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# Molecular Identification and Ochratoxigenic Potential of Black *Aspergillus* from Various Substrates and Indoor Environment

(Pengenalpastian Molekul dan Potensi Okratoksigen *Aspergillus* Hitam daripada Pelbagai Substrat dan Persekitaran Dalaman)

WARDAH ABDUL RAHMAN & LATIFAH ZAKARIA*

ABSTRACT

Aspergillus *section* Nigri *or black* Aspergillus *is characterized by dark brown to black colonies. Several species of this group showcase the ability to produce ochratoxin A (OTA), contaminating various types of food and feed. This study was conducted to identify black* Aspergillus *isolated from rice, groundnuts, spices, corn grains, soil, and indoor environment and determine the ability of the isolates to produce OTA. Based on ITS, β-tubulin and calmodulin sequences, 177 isolates of black* Aspergillus *from various substrates and indoor environment were identified as* A. niger*,* A. aculeatus, *and* A. tubingensis*. By using two sets of primers to amplify two ochratoxin biosynthesis genes (PKS15KS and PKS15C-MeT genes), the* OTA *genes were detected in only six* A. niger *isolates from rice and indoor environment. These six* A. niger *isolates produced 8 to 308 µg/g of OTA as quantified using UPLC analysis. No* OTA *gene was detected in any of the* A. tubingensis *and* A. aculeatus *isolates. In conclusion, three black* Aspergillus *species,* A. niger*,* A. aculeatus, *and* A. tubingensis*, were identified from rice, groundnuts, spices, corn grains, soil, and indoor environment. Only six isolates of* A. niger *produced OTA indicating most of the* A. niger *isolates were non-OTA producers. These results could thus portray the occurrence of OTA in the field. Since both the number of isolates producing OTA and the levels of OTA production were low, it could be possibly assumed that the occurrence as well as the levels of OTA in the field are also low.*

*Keywords:* Aspergillus aculeatus*;* A. niger*;* A. tubingensis*; black* Aspergillus*; ochratoxin A*

ABSTRAK

Aspergillus *seksyen* Nigri *atau* Aspergillus *hitam dicirikan oleh koloni perang gelap ke hitam. Beberapa spesies kumpulan ini mempamerkan keupayaan menghasilkan okratoxin A (OTA) yang dapat dikesan dalam pelbagai jenis makanan dan makanan ternakan. Pengenalpastian ke tahap spesies* Aspergillus *hitam agak sukar kerana persamaan ciri makroskopik dan ciri mikroskopik. Dalam kajian ini,* Aspergillus *hitam dipencilkan daripada bahan makanan (beras, kacang tanah rempah dan bijirin jagung,), tanah dan persekitaran dalaman. Sebanyak 177 pencilan ini dikenal pasti sebagai* A. niger*,* A. aculeatus *dan* A. tubingensis *berdasarkan jujukan kawasan transkripsi penjarak dalaman (ITS), β-tubulin dan kalmodulin. Gen* OTA *hanya dikesan dalam enam pencilan* A. niger *yang diperoleh daripada beras dan persekitaran dalaman. Pencilan* A. niger *ini menghasilkan 8 hingga 308* µg/g *OTA yang dikuantifikasi menggunakan analisis UPLC. Tiada gen* OTA *yang dikesan dalam pencilan* A. tubingensis *dan* A. aculeatus*. Kesimpulannya, tiga spesies* Aspergillus *hitam,* A. niger*,* A. aculeatus *dan* A. tubingensis *dikenal pasti daripada beras, kacang tanah, rempah, bijirin jagung, tanah dan persekitaran dalaman. Hanya 4.2% daripada pencilan* A. niger *menghasilkan OTA dan ini menunjukkan kebanyakan pencilan* A. niger *bukan pengeluar OTA. Keputusan kajian dapat menggambarkan kejadian OTA di lapangan. Oleh kerana bilangan pencilan yang menghasilkan OTA dan tahap penghasilan OTA adalah rendah, kemungkinan kejadian dan tahap OTA di lapangan juga adalah rendah.*

*Kata kunci:* Aspergillus aculeatus*;* Aspergillus *hitam;* A. niger*;* A. tubingensis*; okratoksin A.*

## INTRODUCTION

*Aspergillus* section *Nigri* or black *Aspergillus* is a group of fungi consisting of various species such as *A. niger*, *A. tubingensis, A. carbonarius, A. foetidus, A. awamori,* and *A. japonicus* (Samson et al. 2007). They can be seen naturally occurring on a variety of substrates including

various types of food and feed, different types of soils and diverse indoor environments. Since black *Aspergillus* species showcase a cosmopolitan distribution, they can be seen growing in the tropical regions as well (Pitt & Hocking 2009).

