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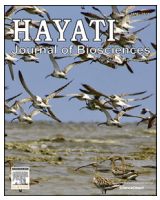

Original research article

Morphological and Molecular Diversity of *Aspergillus* From Corn Grain Used as Livestock Feed

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ABSTRACT

The study of *Aspergillus* from corn grains used as livestock feed is important to ensure the safety of the grains as the occurrence of *Aspergillus* in the corn grain can give an indication of mycotoxin being produced. Morphological and molecular identifications were applied to identify *Aspergillus* isolated from corn grains used as livestock feed. Morphologically, six species were tentatively identified, namely *Aspergillus niger* (Groups I and II), *Aspergillus flavus*, *Aspergillus oryzae*, *Aspergillus fumigatus*, *Aspergillus clavatus* and *Aspergillus terreus*. Isolates of *A. niger* was divided into two groups based on slight differences of their colony appearances. Molecular identification using internal transcribed spacer and β-tubulin sequences supported morphological identification except isolates for *A. niger* Group II isolates, which were molecularly identified as *Aspergillus tubingensis*. Neighbour-joining phylogenetic tree showed that isolates from the same species were grouped in the same clade. The present study showed that diverse species of *Aspergillus* are prevalent in corn grain used as livestock feed. It is also necessary to correctly identify the *Aspergillus* species to employ correct treatment of contaminated corn grains. The occurrence of well-known toxigenic species such as *A. niger*, *A. flavus* and *A. fumigatus* suggested the possible risk of mycotoxin contamination of the corn grain.

1. Introduction

Fungal contamination of corn grains used for livestock feed can be occurred during storage, shipment, and during marketing in the shops and market. Fungal contamination by *Aspergillus* spp. is a worldwide problem, especially corn grains used for livestock feed is imported and exported across the world. The corn grains used for livestock feed in Malaysia is mainly imported from Argentina, India and Brazil (Ghani 2013).

Contamination of corn grains by *Aspergillus* spp. can reduce the quality and nutritional value of the grain as well as lead to mycotoxin contamination. Mycotoxin contamination by toxigenic *Aspergillus* spp. commonly occurs because of high moisture content during storage and presence of insects and mites. Mycotoxin can affect human and animal health as mycotoxin produced can persist in the food chain (Pattron 2006). Therefore, correct species identification and characterization of *Aspergillus* spp. from corn grain are important as it will give clues to the types of mycotoxin produced. Several *Aspergillus* species are well-known mycotoxin producer such as *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus* and *Aspergillus terreus*. The occurrence and contamination of *Aspergillus* in corn grains might pose health risk to the livestock and also to human consuming the livestock products.

To accurately identify *Aspergillus* species to species level, both morphological and molecular identification are applied. Morphological characteristics may not be sufficient as the microscopic and macroscopic characteristics for some species are similar such as the characteristics of black *Aspergillus*. Therefore, internal transcribed spacer (ITS) and β-tubulin sequences are used for molecular identification. ITS region is the universal barcode of fungi and is used for initial identification of *Aspergillus*. β-Tubulin is recommended as secondary identification marker for *Aspergillus* and used mainly for phylogenetic analysis for confirmation of species (Samson *et al.* 2014). Combination of morphological identification and sequencing of ITS and β-tubulin can reliably identify *Aspergillus* isolates to species level. Thus, the present study was conducted to identify *Aspergillus* species contaminated corn grain used as livestock feed using morphological and molecular characteristics.

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2. Materials and Methods

2.1. Isolation and morphological identification of *Aspergillus* isolates

Corn grains used for livestock feed were purchased from 19 sundry shops located in Penang Island, Peninsular Malaysia. From each shop, 100 g corn grains were randomly collected. The corn grains in the sundry shops were kept at room temperature and stored in gunny sacks or storage bins.

For isolation, surface disinfection method by Samson *et al.* (2010) was adopted. About 40 g of corn grains were washed in 360 mL 0.1% ethanol after which the corn grains were disinfected by shaking vigorously in 0.4% freshly prepared sodium hypochloride (house bleach, diluted 1:10) solution for 2 min. About 100 corn grain particles were disinfected at once and rinsed with sterile distilled water. The corn grain particles were plated using sterile forceps onto Malt Extract Agar (MEA) with 5–10 particles per plate, and 10 plates were prepared. The plates were incubated at 27°C for 5 days. Any visible mycelia growth or spores were transferred onto new MEA plates.

Four differential media recommended by Samson *et al.* (2010) were used for morphological identification. The media were MEA, Czapek Yeast Extract Agar (CYA), Dichloran 18% Glycerol (DG-18) Agar and Creatine Sucrose Agar (CREA). The isolates were cultured at three points equidistant from the centre of the plates and identified based on species descriptions by Klich (2002), Samson *et al.* (2007a), Samson *et al.* (2007b) and Samson *et al.* (2010).

2.2. Molecular identification

For molecular identification, 77 isolates were selected from each morphologically identified species as the isolates of each morphologically identified *Aspergillus* species showed the same microscopic and macroscopic characteristics.

The selected isolates were grown in Malt Extract Broth incubated at 27°C for 48 h. The mycelia were then harvested, put on Whatman 2.0 filter paper and kept in a freezer (4°C) overnight before freeze-dried (Labconco, Kansas City, MO, USA) for 48 h. The mycelia were ground using liquid nitrogen into fine powder. The deoxyribonucleic acid (DNA) was extracted from the fine mycelia powder using Invisorb Plant Mini Kit (STRATEC Molecular GmBh, Berlin, Germany) according to the manufacturer's instructions.

2.3. Polymerase Chain Reaction (PCR) amplification of ITS region and β-tubulin gene

The ITS region and β-tubulin gene were amplified using primers ITS1/ITS4 (White *et al.* 1990) and Bt2a/Bt2b (Glass and Donaldson 1995), respectively. For PCR reaction of both ITS and β-tubulin, 25 μL reaction mixture was prepared containing 2.5 μL of PCR buffer (200 mM Tris–HCl), 2.0 μL (2.5 mM) dNTP Mix (Promega, Seattle, WA, USA), 2.0 μL of $MgCl_2$ (2.5 mM), 1.5 μL of each primer (0.5 mM), 0.2 μL *Taq* (5U) DNA polymerase (Promega, Seattle, WA, USA) and 2.0 μL DNA template (5 ng).

