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ELUCIDATION OF MOLECULAR PHYLOGENY OF *RIGIDOPORUS MICROPORUS*: A WHITE ROOT ROT DISEASE FUNGUS OF RUBBER (*HEVEA BRASILIENSIS*) IN MALAYSIA

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White root rot (WRR) disease which is caused by *Rigidoporus microporus* is a major agricultural disease. The disease affects tropical fruits, forest trees, ornamental trees as well as rubber (*Hevea brasiliensis*) trees. Although WRR causes significant economic losses to planters, there is no effective method to control this disease. The understanding on the population genetics of *R. microporus* isolates could mitigate WRR disease in plant management. Therefore, the objective of this work was to elucidate molecular phylogeny of *R. microporus* isolated from rubber trees. Twenty seven *R. microporus* isolates were characterised using internal transcribed spacer (ITS), Beta-tubulin (β-tubulin) and translation elongation factor-α (tef1-α) sequences data. Phylogenetic tree analyses of *R. microporus* isolates were divided into two major groups; the Asian and African groups. However, there was no distinct geographical structuring in the Asian group, suggesting gene flow happened among the populations. Somatic Incompatibility (SI) test and Inter Simple Sequence Repeat (ISSR) marker revealed that the *R. microporus* in the rubber plantations partially clustered based on geographical regions. The genetic diversity of *R. microporus* by ISSR revealed four major groups. The findings of the present study provide a baseline data for breeders and plant pathologists to enable them strategise effective and sustainable WRR disease management.

Keywords: Molecular characterisation, genetic diversity, fungal pathogen, somatic incompatibility, plantation

INTRODUCTION

The rubber industry is one of the most important plantation industries in Malaysia and Southeast Asia (Kaung &Thanate 2020). In January 2020, Malaysia's natural rubber production increased by 13.3% (66,232 tonnes). In 2020, the Malaysian rubber industry is forecasted to contribute USD 9.62 billion to the Malaysian economy. The Malaysian rubber industry is driven mainly by the downstream sector including the rubber glove industry (DOSM 2020). Unfortunately, the decrease in rubber yield was caused by a deadly fungal infection disease or also known as the white root rot (WRR) disease. The WRR disease is caused by a fungal wood degrader known as *Rigidoporus microporus* (Basidiomycota, Agaricomycotina) syn. *Rigidoporus lignosus*. This pathogen is the most destructive rubber root pathogen in tropics and sub-tropics (Guyot & Flori 2002, Jayasuriya & Thennakoon 2007) and spreads rapidly (Zaini & Halimoon 2013). The growing rhizomorphs of the WRR disease attach strongly to the surface of the rubber tree root bark after which they are able to spread several meters below ground and attack other healthy rubber trees (Nandris et al. 1987). In the advanced stage of the disease, a brownish orange basidiocarp will start to appear at the base of the trunk (Omorusi 2012).

The WRR disease affects other economically important agricultural crops such as *Ananas comosus* (Pineapple), *Cocos nucifera* (Coconut), *Arenga pinata* (Sugar palm), *Manihot esculenta*

(Cassava) (Suwandi 2007), *Greenwayodendron suaveolens* (Oghenekaro et al. 2014), *Piper nigrum* (Black pepper) (Suwandi 2003), *Theobroma cacao* (Cocoa) (Azmi 2005) and *Artocarpus nobilis* (Ceylon breadfruit) (Madushani et al. 2013). The WRR disease occurs in forest plantation species and ornamental trees such as *Tectona grandis* (Teak), *Azadirachta excelsa*, *Acacia* sp. and *Delonix regia* (Flamboyant tree) (Suwandi 2007, Oghenekaro et al. 2014).

Rubber plantations in Southeast Asia supply approximately 97% of the world's natural rubber (Fox & Castella 2013, Yasen & Koedsin 2015). The WRR disease attacks rubber tree regardless of the age or health status, causing economic losses to the latex industry in many rubber producing countries such as India, Indonesia, Malaysia, Thailand, Sri Lanka, Ivory Coast, Cameroon, and Gabon (Semangun 2000, Kaewchai & Soytong 2010). Prasetyo et al. (2009) reported that WRR disease caused approximately 3–15% losses in rubber production and financial loss of USD 150,000 yearly. To date, there is no report on the significant economic loss in monetary wise caused by the WRR disease in Malaysia. However, Nandris et al. (1987) stated that the losses can cost up to USD 100,000 per hectare for 25 productive years of a rubber tree. The most common plantation management practices are based on the elimination of the infectious source to reduce the incidence of the WRR disease (Silva et al. 2014). Present management of the WRR disease is in place without sufficient understanding of the population genetics of *R. microporus* isolates. Inter Simple Sequence Repeat (ISSR) is one of the most popular and powerful genetic markers for investigating genetic variation within closely related species other than determining the genetic population on plant pathogenic fungi (Menzies et al. 2003, Chadha & Gopalakrishna 2007, Yu et al. 2008). Outcomes from genetic diversity analysis of plant pathogenic fungi could provide useful information to control plant diseases (Takatsuka 2007). Currently, there is insufficient information on the molecular phylogeny for *R. microporus* in rubber plantations in Malaysia and throughout Asia. Studies on the genetic variability and characterisation (Kaewchai et al. 2009) and somatic incompatibility (Hamidson & Naito 2004) of the WRR pathogen in were reported in Thailand and Indonesia. Considering the limitations of traditional method of identifying fungi, there is a pressing need to establish the molecular identification and genetic variation for the WRR disease in rubber plantations. The outcomes from this approach of managing the WRR disease could serve as a major platform for documenting the genetic variation of *R. microporus* in rubber plantations of Malaysia and elsewhere. Furthermore, molecular markers are important tools that are able to elucidate genetic diversity amongst fungal pathogens where morphological features are either absent or insufficient to permit intra-specific characterisation (Sharma et al. 2005). Therefore, the objective of this study was to elucidate molecular phylogeny of *R. microporus* populations isolated from infected rubber trees in Malaysia.

MATERIALS AND METHODS

Sampling and isolation

Sixty diseased samples including fruiting bodies of infected *H. brasiliensis* were collected from Kedah, Kelantan, Perak, Selangor and Sarawak in Malaysia from July 2017 to May 2018. The samples were wrapped in polythene bags and brought to the Bacteriology Laboratory, Faculty of Agriculture, Universiti Putra Malaysia for further analysis (Kumar & Atri 2019). Four isolates of *R. microporus* were obtained from the Rubber Research Institute Malaysia Research Station in Sungai Buloh, Selangor to serve as reference isolates. Fruiting bodies and infected portions of the rubber roots were cut into pieces of 2–5 mm, rinsed with sterile distilled water and blotted dry on sterile filter paper. The samples were later transferred on to the *Ganoderma* Selective Medium (GSM) plates (Arrifin & Idris 1992) for incubation at 30 °C for three days. Subsequently, the fungal isolates were characterised and identified by preparing slides in Lactophenolcotton Blue. After the identification, pure cultures were transferred to new Malt Extract Agar (MEA) plates supplemented with antibiotics (50 µg/ml chloramphenicol and 250 µg/ml streptomycin sulfate) to resume normal fungal growth. The fungal mycelia from each pure colony culture were inoculated onto the surface of MEA slants, incubated at 30 °C for seven days and stored at 4 °C prior to further studies.

