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New genotypes of aflatoxigenic fungi from Egypt and the Philippines

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Abstract

Aflatoxins (AFs), mainly produced by *Aspergillus* section *Flavi*, are the major natural toxins of crops and commodities in hot climatic geographic regions. These toxins are considered as type A carcinogens. One hundred and sixty single spore isolates of *A.* section *Flavi* were isolated from two different geographical places, Egypt and the Philippines. A quarter (26.5%) of the isolates was able to produce AFs. Four chemotypes of aflatoxin-producing fungi were obtained. Surprisingly, all aflatoxin-producing *A. nomius* isolates produced higher amounts (2400-40400 ng ml^{-1}) of total AFs (AFB1, AFB2, AFG1 and AFG2) than the toxigenic *A. flavus* isolates (<1200 ng ml^{-1}). All isolates producing AFs gave PCR products with the ver-1/ver-2 and ordAF/ordAR primers, which amplify *ver-1* and *ordA* genes in the aflatoxin biosynthetic pathway. Based on PCR products of *ver-1* gene, new genotypes of aflatoxigenic fungi were found which revealed the variability of AFs production between different isolates depending on the region of the isolation.

Key words – PCR – aflatoxin – fungi – *Aspergillus* section *Flavi*

Introduction

Aflatoxins (AFs) are highly toxic secondary metabolites, mainly produced by species in the *Aspergillus* section *Flavi* group, including *Aspergillus flavus* and *A. parasiticus* (Sultan & Magan 2010). *A. nomius* is also found to produce AFs (Kumeda et al. 2003, Ehrlich et al. 2007). They significantly affect food and feed production because of their toxic effects on humans and animals that include carcinogenic, mutagenic, teratogenic, and immunosuppressive effects (Cullen et al. 1987, Iyer et al. 2010). These toxins consist of around 20 related secondary metabolites, although only aflatoxins B_1, B_2, G_1 and G_2 are normally found in foods (CAST 2003). Aflatoxins, especially aflatoxin B_1, have been shown to induce liver cancer (hepatocellular carcinoma, HCC) in laboratory and wild animal species, including subhuman primates (Alberts et al. 2006, Wogan et al. 2012).

Because of health issues, AFB1 and the total AFs in food for direct human consumption are strictly regulated by the EU at 2 and 4 ppb, respectively (European Commission 2010).

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Isolates of *Aspergillus* section *Flavi* can grow over a wide temperature range of 17-42°C but the optimum for AFs production is 25-35°C. For this reason, AFs have often been considered as the major contaminants in countries with hot climates (Cotty & Jaime-Garcia 2007).

Egypt is known to have such a hazard in tropical cereals and other foods or raw materials (el-Tahan et al. 2000). Moubasher et al. (1977) tested 45 isolates of *Aspergillus* species isolated from soil, seed grains (maize, wheat, and peanut) and air for the production of mycotoxins. Thirty isolates exhibited toxicity, whereas three of them were strongly toxigenic.

Since Philippines' weather is always warm coupled with high relative humidity (80-90% at wet season and 50-70% at dry season), AFs contamination in crops, food and feed commodities is considered also a serious problem (Ilag 1984, Garcia & Ilag 1986, Lubulwa & Davis 1994, Arim 2004). Among the five Southeast Asian nations, the Philippines had the highest level of aflatoxin B1 contamination in maize but had the least contaminated samples (Balendres et al. 2019).

The AF biosynthetic pathway in several *Aspergillus spp*. has been well characterized and most of the genes involved have been identified (Prieto & Woloshuk 1997). These genes exist as a gene cluster located in a 70-kb DNA sequence, which contains at least 25 open reading frames (ORFs) (Yu et al. 2004). At least 25 genes are involved in the biosynthesis of AFs and its regulation (Bhatnagar et al. 2006). Several regulator genes such as *aflR* and *aflJ*, involved in the pathway are also reported as a part of this cluster (Cary et al. 2006). In this multistep polyketide pathway, the *NOR1* (*aflD* gene) mediates the first step in the AF biosynthetic pathway (Chang et al. 1992). Primers on sequences of *afl-2, aflD, aflM* and *aflP*, (*apa-2, nor-1, ver-1, omt-A*, respectively) (Geisen 1996, Shapira et al. 1996, Chen et al. 2002) have been used to detect and identify aflatoxigenic strains of *A. flavus* and *A. parasiticus* isolated from different commodities. Multiplex RT–PCR containing 4-5 primer pairs of various combinations of *aflD, aflO, aflP, aflQ, aflR* and *aflS (aflJ)* have also been used to detect toxigenic fungi (Degola et al. 2007).

The biodiversity of aflatoxigenic strains is dramatically affected by climate change overall the world (Cotty & Jaime-Garcia 2007, Magan et al. 2011). Temperature, water activity of the commodities and the carbon dioxide percentage are the most factors affecting the biodiversity. Interaction of such factors was found to stimulate expression of *aflD* and *aflR* during AF biosynthetic pathway by *A. flavus* (Medina et al. 2015). This emphasizes the importance of updating all data belonging to the aflatoxigenic fungi in different climate regions.

The objectives of this study were (a) to generate information on the diversity of *A.*section *Flavi* species isolated from Egypt and the Philippines as different geographical regions and their capacity for AFs production, (b) to test selective primers designed for distinguishing the aflatoxin production efficacy by these isolates, and (c) to utilize PCR results and AFs analysis data to classify the isolated aflatoxigenic fungi into chemo- and genotypes.

