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Characterisation of *Magnaporthe oryzae* Isolates from Rice in Peninsular Malaysia

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Abstract

Abed-Ashtiani F., Kadir J., Nasehi A., Hashemian-Rahaghi S.-R., Vadamalai G., Rambe S.-K. (2016): Characterisation of *Magnaporthe oryzae* isolates from rice in peninsular Malaysia. Czech J. Genet. Plant Breed., 52: 145–156.

The genus *Pyricularia* (anamorph)/*Magnaporthe* (teleomorph) includes important destructive pathogens causing blast disease on various species from the family *Poaceae*. Thirty-five *Magnaporthe* isolates were collected from diseased rice plants (variety MR219) in different rice-growing regions of Malaysia, including the five states of Selangor, Penang, Kedah, Kelantan, and Perak between 2010 and 2014. DNA sequence analyses of the internal transcribed spacer (ITS), actin, β-tubulin and calmodulin gene regions, random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) analyses were conducted to analyse 35 *Magnaporthe* isolates. Phylogenetic analysis of the combined dataset confirmed the identification of all isolates as *M. oryzae* with a high distance from other *Magnaporthe* (*Pyricularia)* species. RAPD and ISSR analyses indicated the existence of a relatively low similarity index value among *M. oryzae* isolates through identification of four main clades. The clustering of RAPD and ISSR analyses demonstrated that there was a correlation between the isolates and their geographical origins.

Keywords: genetic diversity; ISSR; *Oryza sativa*; RAPD; rDNA-ITS; rice blast

Rice (*Oryza sativa* L.) is an important food crop all over the world due to its vast consumption as a primary source of energy and protein (Abed-Ashtiani *et al*. 2012). Rice production of Malaysia in 2013 was 2626.881 tons ranking the country as the 24th rice producing country in the world with 688.207 ha of rice harvested area (Factfish 2015).

Rice blast caused by the fungus *Magnaporthe oryzae* (anamorph, *Pyricularia oryzae* Cavara), is among the most significant diseases affecting rice cultivation, since it is prevalent in most rice-growing regions and causes serious yield losses (Abed-Ashtiani *et al*. 2012). Although the application of huge quantities of fungicides and planting resistant varieties have provided interim relief, the disease still remains as a problem in rice-growing tracts of Malaysia (Personal communication with Department of Agriculture, Malaysia).

Phylogenetic species recognition (PSR) has been used to recognise species on the basis of phylogenetic trees established from DNA data (Taylor *et al*. 2000). Multi-locus sequence analysis has successfully been applied to identify plant pathogenic fungi (Steenkamp *et al*. 2002). Phylogenetic relationships among *Magnaporthe* species have previously been shown (Zellerhoff *et al*. 2006; Hirata *et al*. 2007). The internal transcribed spacer (ITS), actin, β-tubulin and calmodulin gene regions (Hirata *et al*. 2007) have been used for detection, identification and classification of *Magnaporthe* isolates.

Planting resistant varieties and fungicide application to control plant diseases could be affected by

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genetic variability of the plant pathogen (Milgroom & Peever 2003). For instance, the breakdown of plant resistance is attributed to the high genetic variability of the pathogen (Séré *et al*. 2007). In addition, repeated use of the same or similar fungicide can lead to development of resistance to that fungicide due to changes in the pathogen genetic structure. Hence, having the knowledge of genetic variation of plant pathogen populations is needed to provide proper strategies to control and manage plant diseases (McDonald & Linde 2002).

Random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), inter simple sequence repeat (ISSR), and microsatellites or simple sequence repeat (SSR) are the most commonly used molecular markers and they have the capability to show the existing variation in the whole genome of pathogen populations (Mitchell *et al*. 1995).

Genetic diversity among rice blast pathogen populations has been studied using ISSR and RAPD markers (Chadha & Gopalakrishna 2007; Sirithunya *et al*. 2008). The application of RAPD markers combined with other reproducible markers such as ISSR provides more accurate assessments of genetic diversity in pathogen populations (Schlotterer 2004). Identification and grouping of *Magnaporthe* isolates and understanding their genetic diversity are essential for the implementation of efficient and appropriate disease management strategies. However, in Malaysia no study has been critically conducted to identify and characterise *Magnaporthe* isolates and assess their genetic diversity. Therefore, the aims of the present study were to identify and provide a taxonomic position at the species level of rice blast pathogen populations collected from various rice-growing regions of Malaysia using DNA sequence analyses, and assess their genetic variability using RAPD and ISSR markers.

MATERIAL AND METHODS

Fungal material. A total of 35 isolates of *Magnaporthe* spp. were obtained from symptomatic leaves and panicles of rice plants (var. MR219) collected in five main rice-growing regions across Peninsular Malaysia (West Malaysia), including Selangor, Penang, Kedah, Kelantan, and Perak states between 2010 and 2014 (Table 1). Due to plant quarantine rules, no samples were included from East Malaysia. Small segments (5 mm^2) of plant tissues were taken from the edge of the lesions. The specimens were surface sterilised using 1% NaOCl for 2 min, rinsed in sterilised distilled water twice, dried on sterilised filter papers, and eventually transferred to Petri dishes containing potato dextrose agar (PDA) and were incubated at 27 ± 1°C under 12 h photoperiodic conditions for 7–10 days to let the fungus grow. Single spore isolation technique described by Ricker and Ricker (1936) was used to purify the isolates prior to further studies. All of the tested isolates were pathogenic on MR219 rice plants (data not shown).

