MOLECULAR CHARACTERIZATION OF *Aspergillus flavus* TOXIGENICITY IN AGRICULTURAL COMMODITIES IN INDONESIA

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ABSTRACT

Toxigenic *Aspergillus flavus* is a primary producer of aflatoxin in Indonesia, and its presence can lead to the contamination of agricultural commodities. This contamination poses a risk to export-targeted commodities, potentially resulting in their rejection. Therefore, this study aims to characterize the molecular profile of native *A. flavus* isolated from several Indonesian agricultural products, with a major focus on its toxigenicity and toxin production. A total of 18 *A. flavus* collections were isolated from nutmeg, ground peanut, cacao, coffee bean, corn, white pepper, and soil peanut plantation. Species identification was carried out using molecular and morphological approaches. The toxigenicity of isolates was characterized based on the amplification of aflatoxin gene clusters, while toxin production was assessed through growth simulation on a 10% coconut broth media followed by HPLC quantification. The result showed that all isolates were confirmed as *A. flavus* based on the morphological and sequence analysis of the ITS region. A total of 11 isolates (61%) were confirmed as toxigenic and produced 1-2 types of aflatoxin, in varying concentrations of high, moderate, or low levels of AFB1. High levels of AFB1 produced by seven isolates namely BIO3313, BIO33212, BIO3361, BIO33404, BIO3338, BIO3352, and BIO3344, had concentration levels ranging from 76.78 to 2241.06 μg/kg, while three isolates (BIO3314, BIO3312, and BIO3381) produced AFB1 below 1 μg/kg. Twenty-nine pairs of aflatoxin gene-specific sequences were successfully amplified as a single band, while some produced non-specific patterns in several low toxigenic and non-toxigenic isolates. Based on the results, it was concluded that completed gene clusters and variations of gene deletion were observed in both toxigenic and non-toxigenic isolates. However, no specific target gene could effectively distinguish the two groups. Two non-toxigenic isolates namely BIO3393 and BIO33403 exhibited a large deletion and could be potential candidates for biocontrol agents.

Keywords: Aflatoxin, *Aspergillus flavus*, ITS, PCR, Toxigenic

INTRODUCTION

Aflatoxin contamination in agricultural products is a food safety concern in Indonesia and worldwide. This carcinogenic mycotoxin is produced mainly by toxigenic *Aspergillus flavus* and *A. paraciticus* during various stages of agricultural production including pre-harvest, harvest, post-harvest, transportation, and storage. The prevalence of aflatoxin is particularly high in tropical and subtropical regions due to optimal humidity and temperature conditions for toxin production (Bhat *et al.* 2010).

In Indonesia, aflatoxin contamination has been reported in agricultural products such as nutmeg, corn, peanuts, pepper, and cocoa (Dharmaputra 2002). This contamination poses a significant challenge for export-targeted commodities, leading to economic losses for farmers and exporters. For instance, upon arrival in the importing country, nutmeg was found to have a high level of aflatoxin due to the growth of toxigenic *A. flavus* (Anidah *et al.* 2020). The growth was concluded to have

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occurred during transportation based on the low levels of the toxin detected before shipping.

The activities of regulatory proteins and enzymes encoded by more than 25 genes cluster play an essential role in aflatoxin production (Yu *et al.* 2002). Non-toxigenic *A. flavus* was reportedly associated with the deletions of a part or the entire aflatoxin genes cluster (Chang *et al.* 2007). However, the molecular mechanisms underlying the loss of the aflatoxin production ability in *A. flavus* are currently not well understood (Rao *et al.* 2020; Schmidt-Heydt *et al.* 2008). PCR detection for *aflD* (nor1), *aflP* (omtA), *aflM* (ver1), and *aflR* genes have been applied as a marker to differentiate toxigenic and non-toxigenic isolates in different studies. These include isolated native to Indonesia (Anidah *et al.* 2020), animal feed in Iran (Davari *et al.* 2014), *Aspergillus* species in Korea (Kim *et al.* 2011), maize in Italy (Degola *et al.* 2006), and an herbal product in Italy (Criseo *et al.* 2001). The results showed various deletion patterns for non-toxigenic isolates which could be distinguished from toxigenic ones having 4 complete genes or no deletion. However, it was observed that the toxigenic isolate exhibited variations in deletion patterns when amplified using a complete set of aflatoxin biosynthetic genes, rendering the use of 4 genes as a marker irrelevant.

The characterization of deletion patterns within all the aflatoxin biosynthetic gene clusters has been elucidated in non-toxigenic isolates to find and assess the biocontrol agent candidate (Rao *et al.* 2020; Wei *et al.* 2014; Donner *et al.* 2010; Yin *et al.* 2009; Chang *et al.* 2005). However, the genetic variability assessment in toxigenic isolates has not been widely carried out, despite its significance in distinguishing between toxigenic and non-toxigenic isolates. A molecular approach that characterizes the presence or absence of aflatoxin biosynthetic genes can indicate the functional status of the biosynthesis pathway gene (Tran-Dinh *et al.* 2014). This approach could serve as an effective marker for the identification of toxigenic isolates in food commodities. Therefore, this study aims to characterize the molecular profiles of toxigenic and no-toxigenic *A. flavus* isolates from agricultural commodities in Indonesia by using an aflatoxin biosynthetic genes cluster.

