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MOLECULAR PROFILE CHARACTERIZATION OF TOXIGENICITY *Aspergillus flavus*

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1. INTRODUCTION

1.1 Background

Climate change has driver to emerging food and feed safety issue worldwide. Crop production is extremely susceptible to climate change, effect on reduction and damage to production yields. This affects the population of microorganisms in soil, water, and air, which are indicated to contribute to the occurrence of diseases caused by microorganisms such as fungi, bacteria, viruses, and insects (FAO, 2008).

Aflatoxin contamination in agricultural products has become a food safety issue worldwide. Aflatoxin is a carcinogenic mycotoxin produced mainly by toxigenic *Aspergillus flavus* and *A. paraciticus* during harvest, post-harvest, transportation, and storage. Climate factors with high humidity such as in Indonesia and other tropical countries are suitable for mold/fungal growth (Hedayati *et al.,* 2007).

Several studies related to climate change and food safety have reported an increase in toxigenic *A. flavus* infections in sub-tropical countries such as Europe. In the last 15 years there has been severe *A. flavus* infection in maize in several countries in Europe, including Italy, Romania, Serbia, and Spain, due to the very dry conditions in the summer and the dry season in these years (Battilani *et al*., 2016).

Although there is lack of research data regarding the increased risk of toxigenic *A. flavus* infection in Indonesia, but Indonesia also affected by the extreme climatic conditions that have occurred during the last decade. Global climate change is also predicted to support the growth of toxigenic *A. flavus* as major aflatoxin producer in Indonesia. The increase in earth temperature in the future is expected to affect the growth of *A. flavus* and the formation of aflatoxins during food storage in Indonesia (Pratiwi *et al*., 2015).

Aflatoxin contamination in Indonesian agricultural commodities has become problem especially for export targeted commodities. In 2019, the Indonesian Ministry of Agriculture recorded that there were 6 out of 8 rejection cases of Indonesian nutmeg in Europe caused by aflatoxin contamination, even though the six of them had been equipped with health certificate (HC) as mandatory requirements referring to EU Regulation No. 24/2016 (Anidah *et al*., 2019). The HC document only released when the products has aflatoxin level below the maximum level.

The high levels of aflatoxin found in the nutmeg, when it arrived in the export destination country, it was suspected that there was a toxigenic *A. flavus* that produced

aflatoxins inside the container during the transportation. The growth of *A. flavus* during transportation is affected by environmental conditions such as temperature and relative humidity, inside containers and during transportation. Aflatoxin will be increased as soon as arrived in the destination country. Therefore, it is important to ensure the existence of toxigenic *A. flavus* in export targeted food commodities, and apply proper handling before if necessary, as a precautionary of rejection in the destination country.

Aflatoxin production of toxigenic *A. flavus* is determined by the presence of aflatoxin biosynthetic genes, which involve 2 regulatory and 25 structural genes (Yu *et al*., 2002). The four target genes of *aflR, aflD, aflM*, and *aflP*, have succeeded in distinguishing toxigenic and non-toxigenic *A. flavus* isolates of herbal origin in Italy (Criseo *et al*., 2001), maize in Italy (Degola *et al*., 2006), animal feed in Iran (Davari *et al.*, 2014), and the *Aspergillus* species in meju, a starter for soybean fermentation in Korea (Kim *et al.*, 2011). All four genes in have different deletion pattern for non-toxigenic *A. flavus*, while no deletion pattern for non-toxigenic *A. flavus*. Currently, the molecular profile data from *A. flavus* isolates from Indonesia are insufficient to characterize their toxicity. Nagur *et al* (2014) characterized isolates of *A. flavus* from Indonesian peanut using the same four target genes but did not include the aflatoxin production of each isolate. Nurtjatja *et al* (2017) reported there was deletion with a random pattern in some of the amplicons of the four target genes in aflatoxigenic isolates from nutmeg and it was confirmed using TLC.

The study on molecular toxicity characterization using all genes involved in aflatoxin biosynthesis for *A. flavus* isolates from Indonesia has not been carried out. This characterization is needed to obtain a complete of the molecular profile of A. flavus toxicity, as genetic information to determine the candidate markers to differentiate between toxicigenic and non-toxigenic *A. flavus*. This information can be used as screening of toxigenic strain in export targeted comodities using molecular technique.

1.2 State of the Art of The Research

Infection of aflatoxin-producing *A. flavus* in Indonesian agricultural commodities is still an obstacle, especially for export commodities. Aflatoxin contamination occurs due to the growth of toxigenic *A. flavus* which produces aflatoxins during transportation, lead to rejection in the destination country. Global climate change is predicted to affect the growth of *A. flavus* and the formation of aflatoxins during food storage in Indonesia. Characterization of toxigenic and non-toxigenic strains of *A. flavus* from Indonesia, which includes molecular profiles at the DNA level using PCR techniques, is necessary for the

development of specific markers that can be used to detect the presence of toxigenic strains in agricultural commodities.

1.3 Objective

The aim of this study is to explore the diversity of toxicigenic and non-toxigenic molecular profiles of *A. flavus* from Indonesia through:

1. Characterization of the molecular profile of toxigenic and non-toxigenic *A. flavus* using twenty-nine genes involved in aflatoxin biosynthesis using PCR method.
2. Validation of the potential for aflatoxin production using HPLC.
3. Exploration of specific candidate gene between toxigenic and non-toxigenic *A. flavus* from Indonesia.

1.4 Expected Output

The expected output of this study will be a database of molecular profiles of toxigenic and non-toxigenic *A. flavus* based on aflatoxin biosynthesis genes, which will be the basis for the development of molecular markers for the detection of toxigenic strains of *A. flavus* in agricultural commodities.

2. STATE OF THE ART

Aspergillus flavus is one of the fungi that causes of contamination in food commodities, by producing aflatoxins that are carcinogenic to humans and animals. However, not all *A flavus* can produce aflatoxins. The natural toxigenic *A. flavus* is determined by the presence of aflatoxin biosynthesis encoding genes. Characterization of toxigenic and non-toxigenic strains of *A. flavus* from Indonesia, which includes molecular profiles at the DNA level using PCR technique, is necessary for the development of specific markers that can be used to detect the presence of toxigenic strains in agricultural commodities.

3. METHOD

Research activity consist of 4 stages:

(1) Subcultured of toxigenic and non-toxigenic *A. flavus* stains

(2) Total DNA Isolation of *A. flavus* and PCR Amplification using 29 primer pairs of biosynthesis aflatoxin genes

(3) Quantification of Aflatoxin Content by HPLC

(4) Data analysis and reporting

3.1 Materials

Nineteen strains of *A. flavus* isolates collection of Phytopathology Laboratory, SEAMEO BIOTROP, isolated from various foodstuffs and from soil of peanut farm in Indonesia.

Table 1. *A. flavus* stains

No	*A. flavus*	Source of strains
1	BIO 3313	Ground peanut
2	BIO 3338	Ground peanut
3	BIO 3345	Nutmeg
4	BIO 3352	Soil
5	BIO 3361	Soil
6	BIO 3376	Nutmeg
7	BIO 33211	Nutmeg
8	BIO 33212	Nutmeg
9	BIO 33403	Nutmeg
10	BIO 33404	Nutmeg
11	BIO 3312	Cacao
12	BIO 3314	Coffee bean
13	BIO 3334	Ground peanut
14	BIO 3342	Ground peanut
15	BIO 3344	Ground peanut
16	BIO 3381	Ground peanut
17	BIO 3382	Corn
18	BIO 3383	White pepper
19	BIO 3393	Coffee bean

Potato dextrose agar/PDA (Difco, Sparks, USA), and *potato dextrose broth*/PDB (Difco, Sparks, USA) was prepared by suspending 39 g of PDA or PDB in 1000 mL distilled water and boiled to dissolve the medium completely. The media was sterilized by autoclaving in

121˚ C, 5 minutes. DNeasy Plant Kit (Qiagen, Jerman) for DNA extraction. GoTaq Master Mix PCR (Promega, USA) for PCR amplification. Primers were synthesized from Integrated DNA Technologies (IDT), Singapore.