Materials & Methods

Sampling

Thirty five samples were collected from each country. The Egyptian samples were collected from 5 governorates in the north of Egypt between latitudes 28 and 30° North and longitudes 29 and 31° East (Anon 2001) representing nine soil samples, 15 maize, nine wheat samples, one air sample and one lab bench swab sample. From the Philippines, samples were collected from different provinces of Mindanao and Visayas islands which locate between latitudes 5 and 10° North and longitudes 121 and 126° East (Anon 2001). Samples were divided into 23 soil, 6 maize, 2 coconut and 4 peanut samples. Table 1 shows the collection sites and the isolates 'codes.

Fungal isolation using species-specific medium and single-spore preparation

The isolates of *Aspergillus* section *Flavi* were collected using the specific medium *Aspergillus flavus*/*parasiticus* Agar (AFPA) (Cotty 1994). A yellow-orange reverse color was a marker sign of their growth. Direct plating was applied for grain samples. Five grains of either maize or peanut samples and 10 grains of wheat samples were surface disinfected with 5% sodium

hypochlorite (NaOCl) for 1 min followed by rinsing three times with sterile water. The disinfected grains were plated on AFPA medium and incubated for 5 days at 25°C. Serial dilution of soil and coconut samples (2 g sub-samples) using 6 ml sterile distilled water in sterile polystyrene tubes were mixed on a rolling mixer for 20 min (Donner et al. 2009). One hundred microliters of each supernatant were spread onto 90-mm AFPA agar plates. Regarding the air sample, AFPA plates was opened in the collecting place for 5 min. Swab sample was also spread directly on the medium. After incubation, the colonies with yellow-orange reverse color were transferred to PDA plates and single spore isolates were prepared from each isolate by serial dilution of spore suspensions and spreading the spores on water agar Petri plates. The plates were incubated for 12h. After this, three single spores were picked off under a dissecting microscope and inoculated on PDA medium for 24h. The youngest colony was used as a source for inoculation of PDA-Eppendorf tubes, which contained 0.5 ml of PDA medium in 1.5 ml sterilized Eppendorf tubes. All single spore isolates have been preserved in the small culture collection in Department of Biochemistry, University of Turku, Finland and another copy of them have been sent to the culture collection in All-Russian Institute of Plant Protection (VIZR), Petersburg-Pushkin, Russia.

Table 1 Isolates codes, their sources and collection sites

Isolate code	Collection site
1E	Maize sample (Balady*)from Awsiem Tuesday market, Giza
3E	Maize sample (Balady Bahary*) from Awsiem Tuesday market, Giza
16E	Maize sample from El-Monufia
21E	Yellow maize sample from El-Beheira
23E	Maize sample (Hokoma*) from El-Beheira
29E	Soil sample from Eltarbeaa area in Awsiem, Giza
34E	Soil sample from Tanta, El-Gharbia
35E	Soil sample from Eltarbeaa area in Awsiem, Giza
40E	Wheat sample from Awsiem, Giza
41E	Wheat sample from Tanta, El-Gharbia
42E	Soil sample from el-Mansoria, Giza
43E	Yellow maize sample from El-Faiyum
44E	Lab bench swab sample from Mycotoxin lab, Food Toxicology and Contaminants Dept., National Research Centre, Dokki, Giza, Egypt
45E	Air sample from Mycotoxin lab, Food Toxicology and Contaminants Dept., National Research Centre, Dokki, Giza, Egypt.
6P	Soil sample from a field of coconut in Situbo, Tampilisan, Mindanao
7P	Soil sample from a field of coconut in Situbo, Tampilisan, Mindanao
8P	Soil sample from a field of coconut in Situbo, Tampilisan, Mindanao
9P	Soil sample from a field of coconut in Situbo, Tampilisan, Mindanao
10P	Soil sample from a field of coconut in Situbo, Tampilisan, Mindanao
18P	Soil sample from a field of maize in New Barili, Tampilisan, Mindanao
32P	Soil sample from a field of maize in Garimbara, Visayas
33P	Soil sample from a field of maize in Garimbara, Visayas
34P	Soil sample from a field of maize in Garimbara, Visayas
35P	Soil sample from a field of maize in Garimbara, Visayas
36P	Soil sample from a field of peanut in Bagay, Visayas
41P	Soil sample from a field of peanut in Malingin, Visayas
42P	Soil sample from a field of peanut in Malingin, Visayas
43P	Soil sample from a field of peanut in Malingin, Visayas
44P	Soil sample from a field of peanut in Malingin, Visayas
45P	Soil sample from a field of peanut in Malingin, Visayas
59P	Peanut sample from Visayas
86P	Soil sample from a field of maize in Libertad, Visayas
87P	Soil sample from a field of maize in Libertad, Visayas
88P	Soil sample from a field of maize in Libertad, Visayas

Table 1 Continued.

Isolate code	Collection site
89P	Soil sample from a field of maize in Libertad, Visayas
90P	Soil sample from a field of maize in Libertad, Visayas
96P	Soil sample from a field of coconut in Caimbaran, Visayas
97P	Soil sample from a field of coconut in Caimbaran, Visayas
107P	Soil sample from a field of coconut in Kabangkalan, Visayas
108P	Soil sample from a field of coconut in Kabangkalan, Visayas
109P	Soil sample from a field of coconut in Kabangkalan, Visayas
110P	Soil sample from a field of coconut in Kabangkalan, Visayas
112P	Peanut sample from Visayas

E = Egypt, P = Philippines, * = the commercial name of the grain

Fluorescence detection of aflatoxin-producing isolates and phenotypic identification

Coconut agar medium (CAM) was used as a simple detection of isolates producing aflatoxin (Lin & Dianese 1976). Briefly, 100 g of shredded coconut were homogenized for 5 min with 200 ml of hot distilled water. The homogenate was filtered through cheesecloth, and then the agar (2%) was added and the medium autoclaved. The plates were inoculated with PDA plugs of *Aspergillus* strains and incubated at 25°C for 5 days. The reverse side of the plates was periodically observed under 365 nm UV light for blue fluorescence as an indicator of aflatoxins production. The positive aflatoxin-producing isolates were identified according to phenotypic characteristics based on the growth patterns on AFPA, Czapek yeast autolysis (CYA), malt extract sucrose agar (MEA) and yeast extract sucrose agar ,YES after 7 days at 25°C (Rodrigues et al. 2011, Varga et al. 2011).

