UNIVERSITI PUTRA MALAYSIA

GENETIC DIVERSITY OF Fusarium spp. ISOLATED FROM FRUIT ROT OF BANANA IN PENINSULAR MALAYSIA

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FS 2017 33

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By

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

April 2017

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

GENETIC DIVERSITY OF *Fusarium* spp. ISOLATED FROM FRUIT ROT OF BANANA IN PENINSULAR MALAYSIA

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April 2017

Chairman : Nur Ain Izzati Mohd Zainudin, PhD
Faculty : Science

Banana ranks first in global fruit production and economic source for the producing countries, however, bananas are always at high risk for fungal infection dominantly by *Fusarium* species in the field and post-harvest stages. Hence, this study was conducted to understand the etiology of fruit rot disease on banana. The objectives of this study were to isolate and identify the isolates of *Fusarium* species based on gene encoding translation elongation factor 1-α (*tef1-α*) sequence and phenotypic analysis, to determine genetic diversity among isolates and species of *Fusarium* using selected microsatellite markers and to examine pathogenicity of the *Fusarium* isolates in causing fruit rot disease of banana. The justification of the first objective was to provide the information of *Fusarium* species associated with fruit rot disease of banana in Peninsular Malaysia, while for the second objective was to examine the genetic diversity that maybe varies among the individual isolates of the *Fusarium* spp. and for the third objective was to observe the ability of individual isolates of the *Fusarium* spp. to cause fruit rot disease on the inoculated banana fruits in order to fulfil the Koch's Postulate principles. A series of sampling was conducted throughout Peninsular Malaysia and fungal species identification was conducted based on morphological and gene encoding *tef1-α* sequence analysis. Eleven species of *Fusarium* were discovered namely *Fusarium incarnatum, Fusarium equiseti, Fusarium camptoceras, Fusarium solani, Fusarium concolor, Fusarium oxysporum, Fusarium proliferatum, Fusarium verticillioides, Fusarium sacchari, Fusarium concentricum* and *Fusarium fujikuroi*. The genetic diversity of the *Fusarium* isolates was studied using six selected established microsatellite primers for *Fusarium* species. The results showed that all the *Fusarium* isolates were grouped into their respective clades, indicated their similarity and differences in genetic diversity among isolates and species. All 48 *Fusarium* isolates were tested for pathogenicity test by inoculating fungal disc on unwounded banana fruit. The results showed that all *Fusarium* species were causing fruit rot symptom at different level of severity based on Disease Severity Index (DSI) except for non-pathogenic isolates of *F. solani* (isolates B2385 and B2432), *F. oxysporum* (isolate J2421), *F. sacchari* (isolates B2426 and B2427) and *F. equiseti* (isolate B2361). The most virulent isolate was *F. proliferatum* (isolate B2433) with DSI of 100%. The non-

inoculated controls showed no symptom of fruit rot. In conclusion, *F. incarnatum F. solani F. proliferatum*, *F. verticillioides*, *F. oxysporum*, *F. sacchari*, *F. camptoceras, F. concentricum, F. fujikuroi, F. concolor* and *F. equiseti* were successfully identified and some of the *Fusarium* species were confirmed as causal agents of pre- and post-harvest fruit rot on banana in Malaysia.

Keywords: *Fusarium* species, *Gibberella fujikuroi*, microsatellite, translation elongation factor (*tef*)*1-α*, fruit rot

