

IDENTIFICATION OF *Aspergillus flavus* AND DETECTION OF ITS AFLATOXIN GENES ISOLATED FROM PEANUT AND PEANUT PROCESSED PRODUCTS

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ABSTRACT

Aspergillus flavus is one of the main producers of aflatoxin. Therefore, the presence of the fungus is becoming serious problem on food safety. This research was aimed to isolate and identify *A. flavus* from peanut and its processed products collected from several traditional markets in Bogor, Depok and Jakarta, and to detect the aflatoxin genes. Fungal identification was carried out using morphological characteristics and species specific primers of FVAVIQ1/FLAQ2 and AFLA-F/AFLA-R, while detection of aflatoxin genes employed four specific primers of *apa-2* (*afIR*), *nor-1* (*afID*), *ver-1* (*afIM*) and *omt-1* (*afIP*). From 36 samples, the *A. flavus* group was only found in peanut kernels samples with viable count of specific colonies in the range of 0.01-5.52 x 10⁴ cfu/g. Eighteen isolates were identified as *A. flavus* based on species specific primers, FVAVIQ1/FLAQ2 and AFLA-F/AFLA-R by producing amplicons of about 100 and 413 bp, respectively. Based on aflatoxin gene analysis, all 18 isolates successfully amplified by both *apa-2* and *nor-1*, 83.3 % by *omt-1* and 72.2 % by *ver-1* primers which involved in aflatoxin production. The amplicons size of *apa-2*, *nor-1*, *ver-1* and *omt-1* primer pairs were about 1032, 400, 895 and 1024 bp, respectively.

Keywords: aflatoxin genes, AFPA, *Aspergillus flavus*, peanut, species specific primers

INTRODUCTION

Peanut is an important agriculture commodity after rice, maize and soybean in Indonesia. Humidity and tropical climate make peanut kernels and processed products easily infected by fungi particularly during inadequate drying and improper storage which results in physical damage, discoloration, lower quality of nutritional content and mycotoxin contamination of the products (Sauer *et al.* 1992).

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Mycotoxin causes serious problems to human and animal health. Aflatoxin is the most toxic compound in mycotoxin group, it is carcinogenic and teratogenic (JECFA 1997). There are four naturally forms of aflatoxin, namely B₁ (AFB₁), B₂ (AFB₂), G₁ (AFG₁), and G₂ (AFG₂). AFB₁ was the most hazardous because it was very carcinogenic among other form of aflatoxin and usually found at the highest concentration in contaminated food including peanut and processed peanut products (Pitt 2000). Aflatoxin is mostly produced by *Aspergillus flavus* and *A. parasiticus*, which belongs to the *Aspergillus* section *Flavi*. *Aspergillus parasiticus* produces both AFB and AFG, while *A. flavus* produces only AFB₁ and AFB₂, but not all strains of *A. flavus* are able to produce aflatoxin (Pitt & Hocking 2009).

Aflatoxin quantification is commonly done using Thin Layer Chromatography and High Performance Liquid Chromatography but the studies on the level of fungal infection and the identification of aflatoxin producing fungi could be an alternative for indicating the quality of the food and feed products before the toxin was produced. Several studies had been done to detect, quantify and identify species of aflatoxigenic fungi by using specific medium *Aspergillus flavus-parasiticus* agar (AFPA) developed by Pitt *et al.* (1983). This medium is suitable and recommended for determining *A. flavus* and *A. parasiticus* in food and feed due to its simplicity in application since the detection needs only 2 days incubation. The AFPA medium, however, could not differentiate *A. flavus* from *A. parasiticus*, therefore, further differentiation method need to be used such as morphological characteristics by microscopic and culture techniques. These techniques, however, are time consuming and may result in false positive. The AFPA medium also can not differentiate between aflatoxigenic and non-toxigenic *A. flavus*. Several studies had been done by using Polymerase Chain Reaction (PCR)-based methods using full length of ITS regions and species specific primers to identify *A. flavus*. These methods are more sensitive compared to conventional method. There are several species specific primers available to identify *A. flavus* such as FVAVIQ1/FLAQ2 (Sardinas *et al.* 2011) designed from ITS2 rDNA region and AFLA-F/AFLA-R (Hue *et al.* 2013) designed from aflatoxin biosynthesis sequences published on Genbank. These studies indicated that the primers are very specific and able to amplify *A. flavus* only.

