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Molecular Identification and Evaluation of Genetic Variability among Some Aflatoxigenic *Aspergillus flavus* Strains Isolated from Fish Feed in Egypt

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AFLATOXINS are among the most potent carcinogenic, mutagenic and teratogenic natural compounds occurring in food and feed. In this study, molecular typing based on sequence variations in the ITS1-5.8S-ITS2 region was used to identify and genetically discriminate five selected strains of *A. flavus*, four strains (AUMC13909, AUMC13910, AUMC13911 and AUMC13917) were aflatoxigenic isolates recovered from fish feed. *A. flavus* AUMC10311 is a non-aflatoxigenic reference strain which was used as a negative control to investigate the molecular variability of the studied strains concerning their aflatoxigenic potential. The ITS data of the studied strains were submitted to NCBI GenBank with the accession numbers: MK491621, MK491622, MK491623, MK491624 and MK491625. Cluster analysis of these strains was performed depending on their ITS sequences, which discriminate them into three different sub-clusters. The highest genetic similarity (100%) was observed between AUMC13911 and AUMC10311. Proteins banding patterns of the examined strains were analyzed using SDS-PAGE, which resolved six polymorphic protein bands out of a total of 22 bands, ranged from 14 to 125kDa. Interestingly, a protein band (~ 40kDa) was relatively overexpressed in case of the aflatoxigenic strains while was hardly detected in the non-aflatoxigenic strain, suggesting that this protein might be involved in aflatoxin biosynthesis. Cluster analyses based on this banding pattern grouped the tested strains into two major clusters. The highest similarity index (97%) was between AUMC13911 and AUMC10311. These results confirmed the genetic variation among the examined *A. flavus* strains and the necessity for greater insight into cluster assembly using different Omics approaches.

Keywords: Aflatoxin, *Aspergillus flavus*, Genetic variability, DNA barcoding , SDS-PAGE.

Introduction

Aflatoxins are toxic secondary metabolites primarily produced by closely related members of the genus *Aspergillus* in section Flavi (Frisvad et al., 2006; Klich, 2007; Varga et al., 2009). Secondary metabolites are defined as "organic compounds that are not directly involved in the normal growth, development and reproduction of an organism. They are widely believed to be responsible for interactions between the producing organism and its environment" (Li et al., 2018). Toxigenic Aspergilli are ubiquitous and cosmopolitan fungi that produce aflatoxins in a wide variety of agricultural commodities including food and feed crops such as maize, rice, sorghum, nuts and oilseeds (Madbouly et al., 2012; Afsah-Hejri et al., 2013; Lahouar et al., 2015). Aflatoxin consumption causes a wide range of adverse influences including carcinogenic, mutagenic, teratogenic and immunosuppressive effects. Many of these harmful effects on animals and humans have been reported by different authors (Jiang et al., 2008; Wu, 2010; Wild et al., 2015). The global cost of aflatoxin is huge in terms of the trade-related losses, through selling aflatoxin-contaminated commodities and losses in the livestock industry, in addition to the adverse impacts of dietary aflatoxin exposure on the human health. Consequently, aflatoxins represent

a great socio-economic and health issue for both developing and industrialized countries (Wu, 2015).

The term "aflatoxins" refers to a family comprises more than 18 different related compounds, principally, B1, B2, G1 and G2. Aflatoxin B1 is not only the most toxic member of the aflatoxin family but also is one of the most potent naturally occurring carcinogens. *Aspergillus flavus* is the most common species from the section *Flavi* responsible for the production of both aflatoxin B1 and B2 in food and feed (IARC, 2002; Klich, 2007; Bosetti et al., 2008; Lerda, 2010). It is worthy to mention that not all strains of *A. flavus* produce aflatoxins, variation in the aflatoxigenic potential and production levels among isolates of *A. flavus* had been widely reported (Pildain et al., 2004; Okoth et al., 2012).

The application of non-aflatoxigenic strains of *Aspergillus* section Flavi to compete against aflatoxigenic strains has been reported as one of the best management practices for reducing aflatoxins contamination, resulting in successful commercial products for several crops (Zanon et al., 2013; Hruska et al., 2014; Alshannaq & Yu 2017). Before using a non-aflatoxigenic strain as a biocontrol agent, it was important to verify that this strain is differed from aflatoxin producing ones and does not express genes required for aflatoxin production. However, due to the high genetic variability observed within *A. flavus* isolates, not every non-aflatoxigenic strain has deletions in the aflatoxin biosynthesis gene cluster. For example, the commercial biocontrol agents NRRL 30797 and NRRL 18543 have just a few substitutions in the aflatoxin biosynthesis gene cluster (Chang et al., 2005, 2012). Accordingly, the diversity within the *A. flavus* isolates suggests the need for an extended phylogenetic delineation for the benefit of the development of effective and safe prevention programs. Currently, greater attention is being paid to molecularly distinguish true aflatoxigenic from non-aflatoxigenic *A. flavus* strains using multiple Omics technologies which help to decrypt the natural variation of these potential biocontrol agents (Okoth et al., 2018).

