GENETIC DIVERSITY OF *Bacillus pumilus* GROUP CAUSING TRUNK BULGES ON RRIM 3001 SUPERCLONE RUBBER TREE (*Hevea brasiliensis* Müll.Arg.) IN PENINSULAR MALAYSIA

By

AINUR AINIAH BINTI AZMAN HUSNI

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Bacillus pumilus was identified as the pathogen in the initial outbreak of trunk bulges in RRIM 3001 superclone rubber plants in Serdang, Selangor. The occurrences of trunk bulges of RRIM 3001 superclone rubber trees eventually affect the quality and yield of natural rubber. Multilocus sequence analysis (MLSA) of five housekeeping genes (*gyrB, pyrE, aroE*, *rpoB,* and *trpB*) and repetitive elements-based polymerase chain reaction (rep-PCR) using REP, ERIC and BOX primers were conducted to analyze the diversity and systematic relationship of 20 isolates of *B. pumilus* group from four rubber tree plantations in Peninsular Malaysia (Serdang, Tanah Merah, Baling and Rawang). The analysis of all individual phylogenetic trees revealed the nearly congruent topology structure, with 75-100% bootstrap value across all 20 isolates of *B. altitudinis* and *B. safensis.* Both *B. altitudinis* and *B. safensis* are closely related to each other. Interestingly, none of the 20 bacterial isolates collected from the four collection areas were clustered together, indicating that variations between isolates from various geographical locations were significantly smaller than those between isolates from the same locations. Specifically, these 20 isolates were classified into two primary clusters: Cluster A, which contained 17 isolates of *B. altitudinis*, and Cluster B, which contained three isolates of *B. safensis*. The results were validated by a concatenated phylogenetic tree, which revealed that the isolates were divided into two clusters of *B. altitudinis* and *B. safensis*, with 98% and 100% bootstrap values, respectively. The dendrograms generated by REP-, ERIC-, and BOX-PCRs in rep-PCR analysis revealed that both species separated well. Compared to independent rep-PCR experiments, multi rep-PCR generates a high level of discrimination, while isolates of the two species remain separate in the corresponding dendrogram. The similarity coefficient observed

among isolates in Cluster A (*B. altitudinis* isolates from Kedah, Kelantan and Rawang and five isolates from Serdang, ~35%) and Cluster B (three *B. safensis* isolates from Serdang, ~95%) from geographically separated states indicated that the isolates possibly were lineages of a single virulent isolate. The spread of these infections over Peninsular Malaysia was most likely due to transmission via planting stock. REP-PCR is the best approach for species grouping and separation since REP primers produced the most distinct fingerprinting pattern, followed by ERIC primer and BOX primer. The pathogens responsible for trunk bulges on RRIM 3001 superclone rubber trees in Peninsular Malaysia have been reclassified as *B. altitudinis* and *B. safensis* of the *B. pumilus* group. This finding would be a powerful platform for generating detailed documentation on the genetic diversity of *B. pumilus* group associated with RRIM 3001 trunk bulges in Peninsular Malaysia as well as developing disease control strategies to limit the spread of *B. pumilus* group species to new area.

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

KEPELBAGAIAN GENETIK KEATAS KUMPULAN *Bacillus pumilus* PENYEBAB BONJOLAN BATANG KEPADA KLON RRIM 3001 POKOK GETAH (*Hevea brasiliensis* Müll. Arg.) DI SEMENANJUNG MALAYSIA

