification reactions were carried out (Williams *et al*., 1990). Reactions were performed in a 25 µL volume composed of 1x reaction buffer, 0.2 mM of dNTPs, 1.5 mM $MgCl_2$, 0.2 µM of primer, 0.5 unit of Taq polymerase (Qiagen Sciences, Maryland, USA), and 50 ng of template DNA, in sterile distilled water.

Table 1. Name and Sequence of the Primers used in the ISSR analysis

Primer	Sequence
ISSR- 1	5'-AGAGAGAGAGAGAGAGYC-3'
ISSR- 2	5'-AGAGAGAGAGAGAGAGYG-3'
ISSR- 3	5'-ACACACACACACACACYT-3'
ISSR- 4	5'-ACACACACACACACACYG-3'
ISSR- 8	5'-ACACACACACACACACYA-3'
ISSR- 11	5'-ACACACACACACACACYC-3'
ISSR- 12	5'-AGAGAGAGAGAGAGAGYT-3'
ISSR- 13	5'-CTCCTCCTCCTCCTCTT-3'
ISSR- 15	5'-CTCTCTCTCTCTCTCTRG-3'
ISSR- 16	5'-TCTCTCTCTCTCTCTCA-3'

The ISSR-thermocycling profile and the PCR amplification of the DNA have been performed in a Perkin Elmer thermal cycler 9700. The temperature profile in the different cycles was as follows: an initial strand separation cycle for five minutes at 94°C followed by forty cycles comprised of a denaturation step for one minute at 94°C, an annealing step for one minute at 45°C and an extension

step for 1.5 minute at 72°C. The final cycle was a polymerization cycle for ten minutes at 72°C.

PCR products were mixed with a 5 µL gel loading dye and resolved by electrophoresis in a 1.5 % agarose gel containing ethidium bromide (0.5 mg/mL in 1 x TBE buffer at 120 volts). A 100bp DNA ladder was used as molecular-size standard. The PCR products were visualized under UV light, and documented using a ™XR+ Gel Documentation System (Bio-Rad, California 94547, USA).

2.4.1. PCR Fragment Purification

According to the manufacturer's instructions, the PCR products were eluted from agarose gels using Promega®'s Wizard® SV Gel and PCR Clean-Up System.

2.4.2. Data Analysis

The amplified fragments were scored as present (1) or absent (0). Ladder 100 bp DNA was used to identify the molecular weights of fragments. Among the fungal isolates, the similarity matrix was calculated according to Coelho (2001). According to Rohlf (1993), the Unweighted Pair-Group Method with Arithmetical average (UPGMA) were used to design the dendrogram for the similarity coefficient.

3. Results and Discussion

3.1. Ability of Fungal Isolates to Produce Mycotoxins

Fifty fungal isolates, mainly (25) *Aspergillus flavus*, (8) *A. parasiticus*, (8) *A. niger*, (5) *A. ochraceus*, and (4) *A. terreus* were isolated from peanut (*Arachis hypogaea*) samples as mentioned previously (Aly *et al*., 2018). Data in Table 2 show that six out of twenty-five *A. flavus* isolates and four out of eight *A. parasiticus* isolates produced AFs with a percentage of contamination reaching 24.00 and 50.00 % respectively. It was noticed that *A. parasiticus* produced higher concentrations of four types of aflatoxins i.e. AFB_1, AFB_2, ABG_1, and AFG_2 at the concentrations of 53.329, 5.442. 31.746 and 8.408 ppb respectively (Table3). Similarly, *Aspergillus flavus* produced the four types of aflatoxins i.e. AFB_1, AFB_2, AFG_1 and AFG_2 at the concentrations of 1.499, 0.428, 0.806, and 0.452 ppb respectively. *A. ochraceus* produced ochratoxin A at the concentration of 2.775 ppb. Table 3 also shows that *A. niger* and *A. terreus* were not able to produce OTA or territrems, respectively.

The results of this study are in agreement with Sahab *et al*. (2011) who determined that both *A. flavus* and *A. parasiticus* produced AFB_1. Similar observations were reported by Sabry *et al*. (2016) who found that out of fifty *A. parasiticus* isolates, fourteen isolates were able to produce AFB_1. Recently, Al-Hindi *et al*. (2017) reported that 15.4 % of *A. flavus* and 55.0 % of the *A. parasiticus* isolates produced AFB_1in concentrations ranging from 1.6 to 12.4 and from 3.4 to 7.9µg/L respectively. The production of high concentrations of AFB_1 by *A. parasiticus* is highly dangerous as it is considered the most potent carcinogen that causes mycotoxicoses to human and animals (Pildain *et al*., 2008).

Table 2. The ability of isolated fungi to produce mycotoxins.