Genomic DNA extraction. Mycelial disks (5 mm^2) of 10-day-old pure cultures of *Magnaporthe* spp. isolates were transferred to Petri dishes containing PDA. The plates were incubated at 27 ± 1°C under 12 h photoperiodic conditions until the whole surface of the plates was covered with mycelium. After that sterilised distilled water containing 0.05% (v/v) Tween-80 was added into the plates and mycelial mats were gently scrubbed with the spatula and placed in 1.5 ml micro tubes. The mycelial mats were pelleted by centrifugation at 3000 rpm for 5 min at 4°C and the supernatant was carefully removed. Extraction of genomic DNA was carried out following the 3% SDS method (Gonzalez-Mendoza *et al*. 2010). The concentration of obtained genomic DNA was measured using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

PCR amplification and DNA sequencing. Four DNA regions, including the ITS (ITS1, 5.8S rDNA and ITS2), actin, β-tubulin and calmodulin genes, were chosen to characterise *Magnaporthe* spp. isolates using universal primers targeting four DNA regions (Table 2). Polymerase chain reaction (PCR) amplification was conducted in a 25 µl reaction volume containing 2.5 µl of 10× PCR buffer (200 mM Tris-HCl, 500 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM both primers, 1 unit of *Taq* DNA polymerase (Vivantis Technologies, Sdn. Bhd., Subang Jaya, Malaysia) and 2 µl of template DNA (10 ng). The amplification was carried out in a thermal cycler (C1000 Touch™ Thermal Cycler, Bio-Rad Laboratories, Inc., Singapore) programmed for: initial denaturation at 95°C for 8 min; followed by 30 cycles of 95°C for 30 s denaturation, 20 s annealing at 55°C and 72°C for 1 min elongation; and a final extension step of 72°C for 5 min (Hirata *et al*. 2007). The amplification products were resolved in 1% agarose gel using 1× TBE buffer (89 mM Tris base, 89 mM boric acid and 2 mM EDTA pH 8.3) at 70 V for 45 min at

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Table 1. Origin of 35 *Magnaporthe oryzae* isolates collected from rice (*Oryza sativa*) plants in Malaysia in 2010–2014 and their internal transcribed spacer (ITS), actin, β-tubulin and calmodulin regions

Isolate	Plant part	State	Year	ITS[a]	Actin[a]	β-tubulin[a]	Calmodulin[a]
PO-FA01	leaf	Selangor	2013	KM249937	KM249972	KM250007	KM250042
PO-FA02	leaf	Selangor	2013	KM249938	KM249973	KM250008	KM250043
PO-FA03	leaf	Selangor	2013	KM249939	KM249974	KM250009	KM250044
PO-FA04	leaf	Selangor	2013	KM249940	KM249975	KM250010	KM250045
PO-FA05	panicle	Penang	2010	KM249941	KM249976	KM250011	KM250046
PO-FA06	panicle	Penang	2010	KM249942	KM249977	KM250012	KM250047
PO-FA07	leaf	Penang	2010	KM249943	KM249978	KM250013	KM250048
PO-FA08	leaf	Penang	2010	KM249944	KM249979	KM250014	KM250049
PO-FA09	leaf	Penang	2010	KM249945	KM249980	KM250015	KM250050
PO-FA10	leaf	Penang	2010	KM249946	KM249981	KM250016	KM250051
PO-FA11	leaf	Kedah	2014	KM249947	KM249982	KM250017	KM250052
PO-FA12	leaf	Kedah	2014	KM249948	KM249983	KM250018	KM250053
PO-FA13	leaf	Kedah	2014	KM249949	KM249984	KM250019	KM250054
PO-FA14	panicle	Kedah	2014	KM249950	KM249985	KM250020	KM250055
PO-FA15	leaf	Kedah	2014	KM249951	KM249986	KM250021	KM250056
PO-FA16	leaf	Kedah	2014	KM249952	KM249987	KM250022	KM250057
PO-FA17	panicle	Kedah	2014	KM249953	KM249988	KM250023	KM250058
PO-FA18	leaf	Kedah	2014	KM249954	KM249989	KM250024	KM250059
PO-FA19	leaf	Kedah	2014	KM249955	KM249990	KM250025	KM250060
PO-FA20	leaf	Kedah	2014	KM249956	KM249991	KM250026	KM250061
PO-FA21	leaf	Kedah	2014	KM249957	KM249992	KM250027	KM250062
PO-FA22	leaf	Kedah	2014	KM249958	KM249993	KM250028	KM250063
PO-FA23	leaf	Kedah	2014	KM249959	KM249994	KM250029	KM250064
PO-FA24	leaf	Kelantan	2013	KM249960	KM249995	KM250030	KM250065
PO-FA25	leaf	Kelantan	2013	KM249961	KM249996	KM250031	KM250066
PO-FA26	leaf	Kelantan	2013	KM249962	KM249997	KM250032	KM250067
PO-FA27	panicle	Kelantan	2013	KM249963	KM249998	KM250033	KM250068
PO-FA28	leaf	Kelantan	2013	KM249964	KM249999	KM250034	KM250069
PO-FA29	leaf	Kelantan	2013	KM249965	KM250000	KM250035	KM250070
PO-FA30	leaf	Kelantan	2013	KM249966	KM250001	KM250036	KM250071
PO-FA31	leaf	Kelantan	2013	KM249967	KM250002	KM250037	KM250072
PO-FA32	leaf	Perak	2014	KM249968	KM250003	KM250038	KM250073
PO-FA33	leaf	Perak	2014	KM249969	KM250004	KM250039	KM250074
PO-FA34	leaf	Perak	2014	KM249970	KM250005	KM250040	KM250075
PO-FA35	leaf	Perak	2014	KM249971	KM250006	KM250041	KM250076