The development of molecular methods for genetic differentiation of fungal species has advanced their phylogeny as a result of increased sensitivity and specificity. Genotyping techniques have extensively been used to investigate the variability among fungi in both species and subspecies levels (Frisvad et al., 2007; Mahmoud et al., 2014; Jurjević et al., 2015). Nuclear ribosomal DNA has been demonstrated to include regions that are variable within fungal genera. The internal transcribed spacer (ITS) regions of nuclear rDNA, including the intervening 5.8S rRNA gene, ITS1 and ITS2, often varies between different fungal species and within isolates of the same species. Thus, this region was frequently used as a target for studying the phylogenetic analysis among fungal populations (Appiah et al., 2004; Schoch et al., 2012; Heilmann-Clausen et al., 2017).

There is currently a limited understanding of molecular variability among populations of nonaflatoxigenic and aflatoxigenic *A. flavus* isolated in Egypt especially from feed sources including fish feed. Although such differences may further elucidate the evolutionary ancestors of native strains and guide the development of biocontrol strains for reduction of aflatoxin contamination in Egypt. Hence, this study was designed to preliminary evaluate the genetic variability among four aflatoxigenic *A. flavus* strains (isolated and characterized from fish feed in Egypt) and a reference nontoxigenic *A. flavus* strain, concerning their capability to produce aflatoxins by using sequence analysis of the internal transcribed spacers (ITS) region of rDNA. Genotyping based on sequence variations in this region was also used to confirm the morphological identification of the studied strains. In the same context, the protein pattern of these fungal strains was analyzed using SDS-PAGE technique to monitor and survey total cellular protein which can be electrophoresed within the limit of 12% polyacrylamide gel. Utilizing this protein banding profile, a cluster analysis was generated to discriminate the five examined strains.

Materials and Methods

Fungal strains and growth conditions

Five strains of *A. flavus* Link were used in this study, 4 are aflatoxin producing isolates recovered from local fish feed samples. Where the fifth isolate (AUMC 10311), obtained from the Egyptian culture collection of Assiut University Mycological Center (AUMC), was used as a reference strain of *A. flavus*. The mycotoxin profile of the four aflatoxigenic *A. flavus* strains was determined using high-performance liquid

chromatography (HPLC), before deposition at the culture collection of AUMC under the codes: AUMC 13909, AUMC 13910, AUMC 13911 and AUMC 13917 (Sawei et al., 2019). All *A. flavus* strains were grown on potato dextrose broth (PDB; Difco, Sparks, MD, USA) as follows: Each strain was firstly grown on Potato dextrose agar (PDA; Difco, Sparks, MD, USA) at 28ºC for 3 days and a fresh spore suspension was prepared in a 0.05% Tween 80 solution. Aliquots of the spore suspension were inoculated into three 125ml flasks containing 20ml PDB to final concentrations of 10^5 spores/L.All cultures were then grown at 28ºC for 3 days. Mycelia were harvested by filtration and rinsed with sterile distilled water several times before being crushed with liquid nitrogen and stored at -80ºC till use in subsequent extraction processes of DNA and total cellular proteins as previously described by Peterson (2008) and Yin et al. (2018). Potato dextrose agar medium was used for the maintenance and sub-culturing of all isolates.

Assessment of toxigenic potential of the reference strain

To testthe ability of the reference strain (*A. flavus* AUMC 10311) to produce aflatoxins, a method described by Gimeno & Martins (1983) for aflatoxin extraction and purification from the culture filtrate was applied. HPLC was used to analyze the presence of aflatoxins B1, B2, G1 and G2 in the purified extract. The used HPLC system consisted of a Konic KNK 550 D (Konik-Tech, S.A., Barcelona, Spain) chromatograph equipped with a Konic Uvis 200 UV detector (at a wavelength to 365nm), Data jet integrator and 250 x 4.6mm Hypersil ODS column.

Extraction of genomic DNA, PCR amplification and DNA sequencing

A mycelial mate of each isolate was ground under liquid nitrogen to a fine powder. The mycelial powder was transferred to an appropriately sized tube and the DNA extraction was carried out using a DNeasy extraction kit (Qiagen, Germany) according to the manufacturer's instructions, with a final elution volume of 50μl. The universal primers used for amplification and sequencing of ITS regions were ITS1 (*F*:5'- TCCGTAGGTGAACCTGCGG -3') and ITS4 (*R*:5´- TCCTCCGCTTATTGATATGC -3´). PCR amplification was performed in a total volume of 50μl, containing 30ng genomic DNA, 1X reaction buffer, 1.5mM MgCl$_2$, 1U

Taq DNA polymerase (Promega), 2.5mM dNTPs and 30pmol of each primer. PCR amplification was performed in a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems, USA) programmed to fulfill 35 cycles after an initial denaturation cycle for 5min at 94ºC. Each cycle consisted of a denaturation step at 94ºC for 1min, an annealing step at 55ºC for 1min and an elongation step at 72ºC for 1min. The primer extension segment was extended to 7min at 72ºC in the final cycle. PCR products were detected by electrophoresis in a 1.5% agarose gel and purified according to the Molecular Cloning Laboratory Manual (Sambrook et al., 1989). The purified PCR products were subjected to sequence analysis using an automatic sequencer ABI PRISM 3730XL Analyzer and Big Dye TM Terminator Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA) following the protocols supplied by the manufacturer.