DNA extraction, PCR amplification and gene sequencing

Genomic DNA was extracted using a modified CTAB (Cetyltrimethylammonium bromide) buffer protocol as described by Lin et al. (2009). Amplification of the Internal Transcribed Spacer (ITS) region was carried out using a pair of primers; ITS1F (5'-CCGTAGGTGAACCTGCGG-3') and ITS4R (5'-TCCTCCGCTTATTGATATGC-3'). The pairs of the primer used for amplifying the partial Beta-tubulin (β-tubulin) gene region were B36F (5'-CACCCACTCCCTCGGTG-3') and B34R (5'-CCTTCATGGAGACCTT-3') (Oghenekaro et al. 2014). The translation elongation factor-α (tef1-α) region was amplified using the set Efdf (5'-AAGGAYGGNCARACYCGNGARCAYGC-3') and EF1-2218R (5'- ATGACACCRACRGCRAC RGTYTG-3') (Oghenekaro et al. 2014).

PCR amplification were carried out in a 25 µl reaction volume containing 1.0 µl of genomic DNA, 12.5 µl of 2X PCRBIO Taq Mix Red, 1.0 µM of each primer and 10.5 µl of free nuclease water. Thermal cycling conditions for ITS was 94 °C for 4 min, followed by 35 cycles at 94 °C for 30 secs, 55 °C for 30 secs, 72 °C for 1 min and 72 °C for 10 min. For partial β-tubulin, PCR reaction was conducted using an initial cycle of 94 °C for 4 min, 35 cycles of 94 °C for 30 secs, 50 °C for 30 secs, 72 °C for 1 min and a final extension of 72 °C for 10 min. For tef1-α gene, PCR was carried using an initial cycle of 94 °C for 4 min, 35 cycles of 94 °C for 30 secs, 60 °C for 30 secs and 72 °C for 1 min and a final extension of 72 °C for 10 min. All PCR amplifications were performed using a Thermal Cycler. PCR products were detected on 1% agarose electrophoresis gel in 1X TBE buffer and electrophoresed at 80 volts for 30 min. The amplicons were then visualised and analysed using UV transilluminator. Purification and direct sequencing of the PCR products were performed by MyTACG Bioscience Company.

Phylogenetic analysis of *Rigidoporus microporus*

DNA sequences of the isolates were identified using a BLASTN (Basic Local Alignment Search Tool Nucleotide) (Altschul et al. 1990) and the sequence homology was analysed with the same species sequences available in NCBI (National Centre for Biotechnology Institute) GenBank database. The resulting sequences were deposited in GenBank. For phylogenetic analysis, multiple sequence alignments were constructed and the tree was inferred by Maximum Likelihood (ML) method with 1000 bootstrap replications in Molecular Evolutionary Genetic Analysis version 7 (MEGA 7) (Kumar et al. 2016). Phylogenetic analyses were conducted using the Kimura's 2 parameter model (Kimura 1980). The reference sequences used in phylogenetic analysis of ITS, β-tubulin, and tef1-α gene regions were retrieved from NCBI GenBank database (Table 1).

Genetic diversity of *Rigidoporus microporus* using Somatic Incompatibility Test

The isolates were paired in all possible combinations. Self-crosses were performed as controls. Mycelia plugs (5 mm) were transferred onto a standard 90 mm Malt Extract Agar (MEA) plate and placed approximately 2 cm apart. The plates were incubated in the dark at 30 °C and hyphal interaction was observed daily until day 12. The isolates were evaluated as degrees of antagonism using the scores by Adaskaveg & Gilbertson (1987), i.e. 0 = compatible, 1 = weak, 2 = moderate and 3 = strong. Each pairing was repeated twice. The procedures used were similar to those of Latifah & Ho (2005). Incompatibility test data analysis was performed using NTSYS version 2.1 software. Incompatible reaction was scored as (1) and compatible reaction was scored as (0). Subsequently, the binary data were used to construct a dendrogram based on Jaccard's similarity coefficient by unweighted pair-group method with arithmetic means (UPGMA) (Pourmahdi & Taheri 2015).

Genetic diversity of *Rigidoporus microporus* using Inter Simple Sequence Repeat (ISSR)-PCR

Eight Inter Simple Sequence Repeat (ISSR)-PCR primers were synthesized at Apical Scientific Sdn Bhd. The primers were chosen based on the reports of genetic variation among 30 isolates of *R. microporus* by Kaewchai et al. (2009) and Soytong & Kaewchai (2014) (Table 2). PCR amplification of ISSR loci was performed in reaction volumes of 25 µl containing 12.5 µl 2X PCRBIO Taq Mix Red, 1.0 µl of 10 µM primer, 1.0 µl DNA template and complete with 10.5 µl free nuclease water. Optimum annealing temperature was determined for each primer.

Table 1 Country origins of *Rigidoporus microporus* and reference gene sequences from GenBank database

Isolate code	Species	Origin	Host	GenBank accession numbers		
				ITS	ß-tubulin	tef1-α
R01*	*R. microporus*	RRIM Research Station Similajau, Sarawak	*H. brasiliensis*	MH681573	MH796762	MK518054
R02*	*R. microporus*	RRIM Research Station Similajau, Sarawak	*H. brasiliensis*	MH681571	MH796763	MK518055
R03*	*R. microporus*	RRIM Research Station Similajau, Sarawak	*H. brasiliensis*	MH681572	MH807719	MK548888
R04*	*R. microporus*	RRIM Research Station Similajau, Sarawak	*H. brasiliensis*	MH681574	MH796764	MK548889
R05*	*R. microporus*	FELCRA Machang, Kelantan	*H. brasiliensis*	MH681550	MH706765	MK548890
R06*	*R. microporus*	FELCRA Machang, Kelantan	*H. brasiliensis*	MH681551	MH796766	MK548891
R07*	*R. microporus*	FELCRA Machang, Kelantan	*H. brasiliensis*	MH681552	MK285339	MK548892
R08*	*R. microporus*	FELCRA Machang, Kelantan	*H. brasiliensis*	MH681553	MH796767	MK548893
R09*	*R. microporus*	FELCRA Machang, Kelantan	*H. brasiliensis*	MH681554	MH796768	MK548894
R10*	*R. microporus*	FELCRA Machang, Kelantan	*H. brasiliensis*	MH681555	MH796769	MK548895
R11*	*R. microporus*	Smallholder, Kelantan	*H. brasiliensis*	MH681556	MH796770	MK548896
R12*	*R. microporus*	Malaysian Rubber Board, Perak	*H. brasiliensis*	MH681576	MH796771	MK548897
R13*	*R. microporus*	Malaysian Rubber Board, Perak	*H. brasiliensis*	MH681577	MH796772	MK548911
R14*	*R. microporus*	Malaysian Rubber Board, Perak	*H. brasiliensis*	MH681566	MH796773	MK548912
R15*	*R. microporus*	Malaysian Rubber Board, Perak	*H. brasiliensis*	MH681567	MH796774	MK548898
R16*	*R. microporus*	Malaysian Rubber Board, Perak	*H. brasiliensis*	MH681568	MH796775	MK548899
R18*	*R. microporus*	Smallholder, Baling, Kedah	*H. brasiliensis*	MH681563	MH807720	MK548900
R19*	*R. microporus*	UPM Serdang, Selangor	*H. brasiliensis*	MH681562	MH796777	MK548901
R20*	*R. microporus*	RRIM Research Station Sungai Buloh, Selangor	*H. brasiliensis*	MH681558	MH796778	MK548902
R21*	*R. microporus*	RRIM Research Station Sungai Buloh, Selangor	*H. brasiliensis*	MH681559	MH796779	MK548903
R22*	*R. microporus*	RRIM Research Station Sungai Buloh, Selangor	*H. brasiliensis*	MH681560	MH796780	MK548904
R23*	*R. microporus*	RRIM Research Station Sungai Buloh, Selangor	*H. brasiliensis*	MH681569	MH796781	MK548905
R24*	*R. microporus*	RRIM Research Station Sungai Buloh, Selangor	*H. brasiliensis*	MH681570	MH796782	MK548906
REFL20*	*R. microporus*	RRIMINIS Seri Iskandar, Perak	*H. brasiliensis*	MH681565	MH815008	MK548907
REFL21*	*R. microporus*	Sarikei, Sarawak	*H. brasiliensis*	MH681575	MH815009	MK548908