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Bacillus pumilus dikenal pasti sebagai patogen dalam wabak awal bonjolan batang di pokok getah klon RRIM 3001 di Serdang, Selangor. Kejadian bonjolan batang pokok getah klon RRIM 3001 telah mempengaruhi kualiti dan hasil getah asli. Analisis urutan multilokus (MLSA) lima gen pengemasan (gyrB, pyrE, aroE, rpoB, dan trpB) dan reaksi berantai polimerase berasaskan elemen berulang (rep-PCR) menggunakan primer REP, ERIC dan BOX dilakukan untuk menganalisis kepelbagaian dan sistematik hubungan 20 pencilan kumpulan *B. pumilus* dari empat kebun pokok getah di Semenanjung Malaysia (Serdang, Tanah Merah, Baling dan Rawang). Analisis pada semua pokok filogenetik menunjukkan struktur topologi hampir sama, dan hasilnya menunjukkan 75-100% nilai butstrap antara semua 20 pencilan dengan *B. altitudinis* dan *B. safensis*. Kedua-dua *B. altitudinis* dan *B. safensis* berkait rapat antara satu sama lain. Menariknya, tiada satu pun daripada 20 pencilan bakteria yang dikumpul daripada empat kawasan pengumpulan dikelompokkan bersama, menunjukkan bahawa variasi antara pencilan dari pelbagai lokasi geografi adalah jauh lebih kecil daripada pencilan antara pencilan dari lokasi yang sama. Secara khusus, 20 pencilan ini diklasifikasikan menjadi dua kelompok utama: Cluster A, yang berisi 17 pencilan *B. altitudinis*, dan Cluster B, yang mengandungi tiga pencilan *B. safensis*. Keputusan telah disahkan oleh pokok filogenetik gabungan, yang mendedahkan bahawa pencilan dibahagikan kepada dua kelompok, *B. altitudinis* dan *B. safensis* dengan nilai butstrap 98% dan 100% masing-masing. Dalam analisis rep-PCR, dendrogram yang dihasilkan oleh REP-, ERIC-, BOX-PCR menunjukkan bahawa kedua-dua spesies berpisah dengan baik. Berbanding dengan eksperimen rep-PCR bebas, multi-PCR menghasilkan tahap diskriminasi yang lebih tinggi sedangkan pencilan kedua spesies tetap

terpisah dalam dendrogram yang sesuai. Pekali kesamaan yang diperhatikan di antara pencilan di Kluster A (pencilan *B. altitudinis* dari Kedah, Kelantan dan Rawang dan lima pencilan dari Serdang, ~ 35%) dan Kluster B (tiga pencilan *B. safensis* dari Serdang, ~ 95%) dari negeri yang terpisah secara geografi ditunjukkan bahawa pencilan mungkin adalah keturunan dari satu pencilan virulen. Penyebaran jangkitan ini ke seluruh Semenanjung Malaysia berkemungkinan besar disebabkan oleh penularan melalui stok tanaman. REP-PCR adalah kaedah yang paling sesuai untuk pengelompokan dan pemisahan spesies kerana primer REP menunjukkan corak cap jari yang paling jelas, diikuti oleh primer ERIC dan primer BOX. Patogen yang bertanggungjawab untuk membonjol batang pada pokok getah klon RRIM 3001 di Semenanjung Malaysia telah diklasifikasikan semula sebagai *B. altitudinis* dan *B. safensis* dari kumpulan *B. pumilus*. Kajian ini akan menjadi platform utama untuk menghasilkan dokumentasi terperinci mengenai kepelbagaian genetik kumpulan *B. pumilus* yang berkaitan dengan penyakit bonjolan batang pada RRIM 3001 di Semenanjung Malaysia dan dapat digunakan untuk merancang strategi pengendalian penyakit untuk membatasi pengenalan spesies kumpulan *B. pumilus* ke wilayah baru.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirements for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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%	percent
°C	degree Celsius
ANOVA	Analysis of Variance
bp	base pair
DNA	deoxyribonucleic acid
FAO	Food and Agriculture Organization
g	gram
h	hour
ha	hectare
kb	kilobase pair
kg	kilogram
L	litre
LTC	latex timber clones
mL	milliliter
M	molar
min	minutes
MRB	Malaysian Rubber Board
NR	natural rubber
rRNA	ribosomal DNA
RRIM	Rubber Research Institute of Malaysia
sec	second
TBE	*tris-Borate*-EDTA
Taq	*Thermus aquaticus*
TM	melting temperature
NB	nutrient broth
U	unit
µL	microlitre
UV	ultra-violet
V	voltan/volt

CHAPTER 1

INTRODUCTION

1.1 Background of Study

The rubber tree (*Hevea brasiliensis*) is a native tree species of Amazon forests in Brazil and commercially exploited for the production of high-quality natural rubber derived from latex or sap (Priyadarshan and de Gonçalves, 2003; Webster and Paardekooper, 1989). Rubber tree has become an essential commercial crop in many developing countries, especially in South East Asia (Bakri *et al*., 2017; Nath *et al*., 2010; Roy *et al*., 2014). To fulfill the high demand for planting materials, the Malaysian Rubber Board (MRB) has introduced extraordinary rubber tree clones known as latex timber clones (LTC) that can contribute to the remarkable return of latex and timber (Mayati and Izilawati, 2017). RRIM 3001 superclone rubber tree is one of the successful latex timber clones with several vital features, including vigorous growth, high yield latex and timber production (Mokhatar and Daud, 2011). It can generate high production of natural rubber, about 3 tons/hectare/year (Mazlan *et al*., 2019b). Additionally, RRIM 3001 superclone rubber tree has been appointed a new name, “Clone 1 Malaysia” (Borneo Post Online, 2011).