Aflatoxin determination

Each isolate was inoculated on 500 µl of yeast extract sucrose (YES) in an Eppendorf tube and incubated for seven days. Aflatoxins were extracted as follows: 500 µl chloroform was added to each Eppendorf and vortexed well. The chloroform extract was transferred to a new vial and dried gently under air. Dry film was derivatized according to AOAC (2019) and then analyzed quantitatively using HPLC. A 200 µl stock solution of AFs mix standard in methanol (Supelco, Bellefonte, Pa., USA), containing 200 ng B_1, 60 ng B_2, 200 ng G_1 and 60 ng G_2, was dried under nitrogen gas and derivatized. Four different concentrations of working standard solution were used for calibration curve preparation of each AF type.

HPLC conditions

The HPLC system used for AFs analyses was an Agilent 1200 series system (Agilent, Berks., UK) with a fluorescence detector (FLD G1321A), an auto sampler ALS G1329A, FC/ALS therm G1330B, Degasser G1379B, Bin Bump G1312A and a C18 (Phenomonex, Luna 5 micron, 150 × 4.6 mm) column joined to a pre-column (security guard, 4 × 3-mm cartridge, Phenomenex Luna). The mobile phase was water /methanol /acetonitrile (60: 30:10, v/v/v) using an isocratic flow rate of 1 ml min^{-1} at 360 nm excitation and 440 nm emission wavelengths and a 25-min run time for AF analyses. Under these conditions, the LOD levels were 0.042, 0.015, 0.023 and 0.012, whereas LQD levels were 0.131, 0.045, 0.072 and 0.032 (ng $injection^{-1}$) for AFG_1, AFB_1, AFG_2 and AFB_2, respectively.

DNA extraction and PCR

The *Aspergillus* isolates were cultured in 0.5 ml of malt extract broth medium (30 g malt extract and 5 g peptone l^{-1}) in Eppendorf tubes at 25°C for three days, after which the mycelium was transferred to a new Eppendorf tube. DNA was extracted from isolates using both octanol/isopropanol method as described by Paavanen-Huhtala et al. (1999) and GenElute™ Plant Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA) as described by Yli-Mattila et al. (2008). Quality of DNA extraction was confirmed using ITS1 and ITS4 (Table 2) primers amplifying ITS

(the internal transcribed spacer) region of fungi DNA as described by Yli-Mattila et al. (2004). [XE "Multilocus genotyping"] Primers pairs ver-1/ver-2 and ordAF/ordAR (Table 2) which are specific for *ver-1* and *ordA* genes in aflatoxin biosynthetic pathway respectively, were used for the detection of aflatoxin production by aflatoxigenic isolates as described by Färber et al. (1997) and Chang et al. (2005) (Table 2). MJ Research thermal cycler (PTC-200) was used for PCR amplification.

Table 2 Sequences of primer pairs used in PCR: ITS 1/ITS4 for testing the quality of DNA and ver-1/ver-2 and ordA-F/ordA-R for detecting *ver-1* gene and *ordA* gene in aflatoxin-producing fungi respectively.

Primer	5' > 3' sequence	Target sequences and species	Amplicon size (bp)	Authors
ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	Fungal Ribosomal DNA	650-700	Yli-Mattila et al. (2004)
ver-1 ver-2	GCCGCAGGCCGCGGAGAAAGTGGT GGGGATATACTCCCGCGACACAGCC	*ver-1* gene	537	Färber et al. (1997)
ordA-F ordA-R	AAGGCAGCGGAATACAAGCG ACAAGGGCGTCAATAAAGGGT	*ordA* gene	400	Chang et al. (2005)

Determination of the PCR products molecular weight

Aliquots (8 µl) of each PCR product were analyzed by electrophoresis in a TBE buffer in 1.0% agarose gels and visualized by using Alpha Innotech Corporation MultiImage Light cabinet with camera and filters. The gel photos were analysed by using the GEL program (Patzekin and Klopov, Petersburg Nuclear Physics Institute, Russia) to determine the molecular weight of the PCR products and the PCR products profile from different isolates (Yli-Mattila et al. 2004).

Molecular identification of the strains

The identification of ten *Aspergillus* isolates was performed by sequencing their ITS region as a confirmation step of the morphological identification. The gene sequence of each isolate was amplified using the primers ITS1 and ITS4 as described by Yli-Mattila et al. (2004). The PCR mixtures were made to a final volume of 25 µL, involving template DNA (1.0 µL), 1× PCR buffer, 0.25 mM dNTPs, 50 µM of each primer, and 2 U Taq DNA polymerase. The amplified PCR products were sequenced by The Institute for Molecular Medicine Finland (FIMM) and the DNA sequences were aligned using advanced BLAST searches (http://www.ncbi.nlm.nih.gov/). The sequences were submitted to GenBank. Phylogenetic analyses were performed as described by Yli-Mattila et al. (2018).

Statistical analysis

Statistical significance was determined using Statistica Version 9 (StateSoft, Tulsa, OK, USA). The means of AFs concentrations were determined by analysis of variance (ANOVA, two-way analyses) ($P<0.05$). Fisher's LSD method (=0.05) was applied to compare significant differences in AFs production between the strains.