The toxigenic nature of some of the species of black *Aspergillus* is contributed by the production of ochratoxin A (OTA), commonly detected in different types of food and feed worldwide. OTA was either detected in the substrates or produced by the ochratoxigenic strains of black *Aspergillus* (Lasram et al. 2013; Leong et al. 2007). Black *Aspergillus* mediated OTA contamination might pose a potential risk to animal health and food safety owing to the ability of this mycotoxin to be transferred through the food chain. In case of large-scale production, the contamination with OTA can result in serious economic losses to livestock and agricultural products. *Aspergillus carbonarius* and *A. niger* are two most common ochratoxigenic species of black *Aspergillus* (Perrone et al. 2006). There are chances of occurrence of ochratoxigenic strains of these two species in Malaysia due to high relative humidity and temperature in tropical area which are well suited for growth of both the species as well as production of OTA. However, the optimum temperature of OTA production depends on several factors including the ochratoxigenic species, the commodities (substrates), temperature, relative humidity and water activity (Esteban et al. 2006; Magan & Aldred 2005). Generally, temperature at 25-30 °C, relative humidity of 88-95% and water activity greater than 0.78 are favourable for mycotoxigenic fungal growth and associated with mycotoxin production (Schatzmayr & Streit 2013). Various substrates known to support the growth of black *Aspergillus* such as food and feed (rice, groundnuts, spices, and corn grains), and soils were included in this study. In Malaysia, groundnuts and spices are widely used as food commodities while corn grains are widely utilised as livestock feed. Black *Aspergillus* are common soil fungi associated with decomposition of plant materials, and some of the species are known to act as plant pathogen (Hong et al. 2013). Species of black *Aspergillus* can be found frequently growing in indoor environment (Ghosh et al. 2014; Varga et al. 2014), and some of these species are able to produce OTA (Samson et al. 2007).

The ability of black *Aspergillus* to produce OTA and the possibility of occurrence of more than one species in Malaysia necessitate the identification of various black *Aspergillus* species occurring in this region. The aim of the study was to identify the black *Aspergillus* species isolated from various substrates and indoor environments using PCR assay for ITS, β-tubulin and calmodulin sequences. The presence of *OTA* gene was also detected using PCR, and the amount of OTA produced by these isolates was quantified with the aid of ultra-high performance liquid chromatography with fluorescence detector (UPLC-FLR).

## Materials and Methods

A total of 177 isolates of black *Aspergillus* were used in the study. The isolates were either obtained from stock cultures of Plant Pathology Laboratory, School of Biological Sciences, Universiti Sains Malaysia or isolated from rice, groundnuts, spices, corn grains, agricultural soils and indoor environment. The isolates were identified as *A. niger* (153 isolates) and *A. aculeatus* (24 isolates) on the basis of morphological characteristics (Table 1). The molecular identity of these isolates was further established with the aid of PCR assay for ITS, β-tubulin, and calmodulin sequences.

### DNA extraction

For DNA extraction, black *Aspergillus* isolates were cultured in malt extract broth in universal bottle in triplicates for each isolate. After 24-48 h, mycelia were harvested and dried on Whatman No.1 filter paper. The resulting mycelia were stored in a freezer for overnight. This was followed by freeze-drying the samples for 48 h using a Labconco freeze dryer. Further, the freeze-dried mycelia were ground into fine powder in liquid nitrogen using sterile mortar and pestle. Aproximately 60 mg of the powdered mycelia were transferred to 1.5 mL microcentrifuge tubes. DNA extraction was performed using DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol.

### PCR amplification

For PCR amplification, primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') were used for ITS regions (White et al. 1990), primers Bt2a (5'-GGT AAC CAA ATC GGT GCT GCT TTC-3') and Bt2b (5'-ACC CTC AGT GTA GTG ACC CTT GGC-3') were used for β-tubulin gene (Glass & Donaldson 1995), and primers cmd5 (5'-CCGAGTACAAGGAGGCCTTC-3') and cmd6 (5'-CCGATAGAGGTCATAACGTGG-3') were used for calmodulin gene (Hong et al. 2006).

PCR reactions were carried out using a reaction cocktail (25 µL) consisting 4 µL 5X green buffer, 4 µL 25 mM $MgCl_2$, 0.5 µL 10 µM dNTP mix (Promega, USA), 4 µL each primer (5 mM) and 0.15 µL 5 U/µL GoTaq® (Promega, USA). An amount of 0.3 µL DNA template was added for the amplifications of ITS regions and β-tubulin, and 0.1 µL DNA template was added for the amplification of calmodulin. The final reaction volume was adjusted to 25 µL by addition of required amount of deionised distilled water.