[a]GenBank accession number

room temperature. Gels were stained with ethidium bromide solution (10 mg/ml) and visualised under ultra violet (UV) illumination. PCR products were amplified in both directions and were purified using the GF-1 PCR Clean-Up Kit (Vivantis Technologies, Sdn. Bhd.) based on the manufacturer's instructions.

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Table 2. List of primer pairs used for DNA sequencing

Target region, gene	Primers	Primer DNA sequence (5'- 3')	Reference
ITS	ITS5	GGAAGTAAAAGTCGTAACAAGG	White *et al.* (1990)
	ITS4	TCCTCCGCTTATTGATATGC	
Actin	ACT-512F	ATGTGCAAGGCCGGTTTCGC	Carbone and Kohn (1999)
	ACT-783R	TACGAGTCCTTCTGGCCCAT	
β-tubulin	Btla	TTCCCCCGTCTCCACTTCTTCATG	Glass and Donaldson (1995)
	Btlb	GACGAGATCGTTCATGTTGAACTC	
Calmodulin	CAL-228F	GAGTTCAAGGAGGCCTTCTCCC	Carbone and Kohn (1999)
	CAL-737R	CATCTTTCTGGCCATCATGG	

ITS – internal transcribed spacer

The purified PCR products were sequenced with the same forward and reverse primers as in the PCR amplification by a commercial sequencing service provider (NHK Bioscience Solutions, Sdn. Bhd., Kuala Lumpur, Malaysia).

Sequence alignment and phylogenetic analysis. The resulting sequences of each isolate were refined using a BioEdit sequence alignment editor (Hall 1999) in which the sequence got from reverse primer was transformed to reverse complement and aligned with the sequence obtained from forward primer to get consensus sequence. BLASTn alignment was carried out to identify and analyse homologous sequences of fungal isolates used in this study with those of reference *Pyricularia* species deposited in the GenBank by Zellerhoff *et al.* (2006) and Hirata *et al.* (2007). To analyse the relationship of the isolates to known *Pyricularia* species, all 35 sequences from this study and sequences of six reference *Pyricularia* species obtained from the GenBank (Zellerhoff *et al.* 2006; Hirata *et al.* 2007) were initially aligned using the Clustal W multiple alignment (Version 1.8; Thompson *et al.* 1994), checked visually and improved manually where necessary. *Pyricularia higginsii* (HYCI201-1-1) was used as an outgroup. Phylogenetic analysis of the combined data set of the ITS, actin, β-tubulin and calmodulin gene regions using the parsimony optimality criterion was performed in PAUP* 4.0b10 (Swofford 2002). Alignment gaps were treated as missing data. Maximum parsimony (MP) analysis was conducted by heuristic searches consisting of 1000 stepwise random addition replicates and branch swapping by the tree-bisection-reconnection (TBR) algorithm. For each MP analysis, 1000 bootstrap replicates using a heuristic search with simple sequence addition were performed to assess statistical support for branch stability. Concordance between datasets was evaluated by the partition homogeneity test (PHT) implemented in PAUP* 4.0b10 (Swofford 2002).

RAPD analysis. Seven primers including OPA-03, OPA-13, OPF-04, OPM-20, UBC-25, UBC-155, and UBC-173 (Operon Technologies, Inc., Alameda, USA) with reproducible profiles and high polymorphism

Table 3. RAPD and ISSR primers used to identify and assess intraspecific genetic diversity among *Magnaporthe oryzae* isolates

	Primer sequence (5'–3') (5'–3)'	Reference[a]
RAPD primers		
OPA-03	AGTCAGCCAC	Séré *et al.* (2007)
OPA-13	CAGCACCCAC	Séré *et al.* (2007)
OPF-04	GGTGATCAGG	Chadha and Gopalakrishna (2005)
OPM-20	AGGTCTTGGG	Chadha and Gopalakrishna (2005)
UBC 25	ACAGGGCTCA	Sirithunya *et al.* (2008)
UBC 155	CTGGCGGCTG	Sirithunya *et al.* (2008)
UBC 173	CAGGCGGCGT	Sirithunya *et al.* (2008)
ISSR primers		
ISSR5	(CA)8A	Vanaraj *et al.* (2013)
881	(GGGTG)3	Chadha and Gopalakrishna (2007)
890	VHV(GT)7	Chadha and Gopalakrishna (2007)

[a]RAPD and ISSR primers with high polymorphism and reproducible profiles as described by other previous studies on *Magnaporthe* species were selected for this study

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were used to carry out RAPD analysis on 35 *M. oryzae* isolates according to the results of initial screening against a set of representative studied isolates (Table 3). The primers were synthesized by Vivantis Technologies, Sdn. Bhd. PCR amplification was carried out in a 25 µl reaction volume containing 0.5 µM primer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1 unit of Taq DNA polymerase (Vivantis Technologies, Sdn. Bhd.) and 2.5 µl of 10× supportive buffer (100 mM Tris-HCl, 500 mM KCl, pH 9.1) and 10 ng of genomic DNA. RAPD analysis was carried out as described by Nasehi *et al.* (2014). PCR amplification was conducted in a thermal cycler (C1000 Touch™ Thermal Cycler, Bio-Rad Laboratories, Inc.) programmed with the following profiles: 94°C for 4 min initial denaturation, 45 cycles of 94°C for 1 min denaturation, 35°C for 1.5 min annealing and 72°C for 2 min extension and 10 min final extension at 72°C.