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

KEPELBAGAIAN GENETIK SPESIS *Fusarium* YANG DIPENCILKAN DARI PENYAKIT REPUT BUAH PISANG DI SEMENANJUNG MALAYSIA

Oleh

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Pisang menduduki tempat pertama di dalam pengeluaran buah secara global dan merupakan sumber ekonomi buat negara pengeluar pisang, walaubagaimanapun pisang sering kali berisiko tinggi terhadap jangkitan kulat terutamanya oleh spesis *Fusarium* samaada di ladang atau selepas tuai. Oleh itu, kajian ini telah dijalankan bagi mengkaji etiologi penyakit reput buah pisang ini. Objektif kajian adalah untuk memencilkan dan mengenalpasti pencilan tersebut berdasarkan analisis jujukan gen penterjemahan pemanjangan faktor (*tef*)-1α dan analisis fenotip, mengkaji kepelbagaian genetik di kalangan pencilan dan spesis *Fusarium* menggunakan penanda mikrosatelit untuk spesis *Fusarium* yang dipilih dan mengkaji tahap kepatogenan pencilan *Fusarium* tersebut. Justifikasi objektif pertama ialah untuk menyediakan maklumat tentang spesis *Fusarium* yang berkaitan dengan penyakit reput buah pisang di Semenanjung Malaysia, manakala objektif kedua pula dijalankan untuk mengkaji kepelbagaian genetik yang berbeza-beza di kalangan setiap pencilan spesis *Fusarium* yang telah dikenalpasti dan objektif yang ketiga adalah untuk memerhatikan kemampuan setiap pencilan spesis *Fusarium* dalam menyebabkan penyakit reput buah ke atas buah pisang yang telah dijangkitkan di samping untuk memenuhi prinsip Koch Postulate. Satu siri persampelan telah dilakukan di seluruh Semenanjung Malaysia dan pengenalpastian spesis kulat telah dilakukan berdasarkan pemerhatian morfologi dan analisis jujukan gen *tef*1-α. Sebelas spesis *Fusarium* telah dikenalpasti iaitu *Fusarium incarnatum, Fusarium equiseti, Fusarium camptoceras, Fusarium solani, Fusarium concolor, Fusarium oxysporum, Fusarium proliferatum, Fusarium verticillioides, Fusarium sacchari, Fusarium concentricum* dan *Fusarium fujikuroi*. Kepelbagaian genetik pencilan *Fusarium* dikaji dengan menggunakan enam primer mikrosatelit. Keputusan kajian menunjukkan kesemua pencilan *Fusarium* telah terbahagi mengikut kumpulan masing-masing di mana ia menunjukkan persamaan kepelbagaian genetik di kalangan pencilan dan spesis. Kesemua 48 pencilan *Fusarium* telah diuji tahap kepatogenannya dengan cara menjangkitkan kepingan kulat *Fusarium* ke atas buah pisang. Keputusan menunjukkan kesemua pencilan spesis *Fusarium* menyebabkan simptom reput buah pada tahap keseriusan yang berbeza berdasarkan Indeks Keseriusan Penyakit (IKP)

kecuali pencilan yang tidak patogenik iaitu *F. solani* (pencilan B2385 dan B2432), *F. oxysporum* (pencilan J2421), *F. sacchari* (pencilan B2426 dan B2427) dan *F. equiseti* (pencilan B2361). Pencilan yang paling virulen adalah *F. proliferatum* (pencilan B2433) dengan bacaan IKP 100%. Buah pisang kawalan yang tidak diinokulasi tidak menunjukkan sebarang simptom reput buah. Kesimpulannya, *F. incarnatum F. solani F. proliferatum*, *F. verticillioides*, *F. oxysporum*, *F. sacchari*, *F. camptoceras, F. concentricum, F. fujikuroi, F. concolor* dan *F. equiseti* telah dikenalpasti dan beberapa spesis *Fusarium* telah dipastikan sebagai agen penyebab penyakit reput buah pisang sebelum dan selepas tuai di Malaysia.

Kata kunci: Spesis *Fusarium*, *Gibberella fujikuroi*, mikrosatelit, penterjemahan pemanjangan faktor (*tef*)1-α, reput buah

ACKNOWLEDGEMENTS

"In the name of Allah, the Most Merciful and the Most Beneficent "

Alhamdulillah, I am so thankful and grateful to Allah S.W.T for His mercy and guidance, yet, I am gradually being able to finish this report for the Master of Science successfully, although I got some hardness, health problems and difficulties during the process of writing this report. Here, I would like to take the chance to express my million thanks and appreciation to all parties that helping me throughout my Master of Science project period to the members of Mycology Laboratory of Biology Department, Faculty of Science, UPM.

First, I would like to thanks to Dr. Nur Ain Izzati Binti Mohd Zainudin, the supervisor of my project and Assoc. Prof. Dr. Shamarina Binti Shohaimi, the co-supervisor of my project for their guidance and knowledge that has been delivered to me along the progression of this project until it is completed. Then, thank to Dr. Nik Mohd Izham Bin Mohamed Nor for his guidance and knowledge regarding the microsatellite markers analysis used in this study. I also want to express my special thanks to all lab mates in Mycology Laboratory, especially to all the master students as well as not forgettable to my housemates of Casa Impian for their experiences, supports and help when I am in need to boost up my spirit until this project done. In addition, thanks also to my examiner panels for the evaluation of my presentation and report regarding this project.