TABLE 1. List of black *Aspergillus* isolates with their morphological identity

| Isolates | Substrate | Morphologically identified species |
|---|---|---|
| AP1R–AP33R | Rice | *A. niger* |
| AP34R | Rice | *A. aculeatus* |
| AP35R–AP39R | Rice | *A. niger* |
| AR40R–AR41R | Rice | *A. niger* |
| AC42R–AC43R | Rice | *A. niger* |
| AM44R–AM46R | Rice | *A. niger* |
| AN47R–AN48R | Rice | *A. niger* |
| AW49R | Rice | *A. aculeatus* |
| AS50R | Rice | *A. niger* |
| AS51R–AS52R | Rice | *A. aculeatus* |
| AW53R | Rice | *A. aculeatus* |
| AW54R | Rice | *A. niger* |
| AW55R | Rice | *A. aculeatus* |
| AP56R | Rice | *A. aculeatus* |
| AW57R | Rice | *A. niger* |
| AW58R–AW60R | Rice | *A. aculeatus* |
| AP61R–AP62R | Rice | *A. aculeatus* |
| AS63R | Rice | *A. aculeatus* |
| AW64R–AW66R | Rice | *A. aculeatus* |
| AA67N | Groundnuts | *A. niger* |
| AA68N | Groundnuts | *A. aculeatus* |
| AA69N–AA71N | Groundnuts | *A. niger* |
| AA72N | Groundnuts | *A. aculeatus* |
| AA73N–AA82N | Groundnuts | *A. niger* |
| AP83N | Groundnuts | *A. niger* |
| AP84D–AP105D | Spices | *A. niger* |
| AC106B–AC107B | Indoor environment | *A. niger* |
| AD108B | Indoor environment | *A. niger* |
| AK109B | Indoor environment | *A. aculeatus* |
| AM110B–AM111B | Indoor environment | *A. niger* |
| AN112B | Indoor environment | *A. aculeatus* |
| AP113B–AP128B | Indoor environment | *A. niger* |
| AP129B | Indoor environment | *A. aculeatus* |
| AP130B | Indoor environment | *A. niger* |
| AS131B | Indoor environment | *A. niger* |
| AS132B | Indoor environment | *A. aculeatus* |
| AS133B | Indoor environment | *A. niger* |
| AT134B–AT137B | Indoor environment | *A. niger* |
| AP138O–AP1550 | Corn grains | *A. niger* |
| AM156O–AM163O | Corn grains | *A. niger* |
| AP164S | Soil from vegetable farm | *A. aculeatus* |
| AP165S–AP166S | Soil from oil palm plantation | *A. niger* |
| AP167S–AP168S | Soil from paddy field | *A. niger* |
| AP169S–AP177S | Soil from oil palm plantation | *A. niger* |

PCR was performed in a thermal cycler (Bio-Rad Mycycler®) with the following PCR cycles for the amplification of ITS regions: Initial denaturation at 95 °C for 5 min, 30 cycles with each cycle consisting of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min, with final extension at 72 °C for 5 min. For β-tubulin, PCR cycles were as follows: initial denaturation at 94 °C for 5 min, 35 cycles consisting of denaturation at 58.6 °C for 30 s, annealing at 58.6 °C for 30 s and extension at 72 °C for 1 min, with final extension at 72 °C for 5 min. For calmodulin, PCR was carried out with initial denaturation at 95 °C for 5 min, followed by 35 cycles consisting of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. The final extension step was carried out at 72 °C for 5 min.

PCR products for all the DNA regions were separated on a 1% agarose gel, run in 1X Tris-borate EDTA buffer for 90 min at 90 V and 400 mA. The DNA bands were visualised with the aid of Florosafe DNA Stain (1st Base).

### SEQUENCE ANALYSIS AND PHYLOGENETIC ANALYSIS

DNA sequencing on the PCR products was outsourced. The resulting sequences were aligned using ClustalW and edited manually by Molecular Evolutionary Genetic Analysis (MEGA 5) software to create consensus sequence (Tamura et al. 2011). BLAST analysis was used to compare the consensus sequences with the sequences in database of GenBank (http://www.ncbi.nlm.nih.gov). Species were identified on the basis of BLAST results showing 97-100% similarity with the sequences in the GenBank.

Multiple sequence alignment based on combined sequences of ITS, β-tubulin and calmodulin sequences of all isolates were generated to construct a phylogenetic tree using MEGA5 software. Phylogenetic relationships were analysed using Maximum Likelihood (ML), a character-based method. Kimura 2-parameter model was used to construct the ML tree. Model test was run to find the best substitutions DNA models and from the model test result, the lowest AIC scores (Akaike Information Criterion) was chosen. The reliability of the ML tree was estimated using bootstrap method with 1000 replicates. For phylogenetic analysis, complete-deletion option was selected and all positions containing gaps and missing data were eliminated. Ex-type strains for each species were also included for comparison (Samson et al. 2014). To root the tree, *Aspergillus flavus* (NRRL 1957) was used as the outgroup.