ISSR analysis. Three primers including ISSR5, 881, and 890 with reproducible profiles and high polymorphism were used to carry out ISSR analysis on 35 *M. oryzae* isolates according to the results of initial screening against a set of representative studied isolates (Table 3). The primers were synthesized by Vivantis Technologies, Sdn. Bhd. PCR amplification was carried out in the same manner as described for RAPD analysis. ISSR analysis was carried out by the method of Nasehi *et al.* (2014). PCR amplification was conducted in a thermal cycler (C1000 Touch™ Thermal Cycler, Bio-Rad Laboratories, Inc.) programmed with the following profiles: 94°C for 5 min initial denaturation, 45 cycles of 94°C for 1 min denaturation, 55°C for 1.5 min annealing and 72°C for 2 min extension and 10 min final extension at 72°C.

Agarose gel electrophoresis and staining. The amplification products of RAPD and ISSR analyses were size-separated in 1% agarose gel using 1× TBE buffer. The sizes of amplified DNA fragments were estimated using GeneTools (Ver 3.00.13, Syngene Laboratories). Ethidium bromide solution (10 mg per ml) was used for staining. Banding patterns were photographed using a gel documentation system (Molecular Imager Gel Doc™ XR imaging system, Bio-Rad Laboratories, Inc.).

Data analyses. RAPD and ISSR analyses were performed in three replications to confirm the consistency of amplification and only repeatable bands were scored. All monomorphic and polymorphic bands were scored using a binary system. The present band was scored as 1 while the absent band was scored as 0. The scores were then entered into a matrix for analysis using the numerical taxonomy and multivariate analysis system, NTSYS-pc 1.8 program (Applied Biostatistics Inc., Setauket, USA) (Rohlf 1993). Similarity coefficients were calculated for all pairwise combinations of haplotype using the Jaccard coefficient. A dendrogram was generated using the SAHN clustering program with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

RESULTS

DNA sequence analyses. The ITS, actin, β-tubulin and calmodulin gene regions of all *Magnaporthe* spp. isolates were successfully amplified with 505, 336, 536, and 512 bp long, respectively. BLASTn queries based on the four regions indicated that all isolates used in this study were 100% identical to those of *P. oryzae* in the GenBank, except for the three isolates (PO-FA01, PO-FA02, and PO-FA04) collected from the Selangor state which had 99% identity by substitution in one locus at position 397 (C/T) of the calmodulin gene. Sequences of the ITS, actin, β-tubulin, and calmodulin gene regions of all *Magnaporthe* isolates were deposited in the GenBank (Table 1).

The partition homogeneity test (PHT) of the combined alignment conducted in PAUP resulted in a *P* value of 0.034 showing that all four regions were combinable. The final sequence alignment of the combined datasets comprising 41 taxa (including 35 sequences from this study and six *Pyricularia* reference sequences obtained from the GenBank) had 1824 characters, of which 1210 characters were constant, 399 characters were parsimony uninformative and 215 characters were parsimony informative. MP analysis of the combined dataset yielded 2 equally most parsimonious trees (Tree Length (TL) = 859, Consistency Index (CI) = 0.907, Retention Index (RI) = 0.744, Homoplasy Index (HI) = 0.093 and Rescaled Consistency Index (RC) = 0.675), one of which is shown (Figure 1). Phylogenetic analysis of the combined dataset revealed that 35 isolates of this study were clustered into a distinct clade with those of *P. oryzae* (Ken54-20 and FR13, obtained from the GenBank) supported by 100% bootstrap value. Other *Pyricularia* species were clearly separated with high distance from all the isolates used in this study as well as those of *P. oryzae*, and were clustered into other distinct clades. Hence, these results confirmed that all isolates belonged to *M. oryzae*.