PCR amplification of both ITS and β-tubulin was performed in a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA, USA), and run for 35 cycles with an initial denaturation at 92°C for 4 min, denaturation at 92°C for 40 sec, annealing at 55°C for 1 min 30 sec and elongation at 55°C for 2 min and final elongation at 72°C for 5 min.

The amplified bands were subjected to 1% agarose gel electrophoresis, run at 80 V, 400 mA for 60 min. The gel was then stained with ethidium bromide and the bands were viewed under UV light and photographed (Molecular Imager® Gel Doc™ XR System; Bio-Rad, Hercules, CA, USA). The size of the bands was estimated using 100 bp DNA Marker (Fermentas, Vilnius, Lithunia). The PCR products were sent to a service provider for sequencing.

2.4. Phylogenetic analysis

Consensus sequences were obtained from forward and reverse sequences, which were aligned by Clustal W in BioEdit version 7.05 (Hall 1999). The consensus sequences were then BLAST against GenBank database.

Phylogenetic analysis was conducted using Molecular Evolution Genetic Analysis version 5.2 (MEGA 5.2) software (Kumar *et al.* 2008; Tamura *et al.* 2011) by applying neighbour joining (NJ) method on combined ITS and β-tubulin sequences. In the analysis, combined sequences were used to generate phylogenetic tree as the topology, and grouping of the isolates are similar in phylogenetic tree using individual ITS and β-tubulin sequences.

The ex-type strains from each molecularly identified species as described by Samson *et al.* (2014) were used as reference strains in the phylogenetic analysis. The reference strains were *A. niger* NRRL362, *Aspergillus tubingensis* NRRL4875, *A. flavus* NRRL1957, *A. fumigatus* NRRL163, *A. oryzae* NRRL447, *A. terreus* NRRL255 and *Aspergillus clavatus* NRRL1A. Bootstrap analysis using 1000 replications was used to assess the stability of the branches.

3. Results

3.1. Morphological identification

Based on morphological characteristics, 202 isolates were tentatively identified as *A. niger* Group I ($n = 48$), *A. niger* Group II ($n = 68$), *A. flavus* ($n = 32$), *A. fumigatus* ($n = 23$), *A. oryzae* ($n = 14$), *A. terreus* ($n = 15$) and *A. clavatus* ($n = 2$).

The black *Aspergillus* was morphologically identified as *A. niger* based on black colony, biseriate conidial heads and small conidia (2.9–3.9 μm; Figure 1), which was similar with the descriptions of *A. niger* by Klich (2002), Samson *et al.* (2007a) and Samson *et al.* (2010). The *A. niger* isolates were separated into two groups as the colony appearance were slightly different. Group I isolates produced compact black colonies with greyish central region, occasionally produced slightly yellowish colonies and brighter reverse colony appearance, whereas Group II grew maximally on the plate with uniform black to greyish black colonies (Figure 2).

Groups I and II *A. niger* isolates grew well on MEA and CYA, but showed slightly slower growth on CYA37. On DG-18, both groups showed reasonably good growth demonstrates good growth of xerophilic strains. On CREA, both groups have poor growth, but with good acid production (Figure 2).

The *A. flavus* group (including *A. flavus* and *A. oryzae*) were morphologically identified based on yellow-green conidial colour, globose to sub-globose vesicles and biseriate seriations. Morphologically identified *A. oryzae* was characterized by yellowish green to olive green colonies (Figure 3), and *A. flavus* by parrot green to deep green colonies with smaller conidia (3–6 μm) (Figure 3). Although, there were slight differences of the colony appearance from the description by Klich (2002), in general the morphological characteristics of *A. oryzae* and *A. flavus* were similar with the descriptions by Klich (2002) and Samson *et al.* (2010). In this study, colonies of both species appeared compact and floccose, but Klich (2002) described colonies of *A. flavus* as less floccose but compact than *A. oryzae*.

In the present study, both *A. flavus* and *A. oryzae* grew well on MEA and CYA. Sporulation was highly reduced on CYA37 (Figure 4), which was slightly different from the growth described by Klich (2002) and Samson *et al.* (2010) as *A. flavus* and other aflatoxigenic species grew better at 37°C than *A. oryzae* and other non-aflatoxigenic species on the same medium. On DG-18, *A. flavus* grew better than *A. oryzae* (Figure 4), which is consistent with the description by Samson *et al.* (2010). These two species also showed poor growth with no acid production on CREA (Figure 4).

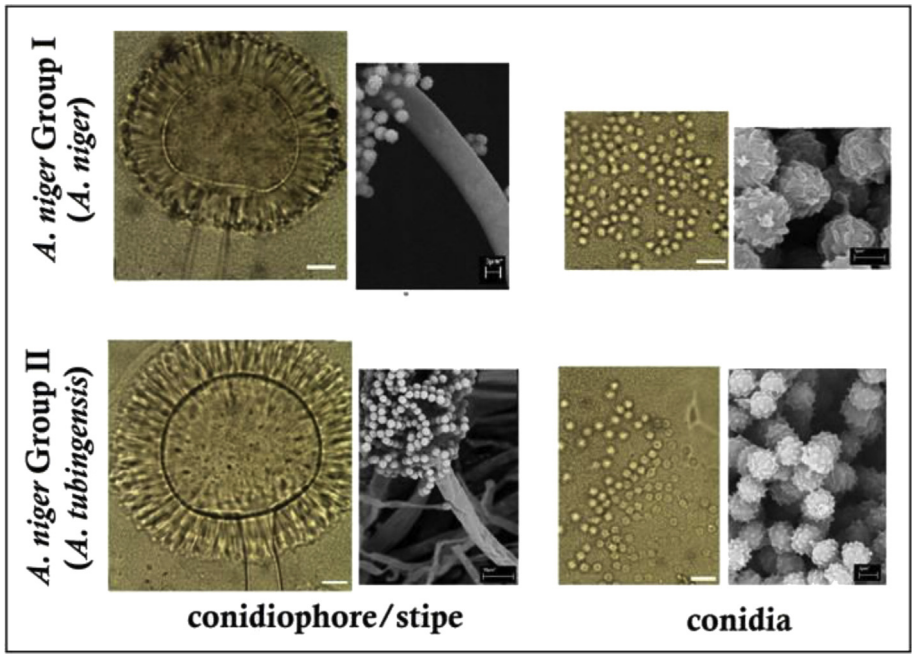

Figure 1. Microscopic characteristics of conidiophores, stipe and conidia of *A. niger* Groups I and II. Scale bar = 10 μm