It has been reported that not all strains of *A. flavus* are aflatoxin producer. It is, therefore, important to detect *A. flavus* carrying aflatoxin gen for controlling the aflatoxin contamination on the products. This determination is also important to be done before the toxin is expressed in early development of the fungi. The aflatoxin biosynthesis pathway involved 25 genes that clustered in a 75-kb DNA region (Bhatnagar *et al.* 2006). There are four pairs of primers, *apa-2*, *nor-1*, *ver-1* and *omt-1*, available to identify aflatoxin genes in *A. flavus*. The *apa-2* gene (*afIR*) involved in regulation of aflatoxin biosynthesis by controlling the expression of the *nor-1* and *ver-1* genes (Liu & Chu 1998; Woloshuk *et al.* 1994). The *nor-1* gene encodes norsolorinic acid reductase and converts norsolorinic acid to averantin (Chang *et al.* 1992). The *ver-1* gene encodes versicolorin A dehydrogenase, and converts versicolorin A to sterigmatocystin (Skory *et al.* 1992). The *omt-1* gene encodes sterigmatocystin-methyltransferase and is required for conversion of demethylsterigmatocystin and dehydrodemethylsterigmatocystin to sterigmatocystin and dihydrosterigmatocystin,

respectively (Yu *et al.* 1995a). All these primers had been studied and used to detect toxigenic *A. flavus* in grains, foods and feeds successfully (Farber *et al.* 1997; Manonmani *et al.* 2005).

Aspergillus flavus is able to grow on various nutrient sources. In Indonesia, many commodities such as peanuts, maize, pepper and feed ingredients were reported to be contaminated by the fungus and caused high level of aflatoxin concentration in the commodities. About 70% of the peanut kernels samples collected from retailers in Bogor, Cianjur and Wonogiri Regencies contained more than 15 ppb of aflatoxin. The highest percentage of peanut kernels genocies infected by *A. flavus* was found at the retails level in the traditional markets (Dharmaputra *et al.* 2005; 2007).

Early detection of aflatoxin producer needs to be done in order to improve the quality of the commodities and to formulate strategy on prevention and control of aflatoxin contamination in the products. The aim of this study was to detect growth of aflatoxin producing fungus, *A. flavus* by isolation and identification of the fungi and detection of the fungal ability to produce aflatoxin from peanut kernel and its processed products. Fungal isolation was done using AFPA specific medium, identification by morphology and molecular methods, and detection of aflatoxin genes involved in aflatoxin biosynthesis pathway was carried out using aflatoxin specific producer primers.

MATERIALS AND METHODS

Isolation of *Aspergillus flavus* group

Thirty six samples of peanut kernels, roasted peanuts with skin pod, flour-coated peanuts, and branded *bumbu pecel* (dry peanut sauce) were collected from traditional markets in Bogor, Depok and Jakarta. Fungal isolation was done by dilution plating method on AFPA medium (NMKL 2004). Twenty five grams of each sample was grounded separately using a blender in medium speed (scale 3 out of 5) (Philips), then suspended in solution containing 0.1% peptone and 0.025% Tween 20 (1:10, w/v). The samples were then homogenized by stomacher for 2 min and treated into several serial dilutions 1:1000. A total volume of 100 µl of each dilution was spread onto duplicate AFPA plates (Oxoid) using sterile glass rod, incubated at 30 ± 1 °C for 48 ± 3 h. Specific colonies with bright yellow orange on lower side of the plates were recorded as the number of colony forming units (cfu). The colonies were cultured and maintained on Potato Dextrose Agar medium for further analysis.