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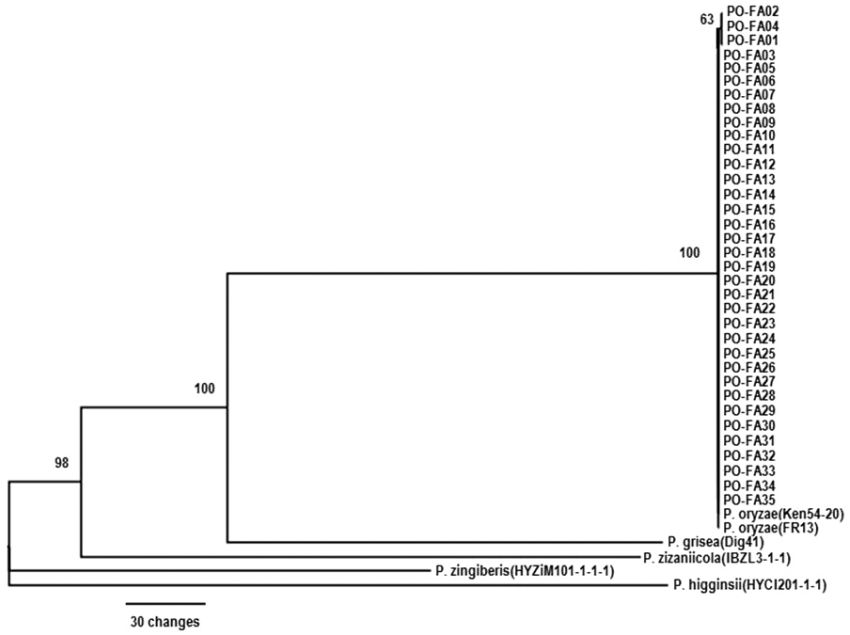

Figure 1. Phylogenetic tree based on the combined dataset of the internal transcribed spacer (ITS), actin, β-tubulin and calmodulin gene regions of 35 isolates used in this study compared to the reference *Pyricularia* species obtained from the GenBank using the maximum parsimony method; the bar indicates nucleotide substitutions per site; number of bootstrap support values ≥ 50% based on 1000 replicates

RAPD analysis. The application of seven RAPD primers generated a total of 95 consistently amplified fragments (Figure 2). Among the seven primers, OPF-04 offered the highest number of amplified DNA bands at 17 while OPA-13 gave the lowest number of bands at 9. The average number of bands per primer was 13.57 and they ranged approximately from 100 to 3000 bp in size. The percentage of polymorphism derived from all the RAPD bands was 90.53 suggesting high diversity within *M. oryzae* populations in Malaysia (Table 4). A dendrogram depicted by UPGMA analysis based on 95 amplified bands and according to the Jaccard similarity coefficient grouped 35 *M. oryzae* isolates into four main clades (A–D) at the Jaccard similarity coefficient approximately 0.83 (Figure 3). The similarity among the isolates ranged from 54% to 100%. Clade A consisted of the isolates obtained from Selangor state. All isolates collected from Perak state, except one isolate (PO-FA03) collected from Selangor state, were clustered in clade B. Clade C was composed of the isolates obtained from Kelantan state, and all isolates collected from Penang and Kedah states, except one isolate (PO-FA24) from Kelantan state, were placed in clade D.

ISSR analysis. The application of three ISSR primers generated a total of 44 consistently amplified fragments (Figure 4). Among the three primers, 881 offered the highest number of amplified DNA bands at 16 while 890 gave the lowest number of bands at 13. The average number of bands per primer

Table 4. Total numbers of amplified fragments and percentage of polymorphic fragments among *Magnaporthe oryzae* isolates generated by RAPD and ISSR analyses

Primer	Amplified fragments	Polymorphic fragments (%)
RAPD		
OPA-03	12	11 (91.66)
OPA-13	9	9 (100.00)
OPF-04	17	16 (94.12)
OPM-20	12	12 (100.00)
UBC-25	15	11 (73.33)
UBC-155	14	12 (85.71)
UBC-173	16	15 (93.75)
Total	95	86 (90.53)
ISSR		
ISSR5	15	15 (100.00)
881	16	15 (93.75)
890	13	12 (92.31)
Total	44	42 (95.45)