## OCHRATOXIN PRODUCTION

### PCR AMPLIFICATION OF *OTA* GENE

PCR amplification of *OTA* gene was performed after molecular identification of the black *Aspergillus* isolates of which three species were identified, *A.niger*, *A. aculeatus,* and *A. tubingensis*. A total of 153 isolates of *A. niger*, 24 isolates of *A. aculeatus* and nine isolates of *A. tubingensis* were used for PCR amplification of the *OTA* gene and quantification of OTA. Two different primer sets, namely PKS15KS primers and PKS15C-MeT primers were used to detect *OTA* gene (An15g07920) (Kim et al. 2014). PKS15KS primer set consisted of PKS15KS-f (5'-CAA TGC CGT CCA ACC GTA TG-3') and PKS15KS-r (5'-CCT TCG CCT CGC CCG TAG-3'), while PKS15C-MeT primer set consisted of PKS15C-MeT-f (5'-GCT TTC ATG GAC TGG ATG-3') and PKS15C-MeT-r (5'-CAT TTC GTT GAT CCC ATC G-3'). These primers were used to amplify the putative *PKS* gene fragments of An15g07920 gene that are involved in OTA biosynthesis. The PKS15KS primer set was used to amplify DNA fragment corresponding to β-ketoacyl synthase (KS) domain, while PKS15C-MeT primer set was used for DNA fragment corresponding to C-methyltransferase (C-Met) domain. Both KS and C-Met domains are reported to be present in the hypothetical protein encoded by An15g07920 gene (Ferracin et al. 2012).

The amplification of *OTA* genes was performed separately in a 25 µL reaction mixture consisting of 4 µL 5X green buffer, 4 µL 25 mM $MgCl_2$, 0.5 µL 10 µM dNTP mix (Promega), 4 µL each primer, 0.15 µL 5 U/µL GoTaq® (Promega) and 0.1 µL DNA template. The final volume of reaction mixture was adjusted to 25 µL with deionised distilled water. Sterile deionised water was used as a negative control. PCR was performed as described above with following PCR cycle conditions: initial denaturation at 95 °C for 4 min, 30 amplification cycles consisting of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, followed by final extension at 72 °C for 7 min.

### AGAR PLUG METHOD FOR IN VITRO MYCOTOXIN PRODUCTION

Black *Aspergillus* isolates were inoculated at three points on Czapek Yeast Extract (CYA) using standard agar plug method (Bragulat et al. 2001) with modifications. After 7 days of inoculation, all the three agar plugs were carefully removed from each plate with the help of an 8 mm cork borer and transferred to a McCartney bottle.

The agar plugs from each isolate were homogenised in 0.5 mL methanol and incubated at room temperature for 60 min. The resulting extracts were filtered by passing through syringe filters (PTFE) with a diameter of 25 mm. These were further injected into 2 mL glass screw neck vials (Waters, USA). The final solution from each isolate was stored at 4 °C until analysed using ultra-high performance liquid chromatography (UPLC) with fluorescence detector. OTA production by each isolate was repeated twice.

### PREPARATION OF OTA STANDARD

OTA was commercially purchased from Sigma Chemical Cp. (St Louis, MO, USA). Standard stock solution (1000 µg/g) was prepared in 1 mL methanol and stored at 8 °C until used. From this, five working stock solutions (2, 4, 6, 8 and 10 µg/g) were prepared in methanol and stored at 4 °C until analysed using UPLC.

### UPLC-FLR ANALYSIS

UPLC system consisting of Waters Acquity UPLC® binary pump equipped with Waters Acquity UPLC® FLR detector was used for quantification of OTA produced by isolates. For detection, the excitation and emission maxima were set at 330 and 460 nm wavelength, respectively. Chromatographic separations were carried out on a C18 reversed-phase column (2.1 × 100 mm, 1.7 µm) (Waters, USA) with isocratic programme of 57% acetonitrile ($CH_3CN$), 41% water and 2% acetic acid (filtered through a PTFE syringe filter). The separation was performed at a flow rate of 0.2 mL/min with total run time of 4 min. For OTA quantification, peak height of the sample was compared with the calibration curve of the standards.