continue

Table 1 *continue*

Isolate code	Species	Origin	Host	GenBank accession numbers		
				ITS	ß-tubulin	tef1-α
REFL25 *	*R. microporus*	Kampung Tohor, Negeri Sembilan	*H. brasiliensis*	MH681561	MH815010	MK548909
ED310**	*R. microporus*	Iyanomo, Nigeria	*H. brasiliensis*	KJ559458	KJ559488	KJ559509
ED331**	*R. microporus*	Benin City,Nigeria	*Greenwayodendron suaveolens*	KJ559461	KJ559489	KJ559510
ED332**	*R. microporus*	Iyanomo, Nigeria	*H. brasiliensis*	KJ559462	KJ559490	KJ559511
ED333**	*R. microporus*	Iyanomo, Nigeria	*H. brasiliensis*	KJ559463	KJ559491	KJ559512
ED334**	*R. microporus*	Iyanomo, Nigeria	*H. brasiliensis*	KJ559464	KJ559492	KJ559513
N401**	*R. microporus*	Cameroon	*H. brasiliensis*	KJ559467	KJ559494	KJ559515
N402**	*R. microporus*	Cameroon	*H. brasiliensis*	KJ559468	KJ559495	KJ559516
N405**	*R. microporus*	Cameroon	*H. brasiliensis*	KJ559471	KJ559497	KJ559518
M13**	*R. microporus*	Malaysia (Ref.)	*H. brasiliensis*	KJ559474	KJ559499	KJ559520
M14**	*R. microporus*	Malaysia (Ref.)	*H. brasiliensis*	KJ559475	KJ559500	KJ559521
M15**	*R. microporus*	Malaysia (Ref.)	*H. brasiliensis*	KJ559476	KJ559501	KJ559522
X1864**	*R. microporus*	Indonesia (Ref.)	*Acacia* sp.	KJ559472	KJ559498	KJ559519
KM178999**	*R. ulmarius*	United Kingdom	*Aesculus hippocastanu*m	KJ559446	KJ559483	KJ559502
M318**	*R. microporus*	Peru	*H. brasiliensis*	KJ559481	-	-
MS564b**	*R. microporus*	Peru	*H. brasiliensis*	KJ559480	-	-
899**	*R. microporus*	Brazil	*H. brasiliensis*	KJ559479	-	-
311**	*R. microporus*	Brazil	*H. brasiliensis*	KJ559478	-	-
X862**	*R. microporus*	Brazil	*H. brasiliensis*	KJ559477	-	-

* = Isolates from rubber trees in Malaysia
** = Isolates from GenBank database

PCR amplification was carried out with initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing temperature ranged from 44–54 °C subject to the 8 primers (Table 2) for 1 min, extension at 72 °C for 2 min and final extension was set at 72 °C for 10 min. All PCR amplifications were performed using Thermal Cycler. In order to separate the amplified DNA, an agarose gel electrophoresis was performed using 2.0% agarose gel and was electrophoresed at 80 volts for 45 min. The gel was photographed using a UV-transilluminator. PCR reactions for each primer were repeated twice to confirm the uniformity and reproducibility of all isolates.

ISSR-PCR analysis

The DNA banding patterns for each isolate generated from ISSR primers were compared visually using UVIDoc software version 99.02. The band sizes were estimated based on 1 kb DNA ladder. The absence and presence of band were scored in a binary model of 0 and 1 respectively. Only repeated and reliable bands were selected. Pairwise comparison between isolates was generated using NTSYS version 2.1 software. Dendrogram was generated using the tree option of NTSYS-PC (Pourmahdi & Taheri 2015).

Table 2 List of eight ISSR primers used in this study

Primers	Sequences (5'-3')	Primer length (bp)	Annealing temperature (°)	References
UBC808	AGAGAGAGAGAGAGAGC	17	48	Kaewchai et al. 2009
UBC810	GAGAGAGAGAGAGAGAT	17	49	Kaewchai et al. 2009
UBC828	TGTGTGTGTGTGTGTGA	17	53	Kaewchai et al. 2009
UBC842	GAGAGAGAGAGAGAGAYG	18	44	Kaewchai et al. 2009
UBC850	GTGTGTGTGTGTGTGTYC	18	54	Kaewchai et al. 2009
UBC860	TGTGTGTGTGTGTGTGGA	18	51	Soytong & Kaewchai 2014
ISSR 7	GGGCGAGAGAGAGAGAGAGA	20	44	Kaewchai et al. 2009
OP-Mic8	CGACGACGACGACGA	15	53	Kaewchai et al. 2009

RESULTS

Phylogenetic analysis of the Internal Transcribed Spacer (ITS) region

Twenty-seven WRR isolates were identified using cultural characterisation followed by molecular characterisation. PCR amplification of ITS region from 27 *R. microporus* isolates formed amplicon size approximately 650 bp. BlastN analysis for all isolates showed 98–100% homology with the existing GenBank sequences in the NCBI database and this shows that they were closely related. The phylogenetic tree (Figure 1) inferred from ITS region sequences and generated from Maximum Likelihood method suggest that the isolates of *R. microporus* were separated into three distinct clades which were clade I (Asia), clade II (Africa), and clade III (South America). This observation was supported by a bootstrap value of 95%. Sub-clade 1 and Sub-clade 2 were two sub-clades in the Malaysian isolates. Sub-clade 1 consists of two isolates (R03 and R18) whereas Sub-clade 2 is made up of isolates R01 and R04. The reference *R. microporus* isolates from Malaysia, Indonesia and Thailand clustered together.