MATERIALS AND METHODS

A. flavus Isolates

The *A. flavus* isolates used in this study (Table 1) were from the Phytopathology Laboratory of SEAMEO BIOTROP. These isolates were sourced from various regions in Indonesia and included samples from nutmeg, ground peanut, cacao, coffee bean, corn, white pepper, and soil peanut plantation. All collected samples were grown in the Potato Dextrose Agar/PDA (HiMedia, India) with parafilm oil as a preservative, and were reinoculation in PDA at 25°C for seven days before use.

Table 1 *A. flavus* isolates from agricultural products

Isolates	Commodities (Origin)
BIO3376	Nutmeg (Manado – North Sulawesi)
BIO33211	
BIO33212	
BIO33403	
BIO33404	
BIO3313	Ground peanut (Bogor – West Java)
BIO3381	
BIO3334	Ground peanut (Wonogiri – Central Java)
BIO3338	
BIO3342	
BIO3344	
BIO3312	Cacao (South Sulawesi)
BIO3314	Coffee bean (Jember – East Java)
BIO3393	Coffee bean (Toraja – South Sulawesi)
BIO3382	Corn (Bogor – West Java)
BIO3383	White pepper (Bogor – West Java)
BIO3361	Soil (Wonogiri – Central Java)
BIO3352	

Morphological Characterization

The morphological characterization of the isolates was carried out according to Samson *et al.* (2004) and Pitt & Hocking (2009). Czapek Yeast Autolysate (CYA) agar was used for macromorphological observation. Each isolate was inoculated at two points, and incubated at temperatures of 25 and 37°C in the dark for seven days. The colony color was observed, and the diameter was measured. Meanwhile, the micromorphological observation was conducted by preparing microscopic mounts in lactic acid with cotton blue from CYA colonies. The excess conidia and air bubbles were removed by adding a drop of 70% ethanol, then conidia morphology and presence, size of sclerotia, and head seriation were analyzed.

DNA Extraction

The mycelia from a 3-day-old culture in 50 mL Potato Dextrose Broth/PDB (HiMedia, India) were harvested and rinsed using sterile distilled water and then dried using filter paper. The sample was crushed using a mortar and pestle with continuous addition of liquid nitrogen until fine mycelia powders were obtained. DNA was extracted using DNEasy Plant Kit (Qiagen, Germany) according to the manufacturer's instructions and the quality was assessed through electrophoresis in 1% agarose gel (Invitrogen, USA) with Sybrsafe dye (Invitrogen, USA) in Tris-acetate-EDTA (TAE) buffer. The absorbance was measured at 260 nm wavelength for quantification, and DNA was stored at -20 °C until further use.

Species Identification

Molecular species identification was carried out using ITS 1 (TCCGTAGGTGAACCTG CGG) and ITS 4 (TCCTCCGCTTATTGATA TGC), as forward and reverse primers against all isolates and sequencing of the ITS region (White *et al.* 1990). The amplification reaction was carried out by mixing 100 ng of DNA template, 0.2 M of each primer, and 1X GoTaq Hot Start PCR master mix (Promega, USA), in a total volume of 40 µL. The amplification program was performed in the GenAmp PCR System 9700 (ABI, USA) thermal cycler with specific conditions including pre-denaturation for 5 minutes at 95°C, followed by 35 cycles of denaturation at 94°C, annealing at 5°C, and extension at 72°C for 30 seconds each. An additional final extension at 72°C for 7 minutes was also included. The amplified product was visualized by 1.2% agarose gel electrophoresis, followed by sequence analysis in 1st Base (Singapore). The sequences were analyzed for similarity using BLAST (http://blast.ncbi.nlm.nih.gov/blat.cgi) and submitted to the NCBI database. The neighbor-joining method with 10000 bootstrap replicates in MEGA X software was used for tree construction (Kumar *et al.* 2018).

Differentiation of Toxigenic and Non-Toxigenic Isolates

Toxigenic and non-toxigenic isolates were determined by growth simulation on an aflatoxin-inducing media (Davis *et al.* 1987). Total aflatoxin was extracted from a ten-day inoculated culture in 10% (v/v) Coconut Broth Media/CBM, according to the AOAC method 991.31 (AOAC, 2000). For extraction, a 25 mL filtrate of the culture was mixed with 5 g NaCl and 125 mL of methanol: water (70:30; v:v). The mixture was filtered through a glass microfiber filter after 2 times dilution with purified water. Aflatoxins B1, B2, G1, and G2 from the extract were selectively isolated using the AflaTest affinity column (VICAM, USA). Furthermore, the content of the extract was determined using HPLC with post-column derivatization (VICAM 2007). All the extraction chemicals and solvents used were from Merck, Germany, while the aflatoxin standard was from SIGMA, USA.