Fungal Isolates	Mycotoxins	No. of fungal isolates	No. of positive isolates	%
A.flavus	Aflatoxins	25	6	24.00
A.parasiticus	Aflatoxins	8	4	50.00
A.niger	Ochratoxin A	8	0	0.00
A.ochraceus	Ochratoxin A	5	2	40.00
A terreus	Territrems	4	0	0.00

Table 3. Mycotoxin concentration (ppb)**.**

Fungi	Mycotoxin concentration (ppb)						
	AFB_1	AFB_2	AFG_1	AFG_2	OTA	TRA	TRB
Aspergillus flavus	1.499±0.758	0.428±0.342	0.806±0.530	0.452±0.426	-	-	-
Aspergillus parasiticus	53.329±3.173	5.442±3.295	31.746±1.560	8.408±1.486	-	-	-
Aspergillus ochraceus	-	-	-	-	2.775±0.264	-	-
Aspergillus niger	-	-	-	-	ND	-	-
Aspergillus terreus	-	-	-	-	-	ND	ND

AFB_1: Aflatoxin B_1; AFB_2: Aflatoxin B_2; AFG_1: Aflatoxin G_1; AFG_2: Aflatoxin G_2; OTA: Ochratoxin A; TRA: Territrems A; TRB: Territrems B. Results are mean ±SD;ND: Not detected

3.2. Molecular Identification of Toxigenic Fungi

For the species-level identification of fungi, the ITS region is the official DNA barcoding marker. To identify fungal species, DNA barcoding systems employ a short standardized region (between 400 and 800 base pairs). For all the isolated fungal strains, the ITS amplification products using the ITS1 and ITS4 primers with a unique band ranging from 500-600 bp were obtained (Figure 1). The amplicons of ITS regions were column-purified and sequenced using a set of primers. The ITS region sequences were aligned using blast algorithm (http://www.ncbi.nlm.nih.gov/ blast/Blast.cgi) and compared with the published sequences of the ITS region gene of several fungi (Table 4). The first fungal isolate of the sequenced 18S rRNA gene was identified as *Aspergillus ochraceus* with a (98 %) similarity with the *Aspergillus ochraceus* isolate Nitaf 24 (Figure 2). The second fungal isolate of the sequenced 18S rRNA gene was identified as *Aspergillus flavus* with a (99 %) similarity with the *Aspergillus flavus* strain UOA/HCPF 5774 (Figure 3). The high similarity between the fungal strains and their closest phylogenetic relatives indicates that the 18S rRNA gene sequence data are helpful for the identification of fungi. The gene sequence was deposited in GenBank database as *Aspergillus ochraceus* Egy2 (Accession No. LC360803.1) and *Aspergillus flavus* Egy3. (Accession No. LC368455.1). Data in Figures 2 and 3 display the phylogenetic tree based on the ITS region sequences, showing the relationship between fungal

isolates and other species. The tree was constructed using the neighbor-joining method.

The results of this study confirm the importance of using molecular methods such as DNA barcoding systems (ITS region) for typing newly-isolated microorganisms. The phenotypic and genotypic methods are part of the first step of the identifications and selection of potential fungal isolates. These results are in agreement with Henry *et al*. (2000) who identified *Aspergillus* at the species level using the 18S and 28S rRNA genes for primer binding sites, to differentiate it from other true pathogenic and opportunistic molds.

The connected ITS region, ITS 1-5.8S-ITS 2, from documented strains and clinical isolates of Aspergilli and different fungi, were amplified, sequenced, and compared with non-reference strain sequences in GenBank. The ITS amplification of the genus *Aspergillus* species ranged from 565 to 613 bp. Similarly, Okoth *et al*. (2018) used ITS to identify and analyze polymorphism in the *A. flavus* ITS region.

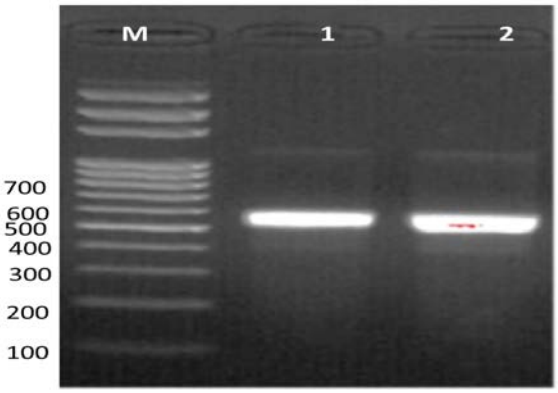

Figure 1. PCR amplification of 18S *rRNA* gene and ITS region ; Lane M: Gene Ruler DNA Ladder 100 bp, Lane 1: 18S *rRNA* gene and ITS region of the isolate *Aspergillus ochraceus*, Lane 2: 18S *rRNA* gene and ITS region of the isolate *Aspergillus flavus*.