Phylogenetic analysis of the Beta-tubulin (ß-tubulin) gene region

PCR amplification of ß-tubulin region from 27 *R. microporus* isolates formed amplicon size approximately 750 bp. Phylogenetic tree inferred (Figure 2) from ß-tubulin region sequences and generated from Maximum Likelihood method revealed that *R. microporus* isolates were separated into two distinct clades which were clade I (Asia) and clade II (Africa). This observation is supported by a bootstrap value of 92%. The reference *R. microporus* isolates from Malaysia and Indonesia were clustered with *R. microporus* sequences. There was no geographical origin structuring among the Asian isolates. However, there were 5 sub-clades in the Malaysian isolates. Sub-clade 1 had three isolates (R03, R18 and REFL25), sub-clade 2 had two isolates from Kelantan (R06 and R07), sub-clade 3 had five isolates from Perak (R12, R13, R14, R15 and R16), sub-clade 4 consisted of three isolates (R01, R04 and R20) and sub-clade 5 had five isolates from Selangor (R19, R21, R22, R23 and R24). These results demonstrated the diversified *R. microporus* isolates in Malaysia.

Phylogenetic analysis of the Translation Elongation Factor-α (tef1-α) gene region

PCR amplification of tef1-α region from 27 *R. microporus* isolates formed amplicon size approximately 1000 bp. The phylogenetic tree (Figure 3) inferred from tef1-α region sequences and generated from Maximum Likelihood method revealed two distinct clades of *R. microporus* isolates which were clade I for Asia and clade II for Africa. This observation was supported by a high bootstrap value of 98%. The phylogram pattern was similar to that of ß-tubulin region and there was no geographical structuring among the isolates of the Asian clade. The reference *R. microporus* isolates from Asia were Malaysia and Indonesia and clustered with *R. microporus* sequences. The tef1-α gene region

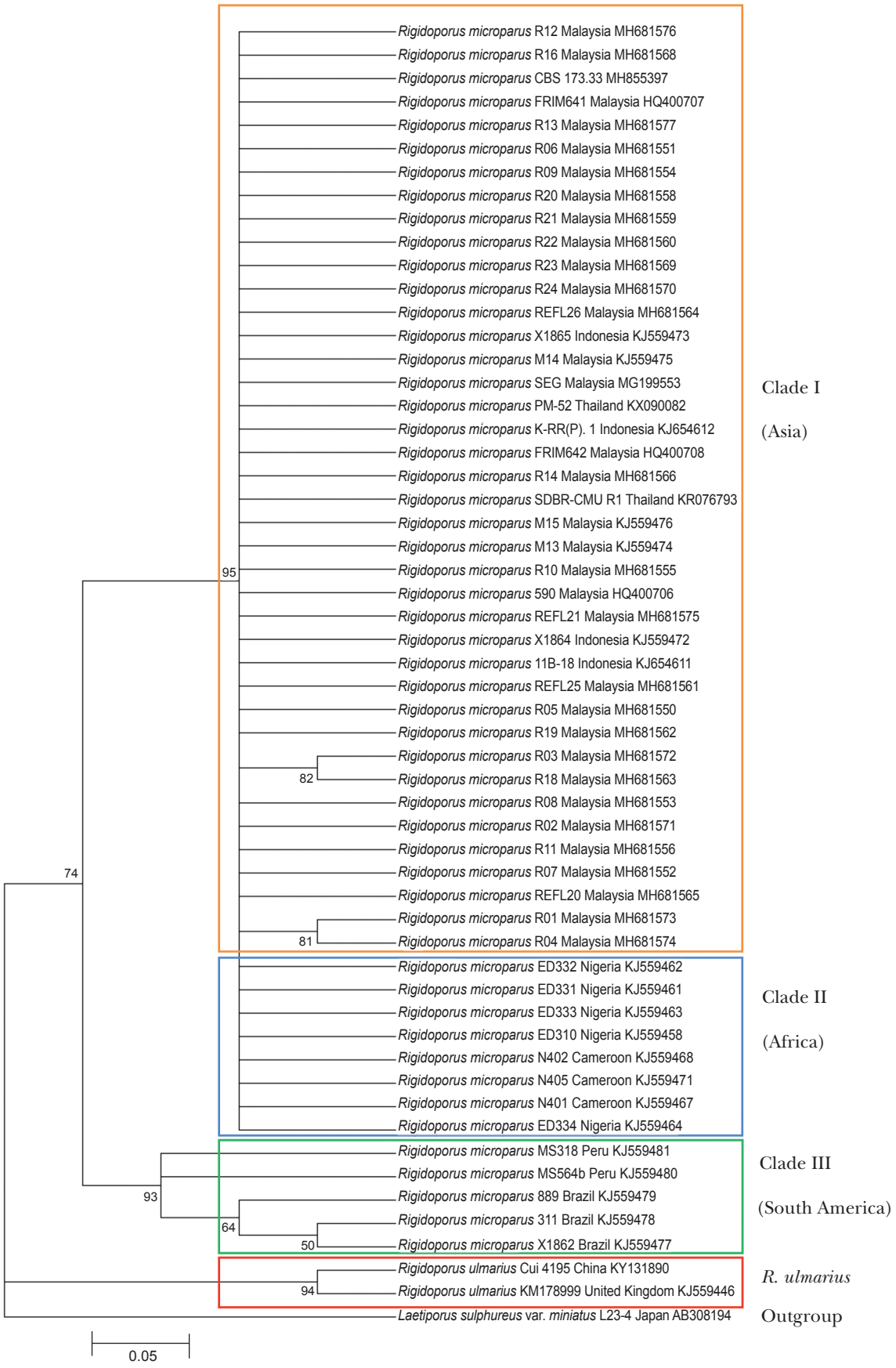

Figure 1 Phylogenetic relationships of ITS sequence of *Rigidoporus microporus* isolates using Maximum Likelihood method based on 1000 bootstrap replications and *Laetiporus sulphureus* var. *miniatus* (L23-4) was used as an outgroup