## RESULTS AND DISCUSSION

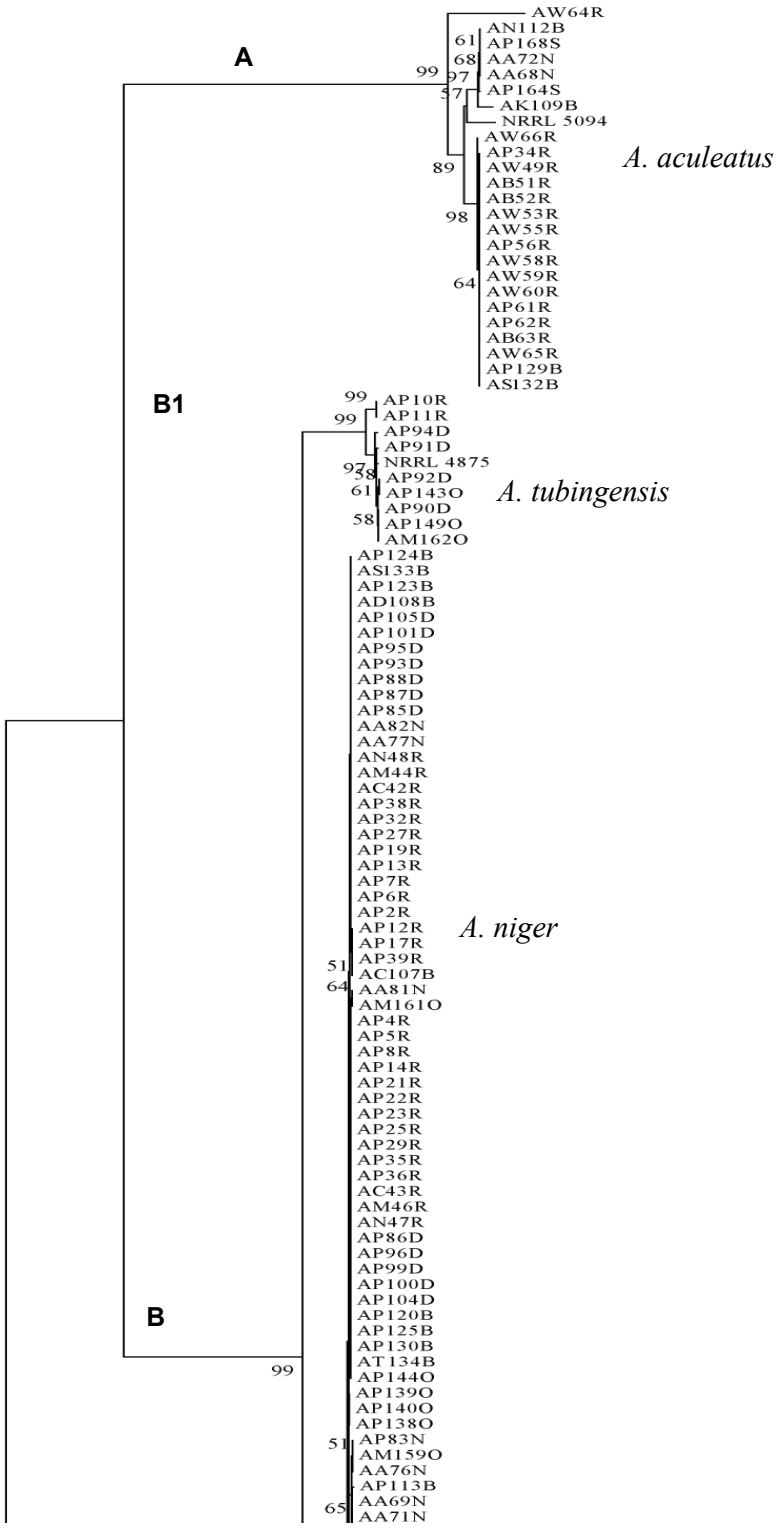
PCR amplification of ITS regions and calmodulin gene produced a fragment of approximately 600 bp of morphologically identified *A. niger* (154 isolates) and *A. aculeatus* (24 isolates). The amplification reaction for *β-tubulin* gene produced a band of 550 bp for all the isolates morphologically identified as *A. niger* and *A. aculeatus*.

BLAST search results for sequences of ITS regions, β-tubulin, and calmodulin expressed as percentage of sequence similarity, and the newly assigned identities for various isolates are shown in Table 2. BLAST search for the sequences of the three markers confirmed the identity of 144 isolates of *A. niger*. However, nine isolates morphologically identified as *A. niger* showed molecular identity of *A. tubingensis*. Molecular study also confirmed the identity of 24 isolates morphologically identified as *A. aculeatus*.

TABLE 2. Percentage of sequence similarity and identity assigned to morphologically identified *A. niger* and *A. aculeatus* isolates obtained from various substrates and indoor environment

| Morphological identification | Sequence similarity (%) | | | Identity assigned |
|---|---|---|---|---|
| | ITS | β-tubulin | Calmodulin | |
| *A. aculeatus* (24 isolates) | *A. aculeatus* (99-100) | *A. aculeatus* (98-100) | *A. aculeatus* (97-99) | *A. aculeatus* |
| *A. niger* (144 isolates) | *A. niger* (99-100) | *A. niger* (98-99) | *A. niger* (98-100) | *A. niger* |
| *A. niger* (9 isolates) | *A. tubingensis* (99) | *A. tubingensis* (99) | *A. tubingensis* (97–99) | *A. tubingensis* |

Maximum likelihood tree generated using combined sequences of ITS, β-tubulin and calmodulin is shown in Figure 1. Isolates from the same species were clustered in the same clade and not grouped according to the locations or substrate. The outgroup, *A. flavus* (NRRL 1957) formed separate clade. Isolates of *A. niger* and *A. tubingensis* were grouped in the same main clade (Clade B) but separated into two sub-clades (B1 and B2). The grouping indicated close relationship between *A. niger* and *A. tubingensis* isolated from different substrates (rice, groundnuts, spices, corn grains, and agricultural soils) including isolates from indoor environment. Isolates of *A. aculeatus* formed separate clade (Clade A) from *A. niger* and *A. tubingensis*.