Identification of *Aspergillus flavus* group

Total of 18 isolates were recovered from the samples. Isolated *A. flavus* groups were identified by morphological and molecular methods. The morphological identification was carried out using the method described by Pitt & Hocking (2009). Molecular analysis was carried out using two species specific primers of FVAVIQ1/FLAQ2 and AFLA-F/AFLA-R developed by Sardinias *et al.* (2011) and Hue *et al.* (2013), respectively. For comparison of PCR analysis, five strains were also

used as standard cultures for positive and negative controls. The strains were *A. flavus* 747 obtained from SEAMEO BIOTROP (Bogor, Indonesia), *A. flavus* NBRC 33021 and *A. flavus* NBRC 30107. Two species, *A. parasiticus* NBRC 33224 and *A. nomius* NBRC 33223 were also included in the analysis.

Fungal DNA was extracted according to the method described by Raeder and Broda (1985) with some modifications by using phenol-chloroform-isoamyl alcohol (PCI) and chloroform-isoamyl alcohol (CI) instead of chloroform and isopropanol. Each fungal strain was grown in 100 ml flask containing 50 ml Potato Dextrose Broth (PDB) in rotary shaker agitated at 100 rpm in room temperature for 3 days. At harvest, the mycelium was filtered using Whatman #2 and washed with sterile distilled water. One gram of washed mycelium was grounded using a mortar and liquid nitrogen. DNA concentration was measured by Nanodrop 2000 Spectrophotometer (Thermo Scientific) and kept in -20 °C for further analysis.

PCR reactions were performed using two pairs of species specific primers for identification of *A. flavus*. The primers pairs were FVAVIQ1/FLAQ2 and AFLA-F/AFLA-R with the sequences 5'-GTCGTCCCCTCTCCGG-3' for FVAVIQ1 and 5'-CTGGAAAAAGATTGATTTGCG-3' for FLAQ2 to amplify a fragment of 100 bp (Sardinas *et al.* 2011); 5'-GGTGGTGA-AGAAGTCTATCTAAGG-3' for AFLA-F and 5'-AAGGCATAAAGGGTGTGGAG-3' for AFLA-R to amplify a fragment of 413 bp (Hue *et al.* 2013). Amplification of fungal DNA was performed in a total volume of 25 µl. The reaction mixtures contained 12.5 µl PCR master mix 2x (Promega), 12.5 pmol of each primer, ±100 ng DNA template and nuclease free water. Amplification reaction was performed as follows: pre-denaturation for 5 min at 94 °C and followed by 35 cycles for 30s at 94 °C for denaturation, 60s at 58 °C for annealing, 90s at 72 °C for extension, and 7 min at 72 °C for final extension by using the Multigene Optimax thermal cycler (Labnet International, Inc).

The PCR products were analyzed on 1.0% agarose gel in 1x TAE buffer, stained with ethidium bromide solution and visualized under UV light illumination (G Box Syngene). A positive control (DNA of *A. flavus* from standard cultures) and negative control (no DNA target and DNA of *A. parasiticus* NBRC 33224 and *A. nomius* NBRC 33223) were included in this analysis.

Detection of Genes Involved in Biosynthesis of Aflatoxin

All isolates showed positive result after amplification using FVAVIQ1/FLAQ2 and AFLA-F/AFLA-R species specific primers were used for further PCR analysis to determine genes involved in aflatoxin production by using 4 primer pairs of *apa-2*, *nor-1*, *ver-1* and *omt-1*. The PCR reactions and conditions were the same as indicated in PCR amplification of using species specific primers, except the annealing was carried out at 68 °C. The sequences and the expected PCR products of each primer are presented in Table 1.

Three isolates were selected for sequence analysis using *omt-1* primers based on sampling location and their ability to be amplified by the four primers tested. The PCR products were sequenced by First Base services (Malaysia) using the same primer. DNA sequences of *omt-1* gene were analyzed with the BioEdit Ver.7.0.0 (Hall 1999)

Table 1. Specific primers used for detection of genes involved in biosynthesis of aflatoxin

