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

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ABSTRACT

Aspergillus flavus is one of the main fungi that are able to produce aflatoxin. The presence of the fungi and its aflatoxin become serious problem on food safety. This research was aimed to isolate and identify *A. flavus* from peanut and its processed products collected from some traditional markets in Bogor, Depok and Jakarta, and detection of their aflatoxin genes. Fungal isolation was using AFPA media. Fungal identification was carried out by combining morphological and molecular analysis using species specific primers FVAVIQ1/FLAQ2 and AFLA-F/AFLA-R, while detection of aflatoxin genes employed four specific primers of *apa-2* (*aflR*), *nor-1* (*aflD*), *ver-1* (*aflM*) and *omt-1* (*aflP*). 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 10^{-4} cfu/g. The total of 18 isolates were identified as *A. flavus* based on species specific primers FVAVIQ1/FLAQ2 and AFLA-F/AFLA-R by producing amplicons about 100 and 413 bp, respectively. Based on aflatoxin gene analysis it was showed that all 18 isolates was successfully amplified by both *apa-2* and *nor-1*, 83.3 % by *omt-1* and 72.2 % by *ver-1* genes which taking part 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.

Key words: 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 be infected by the fungi, particularly during inadequate drying and improper storage which result in physical damage, discoloration, lower quality of nutritional content and mycotoxin contamination of the products (Sauer 1992).

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Mycotoxin causes a serious problem to human and animal health. Aflatoxin is the most toxic compound in mycotoxin group, it is carcinogenic and teratogenic (JECFA 1997). There are four natural forms of aflatoxin, namely B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2). AFB_1 was the most hazardous one because it was very carcinogenic among other forms of aflatoxin and usually found at the highest concentration in contaminated food including peanut and processed peanut products (Pitt 2000). Aflatoxin was mostly produced by *Aspergillus flavus* and *A. parasiticus,* the two species belong to the *Aspergillus* section Flavi. *A. parasiticus* produces both AFB and AFG, while *A. flavus* produces only AFB_1 and AFB_2, but not all strains of *A. flavus* are able to produce aflatoxin (Pitt & Hocking 2009).

The aflatoxin quantification was mostly done by instrument for chemical analysis of the toxin such as TLC and HPLC but the studies for level of fungal infection and the identification of species 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 of the fungi. The AFPA medium, however, could not differentiate *A. flavus* from *A. parasiticus*. Therefore, further differentiation between them need to use 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 that of conventional method. There are several species specific primers available to identify *A. flavus* such as two primer pairs 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 Genebank. Their 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 (= *aflR*) 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-omethyltransferase and is required for conversion of demethylsterigmatocystin and dehydrodemethylsterigmatocystin to sterigmatocystin and dihydrosterigmatocystin,

respectively (Yu *et al.* 1995a). All of 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).

A. 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 fungi 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 Regency contained more than 15 ppb of aflatoxin. The highest percentage of peanut kernels infected by *A. flavus* was found at the retails level in the traditional markets (Dharmaputra *et al.* 2005; 2007).

Early detection of aflatoxin producer fungi needs to be done in order to improve quality of the commodities and to make strategy on prevention and control of aflatoxin contamination in the products. The aim of this study was to detect growth of aflatoxin producing fungi *A. flavus* by isolation and identification of the fungi and detection of fungal ability to produce aflatoxin from peanut kernel and its processed products. Fungal isolation was conducted using AFPA specific medium, identification was done by combining method of morphology and molecular, 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

Total of 36 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 the dilution plating method on AFPA medium (NMKL 2004). Briefly, the method was as follows: twenty five grams of each samples was grounded separately using blender in medium speed then suspended in solution containing 0,1% peptone and 0,025% Tween 20 (1:10, w/v). The were samples then homogenized by stomacher for 2 min and treated into several serial dilutions down to 1:1000. A quantity of 100 µl of each dilution was spread onto duplicate AFPA plates (Oxoid) using sterile glass rod, incubated at 30 ±1ºC for 48±3 h. Specific colonies with bright yellow orange on back side of 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*