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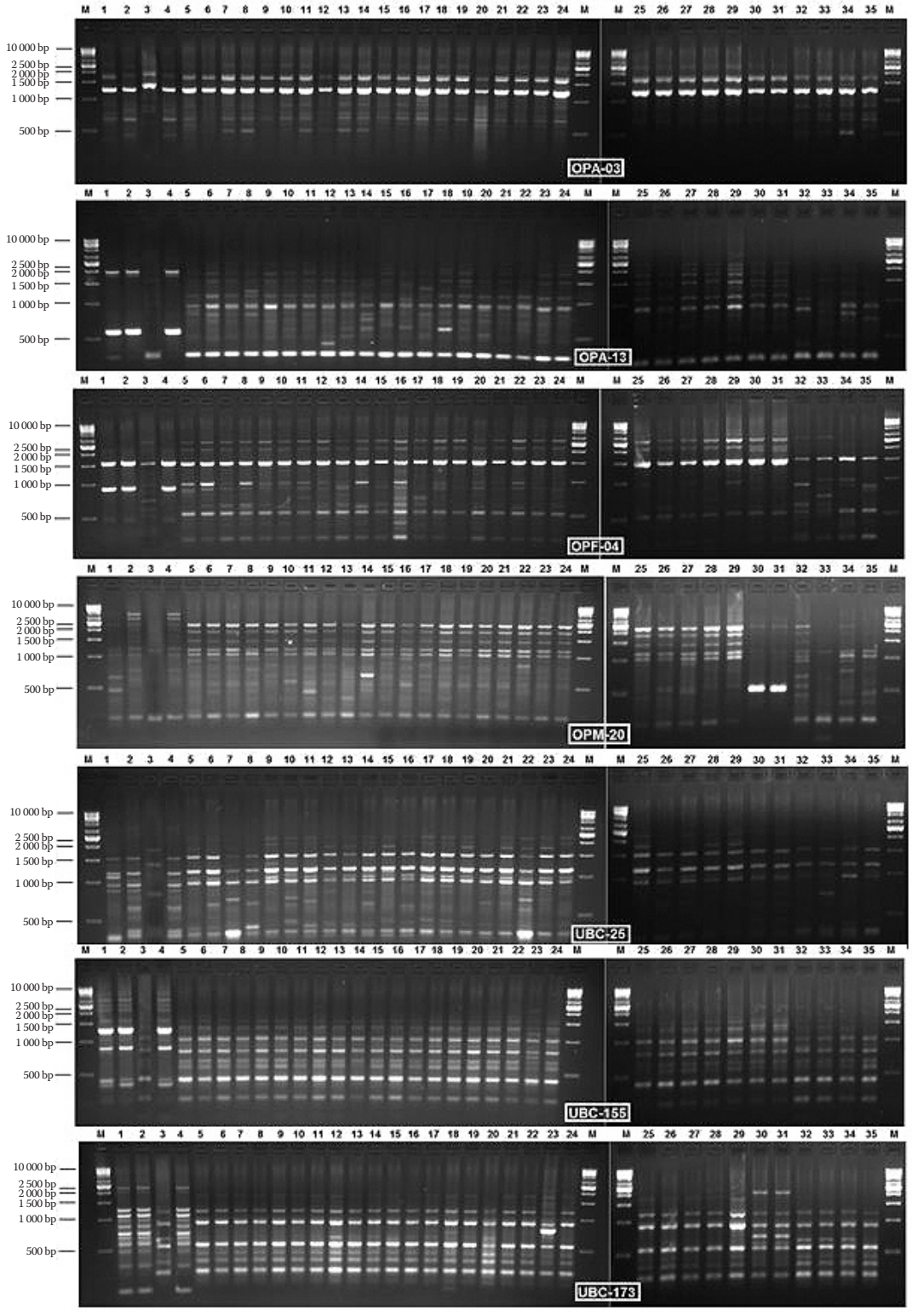

Figure 2. Gel electrophoresis of amplified products of genomic DNA of *Magnaporthe oryzae* isolates using seven RAPD primers; the first and the last lanes (M) are 2-log DNA ladder; the numbering of the isolates is based on Table 1

was 14.66, and they ranged approximately from 100 to 3000 bp in size. The percentage of polymorphism derived from all the ISSR bands was 95.45 suggesting high diversity within *M. oryzae* populations in Malaysia (Table 4). A dendrogram depicted by UPGMA analysis based on 44 amplified bands and ac-

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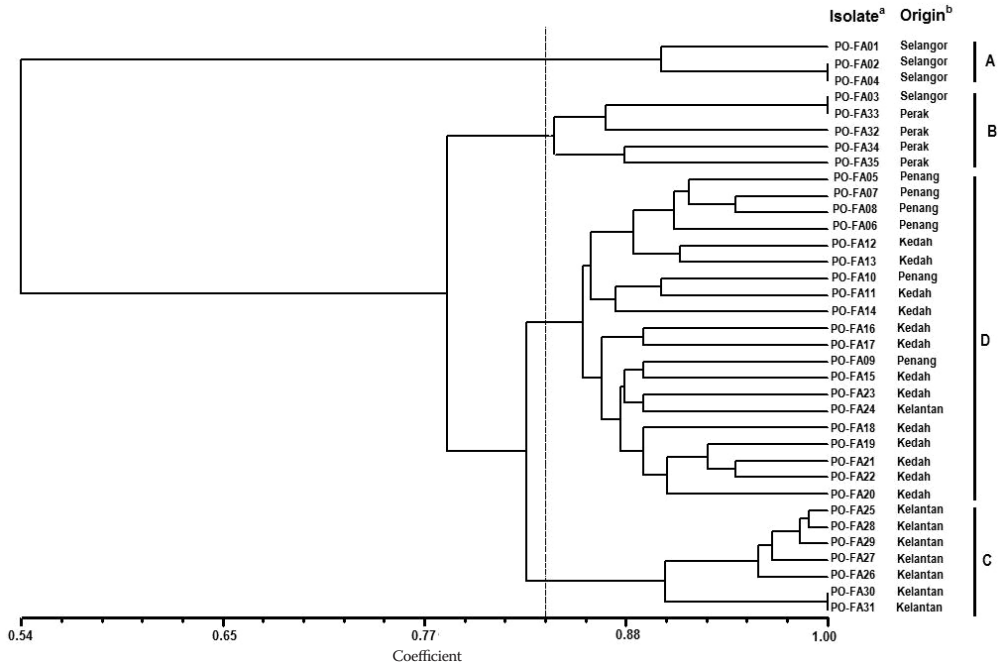

Figure 3. Dendrogram generated by UPGMA showing the genetic diversity of 35 *Magnaporthe oryzae* isolates derived from the combination of seven RAPD primers (OPA-03, OPA-13, OPF-04, OPM-20, UBC-25, UBC-155, and UBC-173); isolates ([a]) and locations ([b]): see Table 1

cording to the Jaccard similarity coefficient grouped 35 *M. oryzae* isolates into four main clades (A–D) at the Jaccard similarity coefficient approximately 0.66 (Figure 5). The similarity among the isolates ranged from 58% to 100%. Clade A consisted of three isolates collected from Selangor state. All isolates collected