Table 4. The nucleotide sequence of two fungal isolates.

Strain	Aligned Sequence Data
Aspergillus ochraceus	CGCGGCGCGCCCCCCCCCCGCCCCCCCGATTCACCCATTTATACCTCCAAACACCCCTTGACCCAAAAAATGC GCGCCTTTGTTCCGGGGGGGTGCGCGCGCTCAACTTTCCTTTCCTTAAGGGGAAACCCTGCGGAAGGATCATT ACTGAGTGAGGGTCCCTCGGGGCCCCAAACCTCCCCACCCCGTGGTATACCGTACCTTGTTGCTTCGGGCGAG CCCCGCCCCCTTTTTTCTTTTAGGGGGCACAGCGCTCGCCGGAGACACCAACGTGAACACTGTCTGAAGTTTT GTCGTCTGAGTCGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAA GAACGCAGCGAAATGCGATAATTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATT GCACCCCCTGGTATTCCGGGGGGTATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGG GTCGTCGTCCCCCCCCCAGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGG GGCTTTGTCACCCGCTCTTGTAGCCCGGCCGGCTGCTGGCCGACGCTGAAAAGCAACCAACTATTTTCCAGGG GACCTCGGATCAGGTAGGATACCCGCTGAATTAGG
Aspergillus flavus	GTGTAACCTGCAGCATGATTCATTACCGAGTGGTAGGGTTCCTTAGCGAGCCCAACCCTCCCCACCCCGTGTT TACTGTACTTTAATTGCTTCGGCGGGCCCCGCCCATTCATGGCCGCCCGGGGGTTTCAGCCCCGGGCCCCGCG CCCCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAAC TTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCA GAATTCCGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAG CGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAA GGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCT TGCCGAACGCAAATCAACTTTCCAGGTTGACCTCGGATCAGGTAGGGAACCCGTGATTAGG

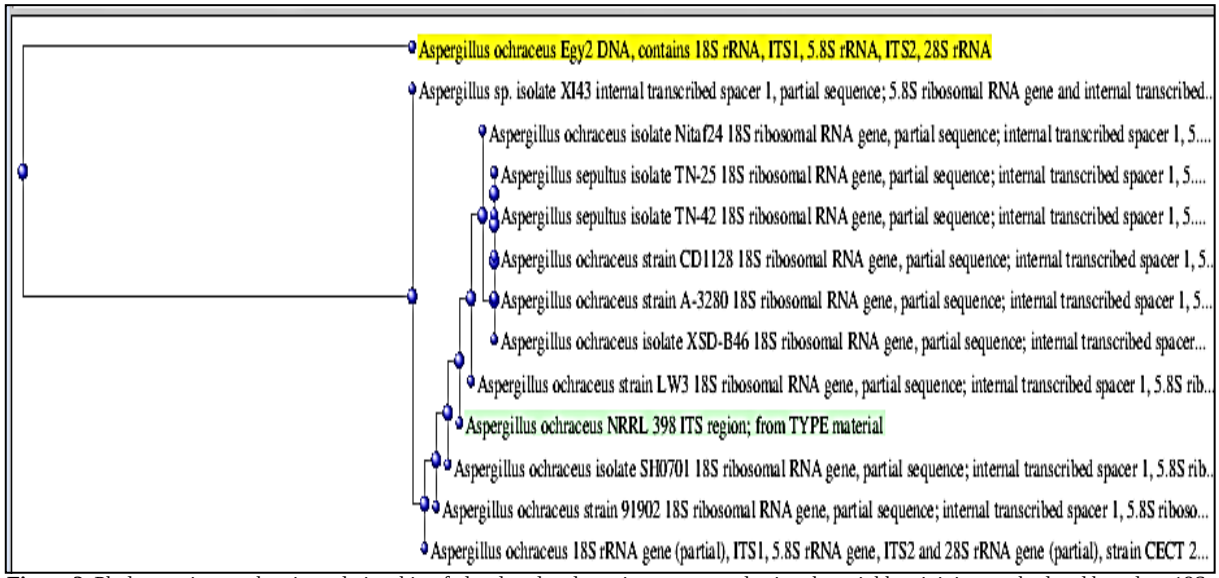

Figure 2. Phylogenetic tree showing relationship of closely related species constructed using the neighbor-joining method and based on 18S *rRNA* gene and ITS region sequences. Isolate is closely related to *Aspergillus ochraceus*

