



UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION AND GENETIC DIVERSITY OF *Rigidoporus microporus* ASSOCIATED WITH WHITE ROOT ROT DISEASE IN RUBBER TREE (*Hevea brasiliensis* Müll.Arg.)

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By

BERNICE ANDREW

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

January 2020

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

CHARACTERIZATION AND GENETIC DIVERSITY OF *Rigidoporus microporus* ASSOCIATED WITH WHITE ROOT ROT DISEASE IN RUBBER TREE (*Hevea brasiliensis* Müll.Arg.)

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January 2020

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White root rot (WRR) disease caused by *Rigidoporus microporus* is a major disease affecting tropical rubber tree (*Hevea brasiliensis*) and some agricultural crops throughout the world. WRR disease causes major financial losses to growers as there is no effective method to control the disease in the field. There is limited information on the genetic variation of *R. microporus*, associated with WRR disease in Malaysia. In view of this, the objectives of this study were to characterize *R. microporus* isolates from rubber tree collected from different regions in Malaysia using morphological and molecular characteristics and to determine genetic diversity of *R. microporus* in Malaysia using somatic incompatibility (SI) test and inter-simple sequence repeat (ISSR). During July 2017 to May 2018, a total of 27 samples showing WRR symptoms such as presence of basidiocarps and rhizomorphs in five states of Malaysia including Kedah (Baling), Kelantan (FELCRA Machang and Smallholder in Machang), Perak (Ipoh) and Selangor (Sungai Buloh and UPM Serdang), Sarawak (Bintulu) were obtained. In the field observation, all sampling areas were infected by the root rot disease. Findings revealed that field disease incidence was in the range of 5-40% based on random sampling. The areas with the highest disease incident (30-40%) were RRIM Research Station Similajau, Sarawak (Nursery area), Kelantan (Smallholder in Machang), RRIM Research Station Sungai Buloh, Selangor (Germplasm) and Universiti Putra Malaysia, Serdang (Germplasm). This data indicated that the disease was prevalent on mature tree, but the disease was also detected on young tree (2 years old) with low disease incidence. Based on cultural morphology data, all the diseased samples collected were *R. microporus*. The isolates were pure white and displayed fluffy mycelial growth when cultured on Malt Extract Agar (MEA) medium. Majority of the isolates grew fast and within 7 - 8 days on MEA plates at 30°C. For molecular identification, BLASTn analysis showed 98 to 100% identical to existing reference sequences in the GenBank database. Phylogenetic analysis based on ITS, β -tubulin and *tef1* gene and the

concatenation of the three genes confirmed that all 27 *R. microporus* isolates in this study were clustered into a clade with the reference sequences from Southeast Asia such as Malaysia, Thailand and Indonesia supported with bootstrap values of 95%, 92%, 98% and 99%, respectively. There was also no geographic structuring observed within the Asian clade, suggesting gene flow among the populations. However, sub-clades were found to form to among the local isolates, suggesting the existence of diversity among the isolates. Results from the SI test indicated that the *R. microporus* in the rubber plantations were clustered according to geographical regions based on a dendrogram generated from Unweighted Pair Group Method with Arithmetic Means (UPGMA) analysis. On the basis of ISSR data, the clustering of isolates revealed a pattern of genetic variation in correlation with the different geographical regions. The dendrogram from SI analysis is in congruent with the dendrogram generated based on ISSR data. These findings provide insights into the studies of genetic and details documentation on *R. microporus* associated with WRR disease of rubber in Malaysia.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

**PENCIRIAN DAN GENETIK DIVERSITI *Rigidoporus microporus*, ASOSIASI
DENGAN PENYAKIT AKAR PUTIH DALAM POKOK GETAH (*Hevea
brasiliensis* Müll.Arg.)**

