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EXPERIMENTAL ARTICLES

Molecular Identification of *Aspergillus* and *Eurotium* Species Isolated from Rice and Their Toxin– Producing Ability[1]

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Abstract—Thirty milled rice samples were collected from retailers in 4 provinces of Malaysia. These samples were evaluated for *Aspergillus* spp, infection by direct plating on malt extract salt agar (MESA). All *Aspergillus* holomorphs were isolated and identified using nucleotide sequences of ITS 1 and ITS 2 of rDNA. Five anamorphs (*Aspergillus flavus, A. oryzae, A. tamarii, A. fumigatus and A. nigef*) and 5 teleomorphs (*Eurotium rubrum, E. amstelodami, E. chevalieri, E. cristatum and E. tonophilum*) were identified. The PCR-sequencing based technique for sequences of ITS 1 and ITS 2 is a fast technique for identification of *Aspergillus* and *Eurotium* species, although it does not work flawlessly for differentiation of *Eurotium* species. All *Aspergillus* and *Eurotium* isolates were screened for their ability to produce aflatoxin and ochratoxin A (OTA) by HPLC and TLC techniques. Only *A. flavus* isolate UPM 89 was able to produce aflatoxins B1 and B2.

Keywords: rice, *Aspergillus, Eurotium*, DNA sequencing, aflatoxin.

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The genus *Aspergillus* is a group of filamentous fungi consist of more than 250 species and the number will continue to grow as scientists discover new species over time. At present, *Aspergillus* is among the most economically important of the fungal genera. They are responsible for production of several toxins, including aflatoxin and ochratoxin A [1].

Identification of the *Aspergillus* species based on the morphological characteristics is not stable because some morphological features do not present in all isolates of a species and their presence can vary among cultures of the same isolate. Besides, physiological characters of *Aspergillus* vary as some metabolites may absent totally in some isolates. Meanwhile, DNA sequence data obtained from molecular approaches are very useful in identification of the *Aspergillus* species. However, there is no strict criterion on the line drawing between phylogenetic species. Therefore, none of the morphological, physiological or molecular methods works perfectly in identification of the *Aspergillus* species [2]. To date, several *Aspergillus* species have been reported in rice. Among them are *E. amstelodami*, *E. chevalieri* [3–5], *A. flavus*, *A. fumigatus*[6–8], and *A. tamarii* [3]. In Malaysia, *A. niger*, *A. candidus*, *A. flavus*, *A. fumigatus* and *A. versicolor* have been reported in rice [8]. Besides, some studies have reported *A. parasiticus* and *A. ochraceus* as the producers of aflatoxin and ochratoxin A, respectively in rice [9, 10]. Rice (*Oryza sativa* L.) is one of the most important staple food crops in Malaysia and approximately 668 000 hectares rice is grown in Peninsular Malaysia [11]. The chemical composition of rice grain make it an ideal substrate for the establishment and growth of some fungal species, especially toxigenic fungi including *Aspergillus* [3]. Moisture content, relative humidity, temperature, period of storage, initial levels of contamination, toxigenic potential of fungal strains influence the production of mycotoxins [12].

Considering the economic and nutritional importance of rice, this research was conducted to determine the *Aspergillus* species contaminating rice under natural conditions in Peninsular Malaysia using nucleotide sequences of the internal transcribed spacers 1 & 2 region of rDNA and determination of their toxin producing ability using thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC), the most common used methods for the detection of mycotoxins [13].

MATERIALS AND METHODS

Collection of Rice Samples. A total of 29 milled rice samples were collected from retailers in four states (Selangor, Perak, Penang and Kedah) of Peninsular Malaysia in October and November of 2008. All samples were stored in nylon bag and kept at 4°C before use.

[1] The article is published in the original.

Isolation of *Aspergilius* spp. From Rice Samples. The *Aspergillus* and *Eurotium* species were isolated from rice grains using agar plating method on malt extract salt agar (MESA: malt extract 20 g, NaCl 75 g, agar 15 g in 1 L distilled water) without surface disinfection. Four hundred seeds of each sample were subjected to MESA plates and incubated at 28°C for 7 days [14].