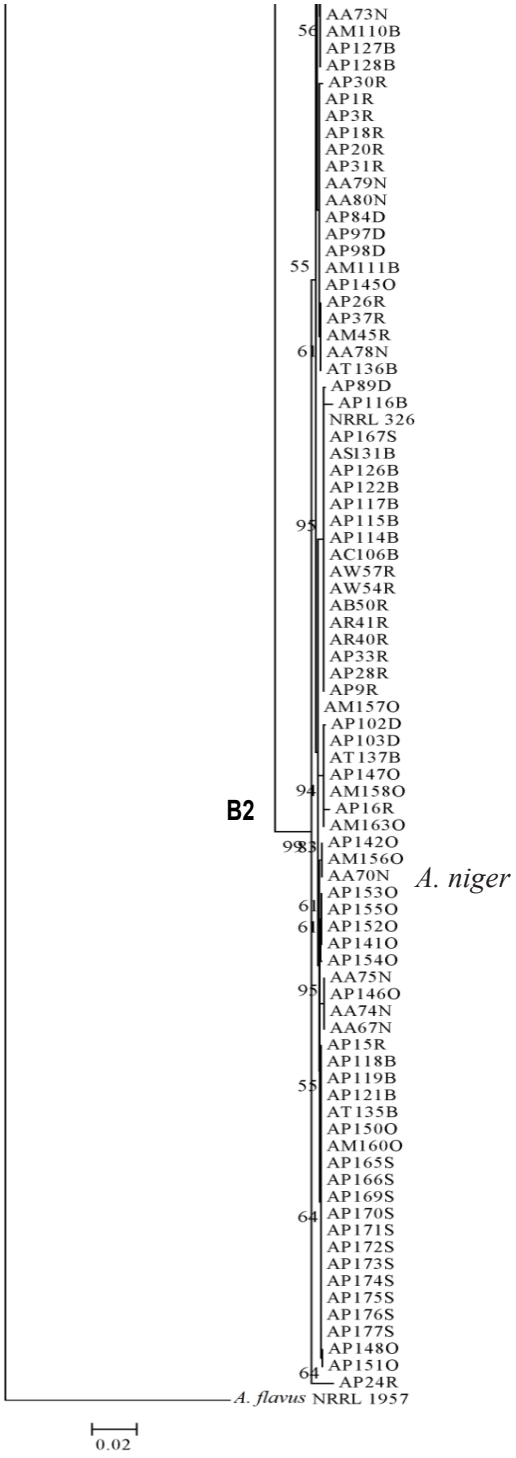



FIGURE 1. Maximum Likelihood tree generated based on combined sequences of ITS, β-tubulin and calmodulin of black *Aspergillus.* Bootstrap values higher than 50% are shown next to the branches. *Aspergillus flavus* is the outgroup

*Aspergillus niger* was found to be the most commonly occurring species of black Aspergilli in Malaysia which was recovered from various types of substrates and indoor environment. Species of the genus *Aspergillus* including *A. niger* are moderately xerophilic owing to their ability to survive in harsh conditions such as high temperature and low water availability (Pettersson & Leong 2011; Pitt & Hocking 2009). The high prevalence of *A. niger* observed in the present study might be contributed by its natural tendency to grow well in warm places with high temperature such as Malaysia. In addition to this, black conidia produced by *A. niger* provide protection against UV light and sunlight (Pitt & Hocking 2009). All these features provide an advantage to *A. niger* to compete with other black *Aspergillus* as well as other fungal species.

Most of the food and feed included in this study were generally dry in nature having low moisture content. There are several reports where rice, groundnuts, spices and corn grains are categorized as dried food and feed having low water content (Beuchat 1979; Pitt & Hocking 2009). The dried substrates thus provide a habitat ideal for survival and growth of *A. niger* conidia. *Aspergillus niger* has been frequently isolated from stored and post-harvest products including onion bulbs and maize (Nyongesa et al. 2015; Shehu & Muhammad 2012). Thus, the occurrence of *A. niger* on different substrates might indicate the possibility of *A. niger* contamination, particularly in the agricultural crops in the field. It is one of the ubiquitous fungi that can be found growing in the soil, thus it can act as a saprophyte.

In case of indoor environment, *A. niger* is a prevalent indoor fungus that can be easily found in any type of buildings. The findings of the present study were in accordance with the previous findings where *A. niger* was found prevalent in different types of soil and indoor environment such as indoor air in a library (Abdullah & Muhammed 2011; Ghosh et al. 2014; Leong et al. 2007; Varga et al. 2014; Zarei et al. 2016). The prevalence of *A. niger* in such environments could be possibly explained by the ability of its conidia to remain dormant in soil and air during the unfavorable growth conditions. Thus, the conidia of *A. niger* are well suited for airborne dispersal and could grow upon availability of favorable environmental conditions (Hayer et al. 2014).