In 2019, the first outbreak of trunk bulges has been reported in RRIM 3001 superclone rubber tree by Mazlan *et al*. (2019b). The occurrences of trunk bulges of RRIM 3001 superclone rubber trees eventually affect the quality and yield of natural rubber. The symptoms can be seen on the whole trunk of the rubber trees, including numerous tumor-like bacteriosis, canker wounds of different sizes at the tapping zone and bleeding lesions (Mazlan *et al*., 2019b). Swelling and cracking of the trunk bulges may happen at the most severe stage (Kovaleva *et al*., 2015; Mazlan *et al*., 2019b). The tapping method on the uneven bark surface could lead to cambium cells' injury and results in bulges on the RRIM 3001 superclone rubber tree (Zarawi, 2018). *Bacillus pumilus* (*B. pumilus*) has been identified as the causative agent of trunk bulges (Mazlan *et al*., 2019b).

1.2 Problem Statement

The occurrences of trunk bulges have further diminished smallholder enthusiasm in the rubber industry since it has become one of the severe trunk diseases in Malaysia. It spreads quickly, produces severe yield losses, and has a serious impact on the rubber industry. Rubber has become the second important commodity crop in Malaysia, with a 4.7% contribution to the national gross domestic products (Sharib and Halog, 2017). The occurrence of trunk bulges causes a significant reduction in latex and rubberwood production in Malaysia (Mokhter and Aris, 2018). Thus, trunk bulges in rubber trees caused by *B.*

pumilus become the primary concern towards latex and rubberwood production. To date, there is no in-depth investigation and research related to this disease and the pathogen, as well as the genetic diversity and genetic relationship between *B. pumilus* group isolates. *Bacillus pumilus* group composed of the species *B. pumilus, B. safensis, B. altitudinis, B. stratosphericus, B. aerophilus, B. xiamenensis and B. invictae* that share sequence similarity value over 99.5% in their 16S rRNA genes (Liu *et al*., 2013). All these species cannot be distinguished by biochemical characteristics, phenotypic tests and 16S rRNA gene sequencing due to high genetic homogeneity. The 16S rRNA gene sequence was introduced by Woese and Fox (1977) and has been widely used as a molecular marker for bacteria identification and to estimate the relationships among bacteria (Amann *et al*., 1995; Stahl, 1991). Unfortunately, 16S rRNA gene sequence is not a perfect measure of overall sequence divergence between some genera such as *Bacillus pumilus* group (Liu *et al*., 2013), *Bacillus cereus* group (Ash *et al*., 1991) and *Burkholderia cepacia* complex (Bcc) (Mahenthiralingam *et al*., 2000; Payne *et al*., 2005).

Hence, this research aims to determine and elucidate the taxonomy, genetic diversity, and genetic profiling between *B. pumilus* group isolated from different geographical locations in Peninsular Malaysia. Indeed, the data obtained from this research will be valuable and useful for quarantine purposes and suppression of trunk bulges in our country and enhance the rubber industry in Malaysia.

1.3 Significance of Study

This study will be a powerful platform for generating detailed documentation of the genetic relationship and diversity of *B. pumilus* group isolates associated with trunk bulges on RRIM 3001 superclone rubber tree in Malaysia. Our study would also provide more information about the evolutionary and population biology studies of *B. pumilus* group species. Moreover, our study is the first attempt to compare multilocus sequence analysis (MLSA) and rep-PCR method on analyzing the genetic diversity of *B. pumilus* group isolates causing trunk bulges of RRIM 3001 superclone rubber tree.

1.4 Objectives of Study

Our study was carried out with the following objectives:

1. To determine the genetic relationship of *B. pumilus* group isolates associated with trunk bulges of RRIM 3001 superclone rubber tree from different geographical areas in Peninsular Malaysia by using multilocus sequence analysis (MLSA).

2. To elucidate the genetic diversity of *B. pumilus* group isolates associated with trunk bulges of RRIM 3001 superclone rubber tree from different geographical areas in Peninsular Malaysia using molecular profiling via rep-PCR.

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