Extraction of Toxins. The isolates of *Aspergillus* were grown on yeast extract sucrose agar (YES; 2% yeast extract, 15% sucrose, 1.5% agar) as a single colony in the centre of plates and incubated at 28°C for 3 days. Extraction was conducted as described [15, 16] with some modifications. Briefly, colony margins together with adjacent surrounding zones of cultures were scraped into a large test tube (32 × 200 mm) containing 10 ml of chloroform: acetone (85 : 15 v/v). The suspension was left at room temperature (25°C) for 15–20 min and agitated every 5 min using a vortex stirrer. The extract was then filtered through Whatman No. 1 filter paper. The filtrate was evaporated to dryness under 40°C in an air circulated oven dryer. The residue was resuspended in 500 µl of methanol and filtered using a 0.2 µm syringe filter (Whatman, GD/x 13 mm). Samples were stored at 4°C before using for TLC and HPLC tests.

TLC Test. TLC was carried out on a silica gel 60 plate 20 × 20 cm (Merck) using toluene: chloroform: acetone (15 : 75 : 10 v/v) for aflatoxin and acetonitrile: acetic acid: methanol (90 : 5 : 5 v/v) as mobile phases for OTA detection. 5 µl of B_1, B_2; G_1 and G_2 aflatoxin (Supelco, USA) and OTA standards (Sigma-Aldrich, Germany) with concentration of 1 µg ml^{-1} each and 20 µl of test samples were spotted on TLC plates and was run for 45 min in a TLC tank at room temperature. Plates were observed under UV light at 254 and 365 nm.

HPLC Test. Confirmation of TLC results were performed using a simultaneous program for determination of aflatoxin and OTA as described by [17, 18]. The toxins were filtered and 20 µl of each extracted toxin were injected into a Shimadzu Liquid Chromatograph LC–20AT fitted with a fluorescence detector (Shimadzu RF–10AXL) and with a column C18 Wakosil II (SGE Analytical Science, Australia), 250 × 4.6 mm. The HPLC was run using a mobile phase with a flow rate of 1 ml min^{-1}1, consisted of three solvents (A: 100% methanol, B: 100% acetonitrileand, C: water with 0.1% acetic acid). The gradient was 0–12 min isocratic 25% A, 15% B, 60% C, 12–14 min linear gradient to 10% A, 50% B, 40% C, 14–24 min held at 10% A, 50% B, 40% C and immediately returned to 25% A, 15% B, 60% C at minute 24 and followed by a 2 min delay for equilibration. The excitation / emission wavelengths for aflatoxin from 0–14 min and ochratoxin from 21–25 min were 365/455 nm and 330/460 nm, respectively.

Identification of Isolates Using Nucleotide Sequences of ITS1 and ITS2

1. Culture Preparation and DNA Extraction. All *Aspergillus* colonies were prepared on CYA at 28°C for 5 days and DNA was extracted according to Liu et al. [19] with some modifications. Fungal mycelium (200–300 mg) except for *A. niger* and *A. fumigatus* were directly collected from culture plates using a sterile toothpick and added to 1.5 mL Eppendorf tube containing 500 ml of lysis buffer (400 mM Tris-HCl [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaCl, 1% sodium dodecyl sulfate). For *A. niger* and *A. fumigatus*, the cultures were prepared in 50 ml tubes containing 20 ml of Potato Dextrose Broth (Difco) with incubation for 48 h in an orbital shaker (300 rpm) at 28°C. Mycelium was then filtered using Whatman No. 1 filter paper and washed with sterile distilled water, frozen in liquid nitrogen and ground to a fine powder using mortar. The powder was added to 1.5 ml Eppendorf tube containing 500 ml of lysis buffer.

The tube was left at room temperature for 10 min. After adding 150 ml of potassium acetate (pH 4.8), the tube was vortexed briefly and spun at 10000 *g* for 1 min. Then, the supernatant was transferred to a new 1.5 ml Eppendorf tube and an equal volume of phenol chloroform isoamyl alcohol (25 : 24 : 1) was added. The tube was vortexed briefly and spun at 10000 *g* for 1 min. This method was repeated before transferring the supernatant to another 1.5 ml Eppendorf tube. It was followed by addition of an equal volume of isopropyl alcohol and the suspension wasmixed by inversion briefly. The tube was spun at 10000 *g* for 3 min andthe supernatant was discarded. The DNA pellet was washed in 300 ml of 70% ice-cold ethanol. After the pellet was spun at 10 000 rpm for 3 min, the supernatant was discarded and this step was repeated. Lastly, the DNA pellet was air dried and dissolved in 50 µl of deionized water. The quality of DNA was determined by agarose gel electrophoresis.