Table 2. Sequence of 29 Primer Pairs of Aflatoxin Biosynthesis (Chang et al., 2005)

no	Gene		Forward primer	Reverse primer
1	aflU	norB-cypA	GTGCCCAGCATCTTGGTCCA	AGGACTTGATGATTCCTCGTC
2	aflT	aflT	ATGACATGCTAATCGACGA	AGGCGCATGCTACGGATC
3	aflC	pksA	ACTTTGAGGGCGTTCTGTGC	CTTTCGGTGGTCGGTGATTC
4	aflD	nor1	AGCACGATCAAGAGAGGCT	GATCTCAACTCCCCTGGTAG
5	aflA	fasA(hexA)	TCCTATCCAGTCCACCTCGT	CACATCTTTGTCTTGCCCGC
6	aflB	fasB(hexB)	ACAATCGAATGACAACACTG	CCACCGAATCCACTACCTACA
7	aflR	aflR	ATGGTCGTCCTTATCGTTCT	CCATGACAAAGACGGATCC
8	aflS	aflJ	CTTCAACAACGACCCAAGG	AGATGAGATACACTGCCGCA
9	aflH	adhA	CCTCGTGGGAGAGCCAAAT	GGAGCAAGAAGGTTACAGCG
10	aflJ	estA	CGATGGGACTGACGGTGAT	ACCACGCCGCTGACTTTAT
11	aflE	norA	GTGTTCGTGTGTCGCCCTTA	GTCGGTGCTTCTCATCCTGA
12	aflM	ver1	CATCGGTGCTGCCATCGC	CCTCGTCTACCTGCTCATCG
13	aflN	verA	CCGCAACACCACAAGTAGCA	AAACGCTCTCCAGGCACCTT
14	aflG	avnA	GCGATAGAACTGACAAAGG	GAATGAGTCTCCAAAGGCGAG
15	aflL	verB	TTCAGTGACAAAGGTCTTCG	GGCAGCGTT ATTGAGCATCT
16	aflI	avfA	ATTCAAATCCTCGTTCGGTC	TAGCCCGTTGGTTGTGTTCC
17	aflO	omtB	ACAGACGATGTGGGCAAAC	ACGCAGTCCTTGTTAGAGGTG
18	aflP	omtA	CAGGATATCATTGTGGACG	CTCCTCTACCAGTGGCTTCG
19	aflQ	ordA	AAGGCAGCGGAATACAAGC	ACAAGGGCGTCAATAAAGGGT
20	aflK	vbs	AACGAGCAGCGTAAGGGTC	TCAGCCAGAGCATACACAGTG
21	aflV	cypX	GGAGCCTACCATTCGCAAC	GGCTTTGACGAACAGATTCCG
22	aflW	moxY	TGCTACTGGAACGAAGACC	CGACGACAACCAAACGCAA
23	aflX	ordB	GCTGCTACTGGAATGAAGA	ATGCGACGACAACCAAACG
24	aflY	hypA	CGCAAGACGGCAGAGATAC	GCTCCTTCAGTTCCACACCA
25	nadA		TGACGAGGCCTGCGAGCTG	AAGCCTCTTCAGAACGGTCA
26	hexA		TGTCCTCACCTCTGGCGTAT	AGACCAACCACTCTTATGGGC
27	glcA		AGACACAGTCATCGCCTGTT	GGTGCGAATAGGTGCAGGTA
28	sugR		TCAGCTGAAGCGCTCGAGA	GTATTGCCGCACTATGTATG
29	C4		ATCGTGCAGACAGGAACAC	GGTGCCTTGGCCTATGCGCT

3.2 Subculture of *A. flavus* strains

Nineteen of *A. flavus* strains were subcultured on potato dextrose agar / PDA (Difco, Sparks, USA), incubation was carried out at 27^0C for 5 days.

3.3 Total DNA Isolation of *A. flavus*

As much as two inoculums (ϕ 5 mm) of each mycelium in previous step were inoculated on 50 mL PDB medium, and incubated at 27^0C in a shaker incubator at 100 rpm, for 3 days. The mycelia that grown on the top of the media were harvested and rinsed using sterile distilled water to remove the remaining media, then dried using filter paper. The mycelia were crushed using a mortar with the addition of liquid nitrogen, until fine mycelia powders were obtained. DNA was extracted using DNEasy Plant Kit (Qiagen) according to the manufacture protocol.

About 100 mg of mycelium powder was lysed with 400 ul AP1 buffer and 4 ul RNase to degrade RNA, then vortexed. The lysate then incubated 10 minutes at 60^0C in a digital heating shaking incubator (Thermolyne, Thermo Scientific, USA) at 3000 rpm. 130 µ Buffer P3 was added to precipitate proteins and other contaminants from the cell, followed by centrifugation for 5 minutes at 11000 rpm using Microcentrifuge Hettich 200R (Germany). The supernatant was filtered using a QIAshredder spin column by centrifugation at 8000 rpm for 1 minute. Filtration results were transferred to another tube and followed by added 1.5x volume of AW 1 buffer to bind the DNA. A total of 600 µL filtrate was transferred into the DNeasy mini spin column and separated by centrifugation at 8000 rpm for 1 minute. The filtration process repeated until all the liquid filtrate was filtered. After the DNA binding step, the membrane was washed with the addition of 2x 500 uL buffer AW2, followed by centrifugation. DNA was eluted by adding 100 µL of AE buffer and centrifuged at 11000 rpm for 2 minutes. Eluted DNAs were stored at -20^0C until the amplification stage was carried out (Qiagen, 2019). The quality and quantity of DNA were carried out by electrophoresis in 1% agarose gel and measuring the absorbance at 260 nm wavelength.

3.4 Species Identification by PCR Amplification using ITS Primers

Molecular species identification was carried out using ITS 1 and ITS 4 primer pairs against all strains of *A. flavus*. The amplification reaction was carried out in a total volume of 40 µl, by mixing 100 ng of sample DNA, with 0.2 M of each primer pairs and a GoTaq Hot Start PCR master mix (Promega) which already contained PCR buffer, DNTPs, MgCl2, and Taq DNA Polymerase enzyme.

The amplification program running on the GenAmp PCR System 9700 (ABI) PCR machine is as follows; pre denaturation of 5 minutes at 95^0C, 35 cycles consisting of 30 seconds at 94^0C, 30 seconds at 55^0C, and 30 seconds at 72^0C. Followed by the final extension at 72^0C for 7 minutes. The 600 bp amplicon was confirmed by electrophoresis 4 L of PCR results on 1.2% agarose gel with Sybr safe dye (Invitrogen) with 1x TAE buffer mobile phase. Then the samples were sent to a third party (Apical Scientific, Singapore) for sequencing.

3.5 Aflatoxin Quantification by HPLC

Aflatoxin production was simulated on 10% (v/v) coconut broth (CB, 100 mL fresh shredded coconut endosperm, 900 mL distilled water, pH 7.0) medium. As much as 2 inoculum (ϕ 5mm) of each toxigenic strains were inoculated on 50 mL 10% (v/v) CB medium, and then agitating at 100 rpm (27^0C, 10 days) in the dark condition. Total aflatoxin were extracted from ten-days-old 10% (v/v) CB medium cultures of all *A. flavus* strains, using AOAC method 991.3125,26. A 25 ml of filtered extract was pipetted and extracted with 5 g NaCl and 125 ml of methanol:water (70:30) ratio into blender jar, and blended for 2 minutes at maximum speed. The filtered extract (15 ml) was diluted using 30 ml of purified water into a clean vessel. The diluted extract was filtered through glass microfiber filter. A 15 ml filtered diluted extract was passed completely through AflaTest affinity column (VICAM, USA) at a rate of about 1-2 drops/second and washed with 2 x 10 ml of purified water at a rate of 2 drops/second. Total aflatoxin was eluted from column with addition of 1 ml HPLC grade methanol (Merck, Germany) at rate of 1 drop/second. Eluted sample was collected in a glass cuvette and added with 1 ml deionized water. Afterward, 20 ul of eluate injected onto HPLC.

Chromatographic analyses were performed with an Agilent 1260 Infinity Isocratic LC (Agilent Technologies, USA), equipped with Photochemical Reactor Derivatization (AURA Industries). Excitation and emission wavelengths were 365 and 465 nm respectively. A Bonclone 10u C18 Column (Phenomex, 3.9 x 150 mm) were used as sepation colomn. The mobile phase will be used methanol: water (60:40) with the flow rate will be 1.3 ml/min. Quantification of aflatoxin was perfomed by comparing the peak areas with the calibration curves of each aflatoxin.