PCR Amplification using Aflatoxin Biosynthesis Genes

PCR amplification using Aflatoxin Biosynthesis Genes was carried out according to Chang *et al.* (2005). The isolated DNA was amplified by PCR to detect the presence or absence of aflatoxin biosynthesis gene fragments using 29 pairs of primers as shown in Table 2.

Table 2 List of aflatoxin biosynthesis primer sequences

Primer Pair	Sequence
aflU (norB-cypA)	F: GTGCCCAGCATCTTGGTCCA R: AGGACTTGATGATTCCTCGTC
aflT	F: ATGACATGCTAATCGACGAG R: AGGCGCATGCTACGGATC
aflC (pksA)	F: ACTTTGAGGGCGTTCTGTGC R: CTTTCGGTGGTCGGTGATTC
aflD (nor1)	F: AGCACGATCAAGAGAGGCTC R: GATCTCAACTCCCCTGGTAG
aflA (fasA/hexA)	F: TCCTATCCAGTCCACCTCGTA R: CACATCTTTGTCTTGCCCGC
aflB (fasB/hexB)	F: ACAATCGAATGACAACACTGC R: CCACCGAATCCACTACCTACA
aflR	F: ATGGTCGTCCTTATCGTTCTC R: CCATGACAAAGACGGATCC
aflS	F: CTTCAACAACGACCCAAGGTT R: AGATGAGATACACTGCCGCA
aflH (adhA)	F: CCTCGTGGGAGAGCCAAATC R: GGAGCAAGAAGGTTACAGCG
aflJ (estA)	F: CGATGGGACTGACGGTGATT R; ACCACGCCGCTGACTTTAT
aflE (norA)	F: GTGTTCGTGTGTCGCCCTTA R: GTCGGTGCTTCTCATCCTGA
aflM (ver1)	F: CATCGGTGCTGCCATCGC R: CCTCGTCTACCTGCTCATCG
aflN (verA)	F: CCGCAACACCACAAGTAGCA R: AAACGCTCTCCAGGCACCTT
aflG (avnA)	F: GCGATAGAACTGACAAAGGCA R: GAATGAGTCTCCAAAGGCGAG
aflL (verB)	F: TTCAGTGACAAAGGTCTTCGC R: GGCAGCGTT ATTGAGCATCT

aflI (avfA)	F: ATTCAAATCCTCGTTCGGTCG
	R: TAGCCCGTTGGTTGTGTTCC
aflO (omtB)	F: ACAGACGATGTGGGCAAACG
	R: ACGCAGTCCTTGTTAGAGGTG
aflP (omtA)	F: CAGGATATCATTGTGGACGG
	R: CTCCTCTACCAGTGGCTTCG
aflQ (ordA)	F: AAGGCAGCGGAATACAAGCG
	R: ACAAGGGCGTCAATAAAGGGT
aflK (vbs)	F: AACGAGCAGCGTAAGGGTCT
	R: TCAGCCAGAGCATACACAGTG
aflV (cypX)	F: GGAGCCTACCATTCGCAACA
	R: GGCTTTGACGAACAGATTCCG
aflW (moxY)	F: TGCTACTGGAACGAAGACCG
	R: CGACGACAACCAAACGCAA
aflX (ordB)	F: GCTGCTACTGGAATGAAGACC
	R: ATGCGACGACAACCAAACG
aflY (hypA)	F: CGCAAGACGGCAGAGATACT
	R: GCTCCTTCAGTTCCACACCA
nadA	F: TGACGAGGCCTGCGAGCTGT
	R: AAGCCTCTTCAGAACGGTCA
hexA	F: TGTCCTCACCTCTGGCGTAT
	R: AGACCAACCACTCTTATGGGC
glcA	F: AGACACAGTCATCGCCTGTT
	R: GGTGCGAATAGGTGCAGGTA
sugR	F: TCAGCTGAAGCGCTCGAGAG
	R: GTATTGCCGCACTATGTATG
C4	F: ATCGTGCAGACAGGAACAC
	R: GGTGCCTTGGCCTATGCGCT

A total of 20 µL of the reaction mixture was prepared for each isolate by mixing 50 ng DNA, 0.2 µM each primer pair, 1X GoTaq Hot Start PCR master mix (Promega, USA), and molecular grade water. PCR amplification was performed in a thermal cycler GeneAmp PCR System 9700 (ABI, USA) with specific conditions of pre-denaturation for 5 minutes at 94°C, followed by 30 cycles of denaturation at 94°C, annealing at 55°C, and extension at 72°C for 1 minute respectively. An additional final extension was carried out at 72°C for 6 minutes. The PCR products were visualized by electrophoresis on a 1.5% agarose gel in TAE buffer, with Sybrsafe dye (Invitrogen, USA). The presence or absence of amplicon was observed, and the results were scored for the presence (1) or absence (0) of aflatoxin genes. To construct the phylogenetic tree, cluster analysis was performed using the unweighted pair-group method with arithmetic mean (UPGMA) functionality in NTSYSpc 2.1 software (Department of Ecology and Evolution, State University of New York, NY, USA).