Lastly, millions of thanks again to all individuals especially to my parents, Dr. Abd Murad Bin Salleh and Puan Natimah Binti Parman as well as my siblings that always keep me in their pray and support me from the back and also any organization that give their contributions and supports either directly or indirectly in helping me until I am be able to complete my project within the fixed time range. May Allah bless all of us.

I certify that a Thesis Examination Committee has met on 13 April 2017 to conduct the final examination of Nur Baiti binti Abd Murad on her thesis entitled "Genetic Diversity of *Fusarium* spp. Isolated from Fruit Rot of Banana in Peninsular Malaysia" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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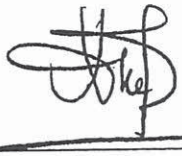

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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Signature: _____
Name of Member of Supervisory Committee: Associate Professor Dr. Shamarina Binti Shohaimi

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BLAST	Basic Local Alignment Search Tool
bp	base pair
Bkt.	Bukit
Bt.	Batu
β-TUB	β-tubulin
CAM	Calmodulin
CFU	Colony Forming Unit
°C	Degree Celcius
dai	day after inoculation
ddH_2O	double distilled water
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
DSI	Disease severity index
EF1/EF2	Elongation Factor 1/Elongation Factor 2
EtBr	Ethidium Bromide
FAOSTAT	Food and Agriculture Organization of the United Nation
f. sp.	formae speciales
GFC	*Gibberella fujikkuroi* complex
g	gram
H3	Histone
ISSRs	Inter-Simple-Sequence Repeats
ITS	Internal Transcribed Spacer
K.	Kuala
kb	kilobyte
L	litre
MAQIS	Malaysian Quarantine and Inspection Service
MARDI	Malaysian Agriculture Research Development Institute
MEGA	Molecular Evolutionary Genetics Analysis
MPCA	Malaysian Phytosanitary Certification Assurance Scheme
mg	milligram
$MgCl_2$	Magnesium Chloride
ml	milliliter
µl	microliter
ML	Maximum Likelihood
mM	millimole
MP-PCR	Microsatellite primers-PCR
Na_2EDTA	Sodium Ethylenediaminetetraacetic acid
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
RAPD	Random Amplified Polymorphic DNA
RNA	Ribonucleic acid
RPB_2	RNA Polymerase II second largest subunit
sp.	species
spp.	S pecies plural
TBE	Tris-Borate EDTA
TEF	Translation Elongation Factor 1-α

Tg.	Tanjong
t/ha/yr	ton/hectare/year
Tm	melting temperature
UV	Ultraviolet
%	percent

CHAPTER 1

INTRODUCTION

Banana and the closely related plantain belong to *Musa* genus of *Musaceae* family (Darvari, Sariah, Puad & Maziah, 2010). They are classically large, tenacious, monocotyledonous herbs for about 6-30 ft. tall arising from large, below ground rhizomes (corms). This group is inherent to the Indo-Malesian, Asian and Australian tropics, but these plants are now planted throughout the tropics and subtropics areas (Nelson, 2008).

According to Ewané, Lepoivre, de Lapeyre de Bellaire and Lassois (2012), banana is cultivated in more than 120 countries over about 10 million hectares. It become the first universal fruit production with an amount of 106 million tons being produced yearly worldwide. The cropping systems used by the banana producing countries are varied and they are focusing on sustainable food source and sale in local and international marketing (Loeillet, 2005). Some bananas including plantains can be eaten either by cooking or raw as dessert. The "Cavendish" subgroup is the important dessert bananas for export purpose at international trade. Banana is presently the first exported fruit in terms of volume and ranks second after citrus fruits. Based on the Food and Agriculture Organization of the United Nation (FAOSTAT) data in 2014, the total world exports of banana were about 17.0 million tons, thus, indicating the banana industry become a priority to banana producing countries. Based on a study conducted by de Lapeyre de Bellaire, Chillet, Lassois and Jijakli (2010), the "Cavendish" subgroup has become a demand in banana industry due to its dynamic source of profits, service, and export for most producing countries, including several countries in Latin America, West Indies, Southeast Asia, and Africa.