Only nine isolates were identified as *A. tubingensis* in this study. Although *A. tubingensis* was not prevalent in this study, this species has been reported as a common contaminant found in food products, feed, soils and indoor air (Garcia-Cela et al. 2014; Lopez-Mendoza et al. 2009; Medina et al. 2005; Varga et al. 2014). In this study, *A. tubingensis* shared several substrates with *A. niger* such as rice, spices and corn grains. Thus, the presence of both the species on the same substrate is a common occurrence. Growth and ecological conditions suitable for *A. tubingensis* also show similarity with *A. niger*. However, *A. tubingensis* was not prevalent in the present study, and this observation lacks a possible explanation. The only possible explanation could be dry nature of the substrates available for growth. There are several reports where *A. tubingensis* preferred cooler temperature like northern Italy where grapes are planted (Spadaro et al. 2012). Nevertheless, both *A. niger* and *A. tubingensis* were found occurred together on the same substrates such as grapes, raisins, cashew nuts, cocoa beans and herbal tea (Bisbal et al. 2009; Lamboni et al. 2016; Palumbo et al. 2011; Vitale et al. 2012).

The presence of *OTA* gene in various isolates was studied by PCR of which 776 bp and 998 bp fragments were obtained, corresponding to KS and C-MeT domains, respectively. Only six isolates of *A. niger* showed the presence of KS and C-MeT domains. These isolates were subjected to OTA quantification using UHPLC-FLR. The results of UHPLC-FLR analysis are summarised in Table 3. The six isolates of *A. niger* showed OTA production levels of 0.26-3.26 µg/g.

TABLE 3. Concentration of OTA produced by six isolates of *A. niger* isolated from rice and indoor environment

| Isolates | Sources | Concentration (µg/g) |
|---|---|---|
| AP33R | Rice | 2.26 |
| AR40R | Rice | 1.21 |
| AW57R | Rice | 0.26 |
| AC106B | Indoor environment | 3.26 |
| AP126B | Indoor environment | 2.56 |
| AS131B | Indoor environment | 2.98 |

UPLC analysis showed that *A. niger* isolates from rice produced OTA in the range of 0.26-2.26 µg/g. This was lower as compared to OTA produced by *A. niger* isolates obtained from indoor environment which showed OTA concentrations of 2.56-3.26 µg/g. Sánchez-Hervás et al. (2008) classified OTA producers having OTA production in the range of 10-100 µg/g as moderately toxigenic isolates. According to the study of Belli et al. (2004), OTA production levels of 0.0-2.5 µg/g were regarded as low. Based on these classifications, the six OTA producing *A. niger* isolates in the present study can be classified as low toxigenic isolates that produce low concentrations of OTA.

OTA produced by *A. niger* isolates obtained from rice in the present study was similar to OTA produced by black *Aspergillus* isolated from rice in Thailand, which produced 0.20- 205.52 ng/g of OTA as studied using HPLC (Kittikamhaeng & Dachoupakan 2011). Even though only three isolates of *A. niger* were found to produce OTA, there is a possibility of rice contamination with OTA. Natural occurrence of OTA in rice has been previously reported in Brazil (Almeida et al. 2012) and India (Priyanka et al. 2014). In Malaysia, OTA has been detected in cereals including rice and quantified by HPLC in two separate studies showing OTA levels of 0.03-5.32 ng/g (Rahmani et al. 2010) and 0.2-4.34 ng/g (Soleimany et al. 2012). In another study, OTA production was also reported in red rice with concentrations in the range of 0.23-2.48 µg/kg (Samsudin & Abdullah 2013).

Since OTA production was detected for *A. niger* isolated from rice, there is a possibility of OTA contamination which might have occurred before harvesting, in between harvesting and drying or during storage (Zheng et al. 2006). Therefore, several measures should be taken to minimize the risk of OTA contamination such as application of Good Agricultural Practise and Good Manufacturing Practise during growth, harvesting and storage of rice. Creating food safety awareness among rice farmers and producers might be instrumental in controlling the entry of contaminated food into dietary channel of humans and animals. Training programs involving direct communication between researchers, farmers and relevant government agencies should be routinely conducted to increase understanding regarding the importance of controlling contamination of rice with OTA and other mycotoxins. Since there are chances of OTA contamination at any stage, regular monitoring should be done at every stage so that necessary steps can be taken at the earliest.

For *A. niger* isolates obtained from indoor environment, only three isolates showed OTA production in the range of 2.56-3.26 µg/g. *Aspergillus* are well-known indoor fungi and inhalation of ochratoxigenic *A. niger* may cause harmful effects in humans (Abbott 2002). In two separate studies, Di-Paolo et al. (1994) and Richard et al. (1999) reported the possibility of ochratoxigenic *A. niger* having detrimental health effects. Current research studies majorly focus on diversity and distribution of black *Aspergillus* without much interest in OTA quantification. Consequently, studies on the production of OTA by black *Aspergillus* from indoor environment are quite limited and poorly understood (Varga et al. 2014; Wardah et al. 2012).