Results

Aspergillus isolates: A total of 160 *A.* section *Flavi* isolates that gave a yellow-orange reverse color on AFPA medium were isolated from 70 samples (32 soil, 21 maize, 9 wheat, 4 peanuts, 2 coconuts,1 lab bench swab and 1 air sample) representing 45 isolates from Egyptian samples and 115 isolates from the Philippines. Single spore isolates of aflatoxin-producing fungi were identified according to their phenotypic properties on AFPA, CYA, MEA, YES and their AFs production.

Aflatoxin production: In general, 26.5% (43) of the tested isolates (Table 3) were able to produce AFs, 14 from Egypt and 29 from the Philippines. All aflatoxigenic Egyptian isolates and 23 from the Philippines were morphologically identified as *A. flavus*, whereas the other 6

Philippine isolates (21%) were *A. nomius*. They looked yellow to light green with white fluffy appearance notably on YES medium, whereas the colonies of *A. flavus* isolates had more greenish color forming intensive conidia on both MEA and YES. Existing uni and bi-seriates in the conidial heads of *A. flavus* isolates was a unique character used for distinguishing them from *A. parasiticus* isolates.

Table 3 Morphological identification, aflatoxins production on CAM and the concentration in YES medium using HPLC. The number of + refers to the density of the blue fluorescence of the reverse side of the colonies under the UV lamb. ND= not detected.

Isolate code	Identification	CAM	Aflatoxins concentration in YES broth (ng ml^{-1} medium ± SE)				
			G_1	B_1	G_2	B_2	Total AFs
1E*	*A. flavus*	+	ND	26.70 ± 2.26	ND	ND	26.70 ± 2.26
3E	*A. flavus*	++	ND	721.90 ± 65.45	ND	6.30 ± 0.93	728.20 ± 66.03
16E*	*A. flavus*	+	ND	22.60 ± 2.52	ND	0.19 ± 0.05	22.79 ± 2.55
21E*	*A. flavus*	+	ND	113.60 ± 9.38	ND	2.30 ± 0.26	115.90 ± 9.63
23E	*A. flavus*	+	ND	45.60 ± 3.99	ND	0.15 ± 0.04	45.75 ± 4.04
29E	*A. flavus*	+	ND	44.50 ± 2.51	ND	ND	44.50 ± 2.51
34E	*A. flavus*	+	ND	0.50 ± 0.07	ND	ND	0.50 ± 0.07
35E	*A. flavus*	+	ND	3.40 ± 0.35	ND	ND	3.40 ± 0.34
40E	*A. flavus*	+	ND	201.00 ± 9.56	ND	9.50 ± 1.07	210.50 ± 10.62
41E	*A. flavus*	+	ND	432.60 ± 20.54	ND	17.91 ± 1.47	450.50 ± 22.31
42E*	*A. flavus*	+	ND	17.70 ± 1.014	ND	ND	17.70 ± 1.01
43E	*A. flavus*	++	ND	803.00 ± 23.99	ND	49.00 ± 4.81	852.00 ± 28.58
44E	*A. flavus*	+	ND	227.00 ± 14.00	ND	17.00 ± 2.65	244.00 ± 15.62
45E*	*A. flavus*	+	ND	1.30 ± 0.23	ND	ND	1.30 ± 0.23
6P	*A. nomius*	+++	26237.20 ± 1951.70	13188.80 ± 1547.29	462.30 ± 31.92	487.97 ± 27.90	40375.47 ± 1957.26
7P*	*A. nomius*	+++	3973.50 ± ± 190.80	1983.00 ± 145.01	70.80 ± 4.62	50.20 ± 7.63	6077.50 ± 347.88
8P	*A. nomius*	+++	2198.50 ± 205.80	1235.60 ± 129.45	41.50 ± 5.785	30.40 ± 5.73	3506.00 ± 342.20
9P*	*A. nomius*	+++	1526.60 ± 115.40	825.20 ± 83.63	26.90 ± 2.57	23.00 ± 2.60	2401.70 ± 203.63
10P	*A. nomius*	+++	12950.30 ± 989.80	5001.30 ± 207.27	224.60 ± 11.56	146.70 ± 6.18	18322.90 ± 1174.09
18P	*A. flavus*	+	4.00 ± 0.24	3.60 ± 0.19	ND	ND	7.60 ± 0.43
32P*	*A. flavus*	+	0.42 ± 0.04	1.60 ± 0.21	ND	ND	2.02 ± 0.25
33P	*A. flavus*	+	0.60 ± 0.04	1.60 ± 0.23	ND	ND	2.20 ± 0.27
34P	*A. flavus*	+	0.30 ± 0.15	1.20 ± 0.17	ND	ND	1.50 ± 0.37
35P	*A. flavus*	+	0.40 ± 0.05	1.30 ± 0.07	ND	ND	1.70 ± 0.06
36P	*A. flavus*	+	ND	1.70 ± 0.15	ND	ND	1.70 ± 0.15
41P	*A. flavus*	++	ND	360.50 ± 17.44	ND	4.80 ± 0.32	365.30 ± 17.69
42P	*A. flavus*	++	ND	822.00 ± 33.86	ND	9.10 ± 0.44	831.10 ± 34.28
43P	*A. flavus*	+	ND	231.20 ± 37.66	ND	1.30 ± 0.18	232.50 ± 13.48
44P	*A. flavus*	+	ND	151.00 ± 16.29	ND	0.40 ± 0.10	151.40 ± 16.58
45P	*A. flavus*	+	ND	135.10 ± 7.64	ND	0.23 ± 0.03	135.33 ± 5.96

Table 3 Continued.