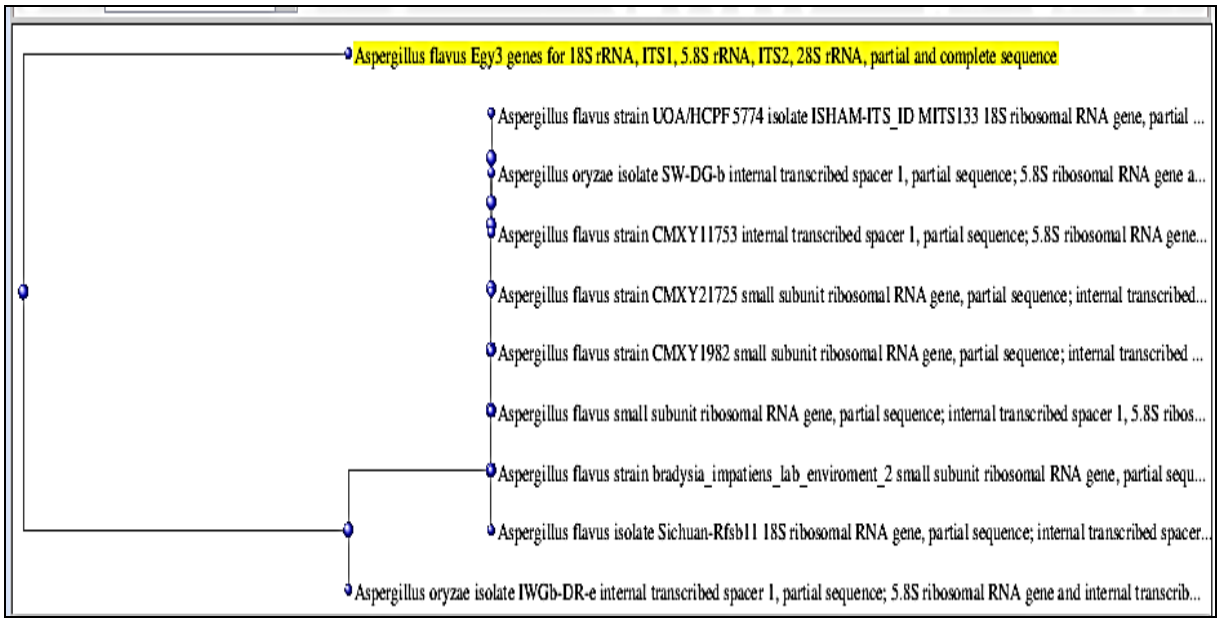

Figure 3. Phylogenetic tree showing the relationship of closely-related species constructed using the neighbor-joining method and based on 18S rRNA gene and ITSregion sequences. The isolate is closely-related to *Aspergillus flavus*

3.3. Molecular Differentiation of Isolated Fungi Using the ISSR Technique

The Inter Simple Sequence Repeat (ISSR) represents genome regions between microsatellite loci. Sequences amplified by ISSR-PCR can be used for delimiting species. ISSR-PCR is a simple, inexpensive, robust, multi-locus marker system which has been used to examine genetic variability among fungal pathogens. The resultant PCR response intensifies the arrangement between two SSRs, yielding a multilocus marker framework helpful for fingerprinting and genome mapping (Chadha and Gopalakrishna, 2007).

In this study, the ISSR PCR technique is used to reveal the genetic diversity between different fungal isolates. Ten ISSR primers produced scorable and reproducible banding patterns. The majority of the primers which produced polymorphic bands had been AC or AG repeats and AT or TC repeats.

A total of 165 full bands were scored from the amplified products with the ten Inter-Simple Sequence Repeat (ISSR) primer; 116 bands were polymorphic, forty-one unique bands with an average range size of (143-1939) bp (Table 5). All primers generated 100 % polymorphism except primers ISSR-14 and ISSR-15 which generated 76.923, and 78.571 % respectively. The obtained results reveal that the primers ISSR-1, ISSR-3 have amplified the maximum number of bands 21-23 respectively. On the other hand primers, ISSR-8 and ISSR-14, have amplified the lowest number of bands (13) (Figure 4). These results indicate that the primers ISSR-1 and ISSR-3 are the most repeated sequences in these fungal isolates compared to other primers. On the other hand, the forty-one unique bands that were detected among the total bands could be considered for marker-assisted selection. The maximum number of unique bands were amplified by primer ISSR-3 which recorded eleven bands, whereas the lowest number of unique bands (two bands) was amplified by ISSR-2, ISSR-8, ISSR-14, and ISSR-15.

These results show that the ISSR primers are robust, informative makers and would be a better tool for genetic divergence and phylogenetic studies. For the production of dendrogram using the UPGMA system, the ISSR primers were used to create a similarity matrix. Mahmoud *et al.* (2016) evaluated the genetic similarity of 30 % among *A. flavus* strains from agricultural crops and air using ISSR markers and proposed that ISSR is an extremely valuable tool for illustrating the genetic similarity of *A. flavus* isolated from several sources.