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Penyakit akar putih yang disebabkan oleh *Rigidoporus microporus* adalah penyakit utama yang menjejaskan pokok getah (*Hevea brasiliensis*) di tropika dan beberapa tanaman pertanian di seluruh dunia. Penyakit akar putih menyebabkan kerugian kewangan utama kepada penanam kerana tiada kaedah yang berkesan untuk mengawal penyakit ini di ladang. Terdapat maklumat terhad mengenai variasi genetik *R. microporus*, yang berasosiasi dengan penyakit akar putih di Malaysia. Oleh itu, objektif kajian ini adalah untuk mencirikan isolat *R. microporus* dari pokok getah yang dikumpulkan dari lokasi berbeza di Malaysia dengan menggunakan ciri morfologi dan molekul, dan menentukan kepelbagaian genetik *R. microporus* di Malaysia dengan menggunakan ujian ketidakerasian somatik dan penanda *inter-simple sequence repeat* (ISSR). Pada Julai 2017 hingga Mei 2018, sebanyak 27 sampel yang menunjukkan simptom penyakit akar putih seperti kehadiran jasad berbuah dan rhizomorph telah disampelkan di lima buah negeri di Malaysia termasuk di Kedah (Baling), Kelantan (FELCRA Machang dan Kebun kecil di Machang), Perak (Ipoh) dan Selangor (Sungai Buloh dan UPM Serdang), Sarawak (Bintulu). Dalam pemerhatian lapangan, semua kawasan persampelan dijangkiti oleh penyakit akar reput. Penemuan mendedahkan bahawa simptom penyakit di lapangan adalah dalam lingkungan 5-40% berdasarkan persampelan rawak. Kawasan yang mempunyai simptom penyakit yang paling tinggi (30-40%) ialah Stesen Penyelidikan RRIM Similajau, Sarawak (Kawasan nurseri), Kelantan (Kebun kecil di Machang), Stesen Penyelidikan RRIM Sungai Buloh, Selangor (Germplasma) dan Universiti Putra Malaysia, Serdang (Germplasma). Data ini menunjukkan bahawa penyakit itu lazim di atas pokok getah yang matang, tetapi penyakit ini juga dikesan pada pokok muda (2 tahun) dengan simptom penyakit yang rendah. Berdasarkan data morfologi, semua sampel yang dikumpul adalah *R. microporus*. Kesemua isolat berwarna putih tulen dan mempamerkan pertumbuhan mycelia yang berbulu di atas agar MEA. Sebahagian besar isolat

tumbuh dengan cepat dan dalam masa 7 - 8 hari di atas plat MEA pada 30°C. Bagi pencirian molekular, analisis BLASTn menunjukkan 98% ke 100% persamaan dengan rujukan jujukan di dalam pangkalan data GenBank. Analisis filogenetik berdasarkan jujukan gen ITS, *β-tubulin* dan *tef1* dan gabungan tiga gen mengesahkan bahawa kesemua 27 *R. microporus* dalam kajian ini telah dikelompokkan bersama gen rujukan dari Asia Tenggara seperti Malaysia, Thailand dan Indonesia, disokong dengan nilai bootstrap 95%, 92%, 98% dan 99%, masing-masing. Analisis filogenetik juga membuktikan bahawa tiada penstrukturan geografi yang dihasilkan dalam kelompok Asia, yang menunjukkan bahawa terdapat aliran gen di kalangan populasi. Walau bagaimanapun, kelompok kecil didapati terbentuk antara isolat tempatan, yang menunjukkan bahawa kewujudan kepelbagaian antara isolat. Hasil daripada ujian ketidakserasian somatik menunjukkan bahawa *R. microporus* di ladang getah dikelompokkan mengikut kawasan geografi berdasarkan dendrogram yang dihasilkan dari analisis *Unweighted Pair Group Method with Arithmetic Means* (UPGMA). Berdasarkan analisis ISSR, klustering isolat menghasilkan corak variasi genetik yang berkaitan dengan kawasan geografi yang berbeza. Dendrogram dari analisis ketidakserasian somatik adalah kongruen dengan dendrogram yang dihasilkan berdasarkan data Pengulangan Jujukan Inter-Mudah. Penemuan ini boleh memberi maklumat kepada kajian genetik dan dokumentasi yang teliti ke atas *R. microporus* yang dikaitkan dengan penyakit akar putih pada tanaman getah di Malaysia.