Isolate code	Identification	CAM	Aflatoxins concentration in YES broth (ng ml^{-1} medium ± SE)				
			G_1	B_1	G_2	B_2	Total AFs
59P	*A. flavus*	+	ND	115.00 ± 93.12	ND	3.50 ± 0.32	118.50 ± 11.28
86P	*A. flavus*	+	ND	385.00 ± 65.19	ND	4.70 ± 0.35	389.70 ± 25.85
87P	*A. flavus*	++	ND	545.70 ± 202.34	ND	7.70 ± 0.32	553.40 ± 18.30
88P	*A. flavus*	++	ND	1136.00 ± 143.61	ND	13.70 ± 1.14	1149.70 ± 56.98
89P	*A. flavus*	++	ND	675.40 ± 116.54	ND	12.00 ± 0.93	687.40 ± 23.28
90P	*A. flavus*	+	ND	319.80 ± 71.77	ND	4.90 ± 0.35	324.70 ± 17.62
96P	*A. flavus*	+	ND	102.50 ± 6.96	ND	0.60 ± 0.09	103.10 ± 5.31
97P	*A. flavus*	+	ND	94.20 ± 14.35	ND	1.20 ± 0.18	95.40 ± 6.13
107P	*A. flavus*	+	ND	128.70 ± 230.26	ND	0.60 ± 0.10	129.30 ± 10.48
108P*	*A. flavus*	++	ND	808.40 ± 205.68	ND	7.60 ± 0.41	816.00 ± 13.94
109P	*A. flavus*	++	553.10 ± 26.70	190.00 ± 374.50	4.80 ± 0.41	2.50 ± 0.18	750.40 ± 36.89
110P*	*A. nomius*	+++	1822.60 ± 64.94	1313.10 ± 432.15	24.40 ± 2.28	22.30 ± 2.11	5621.90 ± 102.13
112P	*A. flavus*	-	ND	1.20 ± 0.10	ND	ND	1.20 ± 0.10

* = Accession number of ITS sequence of the selected isolates
1E: MN511742/ITS, 16E: JF729324/ITS, 21E: MG554234/ITS, 42E: MH595954/ITS, 45E: MH595954/ITS, 7P: MH752557/ITS, 9P: AY510454/ITS, 32P: KF432854/ITS, 108P: MN511745/ITS, 110P: MN511744/ITS

Almost all toxigenic isolates (99.4%) gave different blue fluorescence density under UV lamp at the reverse sides of the CAM medium plates except one isolate (112P). In general, the potential hazard of the Philippines isolates, notably *A. nomius* isolates, was statistically ($P<0.05$) much higher than those from Egypt. The levels of AFG_1 were higher than those of AFB_1 in cultures of all *A. nomius* isolates and the isolate *A. flavus* 109P. All aflatoxin-producing isolates produced AFB_1 in the range 0.5 to 13189 ng ml^{-1} medium. The highest concentration of AFB_1 was produced by isolate 6P which was isolated from a soil sample of coconut field in Philippines. All 14 Egyptian aflatoxin-producing isolates were unable to produce AFG_1 and AFG_2, while 12 isolates from the Philippines were AFG_1 producers and seven of them produced AFG_2 also.

Based on HPLC analyses, there were four chemotypes of aflatoxins producers: (1) isolates producing all four aflatoxin types (16%, seven isolates), (2) isolates producing AFB_1 only (18.5%, 8 isolates), (3) isolates producing AFB_1 and AFB_2 (53.5%, 23 isolates), and (4) those producing AFB_1 and AFG_1 (12%, 5 isolates). All six *A. nomius* isolates belonged to chemotype one and produced all four AFs (total amount 2400-40400 ng ml^{-1}). In contrast, the other isolates (37) which belonged to *A. flavus* produced <1200 ng ml^{-1} total AFs. Only one *A. flavus* isolate from the Philippines was capable of producing all four AFs.

Molecular detection of aflatoxin-producing *Aspergillus* isolates: The results of the tested isolates PCR with primer pairs of ITS1/ITS4, ver-1/ver-2 and ordA-F/ordA-R are summarized in Table 4. All 43 *A. flavus* and *A. nomius* aflatoxin-producing isolates had the ITS1/ITS4 primer pair which referred to how optimal DNA extraction was (Fig. 1a). The PCR with ver-1/ve r-2 primer pair showed three different profiles of PCR products (Fig. 1b); (a) one band at around 537bp (all *A. nomius* and half *A. flavus* isolates), (b) one band at around 700bp (*A. flavus* isolates 88P, 89P and 90P) and (c) two bands: at around 537bp and 700bp (*A. flavus* isolates 23E, 29E, 35E, 41P, 44P, 45P, 59P, 86P, 87P, 96P, 97P, 107P, 108P, 109P, 112P). However, the PCR product of ordAF/ordAR primer pair (Fig.1c) gave one main band at around 400bp. The atoxigenic *A. flavus* isolates produced PCR products with the ITS1/ITS4 primer pair, whereas it had no bands with either ver-1/ver-2 or ordA-F/ordA-R primer pairs.

Based on the ITS sequences, the morphological identification of seven *A. flavus* isolates (1E, 16E, 21E, 42E, 45E, 32P and 108P) and three *A. nomius* isolates (7P, 9P and 110P) were

confirmed by comparing their ITS sequences with known ITS sequences in GenBank (sub-notes, Table 3). In the phylogenetic parsimonious consensus tree (Fig. 2), *A. nomius* isolates formed a clear cluster with known *A. nomius* isolate NRRL 13137 (AF027860) and were separated from *A. flavus* isolates including known *A. flavus* isolate NRRL 1957 (AF027863) and from known *A. parasiticus* isolate NRRL 502 (NR121219).