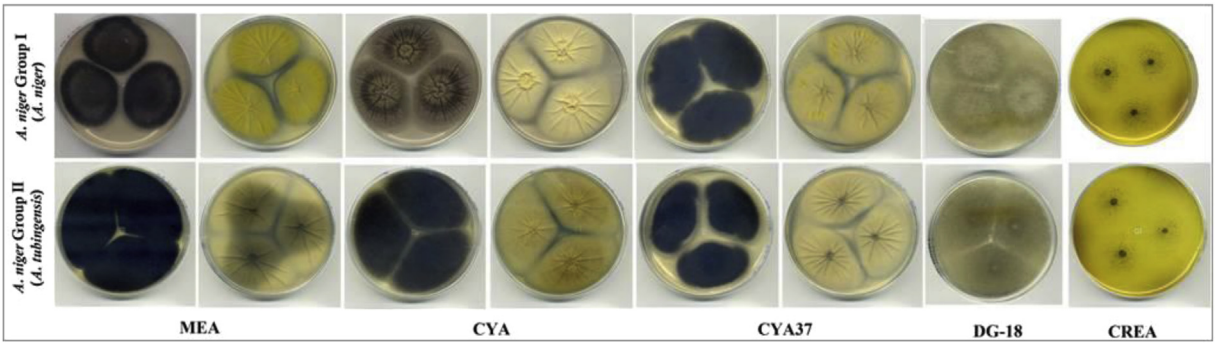

Figure 2. Colony morphology of *A. niger* Groups I and II grown on MEA, CYA, DG-18, and CREA at 27°C, CYA37 at 37°C after 7 days of incubation. CREA = Creatine Sucrose Agar; CYA = Czapek Yeast Extract Agar; DG-18 = Dichloran 18% Glycerol; MEA = Malt Extract Agar.

A. fumigatus isolates were morphologically identified based on velutinous blue-green or dark turquoise colonies and rapid growth on CYA37. Microscopically, *A. fumigatus* isolates have uniseriate conidial heads and curving parallel phialides in a columnar conidial arrangement (Figure 3). These macroscopic and microscopic characteristics were in agreement with the description of *A. fumigatus* by Klich (2002). The *A. fumigatus* isolates grew rapidly on CYA and MEA, but more rapidly on CYA37 (Figure 4). However, poor growth was observed on DG-18 and CREA (Figure 4), which was in agreement with the growth characteristics described by Samson *et al.* (2007b) and Samson *et al.* (2010).

A. clavatus was morphologically identified based on microscopic characteristics of clavate vesicle, uniseriate conidial head with closely packed phialides, radiate conidial arrangement and macroscopically by bright blue green colonies and slow colonies growth (Figure 3). The isolates of *A. clavatus* produced small colony, which indicates slow colony growth, and distinguishable from other species in this study. Although the colonies were small, *A. clavatus* showed good growth on MEA and CYA, and highly reduced sporulation on CYA37 and DG-18 (Figure 4). Isolates of *A. clavatus* also showed good growth with medium acid production on CREA (Figure 4). The microscopic and macroscopic characteristics observed were in accordance with the descriptions of *A. clavatus* by Klich (2002).

The distinguishing morphological characteristics of *A. terreus* were the tan to brown colonies, pyriform vesicles, biseriate conidial heads and columnar conidial arrangement (Figure 3). *A. terreus* produced a good sporulation on MEA and CYA, but sporulation was slightly reduced on CYA37, DG-18 and CREA (Figure 4). The microscopic and macroscopic characteristics observed were in agreement with the descriptions of *A. terreus* by Klich (2002).

3.2. Molecular identification and phylogenetic analysis

All morphologically identified selected isolates showed 100% similarity with ITS and β-tubulin sequences except for *A. flavus* isolates which showed 99% similarity with ITS sequences. In general, molecular identification corroborated with morphological identification except for *A. niger* Group II isolates which were molecularly identified as *A. tubingensis*.

Table 1 shows the molecularly identified *Aspergillus* species recovered from the corn grains.

Based on NJ phylogenetic tree, the isolates of the same species were grouped in the same clade or sub-clade (Figure 5). The NJ tree formed two main clades, I and II with well supported clades and