RESULTS AND DISCUSSION

Morphological and Molecular Identification of *A. flavus*

All isolates were identified as *A. flavus* based on macro and micromorphology observations, following the guidelines of Pitt & Hocking (2009). The colony diameter range was 3.8 - 5.6 cm and 4.2 - 6.4 cm when grown on CYA agar at 25 and 37°C, respectively. The colony colors were greyish-green, yellow-green, and olive-yellow, while the reverse side was uncolored to reddish-brown. Conidia were observed as smooth to finely rough with biseriate conidial heads, globose to subglobose, and 3 - 5 µm in diameter. Moreover, the stipes were hyaline, smooth, variable in length, mostly 350-600 µm, and the diameter just below vesicles was 3 - 8 µm. The vesicles were globose to sub-globose, and 10-30 µm in diameter. Isolates grew well at 25 and 37°C and no sclerotia were observed as shown in Figure 1.
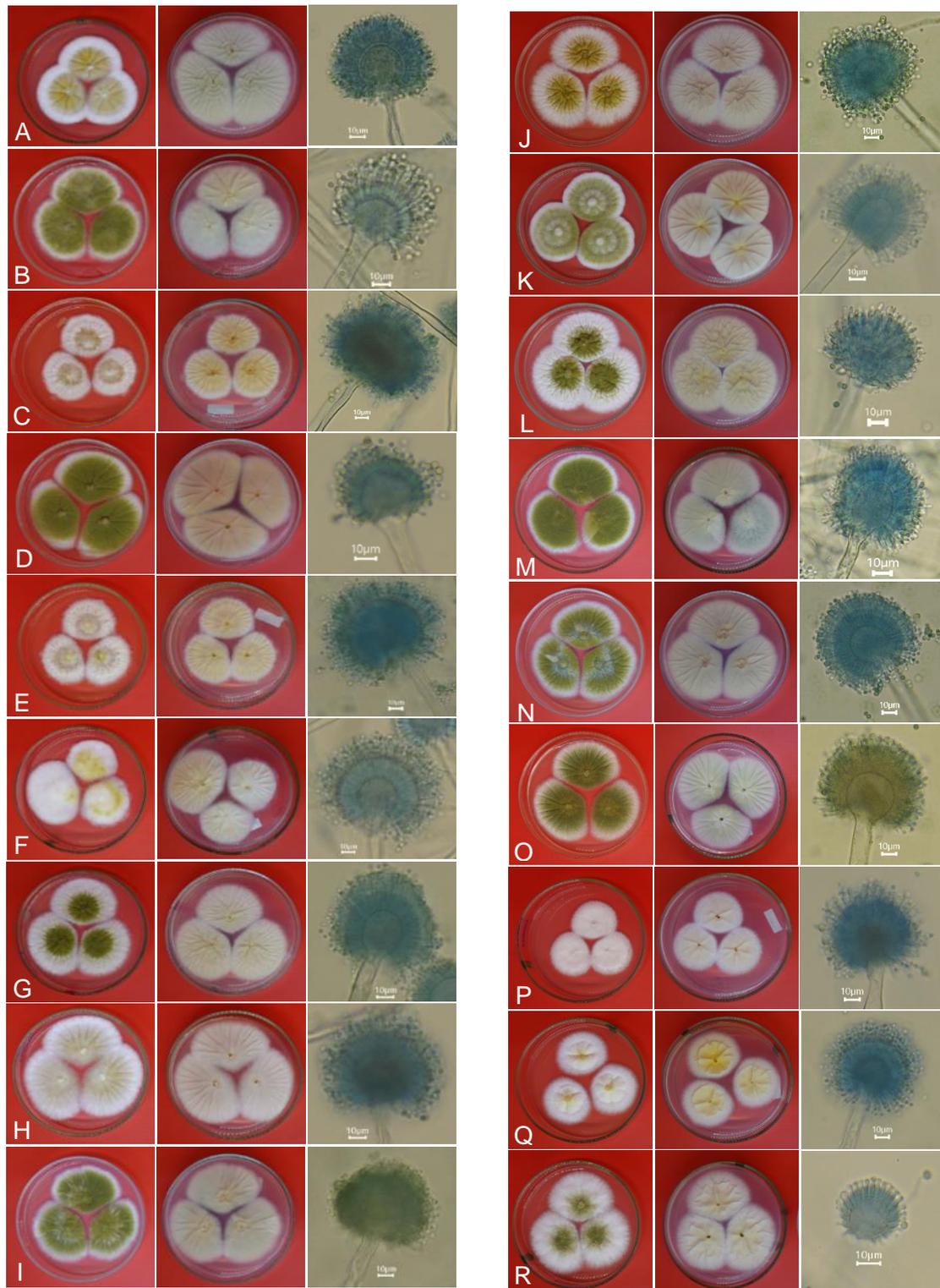
Figure 1 Colonies of the 18 *A. flavus* on CYA (7 days at 25 ºC)

Notes: A = BIO3313; B = BIO33212; C = BIO3361; D = BIO33404; E = BIO3338; F = BIO3352; G = BIO3344; H = BIO3334; I = BIO3314; J = BIO3312; K = BIO3381; L = BIO3382; M = BIO3383; N = BIO3376; O = BIO33211; P = BIO3342; Q = BIO33403; R = BIO3393.