3.6 PCR Amplification using 29 Primer Pairs of Aflatoxin Biosynthesis (Chang *et al.*, 2005)

The isolated DNA were amplified by PCR to detect the presence or absence of aflatoxin biosynthesis gene fragments using 29 pairs of primers (Chang *et al*., 2005). The amplification reactions were made in a total volume of 20 ul, by mixing each of the 50 ng DNA samples, with each 0.2 µM primer pair and GoTaq PCR master mix (Promega) containing PCR buffer, DNTPs, MgCl2, and Taq enzyme. DNA Polymerase. Each PCR mixture was amplified on the PCR GenAmp PCR System 9700 (ABI) machine as follows; pre denaturation 5 minutes at 94^0C. Followed by 30 cycles consisting of; 1

minute at 94^0C, 1 minute at 55^0C, and 1 minute at 72^0C. Final extension will be at 72^0C for 6 minutes. PCR results will be confirmed by electrophoresis on 1.5% agarose gel with Sybr safe dye (Invitrogen) and 1x TAE buffer as mobile phase. The presence or absence of product PCR (amplicon) for each isolate was observed and scored for the presence (1) or absence (0) of aflatoxin genes.

3.7 Data Analysis and Reporting

Molecular profiles of each toxigenic and non-toxigenic isolates were constructed from the scoring results of the resulting amplicon fragments. Each of these fragments represents the presence of the genes that code for them. Cluster analysis was performed using UPGMA functionality in NTSYSpc 2.1 software (Department of Ecology and Evolution, State University of New York, NY, USA) to construct the phylogenetic tree of toxigenic and non-toxigenic isolates.

4. RESULT AND DISSCUSSION

4.1 Peremajaan strain *A. Flavus*

The twenty strains of A. flavus used were from the collection of the Phytopathology Lab, SEAMEO BIOTROP, which were rejuvenated on PDA media with 200 ppm chlorampenichol as an antibacterial. The isolates were grown at room temperature (250C) for 5-7 days.

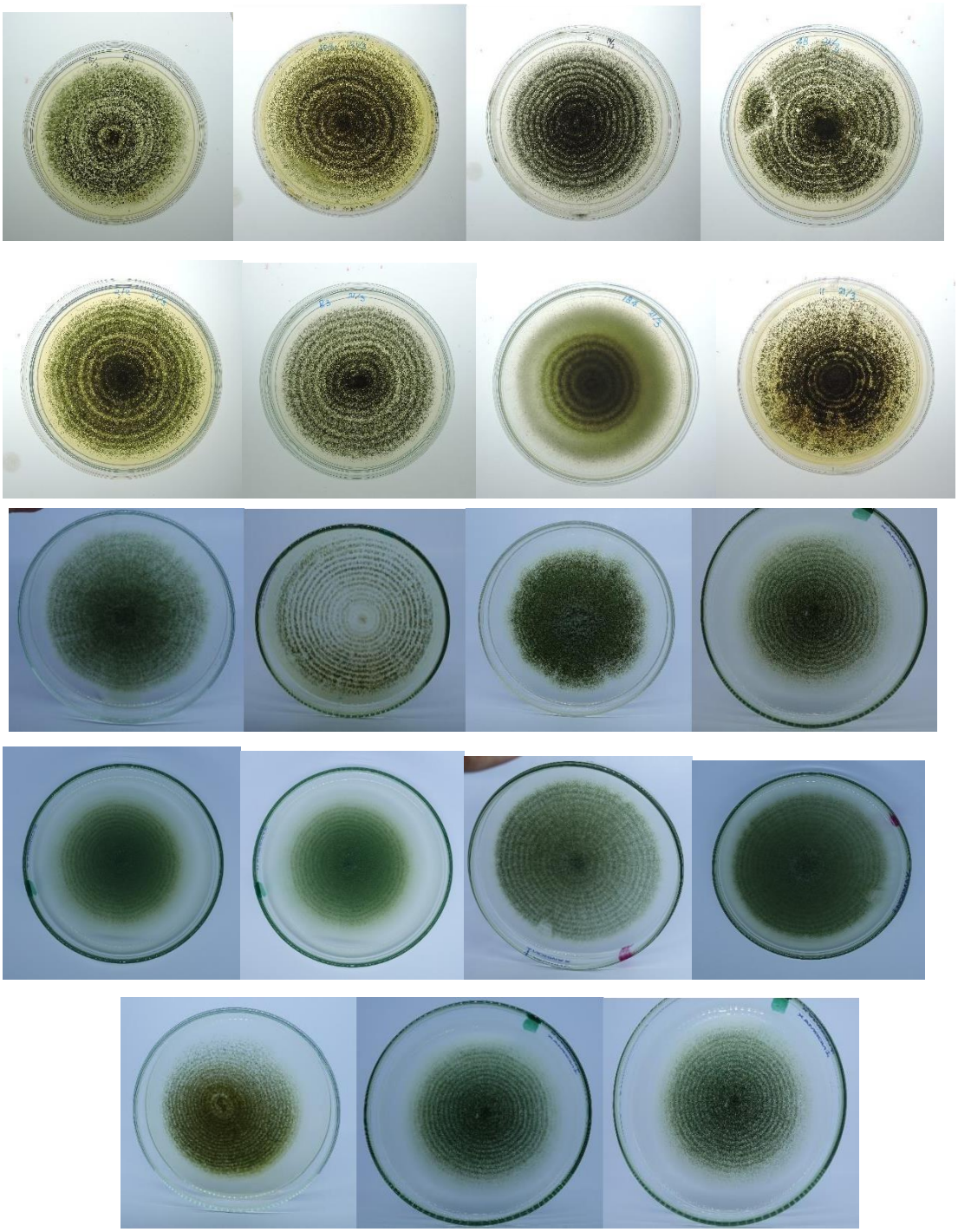

Figure 1. *A.flavus* strain on PDA medium after 7 days inoculation

Based on visual observations, the mycelia appearance of several strains of A. flavus grown on PDA media had different characteristics (Fig. 1).

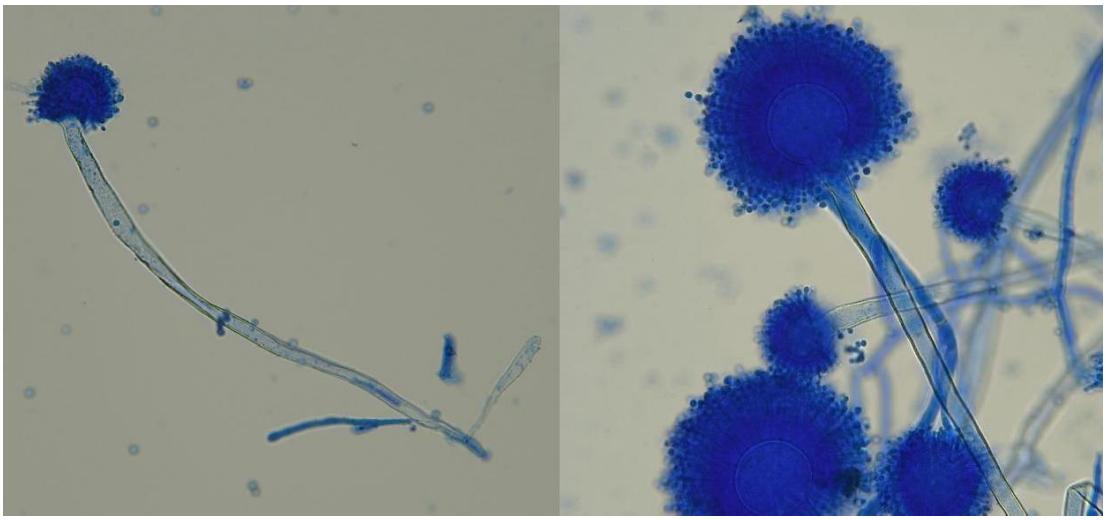

Figure 2. *A. flavus* BIO3313 with 400x. magnification

Subcultured was carried out before the screening stage because the condition of the *A. flavus* strain samples were previously grown in slanted media with the addition of paraffin oil. The existence of paraffin oil aims to preserve the condition of the mold in long-term storage. PDA subcultured aims as readjustment of mold conditions after being stored for a long time in storage media.