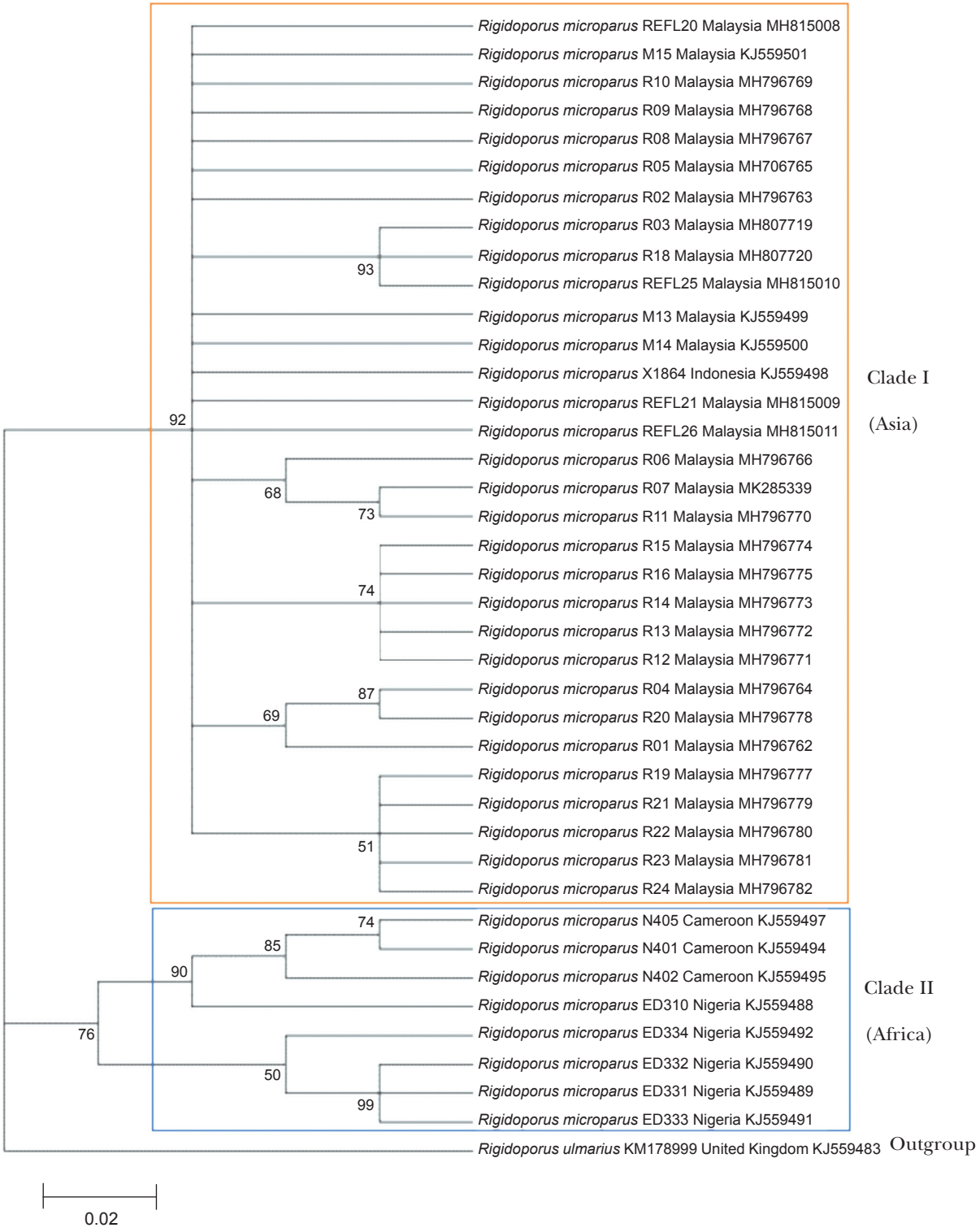

Figure 2 Phylogenetic relationships of ß-tubulin sequence of *Rigidoporus microporus* isolates using Maximum Likelihood method based on 1000 bootstrap replications and *Rigidoporus ulmarius* (KM178999) was used as an outgroup

among Malaysian isolates revealed two sub-clades. Sub-clade 1a consisted of four isolates (R09, R10, R11 and R18) and sub-clade 2a had five isolates (R11, R12, R13, R14 and REFL20). These results demonstrated the diversified *R. microporus* isolates in Malaysia.

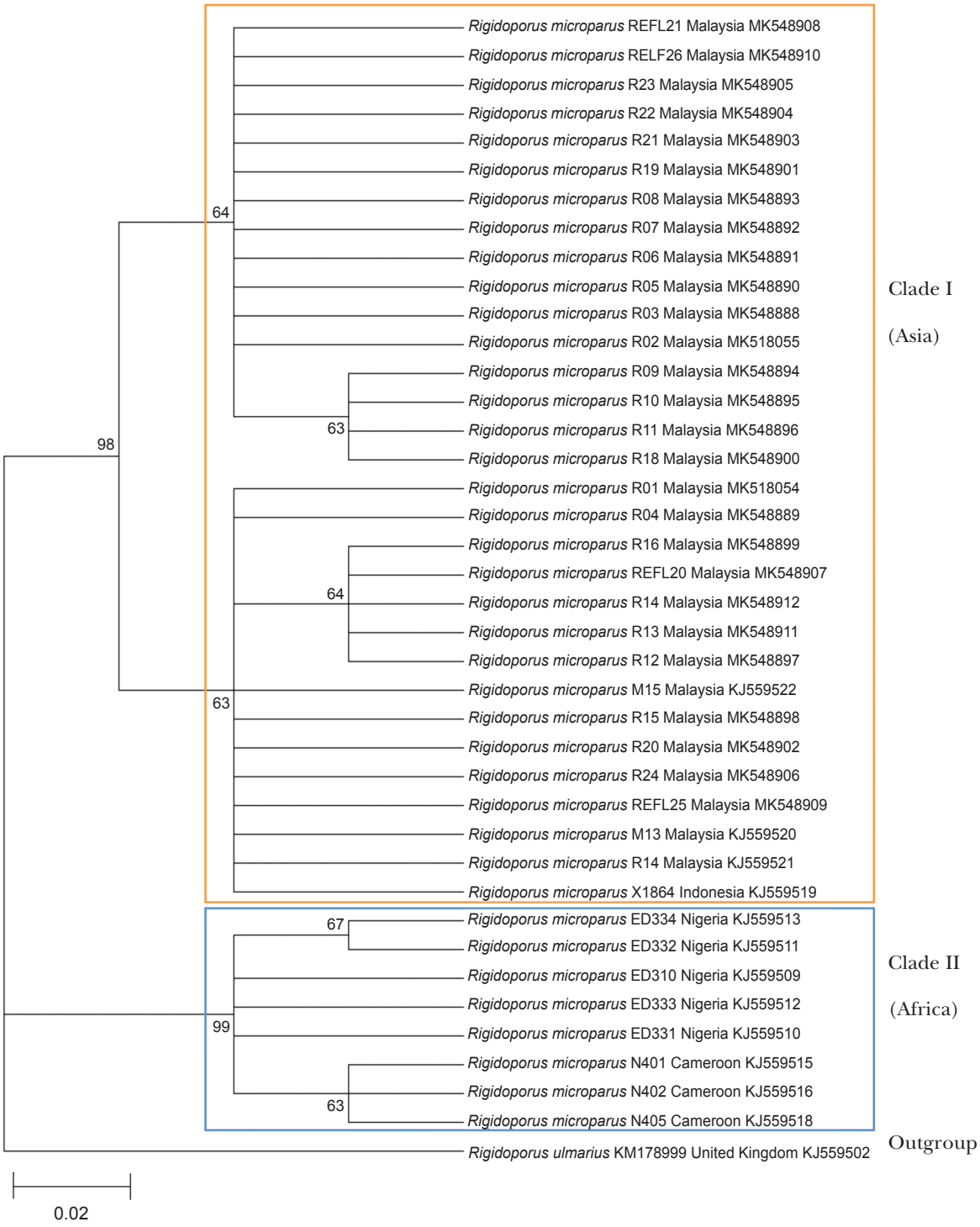

Figure 3 Phylogenetic relationships of tef1-α sequence of *Rigidoporus microporus* isolates using Maximum Likelihood method based on 1000 bootstrap replications and *Rigidoporus ulmarius* (KM178999) was used as an outgroup