Primer codes	Target gen	Sequence (5'→3')	Size products (bp)
apa2-F**	<i>afIR</i>	TAT-CTC-CCC-CCG-GGC-ATC-TCC-CGG	1032
apa2-R		CCG-TCA-GAC-AGC-CAC-TGG-ACA-CGG	
nor1-F*	<i>afID</i>	ACC-GCT-ACG-CCG-GCA-CTC-TCG-GCA-C	400
nor1-R		GTT-GGC-CGC-CAG-CTT-CGA-CAC-TCC-G	
ver1-F**	<i>afIM</i>	ATG-TCG-GAT-AAT-CAC-CGT-TTA-GAT-GGC	895
ver1-R		CGA-AAA-GCG-CCA-CCA-TCC-ACC-CCA-ATG	
omt1-F**	<i>afIP</i>	GGC-CCG-GTT-CCT-TGG-CTC-CTA-AGC	1024
omt1-R		CGC-CCC-AGT-GAG-ACC-CTT-CCT-CG	

References : * Geisen (1996); ** Shapira (1996)

and aligned using Clustal W (Thompson *et al.* 1994). Phylogenetic tree was performed by using Neighbor-Joining method model Kimura 2-parameter using MEGA 5 with 1000 bootstrap replications (Tamura *et al.* 2011). Based on previous study by Varga *et al.* (2011), no outgroup was chosen during the analysis of *omt-1* gene because no sequences are available from any other aflatoxigenic species outside *Aspergillus* section *Flavi*.

RESULTS AND DISCUSSIONS

Isolation of *Aspergillus flavus* group

Based on the data obtained from 36 samples of peanut kernels, roasted peanuts with skin pod, flour-coated peanuts, and branded *bumbu pecel* indicated that fungal colony was only shown on peanut kernels. Number of fungal population based on viable count of specific colony showed that level of fungal infection varied between locations. The number of colonies were ranging from 0.01 - 5.52 x 10⁴ cfu/g, in which the highest population was found on peanut kernels collected from Bogor followed by Depok and Jakarta areas (Table 2). This might be due to Bogor had higher humidity and rainfall compared to the two other locations resulted in higher humidity in room storage and moisture content of peanut kernels. The relative humidity at the time of sampling in Bogor, Depok and Jakarta areas were 84%, 82% and 81%, respectively (BMKG 2013a,b), which would correlate with the moisture content of peanut kernels. Population of *A. flavus* in Bogor obtained in our study was 5.52 x 10⁴ cfu/g, which was 10 fold higher than reported by Dharmaputra (2010) which showed the fungal population collected from the same regency but different traditional markets location was 0.49 x 10⁴ cfu/g. The traditional market (retailers) is the last distribution chain of the commodities before being delivered to consumer. Long chain distribution before being delivered to consumer contributed to the possibility of broken peanut kernels which made it easier for infection of fungi including aflatoxigenic fungi.

Table 2. Population of *A. flavus* group isolated from peanut and its processed products

Samples	Number of colony (viable count, cfu x 10 ⁴ /gram)		
	Jakarta	Depok	Bogor
Peanut kernels	0.01	2.1	5.52
Processed peanut products (roasted peanuts with skin pod, flour-coated peanuts, <i>bumbu pecel</i> (dry peanut sauce))	0	0	0

No colony was found on roasted peanuts with skin pod, flour-coated peanuts, and branded *bumbu pecel* samples in this study. This might be caused by heating of peanut kernels as raw material during the processing which could kill the fungus. Furthermore, this could also be due to standardization method applied to peanut kernels used as raw materials in factory before being processed into peanut products as the peanut kernels were usually obtained directly from the farmers. Dharmaputra *et al.* (2013) reported that the populations of *A. flavus* in processed peanut products were relatively low, which was less than 1 cfu/g in fresh weight basis. *Aspergillus flavus* infection and aflatoxin production particularly in peanut kernels were related to methods of postharvest handling from farmers up to retailers in market and the duration of storage (Dharmaputra *et al.* 2005).

Fungal Identification

Eighteen out of 28 fungal isolates isolated from peanut kernels showed orange yellow reverse coloration on AFPA medium confirming that the isolates were strains of *A. flavus* group (Fig. 1a). The 18 isolates had similar microscopic characteristics such as conidial shapes varied from spherical to elliptical, septate hyphae, and conidial heads uniseriate (phialides only) (Fig. 1b, c, d). The sizes of microscopic morphological structures of each isolate are shown in Table 3. Differentiating *A. flavus* from *A. parasiticus* based on morphological characteristics was not easy since it could give false positive result. Rodrigues *et al.* (2007) reported that the use of Scanning Electron Microscopy (SEM) for the conidial wall ornamentation examination was the primary character analysis for separating of the two species in morphological identification.