Nowadays, the advanced performance of the isolates' characterization is used for the ITS species identification as a result of both traditional techniques combined with molecular markers. For the sake of genetically characterizing the *Aspergillus* species, the internal transcribed spacer, the inter-simple sequence repeats (ISSR), and molecular markers are used.

The similarity matrix between isolated fungi reveals that isolates *A. parasiticus* and *A. niger* showed a 59 % similarity (Table 6). The results also show that both *A. terreus* and *A. niger* had a similarity of 56 %, followed by *A. flavus* and *A. niger* showing a similarity of 54 %. Lower similarities were recorded between *A. flavus* and *A. ochraceus* (34 %), and between *A. flavus* and *A. terreus* (44 %). Similar observations were reported by Rassin *et al.* (2015) who used ISSR to study the genetic diversity between different *A. fumigatus* isolates. Similarly, Yugander *et al.* (2015) also used the ISSR markers to examine the genetic variability of twenty-four strains of *Aspergillus* species isolated from paddy. Recently, Adss *et al.* (2017) used RAPD and ISSR to differentiate between seven isolates of *A.solani* and their pathogenic capability.

Table 5. The ISSR primer names and specific character of the ISSR analysis

NO.	Name of primer	Monomorphic bands*	Polymorphic bands**		Total bands	Polymorphism(%) = (polymorphic bands /total bands)* 100	MW range(bp)	Mean of frequency
			Non- unique bands	unique bands				
1	ISSR-1	0	16	5	21	100	214-1939	0.4
2	ISSR-2	0	17	2	19	100	323-997	0.5
3	ISSR-3	0	12	11	23	100	143-1730	0.4
4	ISSR-4	2	9	6	17	88.235	182-1338	0.5
5	ISSR-8	0	11	2	13	100	227-958	0.5
6	ISSR-11	0	11	3	14	100	145-1365	0.4
7	ISSR-12	0	9	5	14	100	182-1577	0.5
8	ISSR-13	0	14	3	17	100	197-1815	0.4
9	ISSR-14	3	8	2	13	76.923	278-1434	0.6
10	ISSR-15	3	9	2	14	78.571	905-1714	0.6
	Total	8	116	41	165	95.152		