Species identification at the molecular level was conducted by amplifying the ITS region as the official locus for fungal DNA barcoding. Amplification using ITS 1 and ITS 4 yielded a single amplicon of 600 bp in all isolates. The amplified products were sequenced and analyzed for similarity using BLAST (http://blast.ncbi.nln.nih.gov/Blast.cgi), and all the generated sequences were submitted to the NCBI database (Table 3). Afterward, a phylogenetic tree was constructed using MEGA 6, which included sequences from Genbank for *A. flavus* (NR111041.1), *A. paraciticus* (NR151784.1), *A. niger* (NR111348.1), and *A. fumigatus* (NR121481.1). All isolates were found to have 100% homology to *A. flavus* (Figure 2). The neighbor-joining method with 10000 bootstrap replicates in MEGA X software was used for tree construction. Bootstrapping was translated as the accuracy value of the phylogenetic tree against randomization of 10000 repetitions (Tamura *et al.* 2007). The morphological and molecular analysis, both confirmed all isolates identified as *A. Flavus* (Figures 1 and 2).

Toxigenic and Non-Toxigenic Isolate Differentiation

Toxigenic *A. flavus* was induced to produce aflatoxins in the CBM containing 10% coconut milk due to the presence of fat and fatty acids (Lin & Dianese 1976). During incubation, *A. flavus* mycelia grew on the media's surface, releasing the toxin in the solution. The aflatoxin content produced was extracted from the CBM filtrate, followed by identification and quantification by HPLC compared to respective standards as AFB1, AFB2, AFG1, and AFG2.

A total of 11 isolates from *A. flavus* (61%) produced aflatoxin with varying concentrations, while seven (39%) did not, as shown in Table 3. The toxigenic isolates yielded two forms of aflatoxins with a combination of AFB1-AFB2 (three isolates), and AFB1-AFG1 (three isolates), while the remaining five only produced AFB. However, none of the toxigenic isolates produced AFG2 as shown in Table 3.
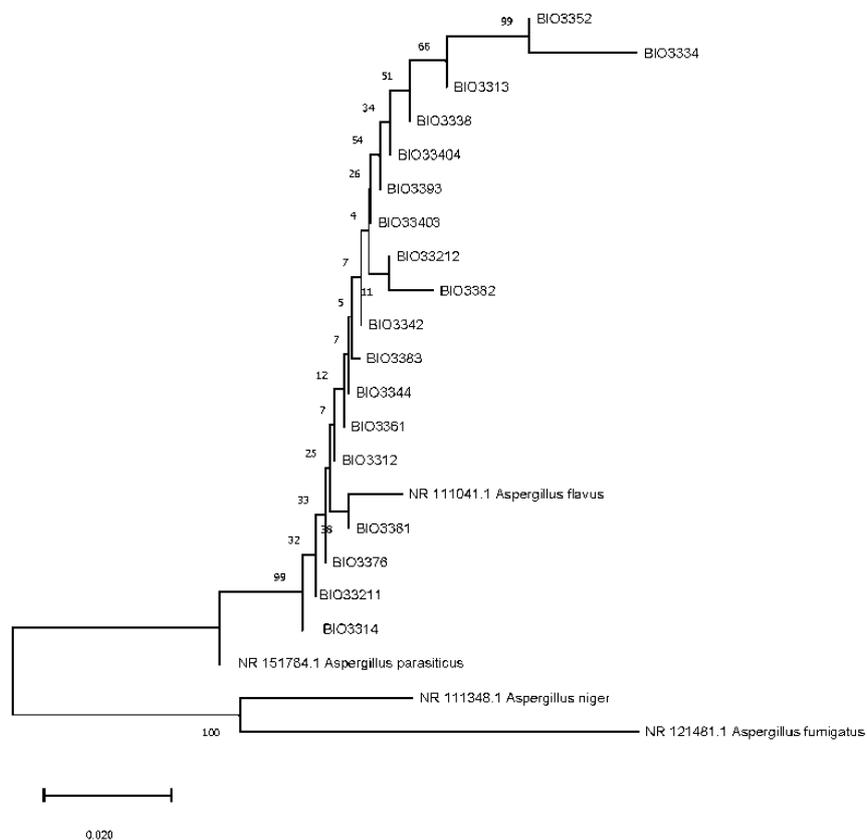
Figure 2 Phylogenetic tree of *A. flavus* isolates using *Neighbor-Joining* method by MEGA X software (10000 X *bootstrap*)