2. PCR Amplification and DNA Sequencing. The amplification of ITS1–5.8S—ITS2 regions of rDNA was performed in 50 µ1 PCR Master Mix (Fermentas, Canada) consisting of 200 nM oligonucleotide primers and 1 µl of DNA template. Oligonucleotide primers ITS1 (5'–TCC GTA GGT GAA CCT GCG G–3') and ITS4 (5'–TCC TCC GCT TAT TGA TAT GC–3') were used [20]. The amplification was performed in a thermal cycler (Biometra® T3, Syngene, UK) programmed for pre-denaturation of 5 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 55°C and 2 min at 72°C. After a final extension of 5 min at 72°C, the samples were cooled to 4°C. The PCR products were analyzed in 1% (w/v) agarose gel in 1 X TBE buffer (Tris-Borate-EDTA, Sigma). The gel was stained in 0.5 µg ml^{-1} ethidium bromide solution and the bands visualized and photographed under UV light using gel documentation system (Syngene, UK). The DNA fragment was purified from the agarose gel using DNA

Table 1. *Aspergillus* and *Eurotium* species isolated from, rice based on classification reported by Balajee (2008) and Peterson et al. (2008)

Genus	Subgenus	Section	Species	Isolates[1]
Aspergillus	*Aspergillus*	Aspergillus	*E. amstelodami*	UPMC 101 and UPMC 106
			E. chevalieri	UPMC 100 and UPMC 104
			E. cristatum	UPMC 102
			E. rubrum	UPMC 98, UPMC 103 and UPMC 105
			E. tonophilum	UPMC 99
	Fumigati	Fumigati	*A. fumigatus*	UPMC 90
	Circumdati	Flavi	*A. flavus*	UPMC 89 and UPMC 92
			A.oryzae	UPMC 95
			A. tamarii	UPMC 93 and UPMC 94
		Nigri	*A. niger*	UPMC 91 and UPMC 96

[1] Identified isolates were deposited in Institute of Bioscience, Microbial Culture Collection Unit (UNiCC), Universiti Putra Malaysia and given codes.

Table 2. Differences in nucleotide numbers in ITS 1 – 5.8S – ITS 2 among *Aspergillus* and *Eurotium* species isolated from rice

Isolate	Accession number	Number of nucleotides (bp)			
		ITS1	ITS2	ITS1-5.8-ITS2	Size (bp)
A. flavus UPM A2	GU 076485	181	169	506	597
A. fumigatus UPM A4	GU 205082	185	168	509	600
A. niger UPM A7	GU 362012	177	169	502	561
A. flavus UPM A8	GU 172440	166	169	491	550
A. tamarii UPM A9	GU 362011	172	171	499	518
A. tamarii UPM A16	HM 116372	183	171	510	588
A. oryzae UPM A22	HM 145964	182	169	507	602
Eurotium rubrum UPM A6	HM 116370	143	167	466	557
E. tonophilum UPM A10	GU 564511	143	167	466	560
E.chevalieri UPM A11	HM 152566	143	167	466	559
E. amstelodami UPM A12	GU 723274	143	167	467	542
E. cristatum UPM A13	GU 784865	141	167	464	532
E. rubrum UPM A14	HM 152565	143	167	466	561
E. chevalieri UPM A15	HM 116371	143	167	466	560
E. rubrum UPM A17	HM 145962	143	167	466	555
A. niger UPM A18	HM 776522	177	169	502	561
E. amstelodami UPM A19	HM 145963	143	167	466	555

extraction kit (Fermentas, Canada) according to manufacturer. DNA sequencing was performed at the Medigen Sdn Bhd on a DNA sequencer machine using the ITS1 and ITS4 PCR primers following the protocol supplied by the manufacturer.