Figure 3. *A.flavus* strains on PDA medium preserved by *Paraffin oil*

4.2 Species Identification of *A. flavus*

The molecular profile of the *A. flavus* strains begins with the preparation of DNA material as a template for PCR amplification. Materials were prepared from cultures of each strain of *A. flavus* in 50 mL PDB medium. After 3 days the mycelia growing on the top of the media were harvested and used as a source of DNA material.

Figure 4. Growth of *A. flavus* on PDB medium after 3 days of inoculation

DNA was extracted from 100 mg of mycelium powder which was ground with the addition of liquid nitrogen. To obtain sufficient DNA yield, each sample was extracted twice with the DNEasy Plant kit (Qiagen).

Table 3. Quality and quantity of DNA as measured by nanophotometer

Isolat *A. flavus*	**Konsentrasi DNA (ng/uL)**	**A_{260}/A_{280}**
BIO3313	33,65	1,85
BIO3338	23,20	1,82
BIO3345	57,10	1,81
BIO3352	10,15	1,76
BIO3361	13,80	1,75
BIO3376	48,55	1,72
BIO33211	30,55	1,85
BIO33212	32,25	1,85
BIO33403	26,95	1,83
BIO33404	56,05	1,85
BIO3312	46,40	1,76
BIO3314	49,50	1,85
BIO3334	61,10	1,84
BIO3342	22,85	1,86
BIO3344	26,75	1,81
BIO3381	38,65	1,79
BIO3382	27,90	1,67
BIO3383	28,75	1,76
BIO3393	42,75	1,72

A_{260}/A_{280}: Perbandingan Absorbansi pada 260 dan 280 nm

The results of the DNA quantity using a nanophptometer and DNA quality with agarose gel electrophoresis are presented in Table 3, Figures 4 and 5. The total DNA obtained from 2x replications varied from each strain, the lowest concentration of BIO3352 was 10.15 ng/uL, and the highest was BIO3334 of 61.10 ng/uL. The level of DNA purity ranged from 1.67 to 1.86, and the quality of the DNA bands obtained was quite good, indicated by a firm DNA band in the agarose gel and not degraded. Degraded DNA will appear in the form of a smear.

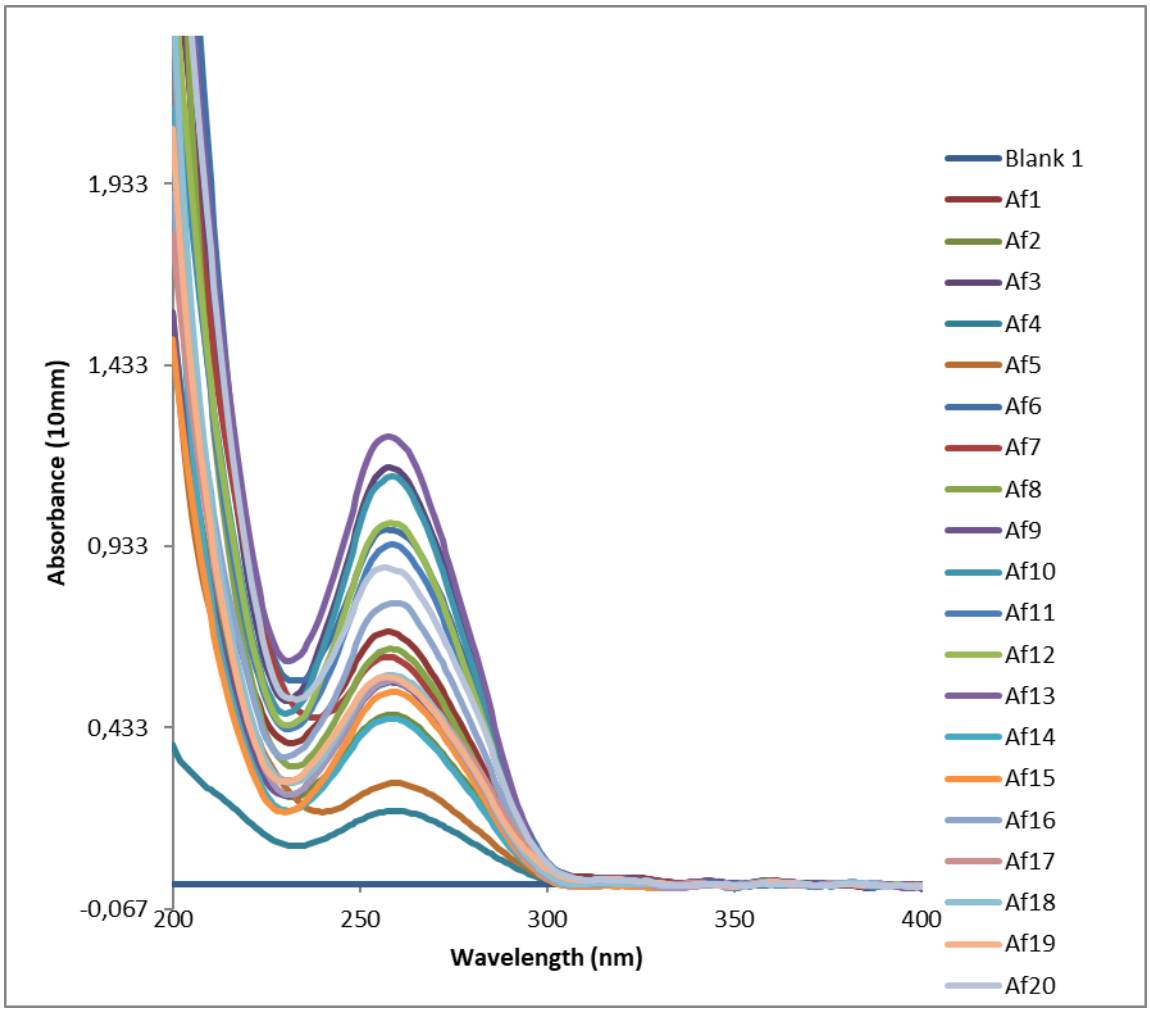

Figure 5. DNA *A. Flavus* absoption at 200 – 600 nm wavelength

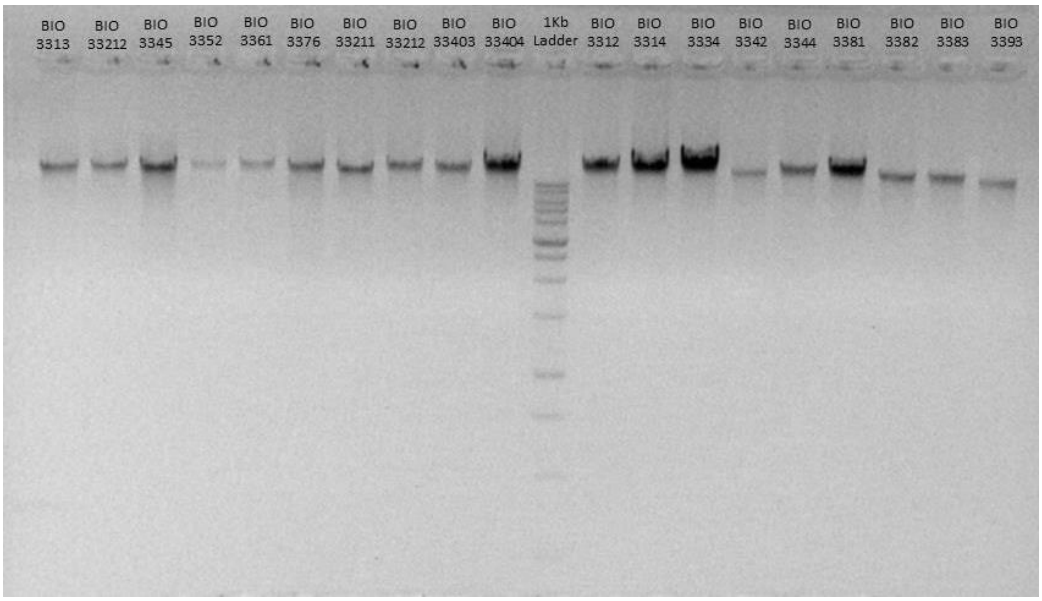

Figure 6. Profile of *A. flavus* DNA on 1% agarose gel electrophoresis

Species identification at the molecular level of the nineteen strains of *A. flavus* by amplifying the internal transcribed spacer (ITS) region was the official locus for fungi species identification. Amplification with ITS 1 and ITS 4 primers resulted in a single amplicon of 600 bp in all strains of *A. flavus* (Fig. 7). The sequencing results of all amplicons were analyzed for diversity using BLAST (http://blast.ncbi.nln.nih.gov/Blast.cgi), and phylogenetic was constructed using MEGA 6. All strains were 100% homolog to *A. flavus.*