Table 3 Aflatoxin contents from 18 isolates of *A. flavus*

A. flavus Isolate	Accession Number	Aflatoxin (µg/kg)			
		B1	G1	B2	G2
BIO3313	ON619457	2241.06	nd	66.8	nd
BIO33212	ON619471	702.72	nd	nd	nd
BIO3361	ON619464	607.67	1081.15	nd	nd
BIO33404	ON619473	255.27	nd	nd	nd
BIO3338	ON619460	217.34	1486.52	nd	nd
BIO3352	ON619463	126.96	200.06	nd	nd
BIO3344	ON619462	76.78	nd	2.60	nd
BIO3334	ON619459	7.68	nd	0.14	nd
BIO3314	ON619458	0.62	nd	nd	nd
BIO3312	ON619456	0.31	nd	nd	nd
BIO3381	ON619466	0.10	nd	nd	nd
BIO3382	ON619467	nd	nd	nd	nd
BIO3383	ON619468	nd	nd	nd	nd
BIO3376	ON619465	nd	nd	nd	nd
BIO33211	ON619470	nd	nd	nd	nd
BIO3342	ON619461	nd	nd	nd	nd
BIO33403	ON619472	nd	nd	nd	nd
BIO3393	ON619469	nd	nd	nd	nd

Notes: nd = Not detected; below the LoQ (B1 = 0.0202; B2 = 0.0171; G1 = 0.0220; G2 = 0.0183) in µg/kg

All toxigenic isolates exhibited the capacity to produce AFB1 in various concentrations, ranging from low, medium, to high levels. A total of seven toxigenic isolates had a high concentration of AFB1 ranging from 76.78 to 2241.06 µg/kg, while one isolate had a moderate concentration of 7.68 µg/kg. Furthermore, three other toxigenic isolates produced AFB1 below 1 µg/kg as shown in Table 3. The presence of toxigenic isolates in food commodities poses a significant threat due to their ability to produce aflatoxins. The maximum value in food commodities permitted by regulations in Indonesia is 15 µg/kg AFB1 (BPOM 2012). Based on the results, the potential of *A. flavus* to produce aflatoxin varied significantly among both strains. In nature, the percentage of the non-toxigenic strain varies from 0 to more than 80% worldwide (Rao *et al.* 2020; Yin *et al.* 2009; Chang *et al.* 2007).

Molecular Profile of Toxigenic and Non-toxigenic *A. flavus* Isolates

Although the species identification by morphological and molecular approaches confirmed the same result, they could not distinguish between the toxigenic and non-toxigenic isolates. The toxigenicity of *A. flavus* was determined by the presence of genes encoding aflatoxin biosynthesis which was detected by PCR (Yu *et al.* 2002). The PCR-based molecular technique offers the advantage of rapid diagnosis with a high level of sensitivity and specificity, compared to the conventional method (Mamo *et al.* 2017). Based on the results, twenty-nine pairs of primers were successfully amplified in 18 *A. flavus* isolates. The majority of the isolates produced single amplicons, while some yielded non-specific amplicons as multiband patterns in several low toxigenic and non-toxigenic isolates (Figure 3). The presence of multiband amplicons indicates the formation of nonspecific product and suggested that the absence of aflatoxin production may be due to base-pair substitution mutations, resulting in the formation of non-functional gene products (Levin 2012; Criseo *et al.* 2001).

The amplification results of 3 toxigenic isolates with high to low levels of AFB1 showed no deletion of the amplicon. BIO3313 which exhibited high AFB1 production, as well as BIO3334 and BIO3381 with moderate and low AFB1 production, respectively, had the complete aflatoxin gene cluster. Similarly, the complete amplicons were also found in BIO3382 as a non-toxigenic isolate. This result was consistent with previous studies which showed a complete set of genes in the non-toxigenic *A. flavus* (Wei *et al.* 2014; Kim *et al.* 2011; Criseo *et al.* 2001). Although amplification using DNA-based PCR confirmed the presence of the target gene in the genomic DNA, it was unable to explain the level of gene expression. This suggested the need for a real-time PCR as a

