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Isolation and identification of *Eurotium* species from contaminated rice by morphology and DNA sequencing

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ABSTRACT

30 milled rice samples were collected from retailers in four states of Malaysia. These samples were evaluated for *Eurotium* spp. contaminations by direct plating on malt extract salt agar (MESA). All *Eurotium* were isolated and identified based on morphology and nucleotide sequences of internal transcribed spacer 1 (ITS1) and ITS2 of the rDNA. Four *Eurotium* species (*E. rubrum, E. amstelodami, E. chevalieri* and *E. cristatum*) dominated seed samples were identified. The main characteristics for morphological differentiation of *Eurotium* species were colony features on different culture media and ascospore surface ornamentations. The PCR-sequencing technique for sequences of ITS1 and ITS2 is a fast technique for identification of *Eurotium* species, but did not work perfectly for differentiating *Eurotium* species from each others. DNA sequence analysis showed a fixed sequence numbers in both ITS1 and ITS2 regions. These results suggest that sequencing of ITS regions could support morphological characteristics for identification of *Eurotium* species.

1. Introduction

Eurotium is one of the teleomorph of the genus *Aspergillus*. Teleomorphs of *Aspergillus* species are considered to belong to different genera of family Tricomaceae of the order Eurotiales, class Eurotiomycetes (Peterson et al., 2008), Phylum Ascomycota (Webster and Webster, 2007). Recent revisions of the Botanical Code have increasingly shown the advantage of the sexual names over the asexual names. By definition, *Aspergillus* is a name referring to the asexual phase and, therefore, according to current rules of nomenclature, any *Aspergillus* with a sexual stage (teleomorph) no longer should be called *Aspergillus* (Baker and Bennett, 2008; Machida and Gomi, 2010).

Eurotium species are saprotrophic and represent some of the most catabolically and anabolically diverse microorganisms known. Some species are capable of growing at extremely low water activities (i.e. xerotolerant and / or osmotolerant), low temperatures (psychrotolerant) and high temperatures (thermotolerant). These properties,

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combined with the ability to produce diverse sets of toxic secondary metabolites such as aflatoxins, ochratoxins and patulins, make these fungi important agents of food spoilage (Geiser, 2006). The chemical composition of rice grain make it an ideal substrate for the establishment and growth of some fungal species, especially toxigenic fungi including *Eurotium* (Lima et al., 2000).

Although molecular methods continue to improve and the advantages of PCR based identification are recognised, microscopy and cultural methods remain the primary laboratory tools for detecting *Aspergillus* (McClenny, 2005). However, morphological characters are not stable, because some morphological features are not always present in all isolates of a species, and also their presence can vary among cultures of the same isolate. Therefore, there is no one method (morphological or molecular) that works perfectly for recognizing *Aspergillus* species (Geiser et al., 2007).

Several *Aspergillus* species have been reported from rice. *Aspergillus niger, A. candidus, A. flavus, A. fumigatus* and *A. versicolor* were reported from rice in Malaysia (Udagawa, 1976), *E. amstelodami* and *E. chevalieri* from paddy grain in Egypt (Abdel-Hafez et al., 1987), in Brazil (Lima et al., 2000), in Uganda (Taligoola et al., 2004) and in USA (Vesonder et al., 1988). Capability of toxin production by *Eurotium* species including *E. rubrum, E. repens* (El-Kady et al., 1994) and *E. amstelodami* (Senyuva et al., 2008) have also been reported. However, previous study (Yazdani et al., 2009) showed that *Eurotium* species were unable to produce aflatoxins or OTA within the incubation period.

Rice (*Oryza sativa* L.) is one of the most important staple food crops in Malaysia. About 668000 hectares of rice are grown in Peninsular Malaysia (FAO, 2010). Considering the economic and nutritional importance of rice, this research was conducted to determine *Eurotium* species that contaminate rice under natural conditions based on morphological characteristics and confirmed by nucleotide sequences of the internal transcribed spacers 1and 2 region of rDNA.