*Monomorphic bands= similar band **polymorphicbands= different band

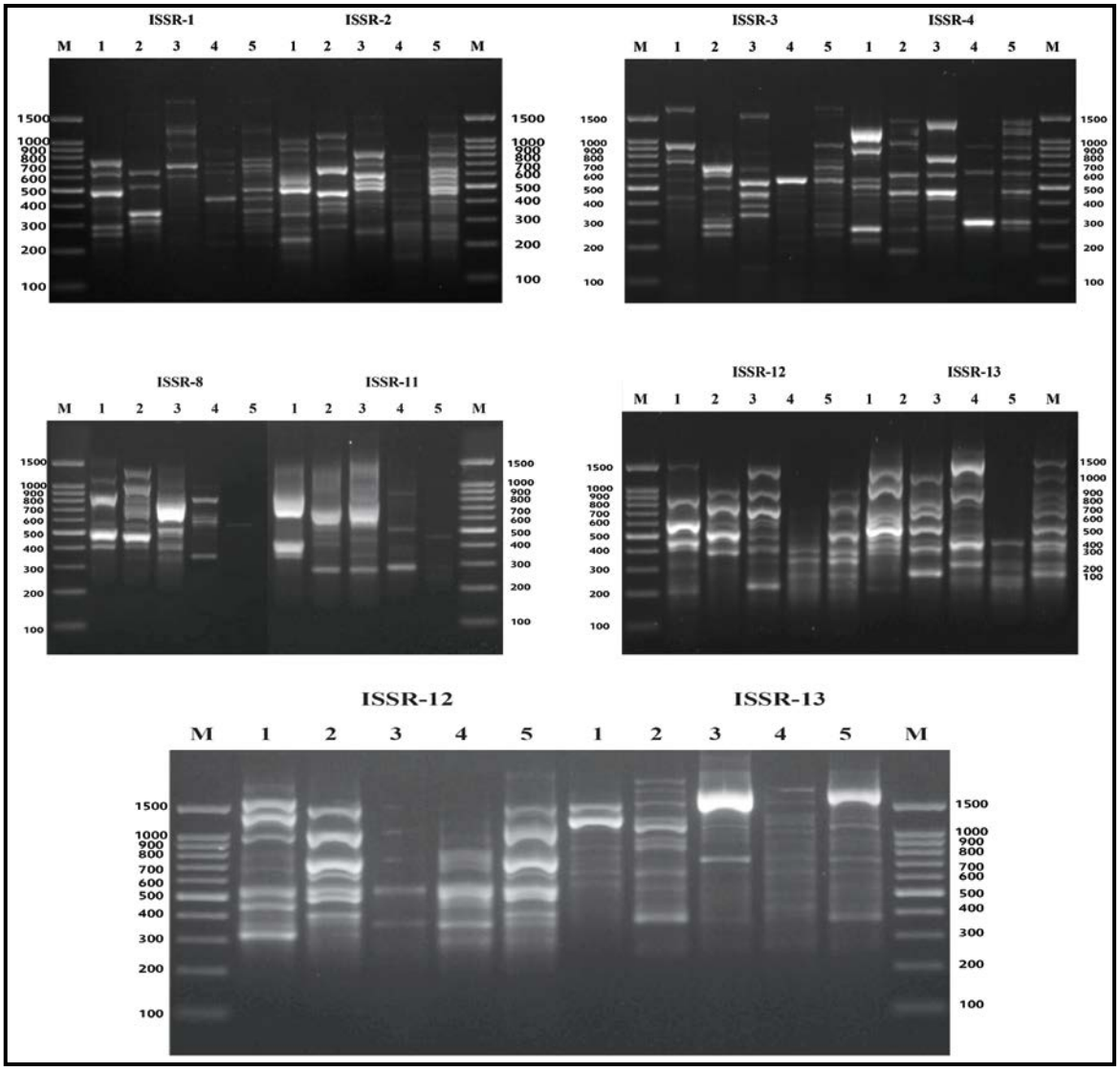

Figure 4. ISSR profile for five isolates of Aspergillus species. Lane 1: Aspergillus flavus; Lane 2: Aspergillus parasiticus; Lane 3: Aspergillus terreus; Lane 4: Aspergillus ochraceus; Lane 5: Aspergillus niger.

Table 6. Similarity matrix of isolated fungi based on ISSR analysis

	Matrix				
Isolates	1	2	3	4	5
1	1				
2	45	1			
3	44	47	1		
4	34	46	45	1	
5	54	59	56	48	1

Isolate 1: Aspergillus flavus; Isolate 2: Aspergillus parasiticus; Isolate 3: Aspergillus terreus; Isolate 4: Aspergillus ochraceus; Isolate 5: Aspergillus niger.

3.4. Cluster Analysis

Dendrogram was constructed using the UPGMA cluster analysis to reveal the genetic relationships among five fungal isolates based on similarity matrix between the isolates. Figure (5) shows three major clusters. The first cluster included the *A. flavus* isolate separate group (1), the second cluster (interposed group) contained the *A. terreus* isolate (3), divided into two subclusters *A. parasiticus* (2) and the *A. niger* isolates (5), and the third group contained A. *ochraceus* isolate (4). Similar observations were reported by Abdulateef *et al*. (2014) who used an ISSR-PCR based technology to document genetic diversity among some local Iraqi isolates through the determination of the ability *A.flavus* isolates to produce aflatoxin B_1.

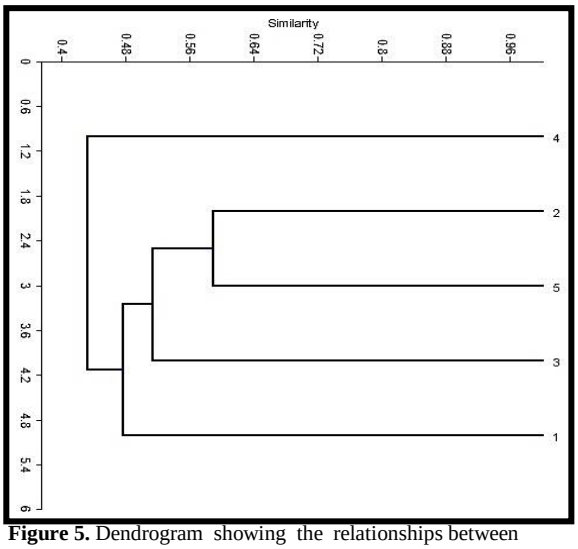

Figure 5. Dendrogram showing the relationships between isolated fungi based on ISSR analysis. Isolate 1: *Aspergillus flavus*; Isolate 2: *Aspergillus parasiticus*; Isolate 3: *Aspergillus terreus*; Isolate 4: *Aspergillus ochraceus*; Isolate 5: *Aspergillus niger*.

4. Conclusion

In this study, results demonstrate the role of ISSR markers, which estimated the genetic diversity for *Aspergillus* genera. In this work, ISSR analysis has provided information on the genetic similarity within selected *Aspergillus* genera. Thus, the present study shows that ISSR is an appropriate and efficient means to estimate genetic similarity amongst the *Aspergillus* genera.