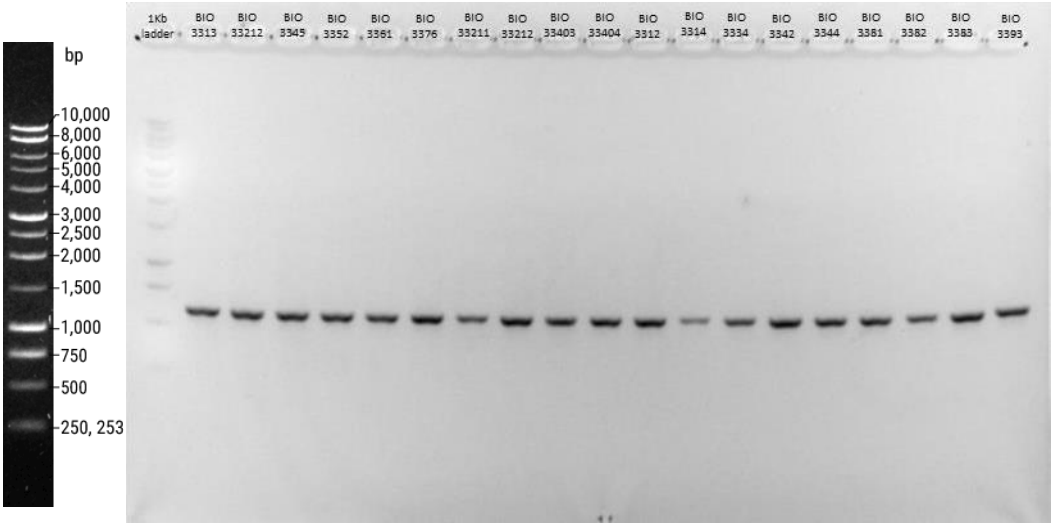

Figure 7. Amplification profile of ITS region in *A. flavus* strains.

The partial ITS gene sequences result from each *A. flavus* strain obtained were compared with the gene sequence database in the NCBI BLAST Genbank. The results showed that all strains were homolog to *A. flavus* species with 100% homology percentage. According to Hagstrom *et al*. (2000), the value of similarity/homologous in genetic sequences if 97% indicates identical species, sequence homology values of 93-97% can be said to be the same genus, but different species. If the homology value is <93%, the novelty of the sequence is only at the genus level, further testing needed to be done through various taxonomic approaches such as phenotype, genotype, chemistry and physiology.

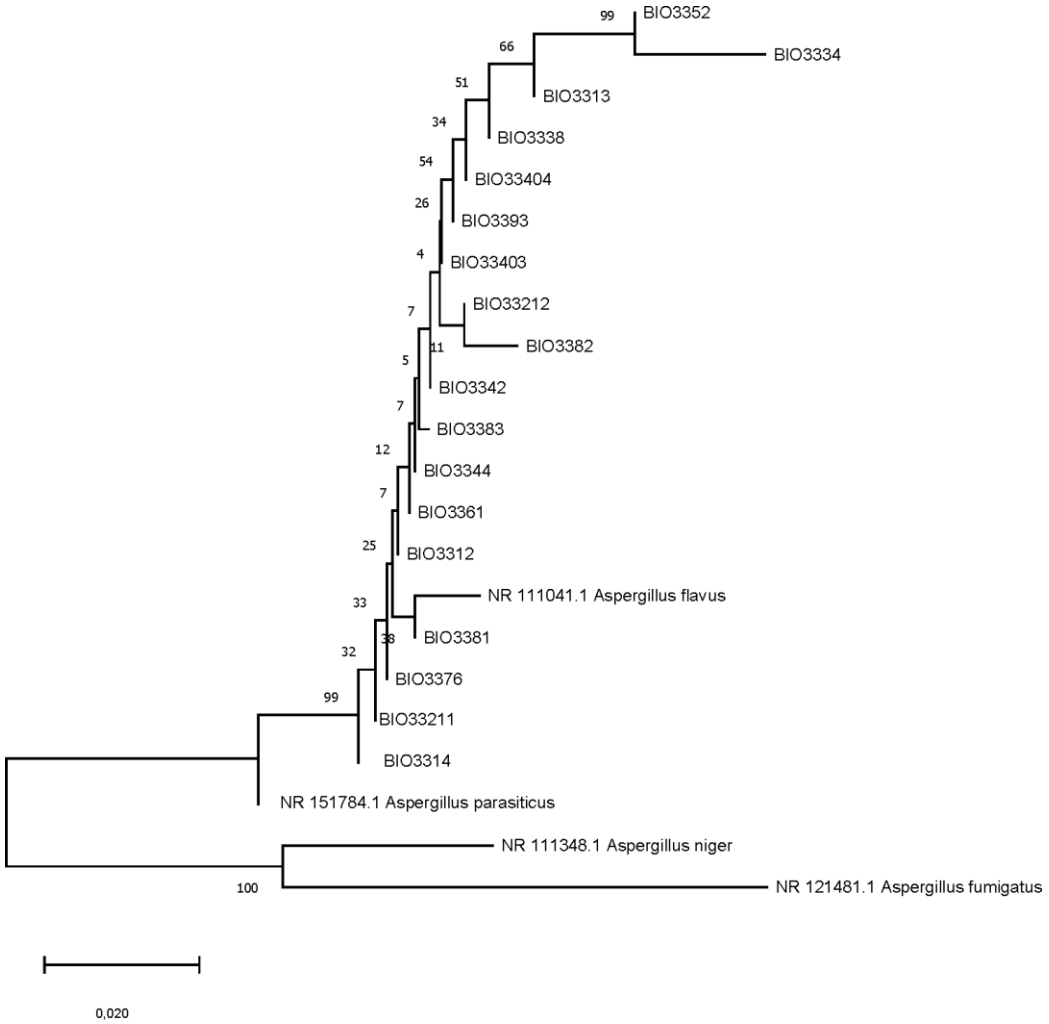

Figure 8. Phylogenetic Tree of *A. flavus* strains using *Neighbour-Joining* method by MEGA X software (1000 X *bootstrap*)

The relationship between all strains of *A. flavus* can be constructed using a phylogenetic tree so that the level of relationship between strains can be analyzed based on DNA sequences. The results of phylogenetic tree constructed using Neighbor-Joining method by

MEGA X software showed that all strains were in the same cluster as *A. flavus* strain (NR 11104.1.1). For comparison, sequences from the NCBI database were included for the strains *A. parasiticus* (NR 151784.1), *A. niger* (NR 111384.1), and *A. fumigatus* (NR 121481.1). If they are in one cluster, it can be concluded that they have close relation with each other. This result is also proven by the percentage value of homolog identity which reaches 100%. Bootstrapping was translated as the value of the accuracy of a phylogenetic tree against randomization of 10000 repetitions (Tamura et al. 2007). The results of the phylogenetic tree are shown in Figure 8.

4.3 Differentiation of Toxigenic and Non-Toxigenic *A. flavus* Strains

Toxigenic *A. flavus* produced aflatoxins when grown on media that induce it, one of which was CBM media containing 10% coconut milk due to the presence of fat and fatty acids (Lin and Dianese 1976). During the incubation process, *A. flavus* mycelia grown on the surface of the media, while the toxin produced was dissolved in the media. The aflatoxin content produced was extracted from the CBM media filtrate, and the content was determined by HPLC.