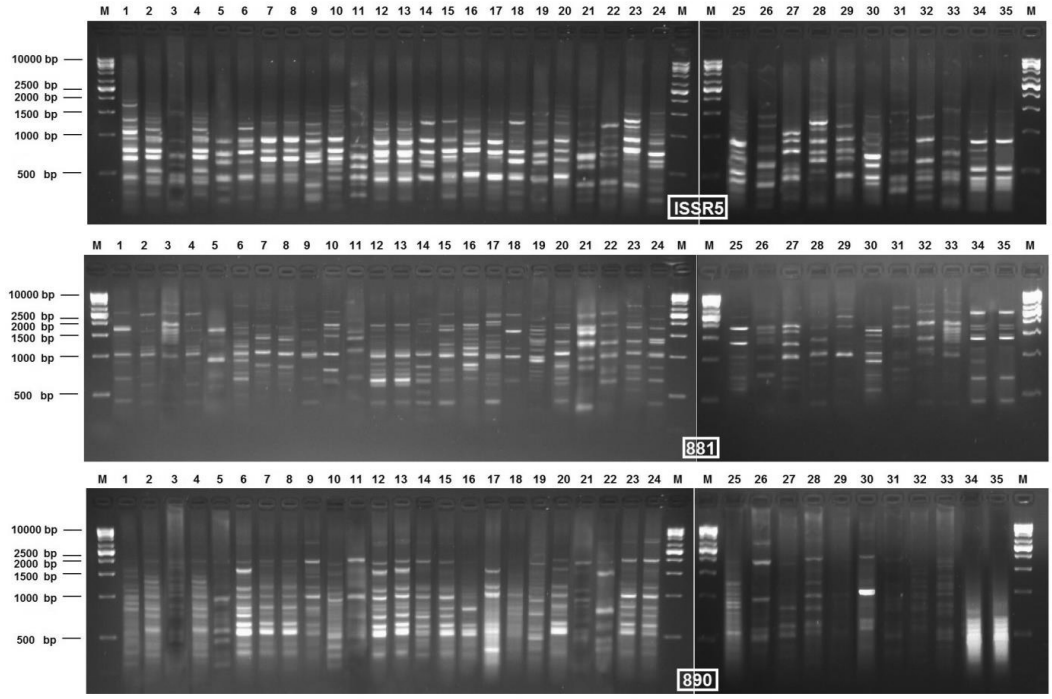

Figure 4. Gel electrophoresis of amplification products of genomic DNA of *Magnaporthe oryzae* isolates using three ISSR primers; the first and the last lanes (M) are 2-log DNA ladder; the numbering of the isolates is based on Table 1

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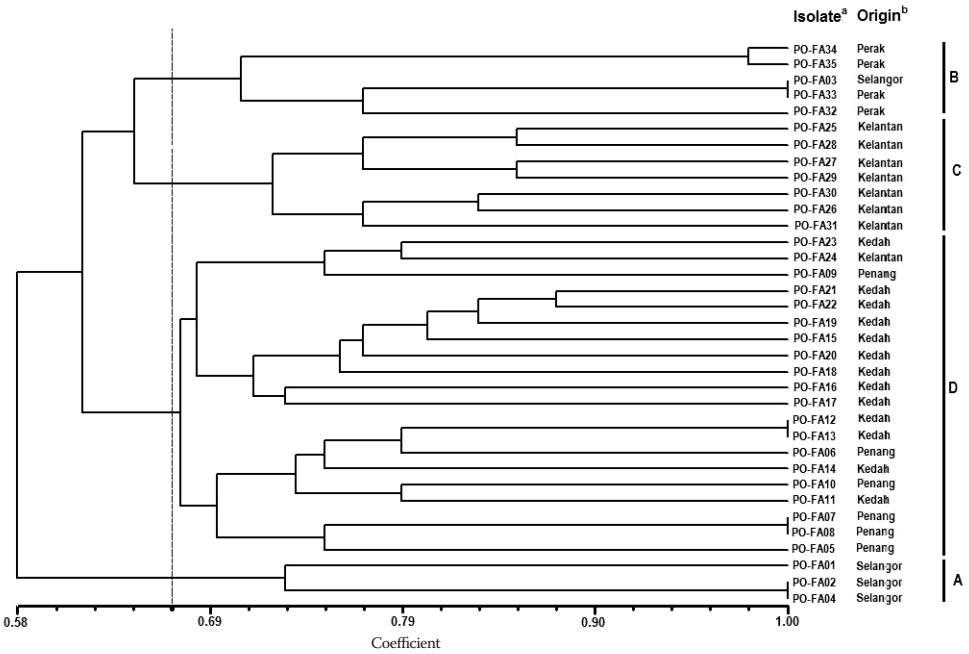

Figure 5. Dendrogram generated by UPGMA showing the genetic diversity of 35 *Magnaporthe oryzae* isolates derived from the combination of three ISSR primers (ISSR5, 881 and 890); isolates ([a]) and locations ([b]): see Table 1

from Perak state, except one isolate (PO-FA03) from Selangor state, were placed in clade B. Clade C was composed of the isolates obtained from Kelantan state. All isolates collected from Penang and Kedah states, except one isolate (PO-FA24) from Kelantan state, were placed in clade D.