Alignments and phylogenetic analysis

The obtained nucleotide sequences were compared with the accessible sequences in the NCBI databases using the BLAST homology search (http://www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignment and percent identity matrix of gene sequences were conducted using Clustal Omega 2.1. The nucleotide sequence data of the studied *A. flavus* strains were deposited in the NCBI GenBank. The genetic similarity coefficient (GS) between the selected genotypes was estimated according to the Dice coefficient. A cluster analysis based on the obtained sequence data was constructed using the unweighted pair-group method with arithmetic average (UPGMA) (Sneath & Sokal, 1973) and a dendrogram was created using MEGA X (Kumar et al., 2018).

Extraction of total cellular proteins (TCPs), SDS-PAGE and data analyses

Fungal mycelia were ground in liquid nitrogen and the obtained powder was homogenized in an equal volume (w/v) of Laemmli's sample buffer supplemented with 1mM PMSF (phenylmethylsulfonyl fluoride) and 2.5 mM EDTA (Laemmli, 1970). Isolated TCPs were extracted following the BIO-RAD general sample preparation guidelines using the manufacturer's instructions, and according to Biggs et al. (1986). Electrophoretic protein fractionation was carried out on 12% polyacrylamide gel and SDS-PAGE technique was performed as previously described by Mehta et al. (1992). An equal percentage of

protein extracts were loaded onto the gel (2% of the total extracted volume/lane). Electrophoresis was performed a 70V for 6hr in Bio-Rad mini vertical electrophoresis cell to retrieve high quality of fractionation and resolution. Separated proteins were visualized by gel staining with Coomassie Brilliant Blue (CoBB), followed by de-staining using the same solution without CoBB till the gel background became stain free. The gel was scanned with a Bio-Rad imaging system and analyzed with PD Quest software (Bio-Rad, USA). According to Sneath & Sokal (1973), protein patterns obtained by SDS-PAGE were clustered to construct a dendrogram using the unweighted pair group with arithmetic mean (UPGMA) by means of GelAnalyzer3 (http://www.geocities.com / egygene, Gel Analyzer Version three, 2007).

Results

Toxigenic potential of the studied strains

Aflatoxins weren't detected in the cultural filtrate of the *A. flavus* AUMC 10311 strain when examining its chromatograms after HPLC analysis (Fig.1). The inability to produce aflatoxins suggested this native Egyptian *A. flavus* AUMC 10311 strain a good candidate for use as a negative control to define the genetic variability between the studied aflatoxigenic *A. flavus* strains. The aflatoxin producing ability of *A. flavus* AUMC 13909, AUMC 13910, AUMC 13911 and AUMC 13917 strains was previously proven (Sawei et al., 2019).The *A. flavus* AUMC13917 strain was found to had the ability to produce both aflatoxins B1 (1.016µg/L) and B2 (0.314µg/L). Whereas, the three remaining fungal strains, AUMC13909, AUMC 13910 and AUMC 13911, were able to produce only aflatoxin B1 but in variable amounts.A characteristic amount (8.082µg/L) was produced by AUMC13909, while AUMC 13910, and AUMC 13911 were able to produce lower levels of aflatoxin B1 (1.617µg/L and 0.141µg/L, respectively).

Genetic diversity among the A. flavus strains as revealed by DNA barcoding of ITS1-5.8S-ITS2 region

Molecular differentiation of the five strains of *A. flavus* based on the DNA sequence analysis of the ITS1-5.8S-1TS2 region was used to confirm their identity and to demonstrate their genetic relatedness/diversity. PCR amplification of the ITS1-5.8S-1TS2 region of the five strains of *A. flavus* using ITS1 and ITS4 primers gave PCR products of approximately 600 base pairs in length (Fig. 2). NCBI GenBank was accessed to identify the investigated strains through BLAST homology search using the obtained ITS data. The nucleotide sequence data of the studied strains of *A. flavus* were deposited in the NCBI GenBank under the following accession numbers: MK491621 (AUMC 13909), MK491622 (AUMC 13910), MK491623 (AUMC 13911), MK491624 (AUMC 13917) and MK491625 (AUMC 10311).Multiple sequence alignment and percent identity matrix of gene sequences were performed based on ITS1-5.8S-ITS2 rDNA region sequences of the studied fungal strains and the reference strains in the GenBank. Clear similarities were observed between the five strains with two different reference strains of *A. flavus* from the GenBank. Four of the studied strains (AUMC 13909, AUMC 13910, AUMC 13911 and AUMC 10311) exhibited relatively high similarity (98%, 97%, 95% and 95%, respectively) with the same *A. flavus* reference strain (GenBank Acc. No. KX898361.1). Whereas, *A. flavus* AUMC 13917 strain was highly identical (98%) to the *A. flavus* reference strains MG736928.1.