3. Sequence Analysis. Sequences from all isolates were aligned using ClustalW 1.8 [21]. Aligned sequence sets were analyzed by maximum parsimony using heuristic search option in Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 [22]. Bootstrap values were determined using heuristic searches with 2000 replications.

RESULTS

Identification of *Aspergillus* and *Eurotium* Species. Amplification of the contiguous region from 3 terminal bases of the 18S rDNA through ITS1, the 5.8S rDNA, ITS2 and part of the 28S rDNA from 17 *Aspergillus* and *Eurotium* isolates generated PCR products ranging in size from 518 to 602 bp (Table 2).

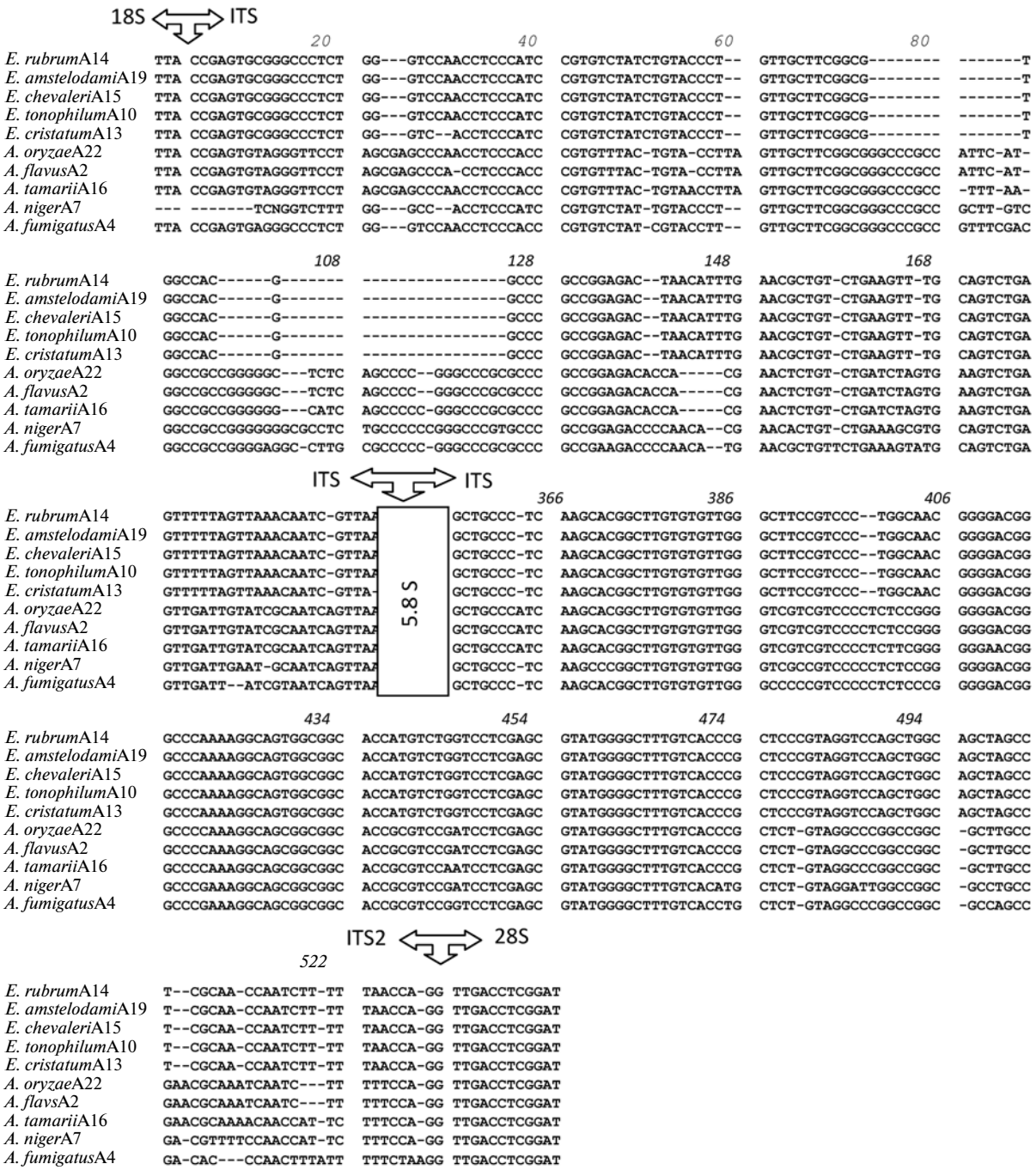

Fig. 1. Nucleotide sequence alignment of *Aspergillus* isolates A2, A4, A7, A16 and A22; *Eurotium* isolates A10, A13, A14, A15 and A19 using Biology Workbench software version 3.2 (Subramaniam 1998). The alignment consists of the 3' end of the 18S rDNA gene, the complete ITS 1, ITS 2 and 5' end of the 28S rDNA gene. The highly conserved 5.8S rDNA gene sequence has been omitted.

All pure fungal cultures and DNA sequences were deposited in the Microbial Culture Collection Unit (Table 1) and GenBank database, respectively (Table 2). ClustaIW (1.8) multiple sequences alignment of *Aspergillus* and *Eurotium* sequences demonstrated that sequence diversity due to insertions or deletions existed in the ITS 1 and ITS 2 regions between the *Aspergillus* and *Eurotium* genera (Fig. 1). The ITS 1 region displayed more variation consisting single-nucleotide and short lengths of sequence diversity (15 and 30 nucleotide sequences from 70–85 and 92–121, respectively in ITS 1 region) as shown in Fig. 1.

All sequences determined in this study yielded top-ranking BLAST scores at the time of this study. None-

theless, analyses of *Eurotium* spp. sequences were indefinite at species level because similar GenBank reference sequences existed for different organisms. A sequence similarity of 98–100% was observed between most *Eurotium* isolates and those obtained from Gene Bank. A lowest similarity was found between *E. cristatus* and others (a deletion of 3 nucleotides in position 26, 27 and 198 in *E. cristatum*).