Combined phylogenetic analysis of *Rigidoporus microporus*

The final gene sequence alignment of the combined dataset of ITS, ß-tubulin and tef1-α comprising of 40 taxa including 27 *R. microporus* isolates used in this study, 12 reference sequences from GenBank and *R. ulmarius* as an outgroup were used to analyse the phylogeny of *R. microphorus* in Malaysia. The phylogeny inferred in Figure 4 was from the concatenation of ITS, ß-tubulin and tef1-α datasets showed

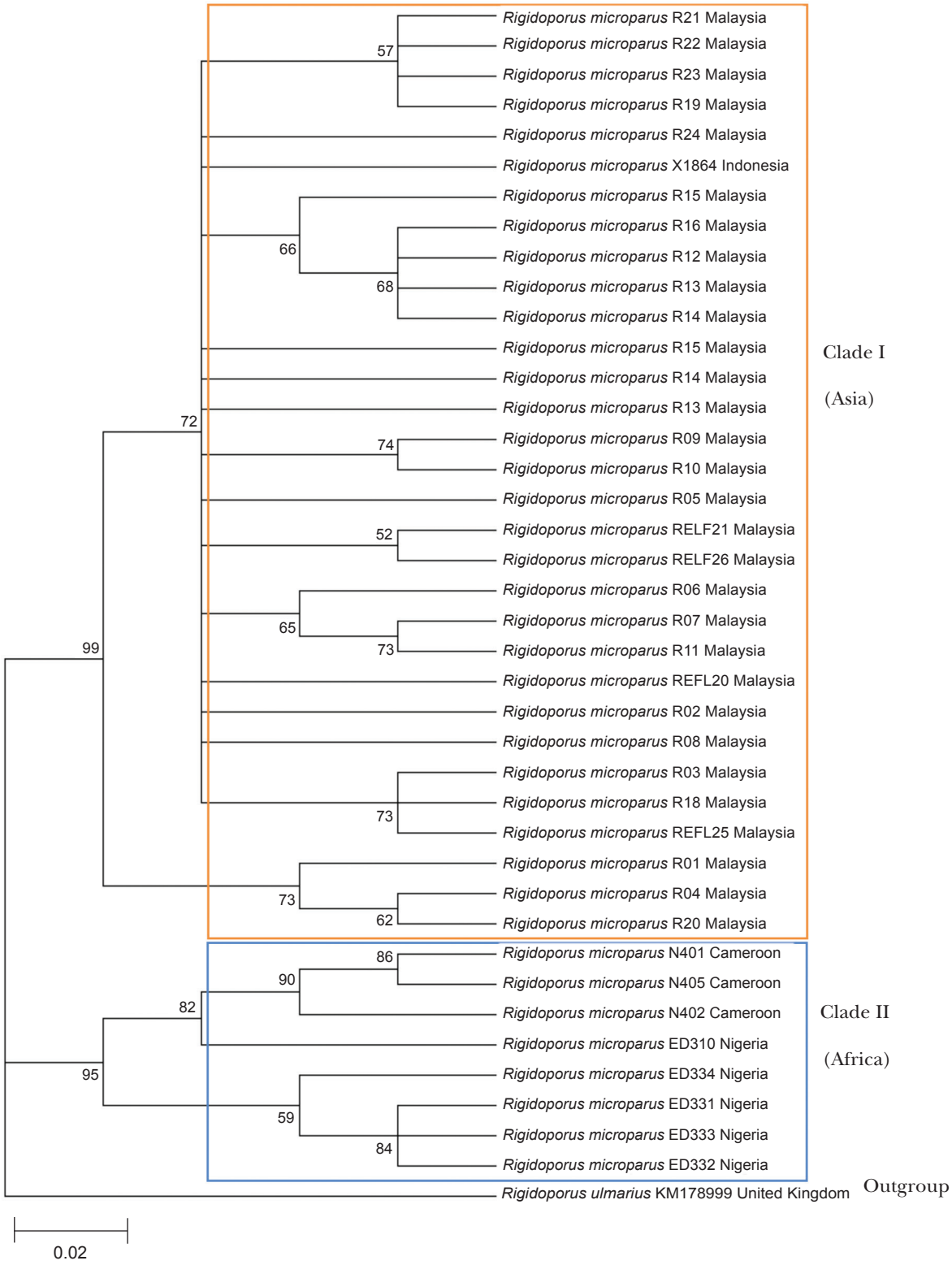

Figure 4 Phylogenetic relationships of combined sequences of ITS, ß-tubulin and tef1-α region of *Rigidoporus microporus* isolates using Maximum Likelihood method based on 1000 bootstrap replications and *Rigidoporus ulmarius* (KM178999) was used as an outgroup

similar clustering output like those of ß-tubulin and tef1-α datasets. All the reference *R. microporus* isolates from Asia such as Malaysia and Indonesia were clustered with *R. microporus* sequences. *R. microporus* isolates demonstrated two distinct clades namely, clade I (Asia) and clade II (Africa) and this finding was supported by a high bootstrap value of 99 %. There were two sub-clades in the Malaysian isolates namely, Sub-clade 1 and Sub-clade 2. Sub-clade 1 had six sub-sub-clades. Sub-sub-clade 1a consisted of three isolates (R06, R07 and R11), sub-sub-clade 1b had two isolates from Kelantan (R09 and R10), sub-sub-clade 1c had five isolates from Perak (R12, R13, R14, R15 and R16), sub-sub-clade 1d consisted of four isolates from Selangor (R19, R21, R22 and R23), sub-sub-clade 1e consisted of three isolates (R03, R18 and REFL25) and sub-sub-clade 1f consisted of two isolates (REFL21 and REFL26). Sub-clade 2 consisted of three isolates (R01, R04 and R20). *Rigidoporus ulmarius* KM178999 was used as an outgroup in the phylogenetic analysis.

Genetic diversity of *Rigidoporus microporus* using Somatic Incompatibility Test

In this test, weak to strong incompatible reactions were formed in all pairings of *R. microporus* isolates except for self-pairs. Isolates collected from the same or different plantations produced incompatible reactions and visible after 7 days of incubation. The interaction was clear after 10 to 14 days. This interaction zone was observed as a line when illuminated from the bottom, and as a raised ridge within a depressed zone from top viewing. The interaction between the mycelia of the two isolates which merged into a single colony was interpreted as an indication of somatic compatibility between the isolates.