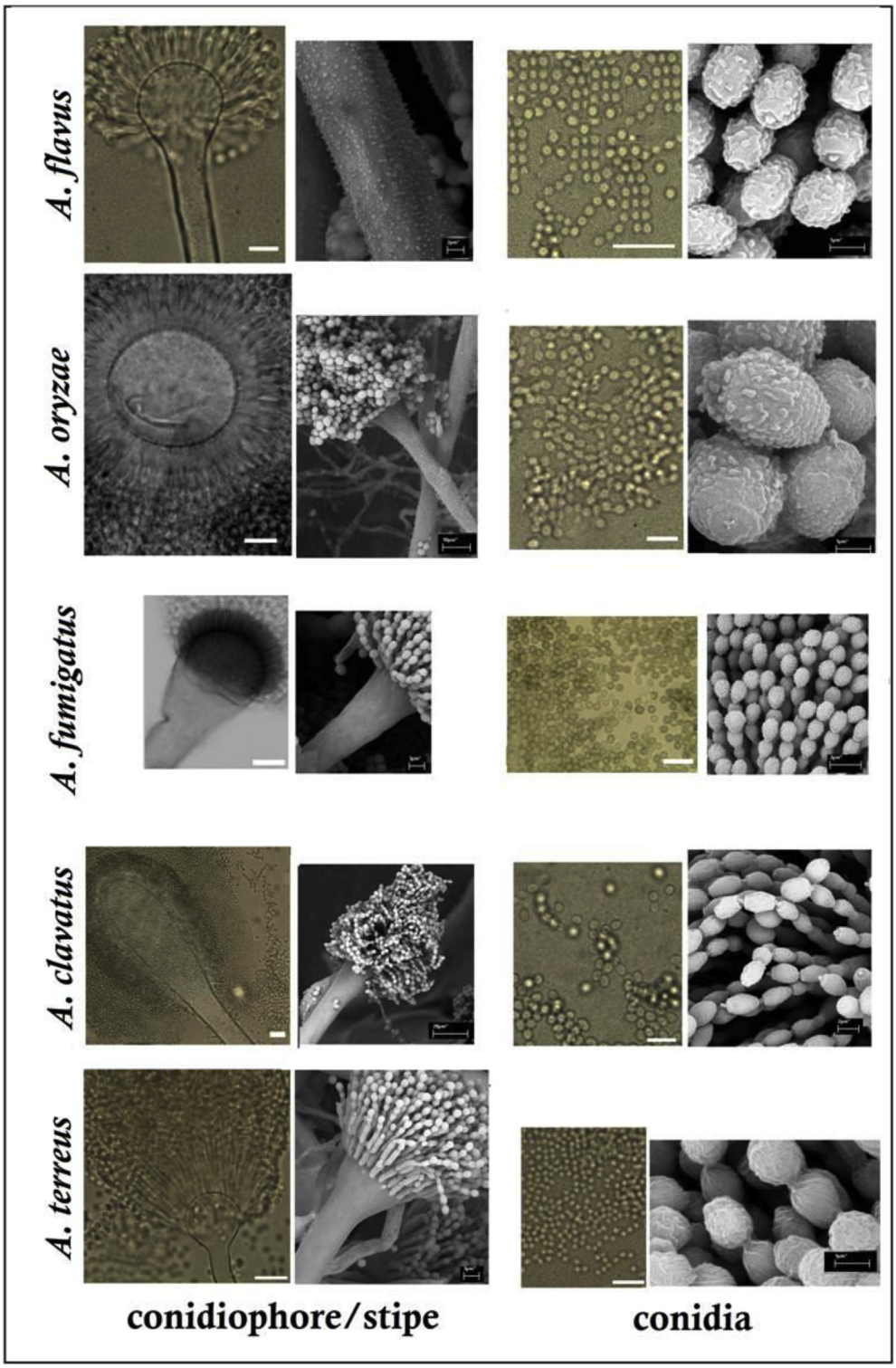

Figure 3. Microscopic characteristics of conidiophore, stipe and conidia of *A. flavus*, *A. oryzae*, *A. fumigatus*, *A. clavatus*, and *A. terreus*. Scale bar = 10 μm.