Amongst the *Aspergillus* anamorphs, the greatest similarity was observed between *A. flavus* and *A. oryzae*, with only 1 nucleotide deletion at sequence number 26 in ITS 1 region (Fig. 1). Although most *Aspergillus* taxonomists believed that *A. oryzae* is a domesticated form of *A. flavus* our experiment has showed that *A. flavus* isolate A2 was able to produce aflatoxin B while *A. oryzae* did not produce aflatoxin under same conditions. *A. fumigatus* sequence alignment showed more similarity to *A. niger* (83%) than *A. oryzae* (80%) and *A. tamarii* (81%), specifically in ITS 1 region.

The evolutionary history was inferred using Maximum Parsimony methods (Fig. 2). The *P. turbatum* NRRL 759 was used as the out-group species. A high bootstrap support (99 and 100%) was found for the branch leading to the clusters of Section Nigri and Flavi (Fig. 2). Furthermore, the uniseriate species (*Eurotium* spp. and *A. fumigatus*) were clearly separated from biseriate species (*A. niger*, *A. flavus* and *A. tamarii*). As shown in Fig. 2, all 5 *Eurotium* species (*E. amstelodami, E. chevalieri, E. cristatum, E. rubrum* and *E. tonophilum*) form a well-supported clade (Section Aspergillus) and, according to ITS1-5.8S rDNA-ITS2 sequences, would represent one species as no differences were observed among the sequences of these species. Our results also showed that the PCR-sequencing technique for sequences of ITS 1 and ITS 2 of the rDNA did not work flawlessly for differentiation of *Eurotium* species.

Toxin-Producing Ability of *Aspergillus* and *Eurotium* Species. The ability of all *Aspergillus* and *Eurotium* species used in this experiment in producing aflatoxin was analyzed using 2 HPLC and TLC techniques. The Rf for the aflatoxin B2, B1 standards and OTA standards in TLC technique were 0.50, 0.55 and 0.87 respectively. The Retention time (RT) for aflatoxin B2, B1 and OTA in HPLC method were 16.068, 18.301 and 26.259 respectively. The results obtained were in agreement with each other. Only *A. flavus* isolate UPM 89 was found to produce aflatoxin B2 and Bl (367 and 91 μg ml^{-1} respectively). None of the other isolates tested gave any positive results.