To investigate the genetic similarity/variability among the five examined *A. flavus* strains based on the nucleotide sequence of the ITS regions of the rDNA, the obtained data were analyzed using the Dice coefficient to compute the similarity matrix. As shown in Table 1, the estimated genetic similarities ranged from 75% to 100% revealing high levels of genetic similarity among the studied *A. flavus* strains. The highest detected genetic similarity (100%) was between AUMC 13911 and AUMC 10311. This was followed by 95% between AUMC 13909 and AUMC 13910, while the lowest genetic similarity (75%) was detected between AUMC 13911 and AUMC 13917 and between AUMC 10311 and AUMC 13917. This sequence data was used to generate a dendrogram using the UPGMA method. According to the obtained dendrogram (Fig. 3), the five examined *A. flavus* strains were grouped into two distinct major clusters. The first major cluster was separated into two sub-clusters (I and II), one of them (sub-cluster I) included *A. flavus* AUMC 13911 and AUMC 10311. Likewise, the second major cluster consisted of two sub-clusters (III and IV). The first one (sub-cluster III) included *A. flavus* AUMC 13910. Whereas, the remaining two strains (AUMC 13909 and AUMC 13917) belonged to sub-cluster IV.
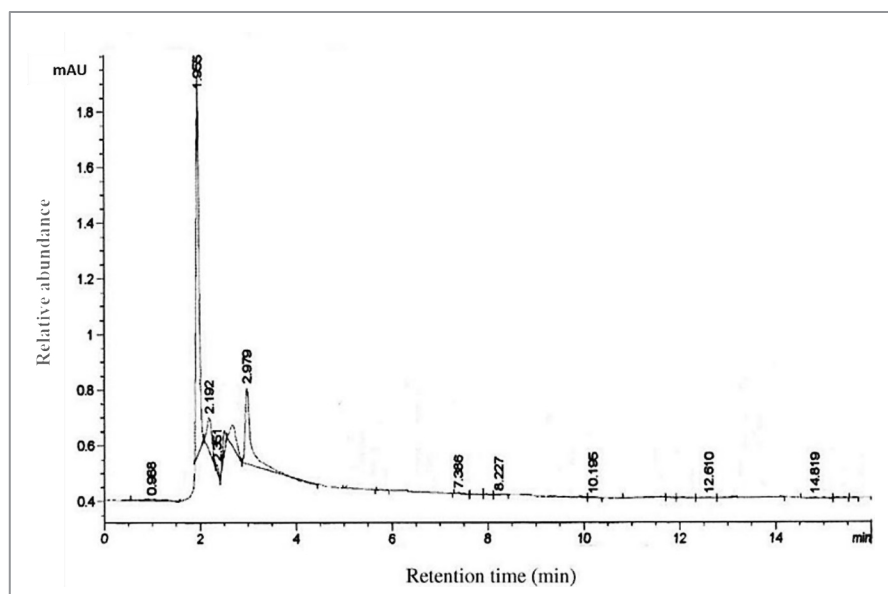
Fig. 1. HPLC pattern of the organic extract of *A. flavus* AUMC10311 culture filtrate showing the absence of detectable peaks of aflatoxins.
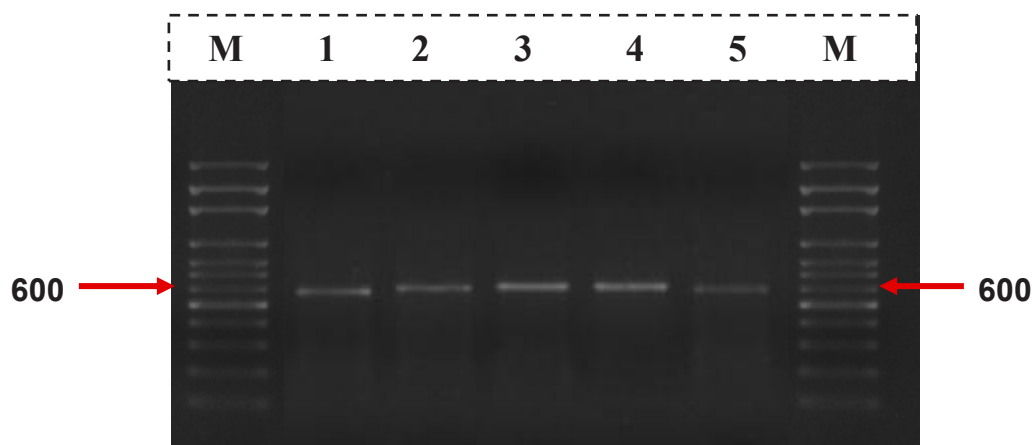

Fig. 2. Gel electrophoresis of the obtained PCR products showing (from left to right) DNA ladder, amplified fragments of lain1 (AUMC 13909), lain 2 (AUMC 13910), lain 3 (AUMC 13911), lain 4 (AUMC 13917) and lain 5 (AUMC 10311) of *A. flavus*.

TABLE 1. Genetic similarity matrix among the five examined strains of *A. flavus* as computed according to Dice coefficient from the nucleotide sequence data of the ITS1-5.8S-ITS2 region.