more proficient method but the gene expression result strongly depends on the reference stain, culture condition, and media used to induce the mRNA expression (Rao *et al.* 2020).
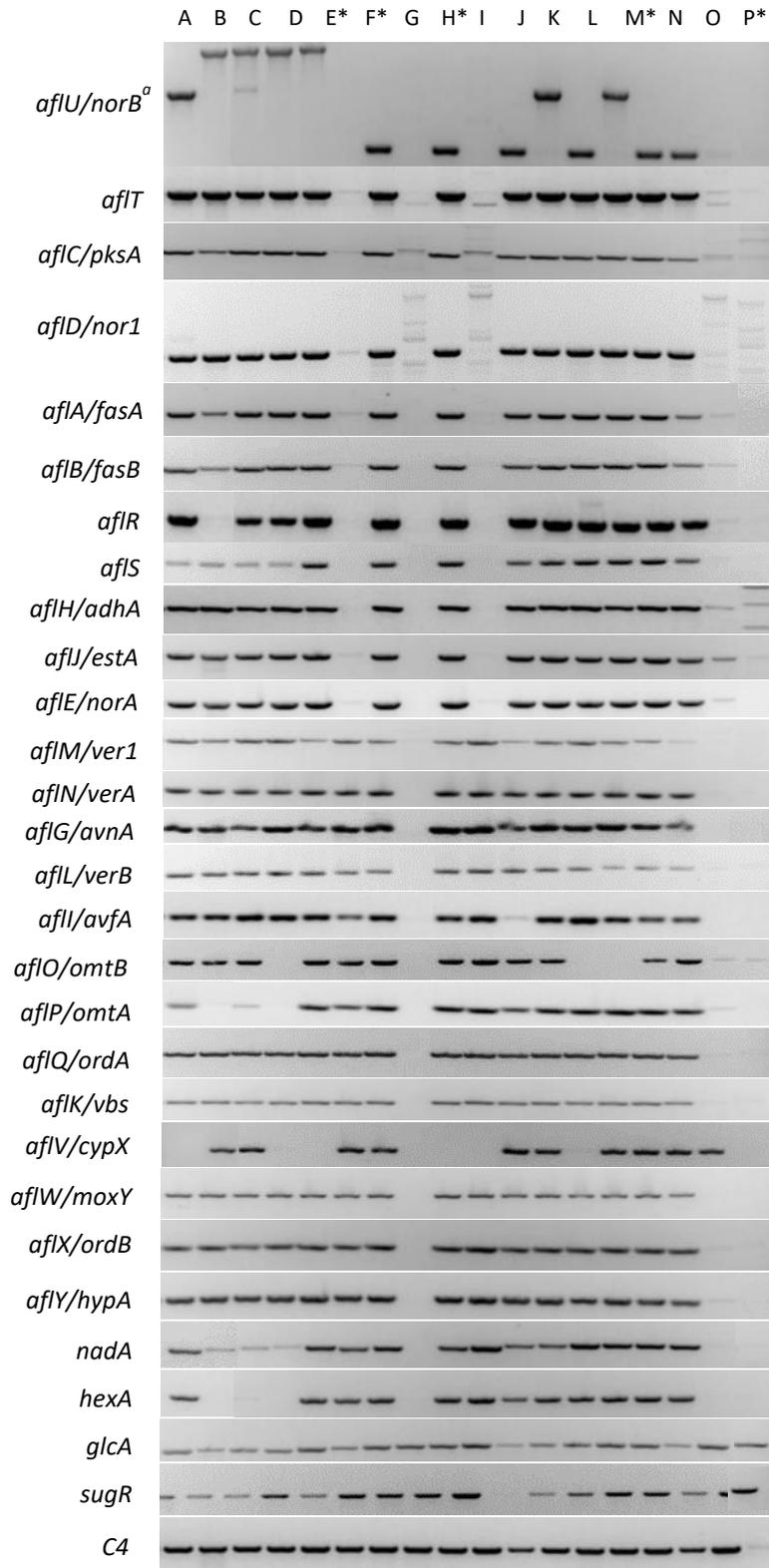
Figure 3 Profile of all amplicons of aflatoxin biosynthesis genes in toxigenic and non-toxigenic isolates

Notes: * = Non-toxigenic isolates; A = BIO3313; B = BIO3338; C = BIO3352; D = BIO3361; E = BIO3376; F = BIO33211; G = BIO33212; H = BIO33403; I = BIO33404; J = BIO3312; K = BIO3314; L = BIO3334; M = BIO3342; N = BIO3344; O = BIO3381; P = BIO3382; Q = BIO3383; R = BIO3393.

Variations in the deletion of aflatoxin biosynthetic genes were observed in both toxigenic and non-toxigenic isolates. The deletion involved one to a maximum of four genes in toxigenic isolates, except for BIO3312 which exhibited a low level of AFB1 production below 1μg/kg and experienced nine genes deletion, with some appearing as non-specific amplicons. Meanwhile, non-toxigenic isolates showed one to 26 gene deletions. Two non-toxigenic isolates, namely BIO 33403 (90% deletion) and BIO3393 (79% deletion) exhibited significant gene deletions, providing scientific data to address the safety issues in the application of biological control. According to a previous study, non-toxigenic isolates with extensive gene deletions are potential candidates for biocontrol agents (Donner *et al.* 2010). Moreover, extensive aflatoxin gene deletion is preferred to prevent adverse genetic recombination (Chang *et al.* 2005).

Molecular characterization of non-toxigenic *A. flavus* using partial or completed aflatoxin biosynthetic genes has been carried out. PCR detection using *aflD* (nor1), *aflP* (omtA), *aflM* (ver1), and *aflR* as partial target genes were used in several studies to discriminate non-toxigenic isolates (Anidah *et al.* 2020; Davari *et al.* 2014; Degola *et al.* 2006; Criseo *et al.* 2001). In this study, *aflD, aflP,* and *aflR* genes were found to be deleted in toxigenic isolates, while the *aflR* gene appeared in several non-toxigenic isolates. Similar results were observed by Rao *et al.* (2020), suggesting that these partial gene detections may not serve as reliable markers.