Table 4. Aflatoxin content from 19 strains of *A. flavus*

Strain *A. flavus*	Aflatoksin (µg/kg)			
	B1	G1	B2	G2
BIO3313	2241,06	nd	66,8	nd
BIO33212	702,72	nd	nd	nd
BIO3361	607,67	1081,15	nd	nd
BIO33404	255,27	nd	nd	nd
BIO3338	217,34	1486,52	nd	nd
BIO3352	126,96	200,06	nd	nd
BIO3344	76,78	nd	2,60	nd
BIO3345	35,09	nd	nd	nd
BIO3334	7,68	nd	0,14	nd
BIO3314	0,62	nd	nd	nd
BIO3312	0,31	nd	nd	nd
BIO3381	0,10	nd	nd	nd
BIO3382	nd	nd	nd	nd
BIO3383	nd	nd	nd	nd
BIO3376	nd	nd	nd	nd
BIO33211	nd	nd	nd	nd
BIO3342	nd	nd	nd	nd
BIO33403	nd	nd	nd	nd
BIO3393	nd	nd	nd	nd

Nd : *Not detected,* under LoQ (B1: 0,0606; B2 = 0,0513; G1 = 0,0660; G2 = 0.0549) in µg/kg

In this study, aflatoxin production was detected using the HPLC method which refers to the verified AOAC 991.31 standard procedure in an ISO/IEC 17025:2017 accredited laboratory. The Limit of Quantification/LoQ values for aflatoxin B1/AFB1, AFB2, AFG1, and AFG2 were 0.0606, respectively; 0.0513; 0.0660; and 0.0549 µg/kg. Compared to the thin layer chromatography (TLC) method, aflatoxin detection with HPLC has a higher level of sensitivity. Park et al (1993) in Wacoo et al. (2014) obtained aflatoxin Limit of Detection (LoD) values ranging from 1 to 20 ng/kg using the TLC method.

A total of 12 strains of *A. flavus* (63%) were able to produce aflatoxin (aflatoxingenic) with varying concentrations, and 7 strains (34%) were not detected to produce aflatoxin (Table 4, Figures 9&10). The aflatoxigenic strains were able to produce 1-2 forms of aflatoxins, 6 strains were able to produce 2 forms of aflatoxins while the other 6 strains only produced 1 form of aflatoxin. Strains BIO3313, BIO3334, and BIO3344 were able to produce AFB1 and AFB2, while strains BIO3361, BIO3338, and BIO3352 produced AFB1 and AFG1, and the rest only produced AFB1.

All aflatoxigenic strains had the ability to produce AFB1 in various concentrations, ranging from low, medium, to high. There were 8 aflatoxigenic strains with high concentration of AFB1 namely BIO3313; BIO33212; BIO3361; BIO33404; BIO3338; BIO3352; BIO3344; and BIO3345 with AFB1 concentration respectively; 2241.06, 702.72; 607.67; 255.27; 217.34; 126.96; 76.78; and 35.09 µg/kg, and 1 strain with a moderate concentration of 7.68 µg/kg (BIO3334). Three other aflatoxigenic strains produced AFB1 below 1 µg/kg, namely BIO3314, BIO3312, and BIO3381, with AFB1 content respectively 0.62; 0.31; and 0.10 µg/kg. AFB1 was the most toxigenic aflatoxin with the minimum value permitted by regulations in Indonesia was 15 µg/kg AFB1 (BPOM, 2012), the presence of toxigenic strains in food was a serious threat because of its ability to produce aflatoxins.

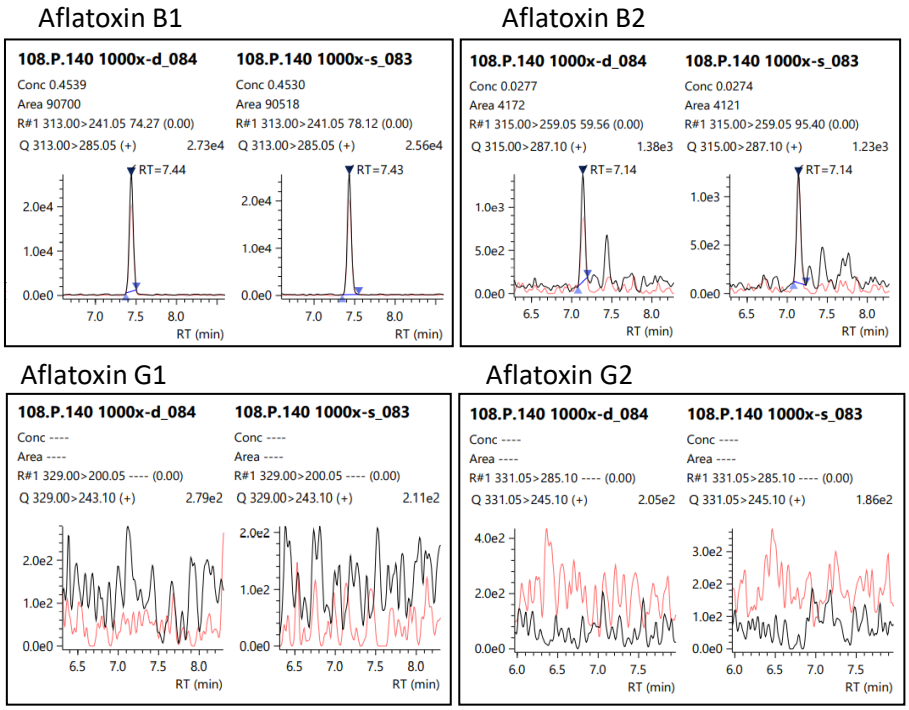

Figure 9. AFB1, AFB2, AFG1, and AFG2 chromatograms of the aflatoxigenic strain BIO3313, with 2 replicates each.

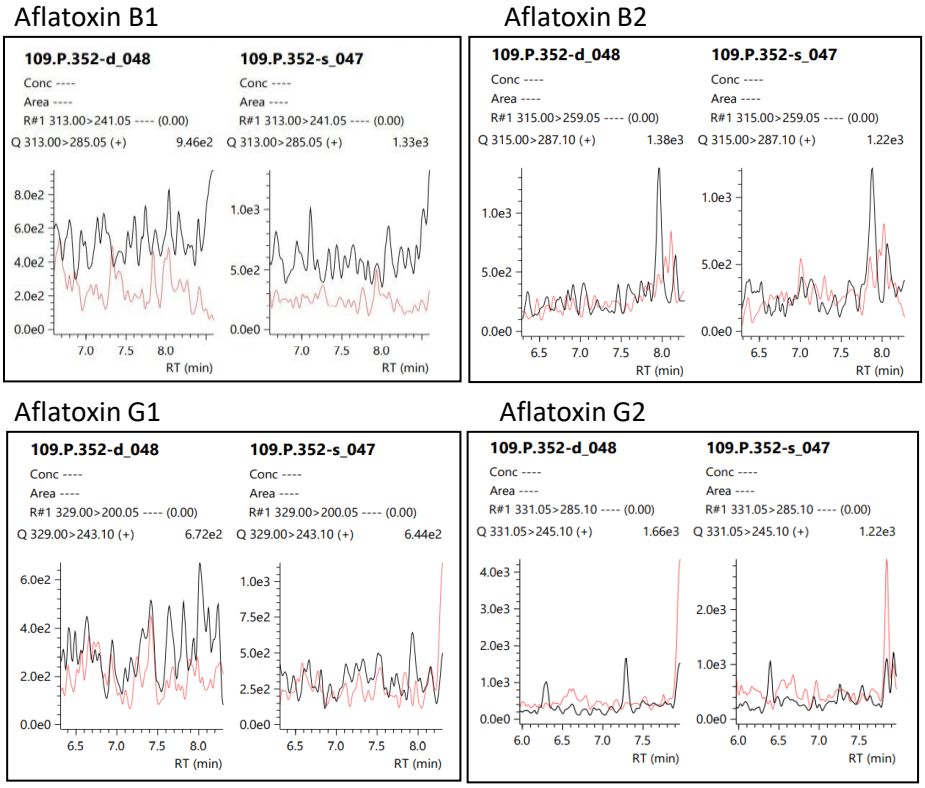

Figure 10. AFB1, AFB2, AFG1, and AFG2 chromatograms of the non-toxigenic strain BIO3393, with 2 replicates each

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

The ability to produce aflatoxins or the toxigenicity of *A. flavus* was determined by the presence of genes encoding aflatoxin biosynthesis that can be detected by PCR (Yu et al. 2002). PCR-based molecular techniques have the advantage of rapid diagnosis with a high level of sensitivity and specificity, compared to conventional techniques using selective media (Mamo et al. 2017). In this study, primers were used that encode 24 aflatoxin biosynthetic genes and 5 sugar clusters flanking areas (Chang et al., 2005). Twenty-nine pairs of primers were successfully amplified in 19 *A. flavus* strains. Most of them produced single amplicons, and some produced multiband amplicon patterns i.e., >1 band in some low toxigenic and non-toxigenic strains (Fig. 11 & Table 5). The amplification of most genes indicates the presence of genes involved in aflatoxin biosynthesis from toxigenic and non-toxigenic strains.