A. flavus strain	AUMC 13909 *(MK491621)	AUMC 13910 *(MK491622)	AUMC 13911 *(MK491623)	AUMC 13917 *(MK491624)	AUMC 10311 *(MK491625)
AUMC 13909(MK491621)	100%				
AUMC 13910 (MK491622)	95%	100%			
AUMC 13911(MK491623)	92%	94%	100%		
AUMC 13917(MK491624)	79%	78%	75%	100%	
AUMC 10311 (MK491625)	92%	94%	100%	75%	100%

AUMC: Assiut University Mycological Center

* NCBI accession number
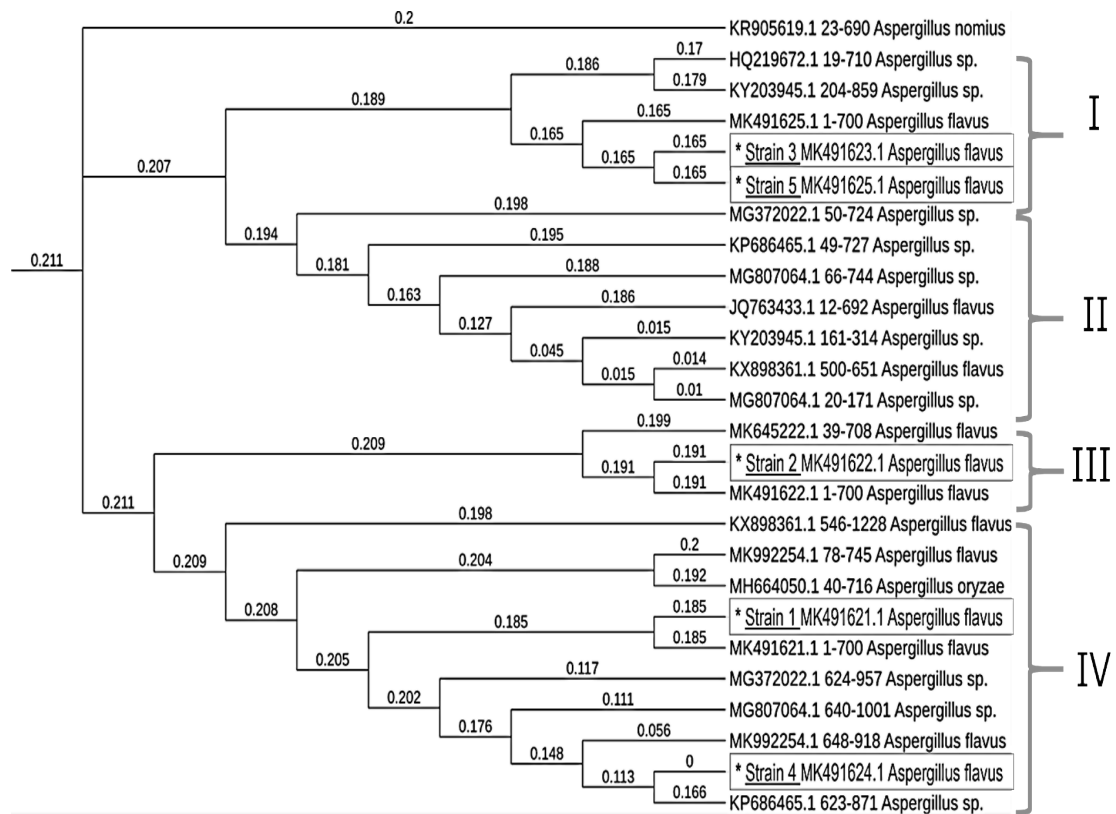
Fig. 3. Dendrogram constructed from the nucleotide sequence data of the ITS1-5.8S-ITS2 region using unweighted pair-group arithmetic (UPGMA) showing the relationship between the examined *A. flavus* strains and reference strains.This dendrogram was drawn to scale using MEGA X software. Strain 1: AUMC 13909 (MK491621), strain 2: AUMC 13910 (MK491622), strain 3: AUMC 13911 (MK491623), strain 4: AUMC 13917 (MK491624) and strain 5: AUMC 10311 (MK491625).

Analysis of TCPs banding patterns of A. flavus strains as revealed by SDS-PAGE technique

Results obtained from the SDS-PAGE electrophoretic fractionation have indicated that extracts obtained from the examined strains of *A. flavus* had a total of 22 protein bands, which ranged from 14 to 125kDa (Fig. 4). Among the studied isolates, *A. flavus* AUMC 13917 strain had the maximum number of protein bands (20 bands) AUMC 10311 had the minimum protein bands (17 bands). There were six Polymorphic bands out of the total of 22 detected bands (polymorphism percentage= 27.3%). Among the Polymorphic protein bands, a unique band with a molecular weight running approximately at 66kDa was detected in aflatoxin producing AUMC 13917 strain (Fig. 4, lane 4, as depicted by a black arrowhead). Interestingly, a protein band running approximately at 40kDa was relatively over expressed in case of aflatoxin producing strains especially the aflatoxigenic