2. Materials and Methods

2.1. Collection of samples

The rice consisted of milled rice collected from retailers in four states (Selangor, Perak, Penang and Kedah) of Peninsular Malaysia sampled in October and November of 2008. All samples were stored in polyethylene bags and kept at 4°C before use.

2.2. Isolation of Eurotium spp. from rice samples

The *Eurotium* spp. were isolated from rice seed samples by the method of Pitt and Hocking (2009) using malt extract salt agar (MESA: malt extract 20g, NaCl 75g, agar 15g in 1L distilled water) without surface disinfection. Four hundred seeds of each sample were cultured on MESA plates and incubated at 28°C for 7 days and purified *Eurotium* colonies were subsequently subcultured on different media.

2.3. Morphological identification

The *Eurotium* strains were grown on 9 cm plastic plates on Czapek Dox agar (CZA; Oxoid), Czapek yeast agar (CYA: CZA 45.4 g , yeast extract 5 g in 1L distilled water) and Czapek sucrose agar (CS20%: CZA supplemented with 20% sucrose) media for 10-15 days at 28°C. Colonies growth rates and microscopic feature were examined. Each species was identified based on specific keys described by Raper and Fennell (1973), Klich (2002) and complementary description was reported by Geiser (2006) and Varga and Samson (2008).

2.4. DNA sequencing identification Isolation and amplification of DNA

All *Eurotium* colonies were cultured on CS20% at 28ºC for 15 days and total DNA were extracted as described by Liu et al., (2000). Fragments containing the ITS region were amplified using two oligonucleotide primers ITS1 and ITS4 (White et al., 1990) to amplify ITS1-5.8S - ITS2 regions of rDNA. Primers were synthesized by Bio Basic Inc. Ontario, Canada. The PCR assay was performed with 1 µl of DNA template in a total reaction

volume of 50 µl PCR buffer (PCR Master Mix, Fermentas International Inc., Canada).

Thirty five cycles of amplification were performed in Biometra T3 Thermocycler, after initial denaturation of DNA at 95°C for 5min. Each cycle consists of a denaturation step at 95°C for 1min, an annealing step at 55°C for 1 min, and an extension step at 72°C for 2 mins, followed by a final extension step at 72°C for 5 mins. The PCR products were electrophoresed on agarose gel 1% (w/v) immersed in TBE 1x buffer (Tris-Borate-EDTA, Sigma) and stained with ethidium bromide (0.5 µg/ml) and photographed under UV light using gel documentation system (Syngene, UK). The DNA fragment was purified from the agarose gel using DNA extraction kit (Fermentas, Canada). DNA sequencing was performed by Medigen Co. Ltd on a DNA sequencer machine using the ITS 1 and ITS 4 PCR primers with protocols supplied by the manufacturer.

2.5. Sequence analysis

Sequence analysis of *Eurotium* species identification were conducted by comparing the DNA sequences against those available in the NCBI GenBank database using a BLASTN search. For phylogenetic analysis, the DNA sequences from all isolates were aligned using ClustalW 1.8 (Thompson et al., 1994) and performed by both neighbor-joining and maximum parsimony in Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (Tamura et al., 2007). Bootstrap value was determined using heuristic searches with 2000 replications. The DNA sequences of ITS region of the examined species were deposited into the GenBank database.

3. RESULTS

3.1. Morphological identification

Seven *Eurotium* spp. were identified based on cultural and microscopic characteristics. The identified isolates were deposited in the Microbial Culture Collection Unit (UNiCC), University Putra Malaysia (Table 1).

Table 1 *Eurotium* species isolated from rice samples

Species	Isolates[1]
E.chevalieri	UPMC A11 and UPMC A15
E. amstelodami	UPMC A12 and UPMC A19
E. cristatum	UPMC A13
E. rubrum	UPMC A14 and UPMC A17

1 Deposited at the Microbial Culture Collection Unit (UNiCC), University Putra Malaysia.

The *Eurotium* species showed low growth rates (3-4cm/10days) with gold yellow to yellowish green color on different media tested. CS20% medium increased growth rates and stimulated conidial head production (Fig 1). The colony color on CS20% after 10-15 days was used as one of the markers for differentiation of species. *E. rubrum* and *E. amstelodami* produced abundant conidial head on CS20% after 7 days incubation. However, only *E. rubrum* is able to produce pink pigments on CS20% media after 10-15 days. *E. cristatum* and *E.chevalieri* lacked and produced limited conidial heads on CS20% after 7 days and are not able to produce red pigments on this media.