Table 4 The results of PCR with ITS 1/ITS4 for confirming the quality of DNA and ver-1/ver-2 and ordA-F/ordA-R for detecting *ver-1* gene and *ordA* gene in aflatoxin-producing fungi respectively.

PCR product	Positive isolates
ITS 1/ITS4 (650-700 bp)	All isolates
ver-1/ver-2 (537bp) only	All isolates except *A. flavus* isolates 23E, 29E, 35E, 41P, 44P, 45P, 59P, 86P, 87P, 88P, 89P, 90P, 96P, 97P, 107P, 108P, 109P and 112P
ver-1/ver-2 (700 bp) only	*A. flavus* isolates 88P, 89P and 90P
ver-1/ver-2 (537bp + 700 bp)	*A. flavus* isolates 23E, 29E, 35E, 41P, 44P, 45P, 59P, 86P, 87P, 96P, 97P, 107P, 108P, 109P, 112P
ordA-F/ordA-R(400bp)	All isolates

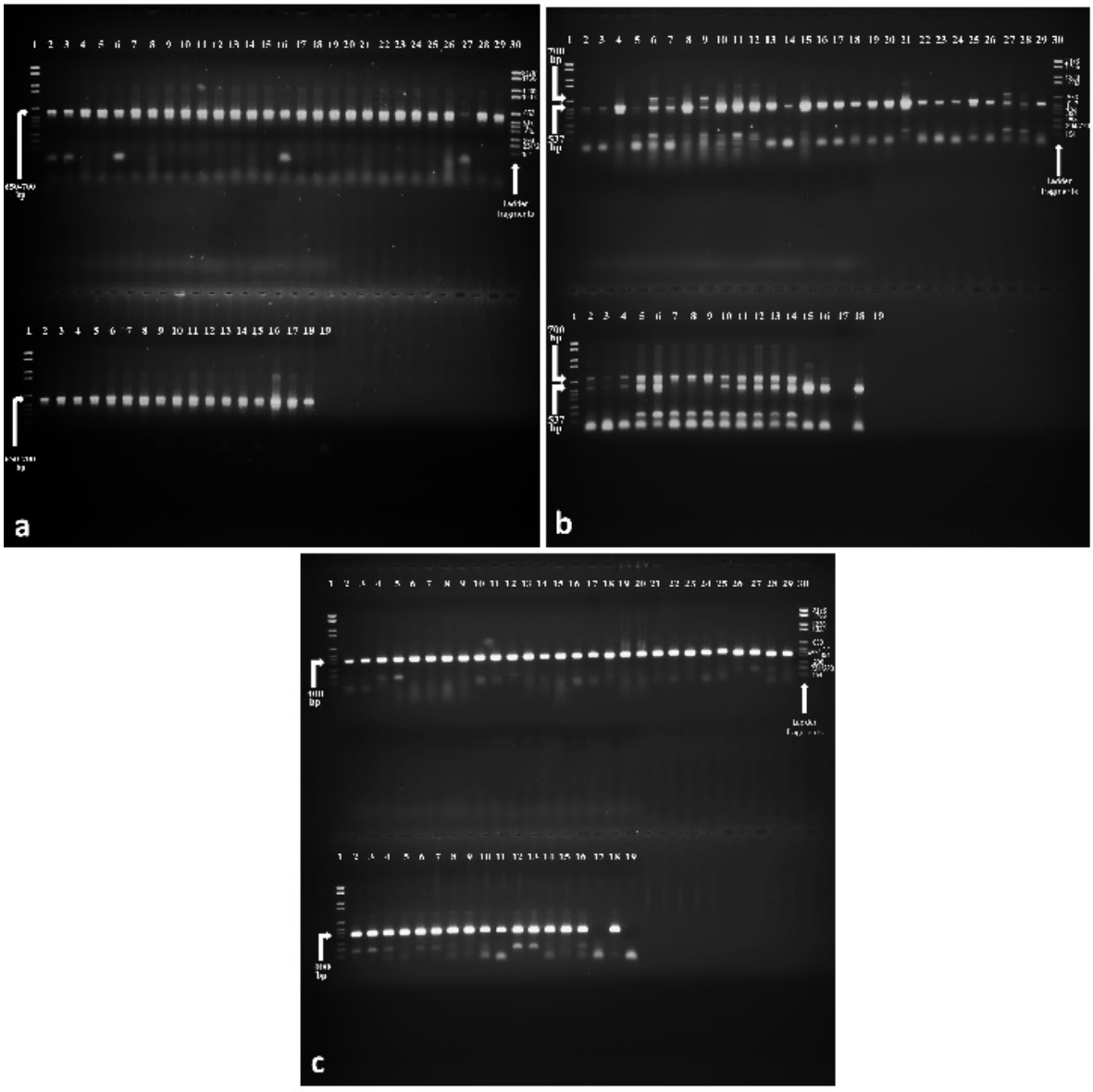

Fig. 1 – Band pattern of different *Aspergillus* species isolates as resulted from PCR reaction primed by primer pairs of a) ITS 1/ITS4 , b) ver-1/ver-2 and c) ordA-F/ordA-R. In the upper part of gel,