strain AUMC 13917 (Fig. 4, lane 4 as depicted by a black arrowhead) while, this polypeptide was hardly detected in the non-aflatoxigenic strain AUMC 10311 (lane 5). As can be seen, faint weak bands were shown in lane 1, which may indicate the need to optimize cultural conditions to maximize protein extraction from *A. flavus* AUMC 13909 strain.Cluster analysis, based on the banding pattern obtained from SDS-PAGE, has grouped the examined strains into two major clusters. One cluster has included *A. flavus* AUMC 13910 and AUMC 13917 strains with about 96% similarity index between them. The remaining three strains (AUMC 13909, AUMC 13911 and AUMC 10311) were grouped in the other major cluster. Unexpectedly, the highest detected similarity index (97%) was observed between the aflatoxigenic *A. flavus* AUMC 13911 and the non-aflatoxigenic AUMC 10311 strains (Fig. 5 and Table 2).
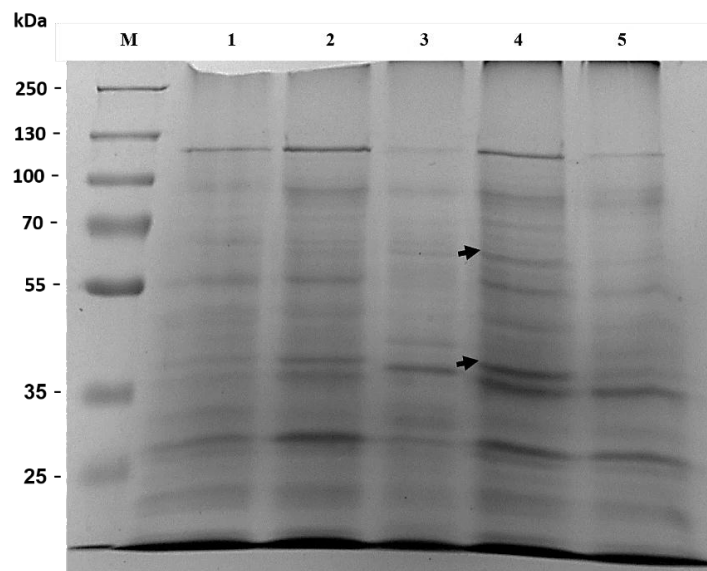
Fig. 4. The electrophoretic banding pattern of extracted total cellular proteins (TCPs) of the five examined strains of *A. flavus* using denaturing SDS-PAGE. Lane 1: AUMC 13909 (MK491621), lane 2: AUMC 13910 (MK491622), lane 3: AUMC 13911 (MK491623), lane 4: AUMC 13917 (MK491624) and lane 5: AUMC 10311 (MK491625). Fermentas PageRuler™ Plus Prestained Protein Ladder (SM1811) was loaded and denoted by numbers left-handed of the figure indicating molecular weight standards in kDa. The SDS-PAGE fractionation was repeated 3 times to confirm the obtained results. Highly expressed and pronounced polypeptides in isolate 4 were denoted by black arrowheads.

Fig. 5. Dendrogram constructed by unweighted pair-group arithmetic (UPGMA) cluster analysis based on the SDS-PAGE profiling of the five examined strains of *A. flavus*, using GelAnalyzer 3 software. Strain 1: AUMC 13909 (MK491621), strain 2: AUMC 13910 (MK491622), strain 3: AUMC 13911 (MK491623), strain 4: AUMC 13917 (MK491624) and strain 5: AUMC 10311 (MK491625).

TABLE 2. Similarity matrix among the five examined strains of *A. flavus* as computed according to Dice coefficient based on the data revealed by SDS-PAGE.

A. flavus strain	AUMC 13909 *(MK491621)	AUMC 13910 *(MK491622)	AUMC 13911 *(MK491623)	AUMC 13917 *(MK491624)	AUMC 10311 *(MK491625)
AUMC 13909(MK491621)	100%				
AUMC 13910 (MK491622)	95%	100%			
AUMC 13911(MK491623)	90%	90%	100%		
AUMC 13917(MK491624)	92%	96%	90%	100%	
AUMC 10311 (MK491625)	95%	95%	97%	92%	100%

AUMC: Assiut University Mycological Center.

* NCBI accession number.

Discussion

Aflatoxins are potent and carcinogenic mycotoxins which contaminate global food and feed supplies, causing widespread health risks to humans and animals (IARC, 2002; Milićević et al., 2010; Wild et al., 2015; Alshannaq & Yu, 2017). *A. flavus* and a few other closely related species of *Aspergillus* belonging to section Flavi are the major producers of aflatoxins (Frisvad et al., 2006; Ehrlich et al., 2007; Klich, 2007). In the present study, four *A. flavus* strains isolated from fish feed were used. The aflatoxin producing ability of these strains was confirmed as reported by Sawei et al. (2019). All the four strains were found to be able to produce aflatoxin B1, but with a difference in the level of toxin production. The amount of aflatoxin B1 produced by the toxigenic strains ranged from <0.141µg/L (detected in AUMC 13911) to 8.08 µg/L (detected in AUMC 13909). On the other hand, *A. flavus* AUMC 10311 was confirmed as a non-aflatoxigenic reference strain using HPLC analysis and was used as a negative control to investigate the molecular variability of the studied strains concerning their capability to produce aflatoxins.Current trends in the qualitative and quantitative analysis of mycotoxins including aflatoxins in food and feed are focused on the application of chromatographic methods such HPLC, due to the fact that these methods represent reliable, accurate and easy to use technologies that are able to detect and quantify simultaneously various mycotoxins with a high sensitivity and selectivity in a single run (Zheng et al., 2006; Pereira et al., 2014 ; Santos et al., 2019). Therefore, HPLC analysis was selected to test the ability of the studied strains to produce aflatoxins.