Based on the somatic incompatibility analysis, 6 distinct groups were identified (Figure 5). Cluster 1 comprised of two isolates from Sarawak (R01 and R04) and cluster 2 consisted of two isolates from Sarawak (R02 and R03). Cluster 3 had four isolates from Kelantan (R05, R08, R09 and R10) and two isolates (R06 and R07) from Kelantan belonged to Cluster 4. Cluster 5 had one isolate from Kelantan (R11) and four isolates from Perak (R12, R13, R14, and R16). Cluster 6 consisted of twelve isolates (R15, R18, R19, R20, R21, R22, R23, R24, REFL20, REFL21, REFL25 and REFL26). The distance similarity coefficients among the 27 *R. microporus* isolates ranged from 0.230 to 1.000 and the cophenetic correlation between the clusters and the data matrix was estimated at 0.90981 and it indicated good fit. The highest genetic similarity (1.000) occurred in isolates R01 and R04, R02 and R03, R06 and R07, R05, R08 and R09, R13, R14 and R16, R21 and R22.

Genetic diversity of *Rigidoporus microporus* using ISSR

The ISSR primers produced polymorphic banding patterns for each of the 27 isolates and generated 257 amplified DNA bands or loci of different sizes. The number of polymorphic bands were 256 and the percentage of polymorphism was 99.61%. In the present study, the highest cophenetic correlation value r = 0.90461 was estimated using Jaccard's similarity coefficient and UPGMA clustering method. The UPGMA cluster analysis of Jaccard's similarity coefficients generated a dendrogram which categorised the 27 *R. microporus* isolates into four major clusters (Figure 6). Cluster I comprised of 10 isolates with two sub-clusters designated as Ia and Ib. Sub-cluster Ia consisted of single isolate (R01) and three isolates (R02, R03 and R04). Sub-cluster Ib consisted of isolates from Kelantan (R05, R06, R07, R08, R09 and R10). Cluster II was split into two sub-clusters, IIa and IIb. Sub-cluster IIa consisted of four isolates (R11, R12, R13 and R14) whereas sub-cluster IIb consisted of two isolates (R15 and R16). Similarly, cluster III which consisted of six isolates had two sub-clusters (IIIa and IIIb). Sub-cluster IIIa comprised of three isolates (R18, R19 and R20) and sub-cluster IIIb consisted of three isolates (R21, R22 and R23). Cluster IV consisted of five isolates (R24, REFL20, REFL21, REFL25 and REFL26). The results suggested that the clusters partially correlated with the geographical origin of the test isolates.

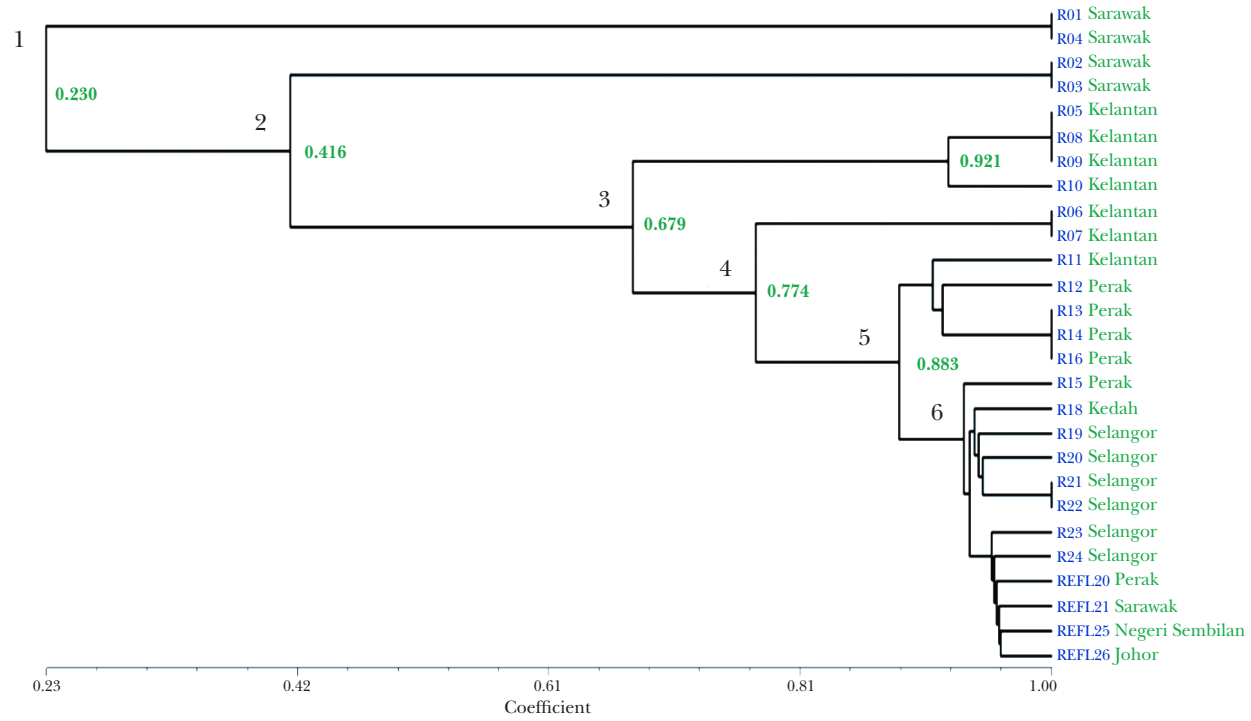

Figure 5 Dendrogram of *Rigidoporus microporus* isolates based on the somatic incompatibility data from different regions in Malaysia