The amplification results of 29 genes in 19 strains of *A. flavus* showed that there were strains that had complete or no deletion of amplicon in 4 toxigenic strains, namely BIO3313 and BIO3345 which were toxigenic strains with high AFB1 production, BIO3334 and BIO3381 as toxigenic strains with moderate AFB1 production and low respectively. AFB1 production of the four in a row was 2241.06; 35.09; 7.68; 0.10 µg/kg. This study also obtained a non-toxigenic strain that produces complete amplicons, namely strain BIO3376. Amplification with 29 target genes using genomic DNA material as a template in the amplification process, explains the presence of the target gene encoding sequences in the genomic DNA of *A. flavus* but has not been able to explain the level of gene expression.

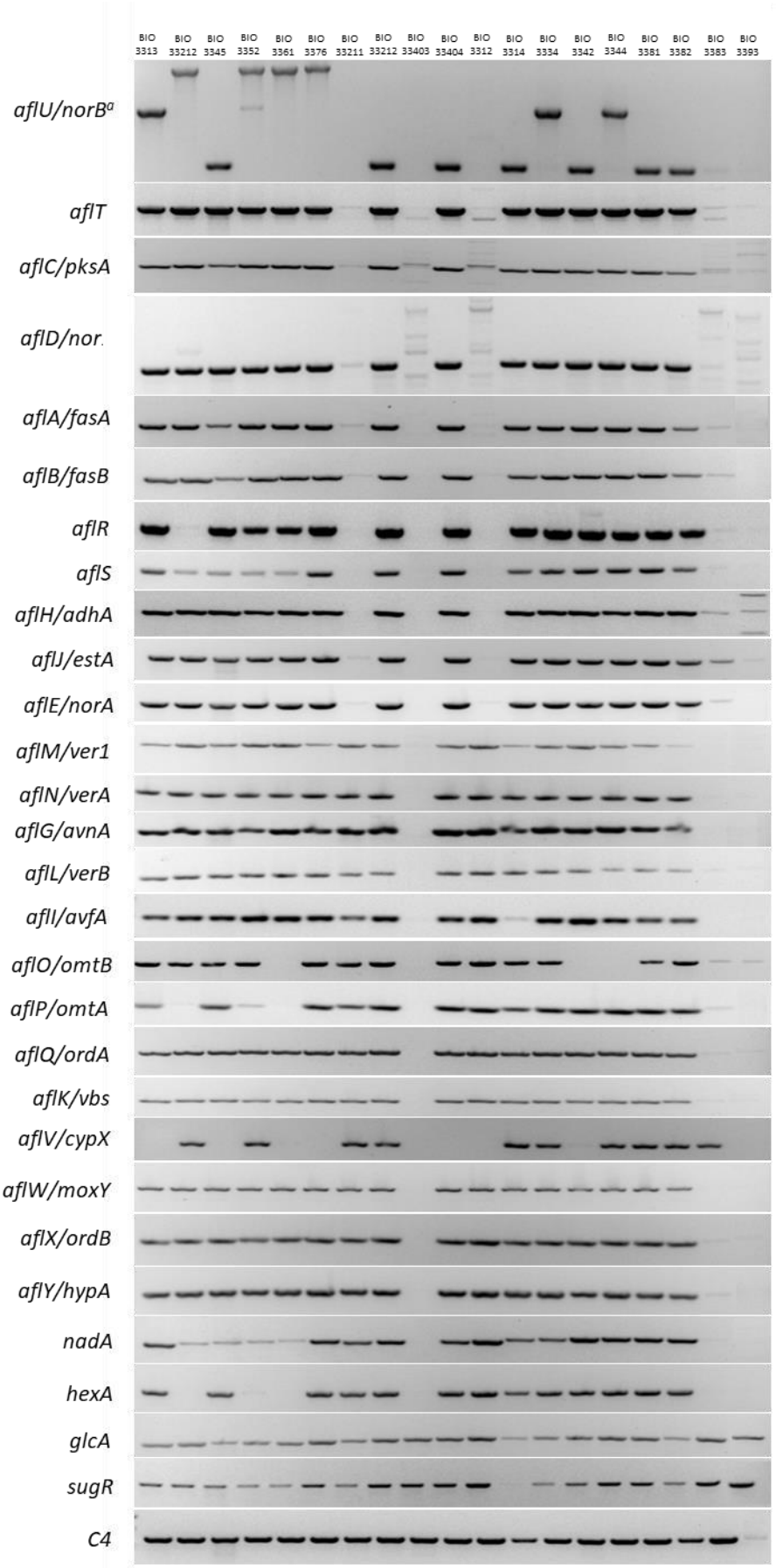

Figure 11. Profile of all amplicons of aflatoxin biosynthesis genes in toxigenic and non-toxigenic strains

Variation of deletion in several target genes were found in both toxigenic and non-toxigenic strains. In high toxigenic strains, the most deletion variations were found in 4 genes (BIO3361, BIO3338, and BIO3352), 2 genes (BIO3338 and BIO2252), and 1 gene (BIO33212, BIO22404, and BIO3344). In low toxigenic strains, several strains experienced 5 genes deletions (BIO3312), and 1 gene deletion (BIO3314).

In toxigenic strains, most of the genes were amplified as single amplicons, except for 4 target genes producing multiband amplicons in several low toxigenic and non-toxigenic strains, namely *aflC, aflD, aflA,* and *aflS* genes. The multiband amplicon variation resulted in low toxigenic strains BIO3312, and 4 non-toxigenic strains namely BIO3382, BIO3383, BIO3393 and BIO33403 (Table 5). However, two other low toxigenic strains, BIO3381 and BIO3314, as well as non-toxigenic strains BIO3312, and BIO3342, BIO3376, and BIO33211, did not produce multiband amplicons. The presence of multiband amplicon variation has not been previously reported in studies related to the profile of aflatoxin biosynthetic genes.

Table 5. Profile of the multiband amplicon of low toxigenic and non-toxigenic strains

Gene	Variasi amplikon *multiband* pada strain *A. flavus*	
	Toksigenik	Non-toksigenik
aflC/pksA	BIO3312	BIO3383, BIO3393, BIO33403
aflD/nor1	BIO3312	BIO3382, BIO3383, BIO3393, BIO33403
aflA/fasA	BIO3312	BIO3393
aflS	BIO3312	BIO3383
nadA	-	BIO3383, BIO3393

Toxigenicity characterization of *A. flavus* using part or all the aflatoxin biosynthetic genes has been widely carried out, Criseo et al. (2001) and Davari et al. (2015) reported the presence of the four target genes *aflP, aflR, aflM,* and *aflD* in all toxigenic isolates from Italy and Iran, while there were variations in deletion patterns in some non-toxigenic isolates with variations of one, two, or three amplicon deletions. A good correlation was also shown in the study of Kim et al. (2011), 6% of the 65 strains of A. flavus from South Korea had complete amplicon from the four pairs of genes confirmed to produce aflatoxins, while the remaining 94% had amplicon variations and were not detected to produce aflatoxins by TLC and HPLC methods. Meanwhile, *A. flavus* strains native to Indonesia, deletion variations were found in all non-toxigenic strains, but also in 4 of the 10 toxigenic strains tested, with deletions in one and two *aflR* and *aflM* gene amplicons (Anidah et al., 2020). The presence of deletion variations in several toxigenic strains

indicated that multiplex PCR with the four target genes *aflP, aflR, aflM*, and *aflD* was not sufficient to distinguish toxigenic from non-toxigenic strains for strains from Indonesia, although the PCR technique had a high level of sensitivity and specificity.

Genetic variations of toxigenic and non-toxigenic strains based on aflatoxin biosynthetic genes have been reported by Chang et al (2005) in the United States, Donner et al (2010) in Nigeria, Wei et al (2014) in China, and Rao et al. (2020) in India, which amplified most of the aflatoxin biosynthetic genes, to study non-toxigenic strains for the purpose of their use as biocontrol agents in the field. All four reported various deletion results in non-toxigenic strains. The non-toxigenic strains with the most gene deletions are potential candidates for biocontrol agents (Donner et al 2010).

Molecular characterization studies based on aflatoxin biosynthetic genes from the Indonesian strain of *A. flavus* have provided an overview of the diversity profile at the molecular level. The genetic variation of each of these toxigenic and non-toxigenic strains is important to know as the first step to search for candidates for selective markers for detection purposes.