DISCUSSION

Because of the low precision and overlapping of some morphological characteristics, more accurate methods such as DNA sequencing have been employed to identify fungal pathogens (Nasehi *et al*. 2014). In this respect, the use of molecular techniques such as sequencing of different regions of DNA is considered as alternatives to morphological methods of identification of *Magnaporthe* species (Hirata *et al*. 2007). A previous study confirmed that the combined dataset of the ITS, actin, β-tubulin and calmodulin gene regions could identify *Magnaporthe* at the species level, and sometimes lower than the species level (Hirata *et al*. 2007). In the present study, a phylogenetic analysis inferred from the combined dataset of the ITS, actin, β-tubulin and calmodulin gene regions confirmed the identification of all 35 isolates as *M. oryzae*. To the best of our knowledge, this is the first report on the phylogenetic analysis of *M. oryzae* isolates collected from different states of Malaysia based on the ITS, actin, β-tubulin and calmodulin gene regions.

Having a deep conception of genetic diversity of a pathogen is essential to be able to understand its contribution to disease epidemiology and management (Scheuermann *et al*. 2012). The importance of the blast disease has been recognised across the world but basic information on fungal diversity and factors affecting the genetic structure of the isolates, which is an important key in response to breeding programmes, is still unknown in Malaysia. The RAPD and ISSR markers have been proved as highly powerful tools in studies of genetic diversity and fingerprinting of *Magnaporthe* spp. isolates (Chadha & Gopalakrishna 2007; Sirithunya *et al*. 2008), as well as other plant pathogenic fungi (Nasehi *et al*. 2014).

In this study, relatively low similarity index values (54% and 58% for RAPD and ISSR markers, respectively) were observed among 35 *M. oryzae* isolates collected across five different rice-growing regions in Malaysia. All isolates were collected from the MR219 rice variety, which is the most popular variety of rice planted on 70% of all rice plantation areas in Malaysia (Hashim *et al*. 2015), and recently it has shown signs of being susceptible to the rice blast disease. Although the results of RAPD analysis showed quite good congruence with the results obtained from ISSR studies, a greater effective ratio of multi-locus

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markers as well as a higher number of private alleles were observed using ISSR markers (Nasehi *et al*. 2014). The clustering of RAPD and ISSR analyses demonstrated that there was a correlation between the isolates and their geographical origins. The results revealed that the isolates collected from the five states of Malaysia have been clustered into four main clads. Clustering of the isolates collected from Kedah and Penang states in the same clade can be explained by the fact that Kedah and Penang states are geographically close to each other and may share the same climatic conditions. Previous studies on the genetic diversity of Asian isolates of *Magnaporthe* spp. also revealed a high extent of diversity in Philippines (Mishra *et al*. 2006), Korea (Park *et al*. 2003), China (Ngueko *et al*. 2004), Iran (Javan-Nikkhah *et al*. 2004) and India (Chadha & Gopalakrishna 2005), which is in agreement with the findings of this study. To the best of our knowledge, this is the first report on the genetic diversity of *M. oryzae* isolates using ISSR and RAPD markers in Malaysia, and evaluating these two types of markers to differentiate rice blast pathogen populations. Despite the high diversity of *Magnaporthe* spp. isolates in Asia, the number of lineages classified in Europe and America was rather low (Consolo *et al*. 2008). Thailand, Malaysia and other Asian countries are considered as ancient rice-growing regions; therefore, the high diversity of the pathogen could be due to the long period of co-existence, development and evolution, while the introduction of rice planting into America and Europe occurred a few centuries ago, leading seldom to the variation of the pathogen (Chadha & Gopalakrishna 2005). Previous studies have also demonstrated that the geographical origin was a dominant factor in determining the genetic diversity of the rice blast pathogen (Sirithunya *et al*. 2008; Srivastava *et al*. 2014). In contrast, an assessment of the genetic diversity of the rice blast fungus in China indicated that the clustering of the isolates was not harmonious with their geographical origins (Ngueko *et al*. 2004). According to RAPD and ISSR analyses, no correlation was observed between the isolates collected from different parts of rice plants, including leaves and panicles. Therefore the high level of polymorphism within *M. oryzae* isolates used in this study is attributed to the fact that isolates were collected from different agro-climatic regions of Malaysia reinforcing the hypothesis of different geographical regions being more important than different plant parts. This result is in agreement with those reported by Sirithunya *et al*. (2008), who did not observe any clear relationship between genetic variability and different plant parts while isolates represented accordance between their genetic diversity and geographical origins.

In conclusion, both RAPD and ISSR markers were useful to differentiate *M. oryzae* isolates into distinct groups. The results demonstrated that the limitations of the DNA sequence method can be compensated using molecular markers such as RAPD and ISSR which were able to precisely explain the interrelationships between the studied isolates. Findings of this study confirmed that there was a correlation between isolates of *M. oryzae* existing in Malaysia and their geographical origins. Therefore an effective extension of the knowledge of genetic diversity of the pathogen in each rice-growing region is required to provide more efficient methods to manage and control the rice blast disease, and to screen rice genotypes for blast resistance. Obtained data from RAPD and ISSR markers could be expanded for more in-depth studies in the genetic diversity of *M. oryzae* isolates collected from different host plants in Malaysia. More investigations can also be conducted on the relationship between the genetic diversity of isolates and their pathotype in order to establish the population structure and define resistance genes in breeding programmes.

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