Isolated *A. flavus* group wes identified by combining morphological and molecular method. The morphological identification followed the method of Pitt & Hocking (2009) by using colony, phialid, vesicle and spore characteristics. Molecular analysis was carried out using two species specific primers of FVAVIQ1/FLAQ2 and AFLA-F/AFLA-R developed by Sardinas *et al.* (2011) and Hue *et al.* (2013). For comparison on PCR analysis, five strains were also used as standard cultures for

positive and negative control. The strains were *A. flavus* 747 obtained from SEAMEO BIOTROP (Bogor, Indonesia), *A. flavus* NBRC 33021 and *A. flavus* NBRC 30107. Two other non *A. flavus, A. parasiticus* NBRC 33224 and *A. nomius* NBRC 33223 were also included in the analysis.

Fungal DNA was extracted from mycelium using SDS lysis buffer according to the method of Raeder and Broda (1985) with some modification 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 mortar and liquid nitrogen. The SDS lysis buffer (200 mM Tris-HCl pH 8.5; 250 mM NaCl; 25 mM EDTA; 0.5% SDS) was followed by PCI (25:24:1) and CI (24:1), then sodium acetate (NaOAc) and absolute ethanol for precipitation. The DNA resuspended **in** 50 µl TE 1x (10 mM Tris-HCl pH 8, 1 mM EDTA) and 0,2 x volume of RNAse 1 mg/ml. DNA concentration measured by Nanodrop 2000 Spectrophotometer (Thermo Scientific) and kept in -20ºC for further analysis.

PCR reactions were performed using two pairs of species specific primers for identification of *A. flavus*. The pair of primers 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 of 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 of 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 explained above, except the annealing was carried out at temperature of 68°C. The sequences and the expected size products of each primer were presented in Table 1.

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

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

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

Three isolates were selected for sequence analysis using *omt-1* primers based on sampling location and their ability to be amplified by four primers tested. The PCR products were sequenced by FirstBase services (Malaysia) using the same primer. DNA sequences of *omt-1* gene were analyzed with the BioEdit Ver.7.0.0 (Hall 1999) 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 available from any other aflatoxigenic species outside *Aspergillus* section *Flavi*.

RESULTS AND DISCUSSION

Isolation of *Aspergillus flavus* group

Based on data obtained from thirty six samples of *peanut kernels,* roasted peanuts with skin pod, flour-coated peanuts, and branded *bumbu pecel* indicated that fungal colony was found only on peanut kernels. Number of fungal population based on viable count of specific colony showed that level of fungal infection was varied between locations. The number of colony range was 0.01-5.52 x 10^4 cfu/g, 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 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), it would correlate with the moisture content of peanut kernels. Population of *A. flavus* in Bogor obtained in our study was 5.52 x 10^4 cfu/g, this was 10 fold higher with that of reported by Dharmaputra (2010) which showed the fungal population

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, *bumbu pecel* (dry peanut sauce)	0	0	0

collected from the same regency but different traditional markets location was 0.49 x 10^4 cfu/g. The traditional market (retailers) is the last distribution chain of the commodities before delivered to consumer. Long chain distribution before delivering to consumer had also made possibility the peanut kernels broken and were easier infected by fungi including aflatoxigenic fungi.

No colony was found in 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 fungi. Furthermore, this could also be due to the peanut kernels used as raw materials in factory that applied standardization method on production of processed peanut products that usually used the kernels directly from the farmers were sorted by the factory before their processing to the final products. Dharmaputra *et al.* (2013) reported that the populations of *A. flavus* in processed peanut products were relatively low, it was less than 1 cfu/g in fresh weight basis. The methods of postharvest handling from farmers up to retailers in market and the duration of storage were related to *A. flavus* infection and aflatoxin production particularly in peanut kernels (Dharmaputra *et al.* 2005).