Genetic variations of non-toxigenic *A. flavus* isolates based on completed aflatoxin biosynthetic genes cluster have also been reported. Chang *et al.* (2005) amplified 25 genes from 38 non-toxigenic *A. flavus* isolates in the United States and found eight deletion patterns. Yin *et al.* (2009) analyzed the presence of 11 genes and found five deletion patterns from 11 non-toxigenic isolates in China. Furthermore, Donner *et al.* (2010) examined 21 non-toxigenic isolates in Nigeria by amplifying 21 genes and found nine deletion patterns. Wei *et al.* (2014) also found 25 deletion patterns by amplifying 29 genes from 76 non-toxigenic isolates in China. All the aforementioned studies focused on the variation of deletion patterns in non-toxigenic isolates with a major emphasis on their use as biocontrol agents in the field. The results indicated that the genetic variability from the aflatoxin biosynthetic gene cluster is diverse in non-toxigenic isolates. A comparison of the deletion patterns might contribute to a better understanding of specific markers for differentiating isolates based on toxigenicity.

A cluster analysis was performed to identify the similarities among the toxigenic and non-toxigenic isolates based on the presence of aflatoxin genes. The genetic similarity coefficients (GSC) ranged from approximately 0.28 to 1.00. The phylogenetic tree assembled using the UPGMA grouped the 18 isolates into two main clusters. One group had the largest number of isolates (16 isolates) including toxigenic as well as non-toxigenic isolates. Another group had 2 non-toxigenic isolates with large deletion, namely BIO3393, and BIO33403. This indicates that no specific genes could distinguish between toxigenic and non-toxigenic isolates (Figure 4).
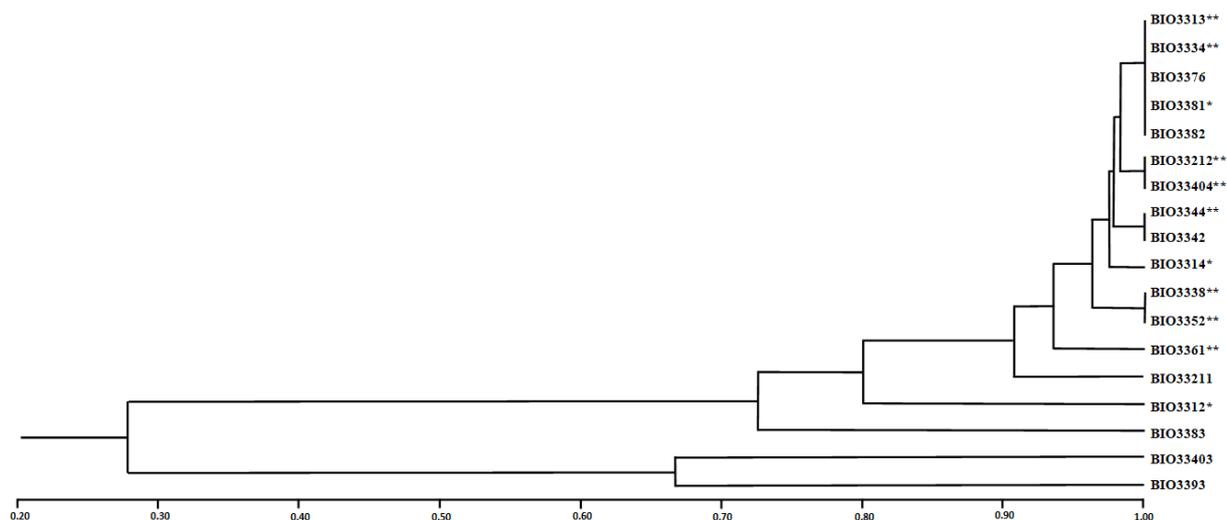
Figure 4 Dendrogram illustrating genetic relationships among 18 isolates of *A. flavus*, generated by the UPGMA cluster analysis (NTSYS) calculated from 29 primers of aflatoxin biosynthesis genes
Notes: ** = highly toxigenic isolates; * = low toxigenic isolates.

A. flavus isolates were identified at the species level through morphological and molecular analysis, (Figures 1 and 2), while the toxin analysis was used to distinguish between toxigenic and non-toxigenic isolates (Table 3). Molecular characterization results based on aflatoxin biosynthetic genes provided an overview of the diversity profile at the molecular level (Figures 3 and 4). The results also contributed to a better understanding of toxigenicity mechanisms. The genetic variations observed in each toxigenic and non-toxigenic isolate are important to explore the specific markers candidate for detection purposes and the development of biological agents.

CONCLUSION

All isolates involved in this study were confirmed as *A. flavus* based on morphological and molecular identifications, while HPLC assays confirmed their ability to produce aflatoxins. Twenty-nine pairs of primers were successfully amplified, and the majority produced a single amplicon in toxigenic isolates. However, certain low toxigenic and non-toxigenic isolates produced non-specific amplicon patterns. Deletion variations in several target genes were found in both isolates but no specific target gene could distinguish the toxigenicity based on the genetic similarity data.

This study provides an overview of the molecular profiles of toxigenic and non-toxigenic *A. flavus* isolates. Two non-toxigenic isolates with significant gene deletion were characterized and identified as potential candidates for biocontrol agents. The information about the molecular characterization of *A. flavus* based on toxigenicity could support the development of effective biological control strategies to prevent aflatoxin contamination.

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