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LIST OF ABBREVIATIONS

%	Percentage
a.i.	Active Ingredient
AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
ANOVA	Analysis of Variance
BLASTn	Nucleotide Basic Local Alignment Search Tool
bp	Base pair
°C	Degree Celsius
CABI	Centre for Agriculture and Bioscience International
CLD	Colletotrichum Leaf Disease
CLF	Corynespora Leaf Fall
cm	Centimetre
CRD	Completely Randomized Design
CTAB	Cetyltrimethylammonium Bromide
DAI	Day after incubation
ddH ₂ O	Double distilled water
dH ₂ O	Distilled water
DI	Disease incidence
DNA	Deoxyribonucleic acid
DOSM	Department of Statistics Malaysia
ED	Effective Dose
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol

g	Gram
GDP	Gross Domestic Product
GNI	Gross National Income
GSM	Ganoderma selective medium
G _{ST}	Coefficient of gene differentiation
H	Nei's Gene Diversity
ha	Hectares
HCl	Hydrochloric acid
He	Expected Heterozygosity
H _s	Diversity within-population
H _T	Total gene diversity
I	Shannon's Information Index
ISSR	Inter-Simple Sequence Repeat
ITS	Internal Transcribed Spacer
kb	Kilobase pair
l	Litre
LSU	Large Subunit
M	Moles/ Litre (Molarity)
MCG	Mycelial Compatibility Group
MEA	Malt Extract Agar
MEB	Malt Extract Broth
mg	Milligram
min	Minute
ML	Maximum Likelihood

ml	Millilitre
mM	Millimolar
mm	Millimetre
MRB	Malaysian Rubber Board
Na	Observed Number of Alleles
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Institute
Ne	Effective Number of Alleles
ng	Nanogram
Nm	Gene flow between populations
nm	Nanometre
NTSYS	Numerical Taxonomy and Multivariate Analysis System
PA	Phenol: Isoamyl alcohol
PCA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
POPGENE	Population Genetic Analysis
r	Correlation Coefficient
RAPD	Random Amplified Polymorphic DNA
rDNA	Ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
RM	Ringgit Malaysia
rpm	Revolutions per minute
RRIM	Rubber Research Institute of Malaysia
SALB	South America Leaf Blight

SAS	Statistical Analysis Software
SDS	Sodium Dodecyl Sulfate
secs	Second
SI	Somatic Incompatibility
SIG	Somatic Incompatibility Group
SM	Similarity Matrix
SSR	Simple Sequence Repeats
TBE	Tris-borate EDTA
<i>tef-1 α</i>	Translation Elongation Factor -1 Alpha
Tukey's HSD	Tukey's Honestly Significant Difference
uHe	Unbiased Expected Heterozygosity
UPMGA	Unweighted Pair Group Methods with Arithmetic Mean
UV	Ultraviolet
V	Voltage
VCG	Vegetative Compatibility Group
<i>β-tubulin</i>	Beta tubulin
μ g	Microgram
μ l	Microliter
μ M	Micromolar
μ m	Micrometre

CHAPTER 1

INTRODUCTION

The rubber tree is a monoclonal crop contributing to the economic growth of Malaysia. Most of the significant raw rubber producers in the world are based in Southeast Asia countries such as Thailand, Malaysia and Indonesia (Killmann, 2001). According to MREPC (n.d.), Malaysia was ranked as the eighth largest consumer of rubber in the world in year 2016. Nevertheless, rubber industry faced a declination of 1.47 million hectares since 1980s. Due to this problem, there were many closures of processing factories due to insufficient of raw materials (Killmann, 2001). Besides, most of the rubber field area were converted for other development such as housing area, commercial and industrial purposes (Vijayaraghavan *et al.*, 2008b). The emergence of other crops such as oil palm and cocoa made the economy more profitable compared to rubber. Consequently, the production of natural rubber plummeted and eventually, Malaysia ranked third among rubber producing countries in Southeast Asia (Vijayaraghavan *et al.*, 2008a).

In 2017, rubber contributed 7.3% to the Gross Domestic Product (GDP) of agriculture in Malaysia. Production of natural rubber also improved by 66.6 thousand tonnes (9.9%) in 2017. The economy growth of natural rubber continued to rise in October 2018 by 8.7% (DOSM, 2018a). Subsequently, natural rubber production increased 1.9% in November 2018 (DOSM, 2019c). Malaysia is known as major supplier for medical gloves in the world, fulfilling more than 50% of demand globally. In 2016, it was reported that rubber gloves boosted up the total exports of rubber products to RM13.3 billion. Despite all the circumstances faced, the rubber industry in Malaysia especially latex goods continued to be the largest contributor to Malaysian rubber export which amounted to RM 9.2 billion in the first six months of 2018 (MREPC, n.d.). In short, there has been positive growth of the rubber industry over the years. The rubber acreage in Malaysia is currently around 1,0830,480 ha (Natural Rubber Statistics, 2018).