Further identification using species specific primer showed that all isolates produced amplicon size of about 100 bp for FVAVIQ1/FLAQ2 primer and 413 bp for AFLA-F/AFLA-R (Fig. 2). Both primers were also successfully amplified the positive control cultures of *A. flavus* 747, *A. flavus* NBRC 33021, and *A. flavus* NBRC 30107, but failed to amplify *A. parasiticus* NBRC 33224 and *A. nomius* 33223 used as negative control (Fig 3a and 3b). Molecular analysis indicated that all 18 isolates were *A. flavus* species. Our results were in agreement with the finding reported by Sardinas *et al.* (2011) and Hue *et al.* (2013) who tested the same species specific primers pairs on several species of *Aspergillus* spp. non *A. flavus*. The FVAVIQ1/FLAQ2 primer pair

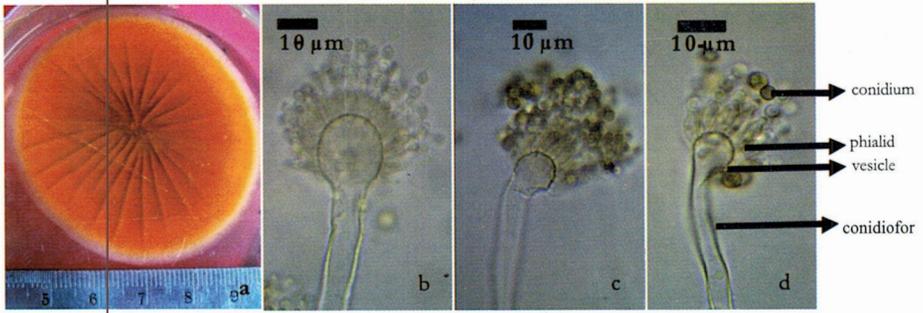


Figure 1. Macroscopic (a) and microscopic (b, c, d) of *A. flavus* group isolated from peanut kernels in Bogor, Depok and Jakarta

Table 3. Size of hypha, vesicle, phialid and conidium of *Aspergillus flavus* group isolated from peanut kernels in Bogor, Depok and Jakarta

Isolates code	size (µm)				Isolates code	size (µm)			
	Conidia	Hypha	Vesicle	Phialid		Conidia	Hypha	Vesicle	Phialid
J1	3-5	5-7	11-13	6-7	B6	3-4	6-7	15-18	5-6
J2	3-4	3-5	11-14	6-7	B7	3-4	6-8	15-17	6-7
D1	3-4	5-8	14-17	7-8	B8	3-4	7-8	18-21	5-6
D2	3-4	6-8	15-20	6-8	B9	3-4	5-7	15-19	5-7
D3	3-4	6-7	16-18	5-7	B10	4-5	7-8	16-19	5-6
D4	3-5	6-8	14-28	6-8	B11	3-4	5-6	15-17	5-6
B1	3-4	5-8	14-17	5-9	B12	3-4	5-6	15-19	6-7
B2	3-4	5-7	11-18	5-8	747*	2-4	4-8	18-26	5-7
B3	3-4	5-6	15-17	5-6	33021*	3-5	4-6	15-22	6-8
B4	3-4	6-8	16-18	5-6	30107*	3-5	4-7	17-21	5-6
B5	3-5	6-8	14-18	5-6					

(*) = standard cultures of positive *A. flavus*

did not amplify DNA of *A. flavus* group such as *A. tamarii*, *A. bombycis*, *A. fumigatus*, *A. terreus*, *A. niger*, *A. tubingensis*, *A. carbonarius*, *A. japonicus* and *A. ochraceus* (Sardinas *et al.* 2011), and AFLA-F/AFLA-R did not amplify the DNA of *A. parasiticus*, *A. oryzae*, *A. niger* and *A. candidus* (Hue *et al.* 2013).