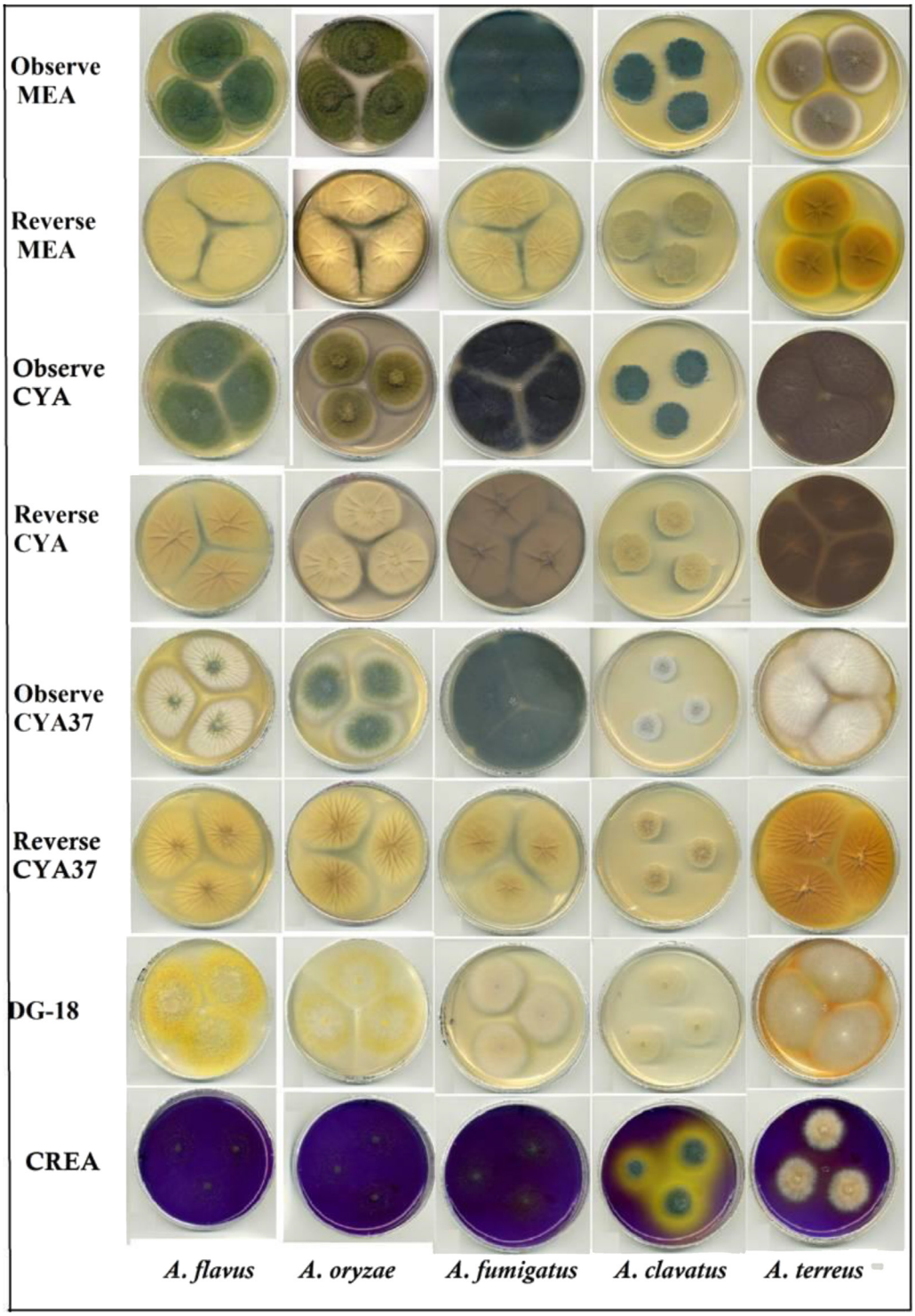

Figure 4. Colony morphology of *A. flavus*, *A. oryzae*, *A. fumigatus*, *A. clavatus*, and *A. terreus* on MEA, CYA, DG-18, and CREA at 27°C, CYA37 at 37°C after 7 days of incubation. CREA = Creatine Sucrose Agar; CYA = Czapek Yeast Extract Agar; DG-18 = Dichloran 18% Glycerol; MEA = Malt Extract Agar.

Table 1. *Aspergillus* species recovered from corn grain with accession number of ITS and β-tubulin sequences

Isolates (species)	Location of sundry shops	Accession number	
		ITS	β-Tubulin
USML03 (*A. tubingensis*)	Pulau Betong	KF669508	KF434104
USMJ02 (*A. tubingensis*)	Balik Pulau	KF669509	KF434116
USML13 (*A. tubingensis*)	Pulau Betong	KF669510	KF434107
USMQ08 (*A. tubingensis*)	Bayan Lepas	KF669452	KF434108
USMQ05 (*A. tubingensis*)	Bayan Lepas	KF669453	KF434106
USMQ03 (*A. tubingensis*)	Bayan Lepas	KF669454	KF434105
USMP06 (*A. tubingensis*)	Teluk Kumbar	KF669455	KF434110
USMN08 (*A. tubingensis*)	Teluk Kumbar	KF669456	KF434102
USMM11 (*A. tubingensis*)	Simpang Empat	KF669457	KF434101
USMM10 (*A. tubingensis*)	Simpang Empat	KF669458	KF434100
USMK07 (*A. tubingensis*)	Pulau Betong	KF669459	KF434099
USMJ13 (*A. tubingensis*)	Balik Pulau	KF669460	KF434112
USMJ11 (*A. tubingensis*)	Balik Pulau	KF669461	KF434117
USMI06 (*A. tubingensis*)	Balik Pulau	KF669462	KF434097
USMI04 (*A. tubingensis*)	Balik Pulau	KF669463	KF434111
USMM05 (*A. tubingensis*)	Simpang Empat	KF669464	KF434098
USMO10 (*A. tubingensis*)	Teluk Kumbar	KF669465	KF434113
USMC09 (*A. tubingensis*)	George Town	KF669466	KF434114
USMP18 (*A. tubingensis*)	Teluk Kumbar	KF669467	KF434109
USMN20 (*A. tubingensis*)	Teluk Kumbar	KF669468	KF434103
USMG08 (*A. tubingensis*)	Sungai Gelugor	KF669469	KF434115
USMI03 (*A. tubingensis*)	Balik Pulau	KF434092	KF434085
USMH06 (*A. tubingensis*)	Balik Pulau	KF434093	KF434084
USMG11 (*A. tubingensis*)	Sungai Gelugor	KF434094	KF434083
USMF11 (*A. tubingensis*)	Sungai Pinang	KF434095	KF434081
USMF07 (*A. tubingensis*)	Sungai Pinang	KF434096	KF434082
USML06 (*A. niger*)	Pulau Betong	KF669432	KF669400
USMQ02 (*A. niger*)	Bayan Lepas	KF669433	KF669403
USML02 (*A. niger*)	Pulau Betong	KF669434	KF669411
USMK10 (*A. niger*)	Pulau Betong	KF669435	KF669410
USME12 (*A. niger*)	Teluk Bahang	KF669436	KF669413
USMF08 (*A. niger*)	Sungai Pinang	KF669437	KF669414
USMC04 (*A. niger*)	George Town	KF669438	KF669404
USMS24 (*A. niger*)	Sungai Pinang	KF669439	KF669402
USMS20 (*A. niger*)	Sungai Pinang	KF669440	KF669409
USMO21 (*A. niger*)	Teluk Kumbar	KF669441	KF669408
USMN04 (*A. niger*)	Teluk Kumbar	KF669442	KF669407
USMG05 (*A. niger*)	Sungai Gelugor	KF669443	KF669405
USMG03 (*A. niger*)	Sungai Gelugor	KF669444	KF669406
USMB20 (*A. niger*)	Pasar Jelutong	KF669445	KF669401
USMD18 (*A. niger*)	Teluk Bahang	KF669511	KF669412
USMC16 (*A. flavus*)	George Town	KF669446	KF669415
USMS06 (*A. flavus*)	Sungai Pinang	KF669447	KF669420
USMO12 (*A. flavus*)	Teluk Kumbar	KF669448	KF669419
USMN03 (*A. flavus*)	Teluk Kumbar	KF669449	KF669418
USMN15 (*A. flavus*)	Teluk Kumbar	KF669450	KF669417
USMM02 (*A. flavus*)	Simpang Empat	KF669451	KF669416
USMJ03 (*A. flavus*)	Sungai Burong	KF434086	KF434075
USMK12 (*A. flavus*)	Pulau Betong	KF434087	KF434076
USMK13 (*A. flavus*)	Pulau Betong	KF434088	KF434077
USMC19 (*A. flavus*)	George Town	KF434089	KF434078
USMG09 (*A. flavus*)	Sungai Gelugor	KF434090	KF434079
USMG16 (*A. flavus*)	Sungai Gelugor	KF434091	KF434080
USME09 (*A. oryzae*)	Teluk Bahang	KF669485	KF669493
USMM03 (*A. oryzae*)	Simpang Empat	KF669486	KF669490
USMN05 (*A. oryzae*)	Teluk Kumbar	KF669487	KF669489
USMO17 (*A. oryzae*)	Teluk Kumbar	KF669488	KF669491
USMM09 (*A. oryzae*)	Simpang Empat	KJ682630	KF669492
USMD06 (*A. fumigatus*)	Teluk Bahang	KF669470	KF669422
USMJ05 (*A. fumigatus*)	Sungai Burong	KF669471	KF669423
USMP16 (*A. fumigatus*)	Teluk Kumbar	KF669472	KF669424
USMJ07 (*A. fumigatus*)	Sungai Burong	KF669473	KF669424
USMP21 (*A. fumigatus*)	Teluk Kumbar	KF669474	KF669426
USMN02 (*A. fumigatus*)	Teluk Kumbar	KF669475	KF669427
USMN10 (*A. fumigatus*)	Teluk Kumbar	KF669476	KF669428
USMP03 (*A. fumigatus*)	Teluk Kumbar	KF669477	KF669429
USMQ20 (*A. fumigatus*)	Bayan Lepas	KF669478	KF669431
USMI02 (*A. fumigatus*)	Balik Pulau	KF669479	KF669430
USMD02 (*A. fumigatus*)	Teluk Bahang	KF669480	KF669421
USMO20 (*A. terreus*)	Teluk Kumbar	KF669494	KF669504
USMO02 (*A. terreus*)	Teluk Kumbar	KF669495	KF669505
USML11 (*A. terreus*)	Pulau Betong	KF669496	KF669502
USMO14 (*A. terreus*)	Teluk Kumbar	KF669497	KF669501
USMR11 (*A. terreus*)	Bayan Lepas	KF669498	KF669500
USMO28 (*A. terreus*)	Teluk Kumbar	KF669499	KF669503
USMA10 (*A. terreus*)	Sungai Ara	KF669506	KF669507
USMO04 (*A. clavatus*)	Teluk Kumbar	KF669481	KF669483
USMO08 (*A. clavatus*)	Teluk Kumbar	KF669482	KF669484