Conventional identification of *Aspergillus* strains based on the phenotypic characteristics has been suffered from some weaknesses including the existence of overlapping morphological features among closely related species. Hence, it was difficult to distinguish and characterize these species from each other depending solely on classical morphological identification. Recently, the use of more than one approach to identify and characterize members of section Flavi, including morphological and molecular characterization has become more popular and reliable than using only a single approach (Baquião et al., 2013; Norlia et al., 2018). Internal transcribed spacer (ITS) regions of nuclear rDNA were widely used to explore the variability within filamentous fungi at species and sub-species levels (Zarrin & Erfaninejad, 2016; Okoth et al., 2018). Henry et al. (2000) confirmed sequence variation in several areas in the ITS regions among referenced and clinical isolates of *Aspergillus* species. Zarrin & Erfaninejad (2016) concluded that ITS-RFLP (restriction fragment length polymorphism) is a valuable tool in screening nucleotide polymorphisms among isolatesof *A. flavus*. Moreover, the use of nuclear ITS sequences had been proposed as an effective approach for identification of DNA barcode(s) of fungal species by Schoch et al. (2012).

In the present study, Genotyping typing based on sequence variations in the ITS1-5.8S-ITS2 region was used to confirm the morphological identification of the five selected *A. flavus* strains. As well as to assess the genetic diversity among these strains concerning their ability to produce aflatoxins. The use of nuclear ITS sequences had effectively confirmed the identification of the five examined *A. flavus* strains. Resulted sequences were deposited in the NCBI GenBank under the following accession numbers: MK491621 (AUMC 13909), MK491622 (AUMC 13910), MK491623 (AUMC 13911), MK491624 (AUMC 13917), and MK491625 (AUMC 10311). Cluster analysis of the five strains was performed depending on the obtained ITS sequences to evaluate the genetic variation between these strains. Although this analysis has clearly separated the five strains into three different genetic groups confirming the genetic diversity among the studied strains of *A. flavus*. The toxigenic isolate (AUMC 13911) was grouped in the same sub-cluster with the non-toxigenic one (AUMC 10311), indicating that the diversity in the nucleotide sequence of the ITS-1-5.8S-ITS-2 region is insufficient to completely discriminatethe isolates of *A. flavus* according to their aflatoxigenic potential. Similar results were reported by Mohankumar et al. (2010) who concluded that no correlationwas observed between cluster analysis based on the ITS region of nuclear rDNA of the isolates of *A. flavus* and aflatoxin potential. The present study has shown that genomes of the aflatoxigenic AUMC 13911 and the non-aflatoxigenic AUMC 10311 strains of *A.flavus* (showing 100% similarity despite different toxigenic potential) require further comprehensive studies in order to confirm and assess the diversity/similarity of these native strains by using more specific molecular markers such as Multilocus Sequence Typing (MST), i.e., associated sequences from two or more DNA loci, e.g., calmodulin

(CaM), β-tubulin (benA), and nuclear ribosomal intergenic spacer region (IGS rDNA).

In this study, proteins banding patterns of the examined strains analyzed by SDS-PAGE have resolved a total of 22 protein bands, which ranged from 14 to 125kDa. A protein band running approximately at 40kDa was relatively over expressed in case of aflatoxin producing strains especially the aflatoxigenic strain AUMC 13917 while, this polypeptide was hardly detected in the non-aflatoxigenic strain AUMC 10311. This result suggests that this protein might be involved in aflatoxin biosynthesis. This assumption is in agreement with the previous finding of Liu et al. (1999) who purified and characterized Sterigmatocystin-O-methyltransferase, an enzyme catalyzing the conversion of sterigmatocystin (ST) to aflatoxin B1 through an O-methyl sterigmatocystin intermediate. Hereby, it might be speculated that due to the high genetic variability observed within *A. flavus* isolates worldwide, not every non-aflatoxigenic strain has deletions in the aflatoxin biosynthesis gene cluster but just a few substitutions in this gene cluster may occur (Chang et al., 2005; Chang et al., 2012), which probably lead to constitutive nonfunctional expression (may appear in SDS-PAG as a lower expression of this protein bandas shown in case of the non-aflatoxigenic strainAUMC 10311). Furthermore, the aflatoxigenic strain AUMC 13917 was characterized by a unique band running approximately at 66kDa, in addition to the overexpression of the polypeptide at ~ 40kDa when compared with the remaining aflatoxigenic strains. Concerning this result, further protein identification using Mass spectrometry (MS) of the aflatoxigenic *A. flavus* strain AUMC 13917 is still required to address why prementioned polypeptides were uniquely distinguished from the other strains.Cluster analysis based on SDS-PAGE data was insufficient to obviously discriminatethe non-aflatoxigenic *A. flavus* AUMC 10311 and the aflatoxigenic AUMC 13911 (with a genetic similarity of 97%). Similarly, presented data based on the nucleotide sequence of the ITS regions has revealed that both strains were very closely related to each other with a genetic similarity of 100%.