In Malaysia, banana has become the second largest planted fruit crop after durian (Ibrahim, Nordin & Mohd Nahar, 2010). Moreover, a great variety of banana are found in Malaysia, as one of the origins of bananas with about 50 types with sufficient volume for exportation purpose. Even some of the banana varieties have been exclusively planted for the exportation, however, the fruit quality is still need to be more improved for extra export in future (Darvari et al., 2010). This is due to the bananas are always at high risk for fungal infection in the field and post-harvest, where caused fungal diseases such as fruit rot.

Fruit rot is referred to the incidence of softening and deterioration of a fruit. Fruit rot can be grouped into two categories, known as field rot (infection occurred before harvest) and storage rot (infection occurred after harvest) (Coates & Johnson, 1997). There are some fungal species classified exclusively as field or storage rot pathogens, while others may cause rot in both field and storage. Since multiple fungal species and other microorganism cause fruit rot, hence it is sometimes called as a 'disease complex' (McManus, 2001). Sangeetha, Usharani and Muthukumar (2009), reported that fruit rot is categorized under post-harvest disease type and the main causes are

fungal infection, physical injury and physiological disorders (Sangeetha, Usharani & Muthukumar, 2009).

There are several factors that may influence the occurrence of fruit diseases such as commodity type, cultivar susceptibility to the disease, storage situation (temperature, relative moisture, and air composition), yield maturity and ripeness phase, handlings used for disease control, yield control approaches and storage hygiene (the tools used, the pickers' hands and containers to keep the yields). The infection mechanism of the fruit rot disease in banana may primarily arise at the opened surface of the crown. Even the rot is limited to the crown at first, but it may blowouts into the pedicles of the fingers at progressive phases. An amount of white fungal mycelia and reproductive bodies may appear on the decayed crown, finger stalks and lastly on the fingers after severe infections (Griffee & Burden, 1976). Poor packaging and improper management may induce the disease occurence. This can cause the fruits that arrive at the market to become welt and squeezed, resulting in different types of rots set under the favorable conditions that will be influenced by the unsuitable temperature and relative humidity for the fruit storage in certain places (Taskeen-un-Nisa, Wani, Bhat, Pala & Mir, 2011; Xie, Tan & Yu, 2012).

Fungal phytopathogens are sources of many plant diseases and cause a great loss of crop yields, especially in tropical and subtropical regions (Morid, Hajmansoor & Kakvan, 2012). This will affect the quality of the tropical fruits that is normally spoiled by postharvest disease such as fruit rot. The presence of the *Fusarium* species on tropical fruits has less known and is not well documented in Malaysia (Latiffah, Mazzura, Heng & Baharuddin, 2012). There are also some problems correlated with improving *Fusarium* species in tropics including the problems of having the fresh isolates from infected sources. Temperature and humid conditions in the tropics, including Malaysia also encourage the decay of the diseased plants to occur rapidly, resulting the plants or the sources become more severely covered by secondary fungal infection and other microorganism invaders (Leslie & Summerell, 2006). Therefore, the possibility of the abundant records of *F. oxysporum* and *F. solani* as plant pathogens of various plants in several tropical countries are due to this secondary infection. Hence, the samples should be taken while the colonization is still at the preliminary stages or when the first disease symptoms observed. Besides, strict pathogenicity tests should be implemented with the recovered isolates that may have uncommon or unpredicted phenotypic features or deviate from the earlier and tested disease profiles (Leslie & Summerell, 2006).

Next, fungal identification procedures are the most important part after fungal isolation obtained from the samples. The fungal taxanomic system is recently identified via morphological means including colony features, pigmentation, macroconidia and microconidia and secondary metabolites. Unfortunately, this method unable to identify the plasticity and intergradation of the phenotypic traits of filamentous fungi (Leslie & Summerell, 2006). The morphological identification has limitation of distinct characters in order to identify *Fusarium* isolates into species-level. Therefore, there is a need for the molecular identification method to observe the morphology features of the closely related fungal species in the same genus. In addition, the DNA markers studies

help to recognize and differentiate cultures that are difficult to be distinguished morphologically (Leslie & Summerell, 2006).