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References

Abdalla EAM, Kheiralla ZMH, Sahab AF and Hathout AS. 1998. *Aspergillus terreus* and its toxic metabolites as a food contaminant in some Egyptian bakery products and grains.*Mycotoxin Res*,**14**:83-91.

Abdulateef SM, Aljubori MH and Abdulbaqi NJ. 2014. Genetic Diversity Among Some *Aspergillus flavus* Isolates by Using Inter-simple sequence repeats (ISSR). *Iraqi J Sci*,**55**: 986-993.

Adejumo TO and Adejoro DO. 2014. Incidence of aflatoxins, fumonisins, trichothecenes,and ochratoxins in Nigerian foods and possible intervention strategies. *Food Sci.Quality Manag*,**31**: 127-146.

Adss AA, Abdel-Gayed MA, Botros W and Hafez E E. 2017.Multilocus Genetic Techniques, RAPD-PCR,and ISSR-PCR Markers and Polygalacturonase Activity as Tools for Differentiation Among Alternaria solani Isolates on Tomato Fruits and Relation to their Pathogenicity in Egypt. *Asian J Plant Pathol*,**11**: 18-27.

Al-Hindi RR, Aly SE, Hathout AS, Alharbi MG, Al-Masaudi S and Al-Jaouni SK, Harakeh SM. 2017. Isolation and molecular characterization of mycotoxigenic fungi in agarwood. *Saudi J. Biol. Sci*, doi.org/10.1016/j.sjbs.2017.07.008

Aly SE, Abo-Sereih NA, Salim, R G and Hathout A S. 2018. Isolation and molecular identification of food grade lactic acid bacteria and their antifungal activity. *J. Biol Sci*,**18**: 260-269.

Aly SE, Hathout AS and Ibrahim MIM. 2012. The stability of some *Lactobacillus* species to remove ochratoxin A from aqueous medium. *J Food Industries Nutr Sci*,**2**: 65-75.

Ashiq S. 2015. Natural occurrence of mycotoxins in food and feed: Pakistan perspective. *Comprehensive Rev. Food Sci Food Safety*,**14**:159-175.

Bruns TD, White TJ and Talyor JW. 1991. Fungal molecular systematics. *Annu Rev Ecol Sys*,**22**: 525-564

Chadha S and Gopalakrishna T. 2007. Comparative assessment of REMAP and ISSR marker assays for genetic polymorphism studies in Magnaporthe grisea. *Curr Sci*,**93**: 688-692.

Chakravarty B. 2011. Trends in Mushroom cultivation and breeding. *Am. J. Agric. Econ*,**2**: 102-109.

Coelho ASG. 2001. Software: Dboot-Evaluation of dendrograms based on estimates of distances / genetic similarities through the bootstrap procedure, version 3.0. Federal University of Goiás, Goiânia.

Dutech C, Enjalbert J, Fournier E, Delmotte F,Barre`s B, Carlier J, Tharreau D and Giraud T. 2007. Challenges of microsatellite isolation in fungi. *Fungal Genet. Biol,* **44**: 933-949.

Frankham R, Ballou JD and Briscoe DA. 2002. **Introduction to Conservation Genetics**. First Edition published by Cambridge University Press, pp. 642

Gramaje D, León M, Santana M, Crous PW and Armengol J . 2014. Multilocus ISSR markers reveal two major genetic groups in Spanish and South African populations of the grapevine fungal pathogen Cadophora luteo-olivacea. *PLoS One*,**9** (10) : e110417.

Hadrich I, Makni F, Ayadi A and Ranque S. 2010. Microsatellite typing to trace *Aspergillus flavus* infections in a hematology unit. *J. Clin. Microbiol*,**48**: 2396-2401.

Henry T, Iwen PC and Hinrichs SH. 2000. Identification of *Aspergillus* species using internal transcribed spacer regions 1 and 2. *J. Clin. Microbiol*,**38**: 1510-1515.

IARC. 2002. Some traditional herbal medicines, some mycotoxins, naphthalene, and styrene. Monograph on the evaluation of carcinogenic risk to humans, **82** (Lyon: IARC).

IARC.2012. Monographs on the evaluation of carcinogenic risks to humans: chemical agents and related occupations. A review of human carcinogens. Lyon, France: Inter Agency for Res Cancer **100F**:224-248

Klaassen CH. 2009. MLST versus microsatellites for typing *Aspergillus fumigatus* isolates. *Med Mycol*,**47**:S27-33.

Kumeda Y and Asao T. 2001. Heteroduplex panel analysis, a novel method for genetic identification of *Aspergillus* section *Flavi* strains. *Appl. Environ.Microbiol*,**67**: 4084-4090.