Detection of Aflatoxin Genes

The population of *A. flavus* is not always correlated with aflatoxin production, as it is depending on the strain whether or not the fungus carried aflatoxin producer gene in their genome. Detection of aflatoxin genes on isolated *A. flavus* were carried out using 4 primer pairs in this study to detect genes fragment involved in the aflatoxin biosynthetic pathway (Fig. 4). The two primers *apa-2* and *nor-1* was successfully produced amplicons from all 18 isolates and standard cultures of *A. flavus*, whereas, *omt-1* amplicons detected 15 isolates and 2 standard cultures, *ver-1* amplicons detected 13 isolates and 2 standard cultures (Table 4).

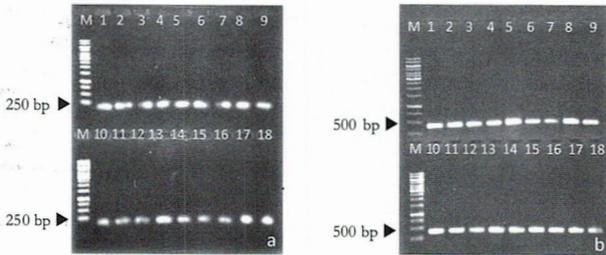


Figure 2. Agarose gel electrophoresis of PCR products from 18 DNA isolates using species specific primers of FVAVIQ1/FLAQ2 (a) and AFLA-F/AFLA-R (b) (M: marker 1 kb; lane 1-18: samples)

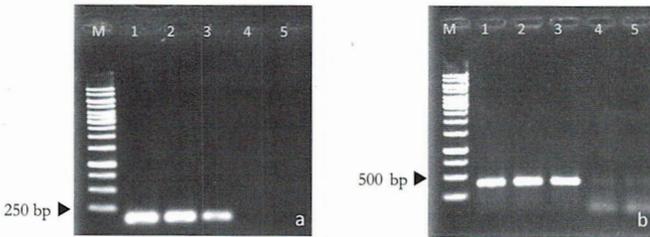


Figure 3. Agarose gel electrophoresis of PCR products from 5 DNA isolates using species specific primers of FVAVIQ1/FLAQ2 (a) and AFLA-F/AFLA-R (b) (M: marker 1 kb; lane 1-2 : standard cultures of *A. flavus*, 3: *A. flavus* from samples; 4-5: standard cultures of *A. parasiticus* and *A. nomius*)

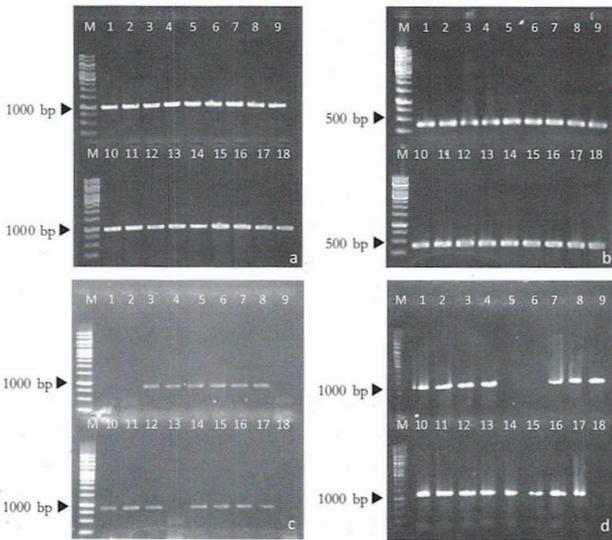


Figure 4. Agarose gel electrophoresis of PCR products from 18 DNA isolates using specific primers. for *apa-2* (a) *nor-1* (b) *ver-1* (c) *omt-1* (d) (M: marker 1 kb; lane 1-18: samples)