Table 6. Scoring of PCR Amplicon from 29 Aflatoxin Biosynthesis Genes

A. flavus Strain	Aflatoxin B1 (µg/kg)	**Scoring of PCR Amplicon from 29 Aflatoxin Biosynthesis Genes**																												
		aflU	*aflT*	*aflC*	*aflD*	*aflA*	*aflB*	*aflR*	*aflS*	*aflH*	*aflJ*	*aflE*	*aflM*	*aflN*	*aflG*	*aflL*	*aflI*	*aflO*	*aflP*	*aflQ*	*aflK*	*aflV*	*aflW*	*aflX*	*aflY*	*nadA*	*hexA*	*glcA*	*sugR*	*C4*
		range[a]	1100 bp	500 bp	600 bp	600 bp	550 bp	600 bp	400 bp	400 bp	600 bp	700 bp	600 bp	400 bp	500 bp	500 bp	500 bp	600 bp	600 bp	500 bp	600 bp	400 bp	600 bp	550 bp	600 bp	600 bp	750 bp	600 bp	700 bp	600 bp
BIO3313	2241,06	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
BIO33212	702,72	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
BIO3361	607,67	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	1	1	1	1	0	1	1	1
BIO33404	255,27	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
BIO3338	217,34	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1[b]	1	1	0	1	1	1	1	0	1	1	1
BIO3352	126,96	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1[b]	1	1	0	1	1	1	1	0	1	1	1
BIO3344	76,78	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1
BIO3345	35,09	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
BIO3334	7,68	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1[b]	1	1	1	1	1	1	1	1
BIO3314	0,62	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1[b]	1	1	1	1	1	1	1	1	1	1	1	0	1
BIO3312	0,31	0	1[b]	M	M	M	0	0	M	0	0	1[b]	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
BIO3381	0,10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
BIO3382	nd	1	1	1	M	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
BIO3383	nd	1[b]	1[b]	M	M	1	1[b]	1	M	1[b]	1	1[b]	0	0	0	1[b]	0	1[b]	1[b]	1[b]	1[b]	0	0	0	1[b]	M	0	1	1	1
BIO3376	nd	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
BIO3342	nd	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1
BIO33211	nd	0	1[b]	1[b]	1	1[b]	1[b]	0	0	0	1[b]	1[b]	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
BIO3393	nd	0	1[b]	M	M	0	0	0	M	0	1[b]	0	0	0	0	0	0	1[b]	0	0	0	0	0	0	0	M	0	1	1	1[b]
BIO33403	nd	0	0	M	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1

Amplicon profile of 29 aflatoxin biosynthetic genes in toxigenic and non-toxigenic *A. flavus* strains. Gene nomenclature refers to Yu, Chang et al. (2004). Amplicon size in base pairs (bp). 1: positive amplicon.. 0 (zero): no amplicon. M: multiband detected amplicon >3 at different sizes, scored as 0 (zero). [a]Three amplicon sizes were detected at 600 bp (BIO3313; BIO3334; BIO3344), 1200 bp (BIO3338; BIO3352; BIO3361; BIO3376), and others at 300 bp. [b]The amplicon band is detected to be thin.

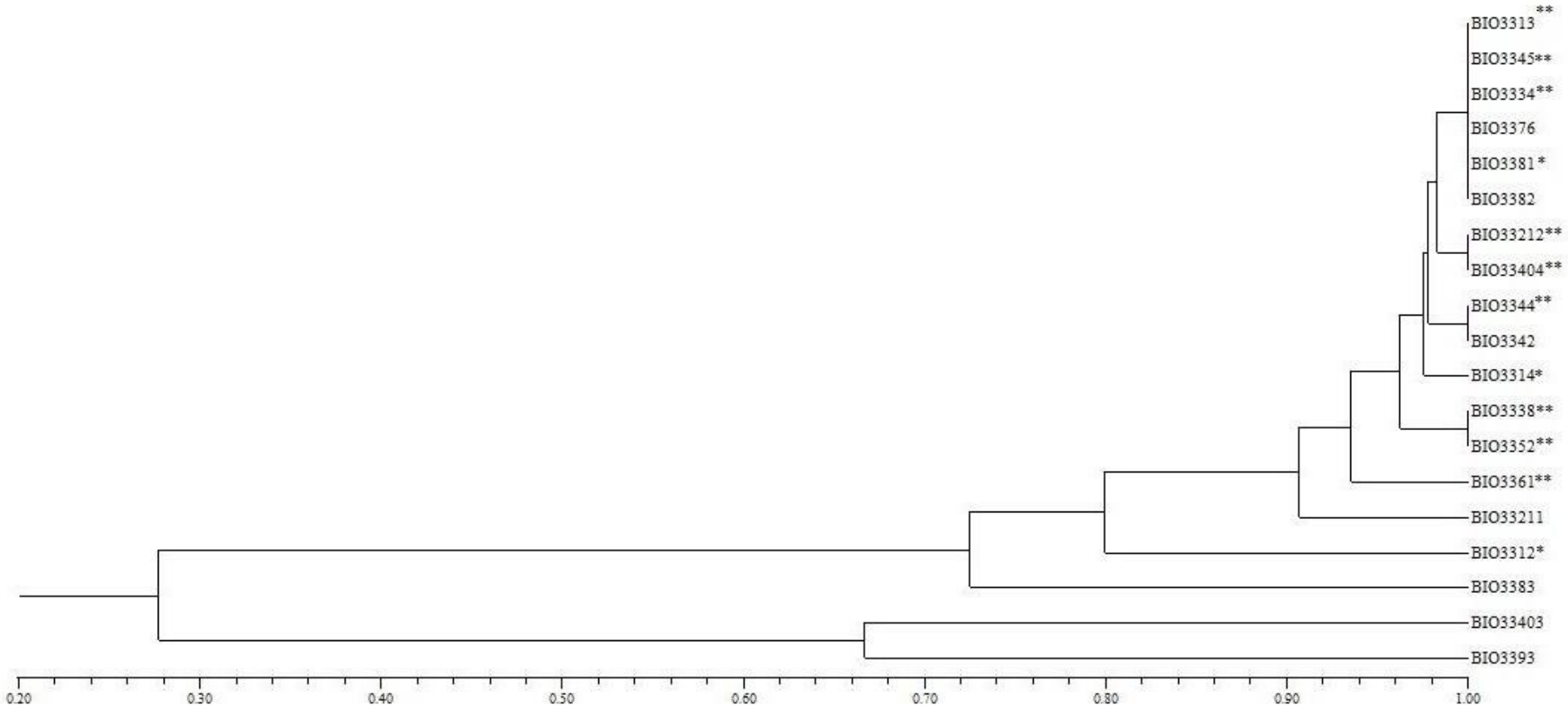

Figure 12. Dendrogram illustrating genetic relationships among 19 strains of *A. flavus*, generated by the UPGMA cluster analysis (NTSYS) calculated from 29 primers of aflatoxin biosynthesis genes. ** highly toxigenic strains; *low toxigenic strains

5. CONCLUSION

Molecular characterization of *A. flavus* strains from Indonesia using 29 genes involved in aflatoxin biosynthesis has been able to provide an overview of the molecular profiles of toxigenic and non-toxigenic *A. flavus*. All strains were confirmed as *A. flavus* through molecular identification, the ability to produce aflatoxins has also been confirmed through HPLC assays. Twenty-nine pairs of primers were successfully amplified, and most of them produced single amplicon in toxigenic strains. Meanwhile, some low toxigenic and non-toxigenic strains produced a multiband amplicon pattern. Deletion variations in several target genes were found in both toxigenic and non-toxigenic strains, so that the twenty-nine target genes were not able to distinguish the toxigenicity of *A. flavus* tested. This study also succeeded in characterizing 2 non-toxigenic candidates of *A. flavus* which lost most of the aflatoxin biosynthetic genes, these characteristics are very useful for use as biocontrol agents in the field against toxigenic *A. flavus*.

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[CODEX] General Standard For Contaminants and Toxins In Food and Feed CXS 193-1995 Adopted in 1995 Revised in 1997, 2006, 2008, 2009 Amended in 2010, 2012, 2013, 2014, 2015, 2016, 2017, 2018: CODEX ALLIMENTARIUS

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