High prevalence of *A. niger* and low levels of OTA production observed in the present study were similar to the findings of Samsudin and Abdullah (2013), where *A. niger* was majorly found to be a non-OTA producer. In Taiwan, *A. niger* was commonly found in green coffee beans but it lacked any OTA production (Leong et al. 2007). Fredj et al. (2009) reported low levels of OTA production by *A. niger* aggregate (consisting of *A. niger* and *A. tubingensis*). The OTA production in grapes was reported only 5% of *A. niger* strains while 80% of *A. carbonarius* strains produced OTA. In Malaysia, some reports on natural occurrence of OTA in association with food products such as white pepper and spices which was probably contributed by OTA-producer, *A. niger* (Ali et al. 2015; Jalili et al. 2010).

The absence of both KS and C-MeT domains and OTA production in 136 isolates of *A. niger* indicated that most of *A. niger* isolates from rice, groundnuts, spices, corn grains, soil and indoor environment were non-OTA producers. Serra et al. (2006) and Susca et al. (2016) described *A. niger* as a low OTA producer while Benford et al. (2001) described it as a less important OTA producer. Existence of non-OTA producing strains of *A. niger* has been previously reported in animal feed, corn and soil (Accensi et al. 2004). The inability of *A. niger* isolates to produce OTA might be caused by gene deletions within the OTA biosynthetic gene clusters (Massi et al. 2016). Frisvad et al. (2007) suggested that the occurrence of silent genes or mutations in the functional or regulatory genes could be responsible for the loss of OTA production. Similarly, a study by Gallo et al. (2012) showed that deletion of a gene from *A. carbonarius* isolate eliminated its ability to produce OTA.

In the present study, *A. aculeatus* was isolated from various sources such as rice, groundnuts, soils and indoor environment. *Aspergillus aculeatus* is also a common contaminant of food and feed that can also be obtained from indoor environment and soil (Dalcero et al. 2002; Kim et al. 2013; Saadabi 2011; Saadullah 2013; Spadaro

et al. 2012; Teh & Latiffah 2015). However, its prevalence was low as compared to *A. niger.* There is a possibility that the substrates of *A. aculeatus* are not as wide as *A. niger*. Besides, there is limited information available for *A. aculeatus,* and it is often misidentified as *A. japonicus* (Parenicová et al. 2001). No *OTA* gene and OTA production was detected in 24 isolates of *A. aculeatus* obtained from various substrates, indicating non-OTA producing nature of this species. In a similar study by Nugroho et al. (2013), *A. aculeatus* was classified as non-OTA producer.

No OTA production was detected in any of the *A. tubingensis* isolates obtained from rice, spices and corn grains. Therefore, it is regarded as a non-OTA producer. This finding corroborated well with the findings of Frisvad et al. (2007), Nielsen et al. (2009) and Varga et al. (2011). However, there are reports for OTA production by *A. tubingensis* (Medina et al. 2005; Perrone et al. 2006). These contrasting reports might be due to misidentification of the species. For instance, six *A. tubingensis* strains deposited in international culture collections by Perrone et al. (2006) were initially described as OTA producer, but were again identified as non-OTA producer by Storari et al. (2012). Moreover, there are many studies where OTA producing strains were denoted by *A. niger* aggregate or black *Aspergillus*, which was used for both *A. niger* and *A. tubingensis*. UPLC analysis showed that only six *A. niger* isolates produced OTA but at low levels. The low levels of OTA production indicated that even though *A. niger* is a prevalent species, but the ochratoxigenic strains of this species are not that prevalent in Malaysia. The differences in the ability of *A. niger* isolates to produce OTA might be caused by the variation in the isolates arising due to differences in the type of substrate as well as the location of occurrence. The absence of OTA genes and OTA production in *A. tubingensis* and *A. aculeatus* isolates in the present study classified these isolates as non-OTA producers. This infrequent occurrence of OTA producing black *Aspergillus* in various substrates and indoor environment might suggest low prevalence of ochratoxigenic forms of black *Aspergillus* in this area. However, accumulation of ochratoxin even at low levels, especially in food might have negative impact on human health.

## Conclusion

The results of this study confirmed three black *Aspergillus* species, *A. niger*, *A. aculeatus* and *A. tubingensis,* were identified from various substrates and indoor environment. Correct species identification of black *Aspergillus* is required to provide early information regarding OTA contamination. The occurrence of black *Aspergillus*, particularly *A. niger*, is relatively high in Malaysia due to favourable growth conditions, but only 4.2% of the *A. niger* isolates produced OTA indicating most of the *A. niger* isolates were non-OTA producers. Since the levels of OTA production were low, it might indicate low occurrence and production of OTA in the field. Even though *A. niger* is not a main OTA-producer, special attention should be given to this species as it is a known for fumonisin $B_2$ production, another mycotoxin having the ability to cause harmful health effects in humans and animals.

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School of Biological Sciences
Universiti Sains Malaysia
11800 USM, Penang
Malaysia

*Corresponding author; email: Lfah@usm.my

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