Lanes: Lane 1 = 1 Kb DNA ladder, Lane 2 = IE, Lane 3 = 3E, Lane 4 = 16E, Lane 5 = 21E, Lane 6 = 23E, Lane 7 = 29E, Lane 8 = 34E, Lane 9 = 35E, Lane 10 = 40E, Lane 11 = 41E, Lane 12 = 42E, Lane 13 = 43E, Lane 14, Lane 15 = 45E, Lane16 = 6P, Lane17=7P, Lane18 = 8P, Lane19 = 9P, Lane20 = 10P, Lane21 = 18P, Lane22 = 32P, Lane23 = 33P, Lane24 = 34P, Lane25 = 35P, Lane26 = 36P, Lane27 = 41P, Lane28 = 42P, Lane29 = 43P, Lane30 = 1 Kb DNA ladder. In the lower part of the gel, Lanes: Lane 1 = 1 Kb DNA ladder, Lane 2 = 44P, Lane 3 = 45P, Lane 4 = 59P, Lane 5 = 86P, Lane 6 = 87P, Lane 7 = 88P, Lane 8 = 89P, Lane 9 = 90P, Lane 10 = 96P, Lane 11 = 97P, Lane 12 = 107P, Lane 13 = 108P, Lane 14 = 109P, Lane 15 = 110P, Lane16 = 112P, Lane17 = atoxigenic *A. flavus*, Lane18 = positive control (*A. flavus*), Lane19 = negative control

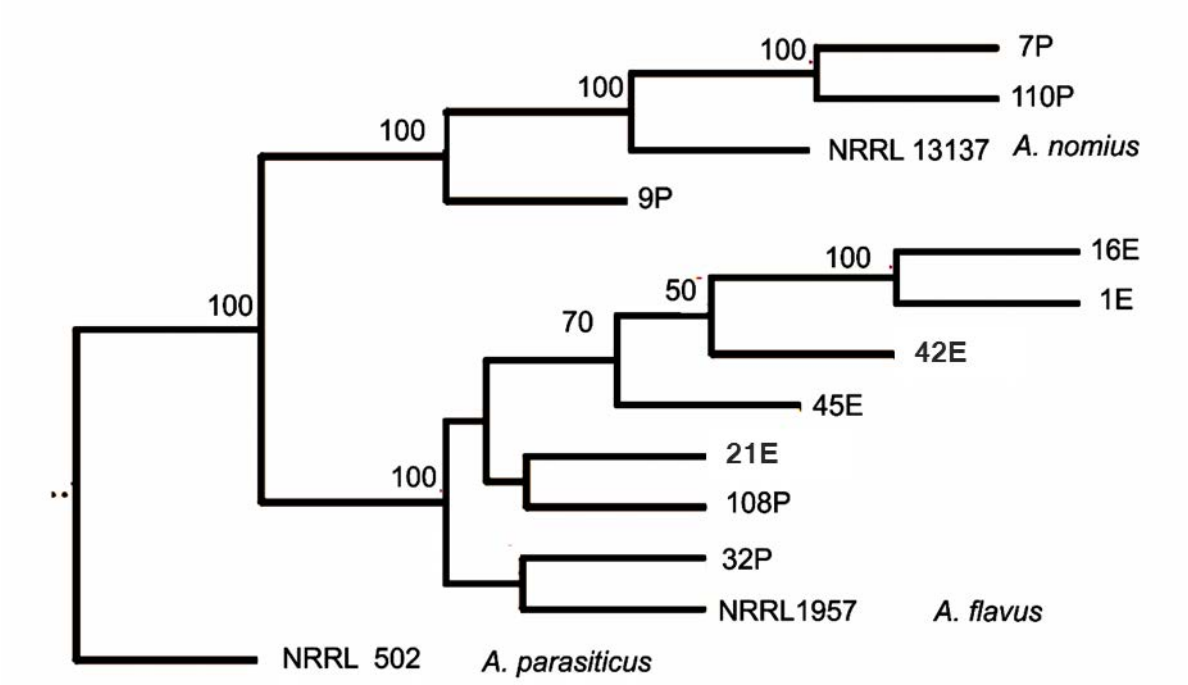

Fig. 2 – Consensus tree of 55 most parsimonious trees based on the ITS sequences of ten *Aspergillus* isolates as compared to reference isolates from GenBank. Only bootstrap values supported by more than 50% of the tree are marked.

Discussion

In the present study, a total of 160 *A. section Flavi* isolates obtained from soil, maize, wheat, coconut, and peanuts showed a characteristic yellow-orange reverse colour on AFPA medium. These results confirmed the effectiveness of this medium for the detection of *A.* section *Flavi* as reported by Pitt et al. (1983). *A. nomius* isolates were identified according to Varga et al. (2011). Visually, they had fluffy shape on YES medium and less sporulation than that of *A. flavus* isolates. The isolates of *A. nomius* were only found in soil samples from coconut fields in the Philippines, while all Egyptian isolates were identified as *A. flavus*. Similarly, *A. nomius* become predominant aflatoxigenic species found in soil samples of Japan (Kumeda et al. 2003) and Thailand (Ehrlich et al. 2007).

About a quarter of the identified isolates were aflatoxigenic according to HPLC analysis (Table 3). The ratio of aflatoxin-producing isolates from Egyptian samples (31%) was higher than that for isolates from the Philippines samples (25%); however most of them were unable to produce AFG_1 and AFG_2. Also, the total AF production by the Egyptian isolates was less than that of the Philippine isolates with values from 0.5 to 852 ng ml^{-1} medium. This finding could be attributed to the differences in the source of isolation; the Egyptian isolates were mainly from maize, wheat, and soil samples while the majority of the Philippines isolates were from soil samples of coconuts and maize fields. Also, because of the location and climate of the Philippines which is characterized by

higher relative humidity (Ilag 1984) than that of Egypt made the soils more susceptible for the fungal invasion at pre-harvest stage.