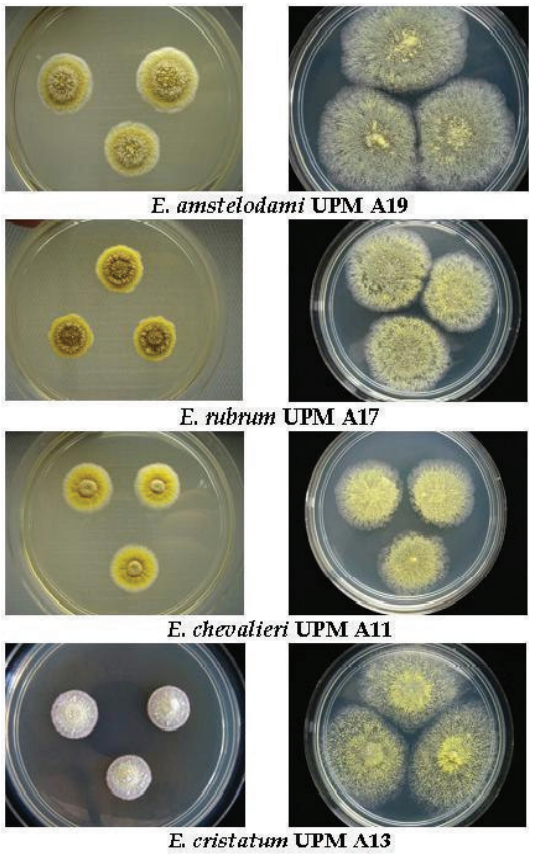

Fig 1. Colony features of *Eurotium* species on MEA (left) and CS20% (right) after 7 days incubation at 28°C

Microscopic characteristics of *Eurotium* species were closely similar and the main markers for differentiation were conidia size and ascospore surface ornamentations. Using identification keys by Raper and Fennell (1973) as reference, a simplified differentiation key based on microscopic characteristics was used in this study for determining species (Table 2).

Table 2. Differentiation keys for identification of *Eurotium* spp. isolated from rice

Ascospore size and surface texture	Equatorial rigids	Colony features on CS20% after 15days incubation		Species
		Conidial head	Cleistothecium	
≤6 µ Smooth or nearly so	Low and rounded, furrow broad and shallow	More or less abundant, pale gray-green	Very abundant, orange-red to red	*E.rubrum*
	Thin and flexuous, ascospore resembling a pulley	abundant , gray-green shade	Yellow, surrounded by orange-red to brown hyphae	*E. chevalieri*
≥6 µ Roughened	V-shape furrow flanked by irregular ridges	Abundant at colony center, olive green	Very abundant, bright yellow	*E. amstelodami*
	Two well seperated and nonflexuous equatorial crests	Lacking or very rare	Abundant, honey yellow to light brownish olive	*E. cristatum*

Table 3. Differences in nucleotide numbers in ITS 1– 5.8S – ITS 2 among *Eurotium* species isolated from rice

Isolate	Accession number	Number of nucleotides (bp)			
		ITS1	ITS2	ITS1-5.8- ITS2	Size (bp)
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
E. amstelodami UPM A19	HM 145963	143	167	466	555

Table 4. Similarity between sequences ***recorded in GenBank*** and ***those*** generated in this study

Eurotium spp. in this study	*E.amstelodami*		*E.rubrum*		*E.chevalieri*		*E.cristatum*	
	Cov.%	Idn.%	Cov.%	Idn.%	Cov.%	Idn.%	Cov.%	Idn.%
UPM A11	96	100	96	100	**96**	**100**	96	100
UPM A13	98	99	98	99	98	99	**98**	**99**
UPM A17	98	100	**98**	**100**	98	100	96	100
UPM A19	**100**	**100**	99	100	100	100	96	100

Cov.= coverage; Idn.=Identity

3.2. DNA sequencing identification

Amplification of the contiguous region of ITS1-5.8S- ITS2 of rDNA from the 8 *Eurotium* isolate generated PCR products ranging in size from 542 to 561 pb (Table 3). All sequences were deposited in the GenBank database under accession numbers listed in Table 3.