ITS = internal transcribed spacer.

sub-clades (90%–100% bootstrap values). Main clade I can be further divided into sub-clades A, B and C. Sub-clade A consists of *A. tubingensis* (A1) and *A. niger* (A2) isolates. Sub-clade B separates into two groups, B1 and B2, of which B1 consists of *A. flavus* isolates and B2, *A. oryzae* isolates. Sub-clade C consists of *A. terreus* isolates. Main clade II can be further divided into sub-clades D and E, which comprise *A. clavatus* and *A. fumigatus* isolates, respectively.

4. Discussion

In the present study, morphological identification was done according to the method and species description by Klich (2002) and Samson *et al.* (2010), of which *A. flavus*, *A. fumigatus*, *A. clavatus* and *A. terreus* were identified to species level. Only black *Aspergillus* cannot be distinguished using morphological characteristics as the microscopic and macroscopic characteristics observed were very similar. In general, morphological characteristics are still widely used for identification of *Aspergillus* as this method is essential to categorize the isolates according to groups or sections, which allows further identification by other methods.

Aspergillus niger Group II isolates were molecularly identified as *A. tubingensis*. Only slight differences of colony appearance were observed between *A. niger* and *A. tubingensis*, of which *A. tubingensis* (morphologically identified as *A. niger* Group II) produced uniformly black to greyish black colonies. Studies of black *Aspergillus* from different substrates and hosts indicated that *A. niger* and *A. tubingensis* were morphologically similar (Perrone *et al.* 2007; Silva *et al.* 2011), but can be differentiated based on sclerotia production. However, sclerotia were not observed in the present study. According to Samson *et al.* (2004), sclerotia are only present in certain *A. tubingensis* isolates.

Yokoyama *et al.* (2001) reported that isolates of *A. niger* and *A. tubingensis* could not be clearly differentiated using phylogenetic analysis of mitochondrial cytochrome b gene. In the present study, phylogenetic analysis of individual and combined sequences of ITS and β-tubulin separated both *A. niger* and *A. tubingensis* isolates into two different clades, and were grouped with the reference strains (*A. niger* NRRL326 and *A. tubingensis* NRRL4875). The results indicated that phylogenetic analysis using ITS and β-tubulin can be used to group or differentiate *A. niger* and *A. tubingensis* isolates from corn grain samples.

Both *A. flavus* and *A. oryzae* are members of section Flavi, which is often characterized by yellow-green conidial colour or colony appearance with globose to sub-globose vesicles and biseriate seriations (Klich 2002). Both species can be distinguished by the colony appearance, of which *A. oryzae* produced yellowish green to olive green colonies, whereas *A. flavus* displayed parrot green to deep green colonies with smaller conidia (3–6 μm). In the present study, colonies of both species appeared compact and floccose, which were in contrast with the colony appearance described by Klich (2002), of which *A. flavus* colonies are less floccose, but compact than *A. oryzae*. In general, microscopic and macroscopic characteristics of colony colour and conidial sizes can be used to differentiate *A. flavus* from *A. oryzae*. However, studies by Payne