The molecular identification method was approached in order to explore the fungal genome that might be useful to discriminate the closely related fungal species in the same genus. The intron-rich parts of protein-coding genes are tremendous markers for species level phylogenetic in fungi (Geiser et al., 2004). According to Nielsen, Friedman, Birren, Burge and Galagan (2004), fungi is an intron rich organism, where the DNA is made up of largely intron parts that are evenly distributed within the gene coding sequence in the intron rich organism. Therefore, this makes the fungal genome is ideal for exploring intron evolution. Gene introns become more practical and precise compared to internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA gene repeat because the gene introns are likely to evolve oftenly. Besides, the non-orthologous copies of the ITS2 may also bring to inappropriate phylogenetic interpretations (Nitschke, Nilhgard & Varrelmann, 2009). Hence, regions identification with long sequence is better since it leads to high phylogenetic efficacy. There are several common DNA markers or genes for *Fusarium* species such as β-tubulin (β-TUB), histone (H3), calmodulin (CAM), RNA polymerase II second largest subunit (RPB2) and translation elongation factor (*tef-1α*) (Geiser et al., 2004). In this study, the *tef-1α* is chosen because high sequence polymorphism among closely related species is shown compared to the other intron-rich portions of protein coding genes. Moreover, non-orthologous copies of the gene have not been detected in the genus (Balajee et al., 2008; Rahjoo et al., 2008; Amatulli, Spadaro, Gullino & Garibaldi, 2010; Walker, Castlebury, Rossman & White, 2012).

To make the study more enlightening, the genetic diversity of the isolated fungi is needed. Microsatellite primers-Polymerase Chain Reaction (MP-PCR) technique is extensively used to study the genetic diversity of different isolates of the same and different fungal species. It is involving the use of a single primer made up of microsatellite sequence. Microsatellites are mainly based on PCR and are known as inter-simple-sequence repeat (ISSR) or MP-PCR (Wunsch & Hormaza, 2002). This molecular technique is also beneficial for the fungal identification at the genus and species level. It is covered a modest, fast, effective and precise method for interspecies studies (Billotte et al., 2001), because no past data of the target species' genomic sequences is required, provide libraries, which are highly supplemented for single locus microsatellite marker development, lessens the cost of single locus DNA microsatellite discovery and high levels of polymorphism can be perceived even when the products of microsatellites amplification are determined on agarose gels without radioactive labeling (Arcade et al., 2000; Sankar & Moore, 2001).

The final step in molecular identification of fungal species will later be followed by analyzing the constructed phylogenetic tree. Phylogenetic tree is created in order to view their genetic relationships to other members of the same species, to unlike species of the same genus and to the out-group belonging to other genera using sequences from the GenBank database. Besides, the phylogenetic analyses of DNA sequence data offer an occasion over which species restrictions can be explored, unknown isolates can be recognized, relationship between species can be well-known and toxin profiles can be mapped to species or group of species. Phylogenetic analyses should always construct

on well-characterized isolates, where the isolates have been prudently identified based on morphology first with proper descriptions about their specific information such as host and geographic origin, secondary metabolites produced and pathogenicity. The importance of preventing the misidentification of isolates is to avoid some of discordance in the classification schemes of fungal species, which can lead to the difficulties of determining consistent delimitation of species and confusing the true evolutionary relationships between species (Kristensen, Torp, Kosiak & Holst-Jensen, 2005).

Realizing the information on diversity of *Fusarium* species associated with fruit rot of banana in tropical area, including Malaysia is insufficient, we set out to study the identification of the isolated fungi based on molecular approaches and examine their genetic diversity using microsatellite markers. The significant of this study are to provide the information on diversity of *Fusarium* species associated with fruit rot disease of banana and contribute to the development of regulatory policies for banana and related tropical fruits for Biosecurity (MAQIS), Phytosanitary (MPCA) and appropriate recommendations of disease control strategies.

The objectives of this study were; i) to isolate and identify *Fusarium* species based on *tef-1α* sequence and phenotypic analysis, ii) to determine genetic diversity among isolates and species of *Fusarium* using selected microsatellite markers and iii) to examine pathogenicity of the *Fusarium* isolates in causing fruit rot disease of banana.

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