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A. flavus is known to produce AFBs (Varga et al. 2009). Most of the A. *flavus* isolates in present study (31) produced AFBs, either AFB_1 alone or both AFB_1 and AFB_2 together, whereas *A. flavus* isolate 109P produced the four types and *A. flavus* isolates 18P, 32P, 33P, 34P &35P produced both AFB_1 and AFG_1 chemotypes. Regarding the *A. nomius* isolates, high levels of aflatoxin were produced in media. The occurrence of such isolates as predominant species was observed for the first time in coconut field soils in the Philippines. This finding is in agreement with the previous records of the highly aflatoxin-contaminated "copra" (dried coconut meat) which has resulted in the suspension of the Philippines from exporting copra meal into Europe in 2004 (Bawalan 2004). In addition, all isolated species from maize, wheat and soil associated with these crops have been identified as *A. flavus.* This suggests that there may be a relationship between the associated crop and the type of aflatoxin-producing fungi. Data obtained by Pildain et al. (2004) showed that the frequencies of *Aspergillus* section *Flavi* varied among both fields and crops being cultivated. Although *A. flavus* is not host specific fungus (St Leger et al. 2000), the distribution of different *Aspergillus section Flavi* species suggests that they may be adapted to specific niches and exhibit competitive advantages in specific soil types, hosts, regions, and seasons (Jaime-Garcia & Cotty 2006). Variation in the quantity and types of AFs produced by each isolate referred to the diversity of the isolated fungi as it has been found previously (Cotty 1989).

Fluorescent detection on CAM was confirmed with the HPLC analysis of aflatoxins except for one isolate (112P), which gave no fluorescence on CMA. Similar results were obtained by Sultan & Magan (2010) who examined the potential aflatoxin production by isolates of *A. flavus* and *A. parasiticus* from Egyptian peanuts. They found that there was 90% compatibility of the results between HPLC and the coconut agar method. Also, Rodrigues et al. (2009) reported that HPLC results had a good correlation with aflatoxin production by fluorescence in CAM.

Concerning the genetic identification of the aflatoxigenic isolates in the current study, a similar study had been made in north-eastern Iran (Davari et al. 2015). They recognized 28 aflatoxigenic strains of *A. flavus* and *A. parasiticus* through amplification of four genes involved in the aflatoxin biosynthesis pathway (*nor1*, *ver1*, *omtA* and *aflR*) followed by thin layer chromatography as a confirmation method. Furthermore, the genetic variability between 109 *A. flavus* strains isolated from maize in Kenya, were analyzed for the presence of four AF genes with their ability to produce aflatoxins, targeting *aflR*, *aflP*, *aflD*, and *aflQ* genes (Okoth et al. 2018).

DNA isolation from the fungal material was performed over a relatively short time period (3 days incubation) in small amounts of broth medium in Eppendorf tubes. This approach reduced the time and amount of the medium necessary for preparing mycelial biomass (20 ml for 7 days) (Rodrigues et al. 2009, Sultan & Magan 2010). The ITS primers were chosen as the standard markers for fungal DNA barcoding of Aflatoxigenic isolates to confirm the existence of enough PCR products for the next molecular identification steps of aflatoxins genes (Yli-Mattila et al. 2004).

For molecular detection of aflatoxin production, two genes were chosen (a) the *ver-1* gene, versicolorin A dehydrogenase, which converts the versicolin A to sterigmatocystin in the middle of the aflatoxin biosynthetic pathway (Yu et al. 2004) and (b) the *ord1* gene which is considered to be

the only gene involved in the last step of transforming O-methylsterigmatocystin into AFB_1, an important step in the aflatoxin biosynthesis pathway that appears to unique to aflatoxigenic species (Prieto & Woloshuk 1997).

Molecular detection of aflatoxin production by using the ver-1/ver-2 primer pair as described previously (Färber et al. 1997) and ordAF/ordAR primers pair as described by Chang et al. (2005) is in accordance with the results obtained from HPLC analyses of AFs and CAM methods. However, the presence of the aflatoxin biosynthetic genes does not always accompany with the occurrence of aflatoxin (Rodrigues et al. 2009). The present observations led to the use of both genes in recognition of the aflatoxigenic isolates on grain samples in a shorter time than the time-consuming traditional methods (Degola et al. 2007). Similar study but on toxigenic *Fusarium* revealed that the detection of the toxigenic strains by molecular approach was more effective than HPLC even at the low or negligible level of fumonisins (Abd-El Fatah et al. 2015).

The PCR products profile with the ver-1/ver-2 primer pair showed differences in *A. flavus* and *A. nomius* isolates. Two new PCR profiles with the ver gene primers were detected in the present isolates. The first profile observed as one band at a molecular weight (700 pb) higher than the reported one and the second profile exhibited as two bands, one at the ordinary (537 pb) and 700pb. Similar PCR products were reported by Geisen (1996), who tested *Penicillium roqueforti* using primers for the same gene. He got also two bands as well and attributed that to the owning genomic sequences similar to that gene. According to these results, the two new PCR profiles with *ver* gene can be considered new genotypes. This revealed to the ability to use this primer pair in identifying the diversity of aflatoxin-producing fungi but not for distinguishing the aflatoxigenic fungi from other species with the same gene. Sequencing of more genes is required to confirm the presence of the 3 genotypes.

In conclusion, the examined aflatoxin-producing fungal isolates showed variability in the types and the quantity of AFs production based on the region of origin. The PCR results with ver-1/ver-2 primer pair were successfully used for studying the diversity of aflatoxigenic fungi from different region, but not for differentiation between fungal species as a molecular marker. On the other hand, ordAF/ordAR primers pair was used for the screening of the contaminated grain samples for the presence of *Aspergillus* section *Flavi* species. Finally, further work is required using RAPD and ISSR PCR to confirm the separation of aflatoxin-producing isolates into different genotypes.

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