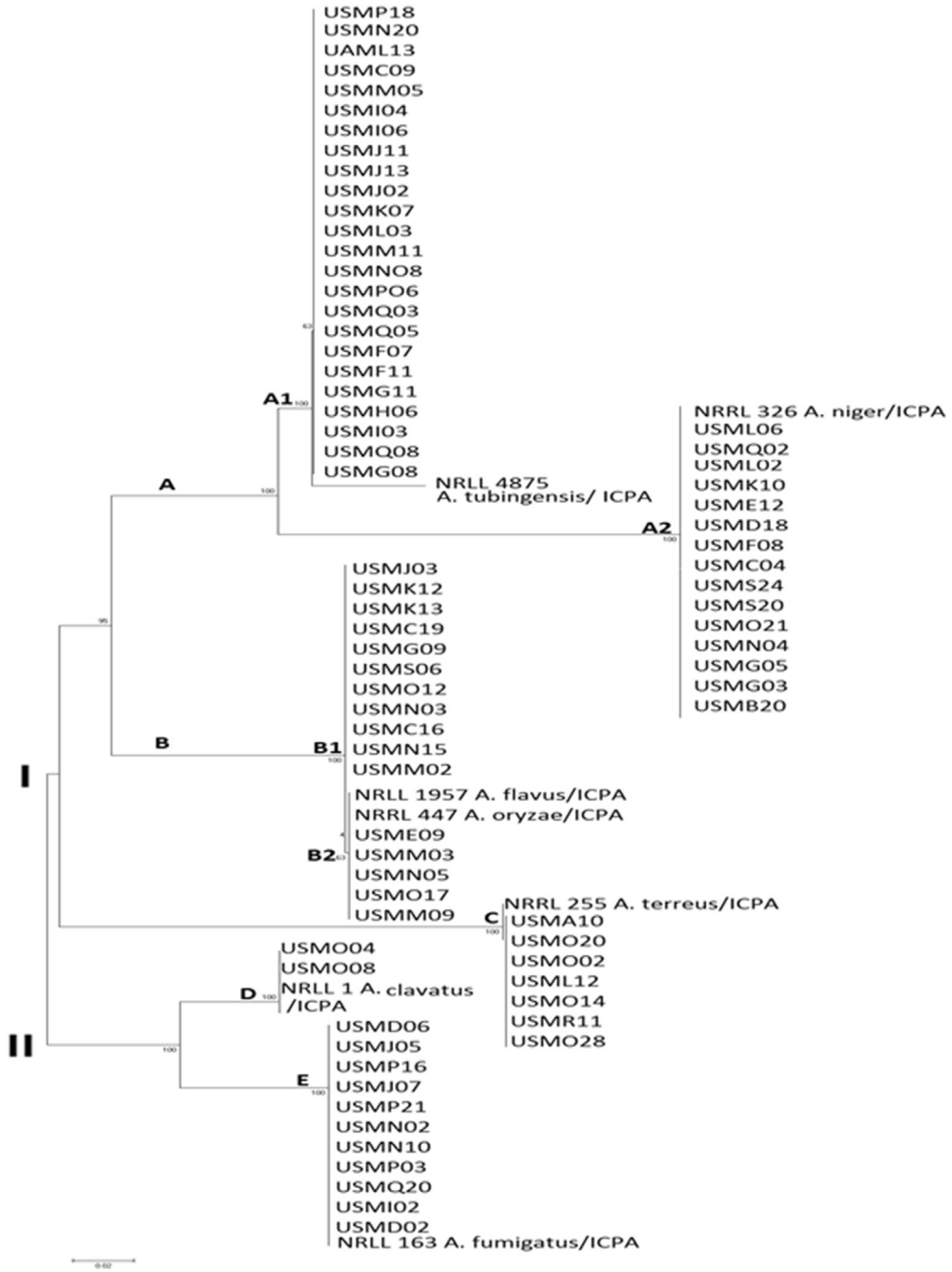

Figure 5. Neighbour-joining tree generated based on combined sequences of ITS and β-tubulin of *Aspergillus* species isolated from corn grain. ITS = internal transcribed spacer.

et al. (2006) and Chang and Ehrlich (2010) indicated that morphological characteristics used to differentiate *A. flavus* and *A. oryzae* can be subtle and can lead to misidentification.

Although isolates of *A. flavus* and *A. oryzae* from corn grains were separated into two separate sub-clades, surprisingly the reference strains (*A. flavus* NRRL1957 and *A. oryzae* NRRL447) were grouped together. Previous studies have reported that *A. flavus* and *A. oryzae* were genetically similar and have a close phylogenetic association (Geiser *et al.* 1998; Chang and Ehrlich 2010). Rodrigues *et al.* (2007) reported that several genes including β-tubulin, calmodulin and topoisomerase II have low variability to separate *A. flavus* and *A. oryzae*, and recommended regulatory gene locus, *afl*R or several structural genes, *pks*A, *nor*-1, *ver*-1, *uvm*8, and *omt*A to be used to separate the two closely related species. Kurtzman *et al.* (1986) also reported 100% DNA hybridization between *A. fla*vus and *A. oryzae*, which showed that both species were genetically similar.

In the present study, phylogenetic analysis of ITS and β-tubulin sequences showed that isolates of *A. flavus* and *A. oryzae* from corn grains were grouped into separate clades. Therefore, both ITS and β-tubulin sequences can be used to differentiate *A. flavus* and *A. oryzae* from corn grain which corroborate with morphological identification.

Based on colony colours, uniseriate conidial heads and the phialides, *A. fumigatus* can be easily differentiated from other species in the present study. However, *A. fumigatus* is

morphologically very similar to other related species such as *Aspergillus lentulus*, *Aspergillus fumigatiaffinis* and *Aspergillus novofumigatus* (Hong *et al.* 2005). In this study, *A. fumigatus* isolates have pyriform vesicle, showed rapid sporulation and rapid colony growth rate, which may eliminate the existence of other related species.

Morphologically identified *A. fumigatus* isolates from corn grain have 100% similarity with *A. fumigatus* NRRL163 reference strain for both ITS and β-tubulin sequences. In phylogenetic tree, all the isolates were grouped in the same clade, and genetic variation was not observed. Low genetic variation among *A. fumigatus* isolates have been reported in several studies using other genes as well as using other molecular methods (Burnie *et al.* 1992; Rinyu *et al.* 1995; Wang *et al.* 2000; Hong *et al.* 2005). Therefore, for identification of *A. fumigatus* isolates from corn grains, morphological characteristics and molecular identification using ITS and β-tubulin sequences should be applied.