DISCUSSION

Aspergilli are truly diverse organisms and identification of *Aspergillus* isolates based on the phenotype has been plagued by many factors including the presence of overlapping morphological features among closely related species. In contrast, molecular methods for differentiation of species within the genus *Aspergillus* using DNA sequence based methods and their potential that can be used for genus and species level identification has entered an exciting era [23]. In this study, we identified the *Aspergillus* and *Eurotium* species isolated from milled rice in Malaysia using the ITS 1-5.8S-1TS2 sequences as targets for the differentiation of these species.

The genus *Aspergillus* was divided into 7 subgenus, subgenus was divided into sections and species were placed within the sections [23, 24]. Based on this classification, the members of the genus *Aspergillus* and *Eurotium* which were identified in present study were listed in Table 1. The present investigation revealed 65% of tested J rice samples collected from different regions in Malaysia was infected by *Aspergillus* or *Eurotium* species. The frequency of *Aspergillus* and *Eurotium* genus among tested samples was similar, 50% of tested samples were contaminated by *Aspergillus* spp. and the remainder of 50% by *Eurotium* spp. *A. flavus* and *A. niger* with 17% contamination in rice samples were predominant species among the *Aspergillus* spp. These results confirmed previous observations [6, 9, 10] which have reported *A. flavus* and *A. niger* as the predominant fungus in the grain samples of paddy. The presence of *Eurotium* spp. in rice samples also was reported [5, 9]. A survey in Southeast Asia showed that *A. niger*, *A. candidus*, *A. flavus*, *A. fumigatus* and *A. versicolor* were the predominant species on milled rice in Malaysia [8]. The results obtained in our study were different from those obtained previously [8]. In these sampling regions, *A. candidus* and *A. versicolor* were not isolated and the *Eurotium* spp. was the most prevalent species (Table 1).

In the analyses of DNA sequences, most of the ITS amplicons showed a consistent sequence length among species of the same section in one or both spacer regions. However, sequences of *A. flavus* and *A. tamarii* within Section Flavi showed an inconsistent sequence in ITS 2 region. The *Eurotium* species have shown a fixed sequence number in both ITS 1 and ITS 2 regions (Table 2).

However, ITS 1 and ITS 2 of the rDNA sequence analysis were reliable for Sections differentiation and for species within sections at times. *A. flavus* and *A. tamari* (Section Flavi), *A. fumigatus* (Section Fumigati) and *A. niger* (section Nigri) were differentiable from each other using DNA sequences of ITS regions (Fig. 2). Differentiation between some of the more closely related *Aspergillus* species, especially among *Eurotium* may require analysis of other targets such as the ribosomal external transcribed spacer regions [23].

Only 4% of tested samples was contaminated by an aflatoxin producing strain (*A. flavus* UPMC 89). Despite several reports on aflatoxin production by