In previous study, *A. flavus* isolated from peanut from north Vietnam showed that the *ver-1* gene was the most representative (82%) gene followed by *nor-1*, *omt-1* and *apa-2* genes (73, 70 and 67%, respectively) (Pham & Dam 2010), while in India, *afIR* (*apa-2*) and *omt* genes appeared on 80% of peanut samples (Somashekar *et al.* 2004). Our study indicated that all 18 isolates carried aflatoxin producer genes. The information from this study could be used as early detection to determine which strain of *A. flavus* that has potential as aflatoxin producer. Further study is needed to analyze the aflatoxin expression genes of *A. flavus*. The gene expression depends on several conditions such as transcriptional regulatory factors, physiological response and environmental factors (pH, water activity (a_w) and temperature) (Schmidt *et al.* 2009; Abdel-Hadi *et al.* 2010).

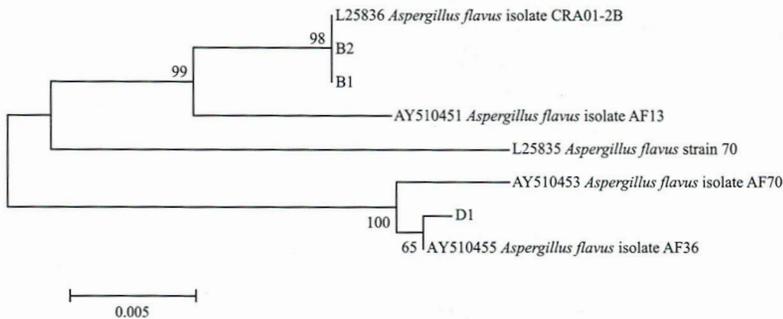


Figure 5. The phylogenetic tree of *A. flavus* B1, *A. flavus* B2 and *A. flavus* D1 based on *omt-1* sequence by Neighbor-Joining method model Kimura 2-parameter with 1000 bootstrap replications. Bootstrap values (<50%) are not shown

Table 4. The presence of the target genes involved in aflatoxin biosynthesis of *Aspergillus flavus* species

No	Code of <i>A. flavus</i>	Ttarget gene				No	Code of <i>A. flavus</i>	Target genes			
		<i>apa-2</i>	<i>nor-1</i>	<i>ver-1</i>	<i>omt-1</i>			<i>apa-2</i>	<i>nor-1</i>	<i>ver-1</i>	<i>omt-1</i>
1	J1	+	+	-	+	11	B5	+	+	+	+
2	J2	+	+	-	+	12	B6	+	+	+	+
3	D1	+	+	+	+	13	B7	+	+	-	+
4	D2	+	+	+	+	14	B8	+	+	+	+
5	D3	+	+	+	-	15	B9	+	+	+	+
6	D4	+	+	+	-	16	B10	+	+	+	+
7	B1	+	+	+	+	17	B11	+	+	+	+
8	B2	+	+	+	+	18	B12	+	+	-	-
9	B3	+	+	-	+	19	747*	+	+	+	+
10	B4	+	+	+	+	20	33021*	+	+	+	+

(+) : detected; (-) : not detected; (*) : standard cultures of positive *A. flavus*

Further analysis using sequences analysis for *omt-1* genes which taking part at the end of aflatoxin biosynthesis pathway revealed that isolates *A. flavus* B1 and *A. flavus* B2 were closely related to *A. flavus* isolate CRA01-2B, while isolate *A. flavus* D1 was closely related to *A. flavus* isolate AF36 (Fig. 5). *A. flavus* isolate CRA01-2B has been used to study biosynthesis aflatoxin pathway while *A. flavus* isolate AF36 is non-carcinogenic and has been used as biopesticides for controlling aflatoxin contamination in cotton seed (Yu *et al.* 1995b, Ehrlich & Cotty 2004). The *omt-1* gene (*omtA*) was one of many genes involved in the aflatoxin biosynthetic pathway.

CONCLUSIONS

Eighteen isolates of *A. flavus* isolates were isolated from peanut kernels samples and identified based on morphological and molecular analyses. The *apa-2* and *nor-1* were detected in 18 isolates, *omt-1* in 15 isolates (83.3%) while *ver-1* gene in 13 isolates (72.2%). Only 11 isolates had all the 4 genes involved in biosynthesis aflatoxin pathway.

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