Mahmoud MA, Ali HM, El-Aziz ARM, Al-Othman MR and Al-Wadai SA. 2014. Molecular characterization of aflatoxigenic and nonaflatoxigenic*Aspergillus flavus* isolates collected from corn grains. *Genet. Mol. Res*,**13**: 9352-9370.

Mahmoud MA, EL-Samawaty AMA,Yassin MA and Abd EL-Aziz ARM. 2016. Genetic diversity analysis of *Aspergillus flavus* isolates from plants and air by ISSR Markers. Genet. *Molecul. Res*,**15**: 15028-081.

Morgante M, Hanafey M and Powell W. 2002. Microsatellites are preferentially associated with nonrepetitive DNA in plant genomes. *Nature Genet*,**30**: 194-200.

O´Donnell K. 1992. Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (Gibberella pulicaris*). Curr. Genet*, **22**: 213-220.

Okoth S, De Boevre M, Vidal A, Diana D M J, Landschoot S, Kyallo M, Njuguna J, Harvey J and De Saeger S. 2018.Genetic and toxigenic variability within *Aspergillus flavus* population isolated from maize in two diverse environments in Kenya. *Front. Microbiol*,**9**: 57.

Paplomatas EJ. 2004. Molecular diagnostics for soil-borne fungal pathogens. *Phytopathol. Mediterr*,**43**: 213-220.

Pildain MB, Frisvad JC, Vaamonde G, Cabral D. Varga J and Samson RA. 2008. Two novel aflatoxin-producing *Aspergillus* species from Argentinean peanuts. *Int. J. Syst. Evol. Microbiol*,**58**: 725-735.

Priyanka SR, Uppalapati SR, Kingston JJ, Murali HS and Batra HV. 2014. Development of ISSR-derived SCAR marker-targeted PCR for identification of *Aspergillus* section Flavi members. *Lett. Appl. Microbiol*,**58**: 414-42.

Rampersad SN. 2013. Genetic structure of Colletotrichum gloeosporioidessensulato isolates infecting papaya inferred by multilocus ISSR markers. *Phytopathol*,**103**: 182-189.

Rassin NK, Al- Judy NJ and Dheeb BI. 2015. Molecular Identification of *Aspergillus fumigatus* Using ISSR and RAPD Markers. *Iraqi J. Sci*,**56**: 2788-2797.

Ratnaparkhe MB, Tekeoglu M and Muehlbauer FJ. 1998. Inter-simple-sequence-repeat (ISSR) polymorphisms are useful for finding markers associated with disease resistance gene clusters. *Theor. Appl. Genet*,**97:** 515-519.

Reischl U and Lohmann C P. 1997. Die Polymerase-Kettenreaktion (PCR) und ihre Anwendungsmöglichkeiten zur infektiologischen Diagnsotik in der Opthalmologie. Klin. Monatsbl. *Augenheilkd*,**211**: 227-234.

Richard JL. 2007. Some major mycotoxins and their mycotoxicoses-An overview. *Int. J. Food Microbiol*,**119**: 3-10.

Rohlf FJ. 1993. NTSYS-pc: Numerical taxonomy and multivariate system. Version 2.9. New York. *Applied Biostatistics.*

Sabry BA, Hathout AS, Nooh A, Aly SE and Shehata M. 2016. The prevalence of aflatoxin and *Aspergillus parasiticus* in Egyptian sesame seeds. *Int. J. ChemTech Res*,**9:** 308-319.

SahabAF, Aly SE, Nawar LS and El-Faham SY. 2011. Fungal occurrence in physic nut (*Jatropha curcas*) seeds during storage and possibility aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus* isolates. *J. Am. Sci*,**7**:511-516.

Sahab AF, Hathout AS, Sabry BA and Aly SE. 2014. Application of some plant essential oils to control *Fusarium* isolates associated with freshly harvested maize in Egypt. *J. Essential Oil Bearing Plants*,**17**:1146-1155.

White TJ, Bruns T, Lee S and Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, and White TJ, (Eds.), **PCR Protocols: A Guide to Methods and Applications**. Academic Press Inc., New York, pp. 315-322.

Williams JG, Kubelik AR, Livak KJ, Rafalski, JA, and Tingey SV.1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res*,**18**: 6531-6535.

Yu J, Cleveland TE, NiermanWC and Bennett JW. 2005. *Aspergillus flavus* genomics: gateway to human and animal health, food safety, and crop resistance to diseases. *Rev. Iberoam. Micol*,**22**: 194-202.

Yugander A, Kiran S, Surekha M, babupK, LahaGS, Madhav M S and Reddy S M. 2015. determination of genetic variation by using ISSR markers in toxigenic strains of *Aspergillus* in paddy from Telangana state India. *Int. J. Agricult. Sci. Res*, **5**:193-202.