Conclusion

In conclusion, the results obtained have revealed that both analyses based on the nucleotide sequence of the ITS regions and on SDS-PAGE data of the examined strains were insufficient to clearly establish the genetic variation between non-aflatoxigenic and aflatoxigenic strains of *A. flavus*. However, this study has formed a basis and paved a way for further investigations to assess and establish the genetic diversity among different *A. flavus* strains regarding their ability to produce aflatoxin using different Omics approaches. The intended future perspectives of the present study include the following: (a) Verifying the genetic variation among the examined *A. flavus* strains by using Multilocus Sequence Typing [associated sequences from two or more DNA loci using calmodulin (CaM), β-tubulin (benA), and nuclear ribosomal intergenic spacer region (IGS rDNA)], and (b) Fractionating the extracted total cellular proteins using gradient PAGEs (denaturing & native) to have a more conclusive point of view about protein/protein complexes, respectively, of the studied strains to make clearer discrimination between aflatoxin producing and non-producing strains.

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التعريف الجزيئي وتقييم التباين الوراثي لبعض سلالات أسبرجيلوس فلافوس المنتجة للأفلاتوكسين المعزولة من أعلاف الأسماك في مصر

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الأفلاتوكسينات هي من أكثر المركبات الطبيعية المسببة للسرطانات والطفرات و تشوهات الأجنة انتشاراً في الأطعمة والأعلاف. في هذه الدراسة، تم استخدام التحليل الجزيئي المستند إلى اختلافات تسلسل النيوكليوتيدات في منطقة ITS1-5.8S-ITS2 في تأكيد تعريف خمس سلالات مختارة من الأسبرجيلوس فلافوس وتمييزها وراثياً، أربعة من هذه السلالات (AUMC13909، AUMC13910، AUMC13911،AUMC13917) هي سلالات منتجة للأفلاتوكسينلت معزولة من أعلاف الأسماك. أما السلالة الخامسة (AUMC10311) فهي سلالة مرجعية غير قادرة على إفراز الأفلاتوكسينات والتي تم استخدامها كعنصر تحكم سلبي لاستقصاء التباين الجزيئي بين السلالات محل الدراسة فيما يتعلق بقدرة هذه السلالات على انتاج اللأفلاتوكسين. تم إيداع بيانات ITS الخاصة بالسلالات المدروسة في بنك الجينات NCBI GenBank تحت أرقام انضمام هي: MK491621وMK491622 و MK491623 و MK491624 و MK491625. وتم إجراء تحليل عنقودي cluster analysis لتلك السلالات اعتمادًا على تتابع النيوكليوتيدات لمنطقة ITS الخاصة بها، حيث وزعت السلالات الخمسة في ثلاث مجموعات ثانوية مختلفة. وقد لوحظ أن أعلى تشابه جيني (100%) كان بين سلالتي أسبرجيلوس فلافوس رقم AUMC13911 وAUMC10311. هذا وقد تم تحليل أنماط توزيع نطاقات البروتينات Proteins banding patterns المستخلصة من السلالات التي تم فحصها باستخدامSDS-PAGE ، ولوحظ تواجد ستة نطاقات بروتين متعددة الأشكال من إجمالي 22 نطاقًا، وتراوحت الأوزان الجزيئية لإجمالي النطاقات بين 14 و 125 كيلو دالتون. ومن المثير للإهتمام ظهورنطاق بروتيني (Protein band) وزنة الجزيئي ~ 40 كيلو دالتون بشكل مفرط إلى حد ما في السلالات المنتجة للأفلاتوكسين في حين تم اكتشافه بالكاد في السلالة الغيرمنتجة للأفلاتوكسين، مما يوحي بأن هذا البروتين قد يكون له دور في التخليق الحيوي للأفلاتوكسين. قسم التحليل العنقودي -المعتمد على هذا النمط من نطاقات البروتينات ـ السلالات الخمس المختبرة إلى مجموعتين رئيسيتين. وكانت أعلى نسبة تشابه (97%) تم رصدها بين السلالتين رقم AUMC13911 و AUMC10311. أكدت نتائج الدراسة التباين الوراثي بين سلالات فطرة أسبرجيلوس فلافوس التي تم فحصها، كما خَلُصَت الدراسة إلى ضرورة إجراء المزيد من البحث المتعمق لرسم صورة مكتملة عن الأختلافات الوراثية بين هذه السلالات اعتماداً على التقنيات الجينومية والبروتيومية و الميتابوليومية (Omics) المختلفة.