All sequences determined in this research yielded top-ranking BLAST scores at the time of this study. However, sequences were indefinite at the species level because similar GenBank reference

sequences existed for different organisms (Table 4). A sequence similarity of 98-100% was observed between most *Eurotium* isolates and those obtained from Gene bank. The lowest similarity was found between *E. cristatum* and others (a deletion of 3 nucleotides in position 26, 27 and 198 in *E. cristatum*).

The evolutionary history was inferred using Maximum Parsimony methods (Fig 2). The *Aspergillus niger* GU 362012 was used as the outgroup species. As shown in Figure 1, all 5 *Eurotium* species (*E.amstelodami, E.chevalieri, E.cristatum* and *E.rubrum*) form a well-supported clade and, according to ITS1-5.8SrDNA-ITS2 sequences, would represent one species as no differences were observed among the sequences of these species. The results indicated that PCR-sequencing technique for sequences of ITS1 and ITS2 of the rDNA was not strong enough for differentiation of *Eurotium* species.

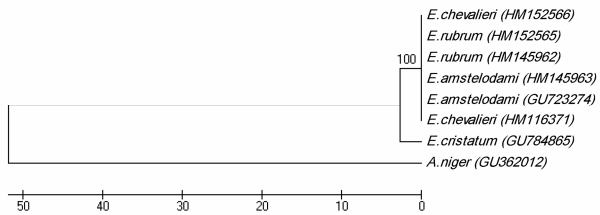

Fig 2. Maximum parsimony (MP) tree of *Eurotium* isolates was inferred based on the ITS1, 5.8S rDNA, ITS2 and 28S rDNA (partial) sequences, using *A.niger* GU362012 as the outgroup species. A most parsimonious tree was generated using MEGA 4.0. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with bootstrap test (2000 replicates). Number over tree node is the bootstrap value. The scale bar at the bottom refers to branch length in terms of the number of steps in the tree construction.

4. DISCUSSION

Aspergilli are truly diverse organisms and identification of isolates based on phenotype has been plagued by many factors including the presence of overlapping morphological features among closely related species. However, in concordance with Geiser et al., (2007) we believed there is no one method (morphological, physiological or molecular) that works perfectly in recognizing *Aspergillus* species. In the present study we identified *Eurotium* species isolated of milled rice in Malaysia using their morphological characteristics and verified using DNA sequencing.

The present investigation revealed that rice samples collected from different regions were contaminated by *Eurotium* species. These confirmed previous observations by Vesonder et al., (1988), Taligoola et al., (2004) and Park et al., (2005) the presence of *Eurotium* spp. from rice samples. A survey by Udagawa (1976) 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. The results obtained in our study showed differences from Udagawa (1976) where *Eurotium* spp. were found to be the most prevalent.

From the analyses of DNA sequences, *Eurotium* species showed a fixed sequence numbers (143 bp in ITS1 and 167 bp in ITS2 regions). The ITS1 and ITS2 of the rDNA sequence analysis are reliable for Sections differentiation and sometime for species within Sections in Aspergilli (Yazdani et al., 2011). Differentiation between some more closely related *Aspergillus* species, especially among *Eurotium* may require analysis of other targets such as the ribosomal external transcribed spacer regions (Balajee, 2008) and also using morphological and physiological characteristics (Geiser et al., 2007).

The major markers for morphological differentiation of *Eurotium* are colony color on media cultures, while for microscopic differentiation the shape and color of conidia heads and ascospore surface ornamentations.

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