Apart from that, the Malaysian rubber industry also provided job opportunities. There were about 47.3% smallholders involve in this industry based on the report in four states of Malaysia (Negeri Sembilan, Perak, Sabah and Sarawak). The rubber industry is expected to continue to produce high value products in order to achieve the government's objective of transforming Malaysia into a developed nation with high income by 2020. However, the objective can be achieved if farmers still persist to plant rubber. There are many different schemes and assistance that have been implemented to assist smallholders. For example, smallholders have the privilege to get special cash payments and the fertiliser for rubber tree being subsidised (Wee and Singaravelloo, 2018).

Nonetheless, one of the major constraints of the rubber cultivation and dropping of rubber yield are mostly due to pathogens that affect the rubber tree in plantations. The pathogens caused deterioration of yield by reducing the quality and quantity of crops. Diseases caused by fungi are common in country having tropical climate. According to Ogbebor *et al.* (2010), there are three most significant fungal root diseases: white root rot (*Rigidoporus microporus* (Fr.) Overeem), brown root rot (*Phellinus noxius* (Corner) G. Cunningham), and red root rot (*Ganoderma pseudoferreum* (Wakefield) Overeem and B.A. Steinmann). Based on the previous studies by Omo-Ikerodah *et al.* (2012), the authors mentioned that root diseases of rubber tree is the most serious disease as these diseases often fatal. WRR is the most destructive root disease and accountable for about 96% of root diseases occurrence in West Africa. Jayasuriya and Thennakoon (2007) also pointed out that WRR disease is the most threatening root disease in many tropical countries such as Sri Lanka, Thailand and Indonesia. There is no report on the significant economic loss in monetary terms caused by WRR disease in Malaysia. Previous assumption stated by Nandris *et al.* (1987) regarding the losses can cost up to hundred thousand dollars per hectare for 25 productive years of a rubber tree.

Rigidoporus microporus survives by producing plethora of white mycelium and flattened rhizomorphs which eventually would colonize the surface of a root bark and infect a healthy tree when in contact. According to Omorusi (2012), in the advanced stage of maturity, the WRR fungi can form a brownish-orange basidiocarp, with a bright yellow margin when fresh, while the lower surface is reddish-brown which surrounds the base of the trunk. At the late stages, the infection followed by yellowing of leaves, canopy and root system are being damaged and eventually, the tree falls (Nandris *et al.*, 1987; Omo-Ikerodah *et al.*, 2012). In Malaysia, WRR disease was a major problem for 43% of farmers in a smallholdings survey conducted (Sail and Ahmad, 2009).

Several studies have been conducted to solve the economically important disease in rubber plantations. Cultural and chemical means such as removal of infected stumps and using chemical fungicides are commonly used to control WRR disease, however, it may not be effective as the diseases spreads rapidly (Kaewchai *et al.*, 2009). According to Guyot and Flori (2002), one of the most important control strategies of WRR disease is to reduce the inoculum sources to prevent the disease from spreading further. In order to implement that strategy, Bayraktar *et al.* (2008) proposed that the knowledge of genetic variation among the populations in pathogenic fungi would be beneficial in the enhancement of disease management system and breeding purposes. Similar suggestion also emphasized by McDonald (1997) on the importance of study the population biology of fungal pathogen.

In Asia, the characterization of *R. microporus* obtained from rubber trees were done in Thailand by using sequence analysis of internal transcribed spacer (ITS) region and inter-simple sequence repeat (ISSR) as reported by Kaewchai *et al.* (2009; 2010). The traditional identification of fungi has limitation and

therefore, it is necessary to establish the molecular identification and genetic variation study related to WRR disease in rubber plantation. The results from this study will be a major platform on generating details documentation on the genetic variation of WRR disease of rubber in Malaysia by using molecular approaches. Besides, the output from this research provides useful information to overcome the disease, hence improving the rubber production in Malaysia. Therefore, this study was carried out based on the following objectives:

- i. To characterize *Rigidoporus microporus* isolates from rubber tree (*Hevea brasiliensis*) collected from different regions in Malaysia using morphological and molecular characteristics; and
- ii. To determine genetic diversity of *Rigidoporus microporus* in Malaysia using somatic incompatibility (SI) and inter-simple sequence repeat (ISSR).

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