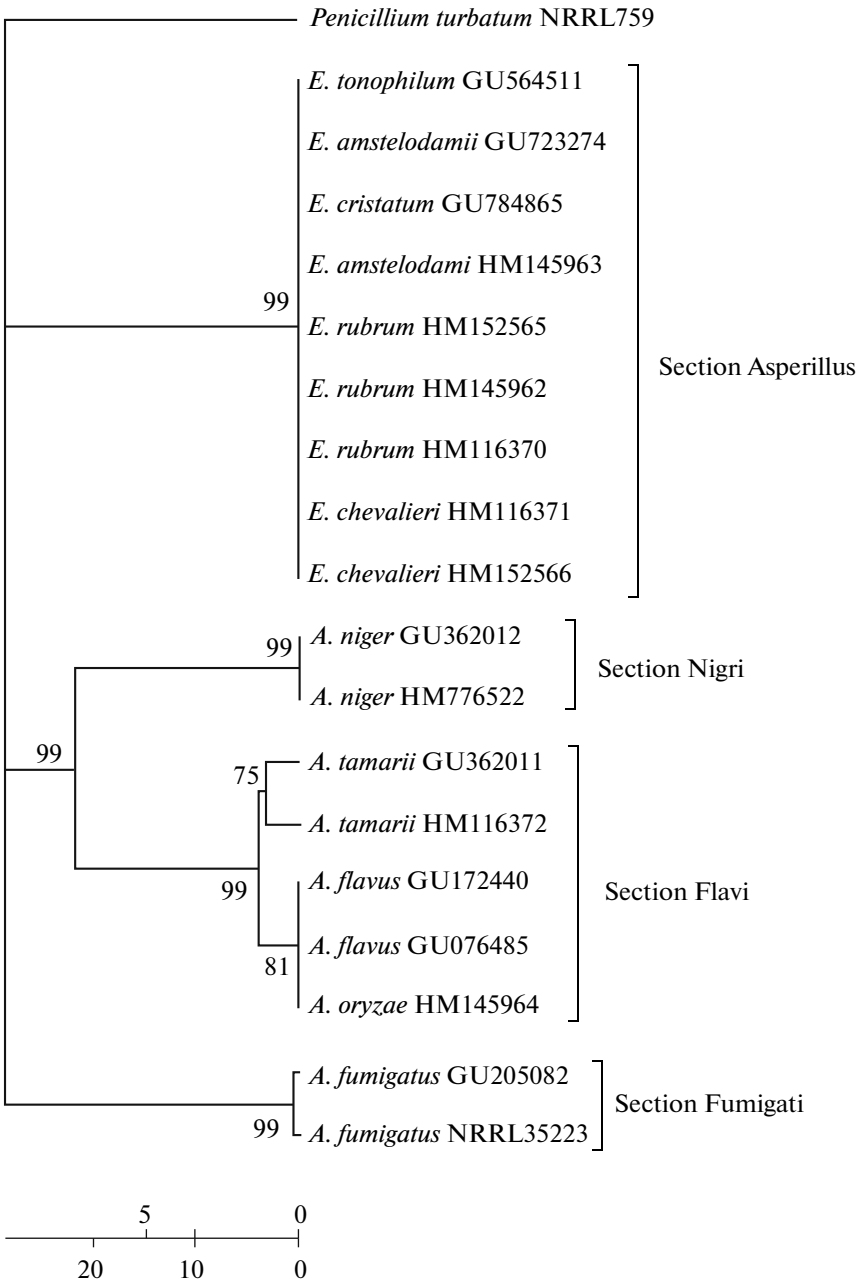

Fig. 2. Maximum parsimony tree of *Aspergillus* and *Eurotium* islates based on the ITS1, 5.8S rDNA, ITS2 and 28S rDNA (partial) sequences, using *P. turbatum* NRRL 759 and *A. fumigatus* NRRL 35223 as the outgroup and reference species respectively. A most parsimonious tree was generated using MEGA 4.0 with the heuristic search of the data and 2000 bootstrap samples option. The consistency index (CI) was 0.9078, retention index (RI) was 0.9735 and the rescaled consistency index (RCI) was 0.8754. Numbers over tree nodes are the bootstrap value. The scale bar at the bottom refers to branch length in terms of the number of steps in the tree construction.

A. tamari [25], this isolate was not found to produce aflatoxin in this study, concurring with the findings of Frisvad et al. [27]. Our findings concur with previous reports [26, 27] demonstrating that all *Eurotium* species tested were unable to produce aflatoxin or OTA within the incubation period. The *Eurotium* species are xerophilic fungi and although are not able to produce aflatoxin or OTA, but they are common on mould damage and produce several metabolites such as neoechinulin A, B and echinulin [28]. *E. chevalieri* is heat resistant so that 18–25% of ascospore can survive 10 minutes of heating at 70°C and was reported to cause spoilage on different kinds of dried food product [29].

Sixty-five percent of tested milled rice was contaminated by *Aspergillus* or *Eurotium* species. The isolates

belonging to Section Aspergillus and Flavi were dominant amongst the identified species. This is the first report of *A. tamari, E. rubrum, E. tonophilum, E. chevalieri, E. amstelodami* and *E. cristatum* isolated from milled rice in Malaysia. None of isolates tested gave any positive results against OTA test, but 4% of tested samples were contaminated by an aflatoxin producing strain (*A. flavus* UPMC 89). Sequence analysis of ITS1-5.8S-ITS2 regions using ITSl and ITS4 primers are reliable for identification of *Aspergillus* in species level among Sections: Fumigati, Flavi and Nigri, but differentiation among *Eurotium* species in Section *Aspergillus* requires analysis of other targets such as the ribosomal external transcribed spacer regions.

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