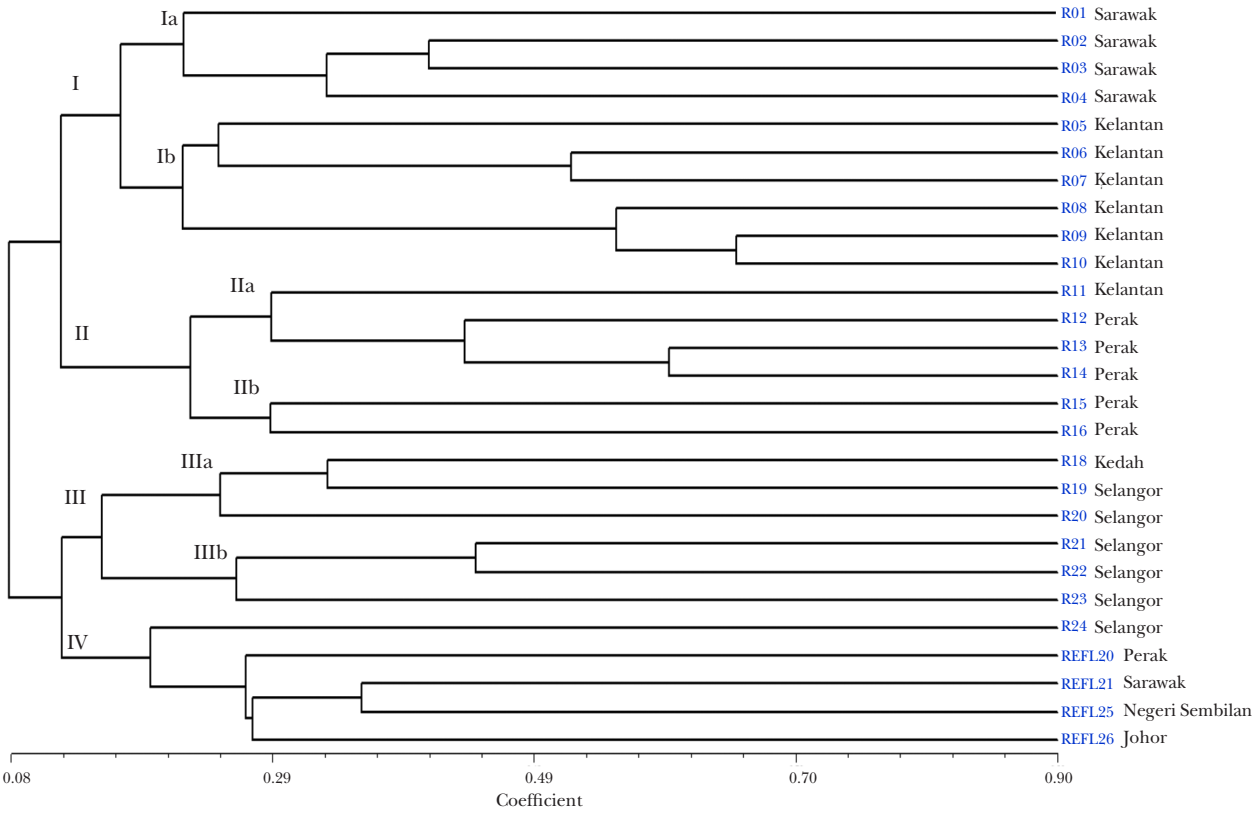

Figure 6 Dendrogram derived from UPGMA cluster analysis showing the genetic relationships among 27 *Rigidoporus microporus* isolate

DISCUSSIONS

In their natural habitat, plants are constantly challenged by disease-causing pathogens. In the case of rubber plantation, the WRR disease causes significant yield loss at about 15% in addition to the increasing operational cost. Moreover, by adopting the mono-cultural systems in rubber plantation, severe disease outbreaks can cause significant economic loss through wiping out rubber plantations. Therefore, knowledge on genetic diversity plays an important role in revealing and confirming the evolutionary relationships among different species of fungal pathogens on rubber plants (Hibbett & Donoghue 2001). The ISSR technique was used to examine the genetic diversity and population structure of several pathogenic fungi including *Fusarium oxysporum* f. sp. *melongenae* isolates (Altinok et al. 2018), *Rhizoctonia bataticola* (Walunj et al. 2018), *Puccinia polysora* (Unartngam et al. 2011), *Auricularia polytricha* (Yu et al. 2008), *Melampsora Larici-populina* (Yu et al. 2006) and *Beauveria bassiana* (Wang et al. 2005).

In the present study, 27 *R. microporus* isolates were isolated from various locations in Malaysia to determine their genetic diversity for the development of effective and sustainable control measures for the WRR disease. The primary aim of the present study is to produce fungal resistance rubber clone through breeding and to achieve disease resistant crop. However, it is important to identify the pathotypes to be used in the preliminary screening process and to know by what means the resistance is expressed and inherited (Thakur 1999). Currently, the collection of molecular entries of *R. microporus* for β-tubulin and tef1-α genes are limited in the NCBI GenBank database. Nevertheless, there is relatively a high number of ITS gene sequence data for *R. microporus* available in the GenBank database. The phylogenetic tree generated from the combination of the ITS, β-tubulin and tef1-α datasets suggest that there are two major groups of *R. microporus* world-wide and they are the Asian and African groups. There is no geographic structuring within the Asian clade. The African and Asian clades appear to be sister clades. These results are consistent with those of Oghenekaro et al. (2014). The Asian and African groups are known to cause serious damage in rubber plantations, and they are also recognized of having wide range of hosts other than *H. brasiliensis*. The existence of various sub-groups and sub-sub-groups of *R. microporus* both in Asia and Africa could be explained based on the variations in field symptoms and development of the WRR disease in rubber plantations world-wide. This variation might be due to the different environmental conditions and agronomic practices in the respective regions (Riggenbach 1960).

The phylogenetic tree revealed 2 sub-groups occurred in the Asian group although there was no clear geographic origin pattern in the distribution of *R. microporus* isolates. These findings suggested gene flow effect in the Asian population because of planting materials exchanges the neighbouring Southeast Asian countries such as Malaysia, Indonesia and Thailand. The sequences of *R. microporus* isolates obtained from rubber trees were analysed together with the sequences of *R. microporus* isolates collected from other hosts such as *Acacia* sp. and *Aesculus* sp. *R. microporus* isolates from rubber trees exhibited close relationship with those isolates isolated from other hosts suggesting that genetic variation was not affected by the host plant.

The ISSR markers and Somatic Incompatibility Test were used in this present study to further evaluate the levels of genetic relatedness among the 27 *R. microporus* isolated from the five states of Malaysia. Findings of these two studies were consistent. Eight ISSR primers detected sufficient genetic variation and relatedness among the isolates for complete differentiation. The generated banding patterns were reproducible, suggesting the suitability of these primers for the genetic studying of *R. microporus* isolates. There was high level of genetic diversity with 99.61% of polymorphism. Previous studies by Kaewchai et al. (2009) using ISSR markers indicated that there was a high level of genetic variation among the isolates of *R. microporus* collected from different sites in Thailand. ISSR-PCR method is a suitable and reliable tool for studying the population structure and differences in fungal isolates (Rodrigues et al. 2004, Altinok et al. 2018). In ISSR, genetic polymorphism suggests evolutionary adaptation which plays a role for the survival of a population in an environmental change (Stevens et al. 2007).

Based on the ISSR analysis, the clusters were consistent with the geographical boundary of

Peninsular Malaysia and East Malaysia. Four isolates from Sarawak were clustered and were clearly distinct from the isolates collected in other states such as Kedah, Kelantan, Perak and Selangor. Sarawak is located in East Malaysia and separated from Peninsular Malaysia by the South China Sea. The *R. microporus* isolates of Kelantan were also clustered and they were clearly distinct from the isolates of Kedah, Perak and Selangor. The boundary separating the west and east coast of Peninsular Malaysia is the Titiwangsa Range. Likewise, a geography-based separation of *Colletotrichum truncatum* populations had been reported (Mahmodi et al. 2014). These findings are consistent with those of Silva et al. (2012) who demonstrated that there was geographical differentiation between the populations of anthracnose pathogen, *C. kahawae* in eastern and western Africa. In their study, all the isolates from eastern Africa were grouped together and isolates from western African were found to be more closely related. This phenomenon was the results of the separation between western and eastern populations by lowland areas, where it could be a representative of an effective barrier for gene flow.

CONCLUSIONS

In conclusion, it is confirmed that rubber trees with WRR symptoms surveyed in the five states of Malaysia are infected by *R. microporus*. The results from this present study suggest the importance of a better understanding of *R. microporus* in Malaysia because it affects the rubber industry which contributes to the economy of Malaysia. The results of Somatic Incompatibility Test and those of ISSR analysis are comparable. The results obtained also suggest that ISSR marker is an informative marker and can be used for the assessment of genetic diversity among *R. microporus* isolates.

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