Fungal identification

Eighteen out of 28 total fungal strains isolated from peanut kernels showed characteristic 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 vary from spherical to elliptical, septate hyphae, and conidial heads uniseriate (phialides only) (Fig. 1b, c, d). The size of microscopic morphological structures of each isolates was shown in Table 3. To differentiate *A. flavus* from those of *A. parasiticus* based on morphological characteristics was not ease since it could give false positive result. Rodrigues *et al.* (2007) reported that the use of Scanning Electron Microscopy (SEM) for the conidial wall ornament examination was the primary character analysis for separation of the two species in morphological identification.

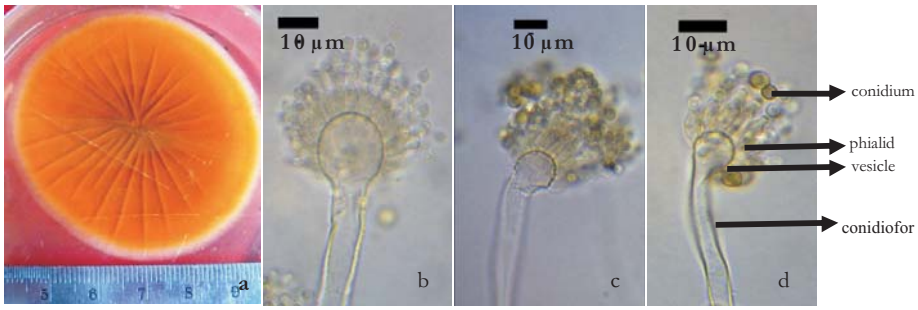

Figure 1. Macroscopic (a) and microscopic (b, c, d) of *A. flavus* group isolated from peanut kernels in Bogor, Depok and Jakarta

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*

Further identification using molecular analysis by 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 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 repoted 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 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).

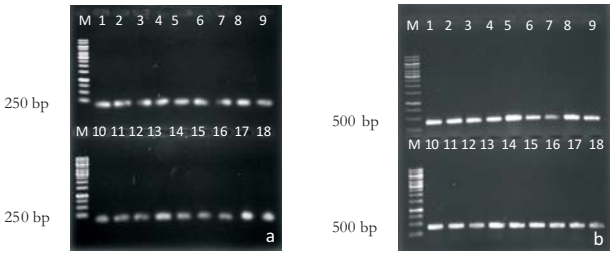

Figure 2. Agarose gel electrophoresis of PCR products from 18 DNA isolates using species specific primers of FVAVIQ1/FLAQ2 (a) and AFLA-F/AFLA-R (b) (M: marker 1 kb; lane 1-18: samples)

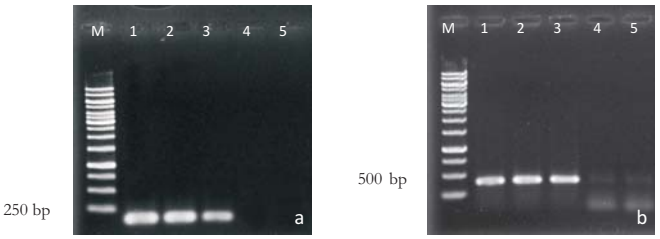

Figure 3. Agarose gel electrophoresis of PCR products from 5 DNA isolates using species specific primers of FVAVIQ1/FLAQ2 (a) and AFLA-F/AFLA-R (b) (M: marker 1 kb; lane 1-2 : standard cultures of *A. flavus*, 3: *A. flavus* from samples; 4-5: standard cultures of *A. parasiticus* and *A. nomius*)