Morphologically, *A. terreus* isolates were identified based on tan to brown colonies with columnar biseriate conidial heads. Several studies have also identified *A. terreus* from different substrates based on these macroscopic and microscopic characteristics (Diba *et al.* 2007; Samson *et al.* 2011; Afzal *et al.* 2013) which indicated *A. terreus* from different substrates produced similar morphological characteristics. Two varieties of *A. terreus* which are *A. terreus* var. *aureus* and *A. terreus* var. *africanus* have been reported (Balajee 2009) and produced bright orange colonies or sclerotium-like bodies. In the present study, none of the *A. terreus* isolates from corn grains produced these structures which excluded the presence of the two varieties.

Molecular identification of *A. terreus* isolates corroborated with morphological identification. *A. terreus* isolates showed 100% similarity with *A. terreus* NRRL255 reference strain, and in phylogenetic analysis all the isolates were grouped in the same clade. Phylogenetic studies by Varga *et al.* (2005) and Samson *et al.* (2011) have also separated *A. terreus* into a distinct clade from other *Aspergillus* species.

Aspergillus clavatus isolates from corn grains were identified morphologically by the clavate vesicle. Microscopic character of clavate vesicle has also been used to identify *A. clavatus* from several substrates (Varga *et al.* 2007; Muthomi *et al.* 2012; Krimitzas *et al.* 2013). Clavate vesicle is the prominent feature for identification of species within section Clavati, but other species in this section produced clavate vesicle with different shapes (Varga *et al.* 2007). In this study, molecular identification and phylogenetic analysis of *A. clavatus* corroborated with morphological identification. Phylogenetic analysis of β-tubulin and ITS as well as calmodulin have been applied to separate species in section Clavati into six species including *A. clavatus*, as separate species and synonyms to *Aspergillus apicalis* and *Aspergillus pallidus* (Varga *et al.* 2007). The study suggested that ITS and β-tubulin are among suitable markers to be used for molecular identification of *A. clavatus*.

Several species of *Aspergillus* including *A. flavus*, *A. oryzae*, *A. fumigatus*, *A. clavatus* and *A. terreus* from corn grains can be identified using morphological characteristics, and the identification was in agreement with molecular identification and phylogenetic analysis based on ITS and β-tubulin sequences. Although, morphological identification can be used for species differentiation, molecular identification is still needed for accurate species identity because of the existence of cryptic species (Alastruey-Izquierdo *et al.* 2012), and not all *Aspergillus* species can be identified accurately using macroscopic and microscopic characteristics. Moreover, morphological identification of *Aspergillus* requires skill, extensive knowledge on the microscopic characters and patience as minor differences in medium composition, inoculation technique and incubation conditions can affect the formation of morphological features (Larone 1995; Okuda *et al.* 2000).

The present study showed that diverse species of *Aspergillus* were prevalent in corn grains used for livestock feed. The occurrence of several *Aspergillus* species may be related to high temperature and high relative humidity as Malaysia is located in the tropics. Higher relative humidity as well as conducive temperature can significantly support the growth of *Aspergillus* (Shehu and Bello 2011). Therefore, storage conditions of corn grains need to be under suitable environmental conditions to prevent the growth of *Aspergillus* as well as the growth of other storage fungi.

So far, there is no report of livestock poisoning due to mycotoxin contamination in Malaysia. Nevertheless, the occurrence of toxigenic species such as *A. niger*, *A. flavus*, *A. fumigatus* and *A. terreus* in the present study, suggested the potential risk of mycotoxin contamination of corn grains used as livestock feed. Several studies have reported the occurrence of mycotoxin in feed and livestock products in Malaysia. A study by Reddy and Salleh (2011) reported that 81.2% corn feed was contaminated with aflatoxins, which are commonly produced by toxigenic strains of *A. flavus* and *Aspergillus parasiticus*. Aflatoxins are frequently detected in feed and grains, in meat, dairy cattle milk and milk products, and from other mammals that consume aflatoxin-contaminated feed (Afsah-Hejri *et al.* 2013).

Ochratoxins have also been detected in stored grains and livestock feed, and are commonly produced by toxigenic strain of *A. niger*. Several studies have demonstrated that ochratoxins can be transferred into milk in humans, rabbits and rats, and also blood and internal organs of livestock that fed on contaminated feed (Jorgensen and Petersen 2002; Bhat *et al.* 2010; Afsah-Hejri *et al.* 2013). Healthy cattle may able to tolerate high ochratoxins concentration and if undetected, contaminated dairy products may be consumed by humans (Hult *et al.* 1976). Thus, mycotoxin contamination not only can affect livestock consuming the feed, but also human consuming the livestock products. Even if the level of mycotoxins produced is not sufficient to cause adverse effects, it is an indication that livestock feed contains lower nutrients (Jones *et al.*, 1982).

Mycotoxin contamination not only threatens livestock, but also humans as mycotoxin can resist decomposition, can persist in the food chain and end up in meat and dairy products (Sabran *et al.* 2012). Thus, the occurrence of ochratoxins and aflatoxins in corn grains intended for livestock feed needs to be surveyed and establish livestock feed toxicological risk. In addition, comprehensive regulation, inspection and prevention of fungal growth and contamination in corn grain used for livestock feed are needed. Co-occurrence of mycotoxins is another aspect that needs to be highlighted as most of the co-occurrences of mycotoxin lead to additive or synergistic effects, which can cause significant threat to human and animal health (Smith *et al.*, 2016).

Correct and rapid identification of *Aspergillus* from corn grains are important particularly to detect and identify toxigenic species to employ correct treatment of contaminated corn grains. Therefore, both morphological and molecular identifications need to be applied to avoid species misidentification. A few studies available on fungal contamination and the presence of mycotoxins in livestock grains in Malaysia (Reddy and Salleh 2011; Afsah-Hejri *et al.* 2013; Leong *et al.*, 2014) enhance the contribution of this study, which suggest that there is a potential risk of mycotoxins contamination in corn grains used for livestock feed as several toxigenic *Aspergillus* species were recovered.

Conflict of interest

There is no conflict of interest to declare.

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