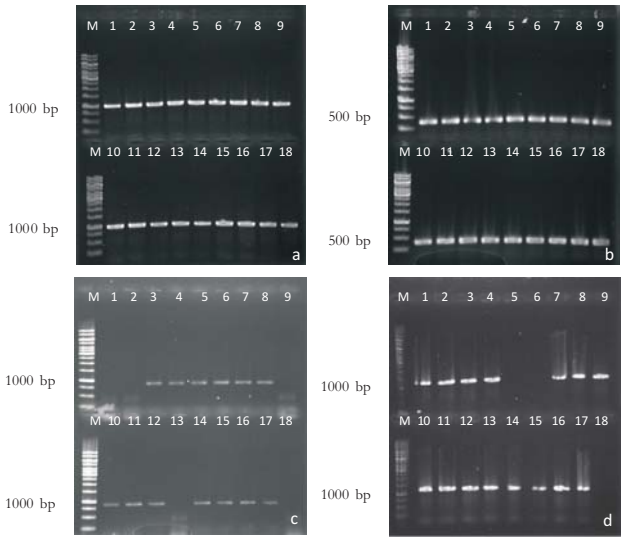

Figure 3. Agarose gel electrophoresis of PCR products from 18 DNA isolates using specific primers. for *apa-2* (a) *nor-1* (b) *ver-1* (c) *omt-1* (d) (M: marker 1 kb; lane 1-18: samples)

Detection of aflatoxin genes

The population of *A. flavus* is not always correlated with aflatoxin production, it depends on the strain whether or not the fungi carried out 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 involved in the aflatoxin biosynthetic pathway (Fig. 4). The two primers *apa-2 and nor-1* was successfully amplified all 18 isolates and standard cultures of *A. flavus*, whereas, *omt-1* genes detected 15 isolates and 2 standard cultures, *ver-1 genes detected 13 isolates* and 2 standard cultures (Table 4).

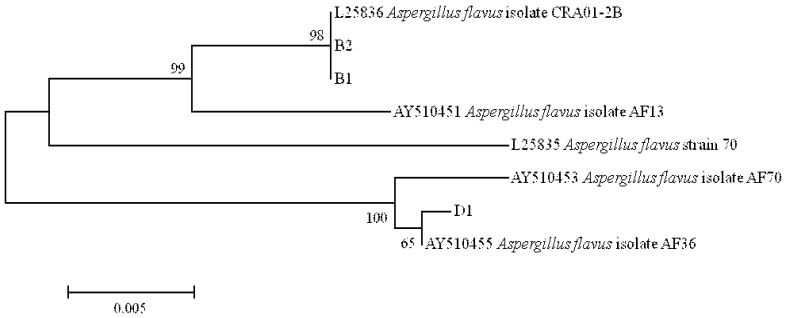

Figure 4. The phylogenetic tree of *A.flavus* B1, *A. flavus* B2 and *A. flavus* D1 based on *omt-1* sequence by Neighbor Joining method model Kimura 2-parameter with 1000 bootstrap replications. Bootstrap values (<50%) are not shown

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

No	Code of *A. flavus*	Ttarget gene				No	Code of *A. flavus*	Target genes			
		apa-2	*nor-1*	*ver-1*	*omt-1*			*apa-2*	*nor-1*	*ver-1*	*omt-1*
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*

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

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 more closely related to *A. flavus* isolate CRA01-2B, while isolate, *A. flavus* D1 was more closely related to *A. flavus* isolate AF36 (Fig. 5). *A. flavus* isolate CRA01-2B is isolate that had been used for study on biosynthesis aflatoxin pathway while *A. flavus* isolate AF 36 is non-carcinogenic and had been used as biopesticides for controlling aflatoxin contamination in cotton seed (Yu *et al*. 1995b, Ehrlich & Cotty 2004). The *omt-1* gene (= *omt* A) was one of many genes involved in the aflatoxin biosynthetic pathway.

DISCUSSION

A. flavus was only found at peanut kernels samples and 18 isolates were obtained in this study identified by morphological and moleculer analysis. The *apa-2* and *nor-1* detected in 18 isolates, the *omt-1* detected in 15 isolates (83.3%) while *ver-1* gene detected in 13 isolates (72.2%). Only 11 isolates had all 4 genes involved in biosynthesis aflatoxin pathway.

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