CHARACTERIZATION OF FUNGI ASSOCIATED WITH LEAF SPOT OF MANGO (*Mangifera indica* L.)

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CHARACTERIZATION OF FUNGI ASSOCIATED WITH LEAF SPOT OF MANGO (*Mangifera indica* L.)

by

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LIST OF ABBREVIATIONS

ACT	Actin
ANOVA	Analysis of Variance
AFLP	Amplified-fragment Length Polymorphism
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C_2H_5OH	Ethanol
CAL	Calmodulin
CHS	Chitin synthase
CLA	Carnation Leaf Agar
d	Day
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DS	Disease severity
EtBr	Ethidium bromide
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GS	Glutamine synthetase
Ha	Hectare
HIS3	Histamine
ITS	Internal Transcribed Spacer
LSU	Large-subunit
MA	Mango clones
MARDI	Malaysian Agricultural Research and Development Institute

MEGA	Molecular Evolutionary Genetics Analysis
ML	Maximum Likelihood
Mt	Metric ton
NaOCl	Sodium hypochlorite
OsO4	Osmium tetraoxide
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
RAPD	Random-amplified Polymorphism DNA
rDNA	Ribosomal DNA
SEM	Scanning Electron Microscope
SSU	Small-subunit
TBE	Tris-Boric acid-EDTA
TEF-1α	Translation Elongation Factor 1α
WA	Water Agar

PENCIRIAN KULAT BERASIOSIASI DENGAN BINTIK DAUN MANGGA (*Mangifera indica* L.)

ABSTRAK

Mangga (*Mangifera indica* L.) merupakan tanaman buah-buahan yang popular di Malaysia dan mudah terdedah kepada penyakit bintik daun disebabkan oleh pelbagai kumpulan kulat Askomiset dan mitosporik. Penyakit ini akan mengganggu fotosintesis dan mengurangkan pertumbuhan pokok mangga. Kajian ini dijalankan untuk mengenalpasti dan mencirikan kulat yang berasiosiasi dengan penyakit bintik daun mangga. Ujian kepatogenan telah dijalankan untuk menentukan patogen penyebab penyakit ini. Berdasarkan pengecaman secara morfologi, 264 pencilan kulat telah dikenalpasti secara tentatif kepada 11 genus dan 18 spesies, iaitu *Colletotrichum* (n = 93, *C. acutatum* dan *C. gloeosporioides*), *Fusarium* (n = 90, *F. proliferatum, F. semitectum, F. mangiferae, F. solani* dan *F. chlamydosporum*), *Pestalotiopsis* (n = 28, *P. theae* dan *P. mangiferae*), *Phomopsis* (n = 13, *Phomopsis* sp.), *Curvularia* (n = 12, *Cur. geniculata* dan *Cur. lunata*), *Guignardia* (n = 9, *G. mangiferae*), *Lasiodiplodia* (n = 9, *L. theobromae)*, *Nigrospora* (n = 5; *N. sphaerica*), *Botryosphaeria* (n = 3, *B. dothidea*), *Nodulisporium* (n = 1, *Nodulisporium* sp.) dan *Corynespora* (n = 1, *Cor. cassiicola*). Sejumlah 151 pencilan kulat daripada spesies yang sama menunjukan ciri-ciri morfologi yang serupa dipilih sebagai wakil pencilan untuk pengecaman secara molekul. Bergantung kepada genus kulat, penjujukan DNA dan analisis filogenetik berasaskan Kawasan Transkripsi Dalaman (ITS), gen β-tubulin dan gen Faktor Pemanjangan Translasi 1α (TEF-1α) telah digunakan untuk pengesahan spesies. Dua kompleks spesies *Colletotrichum* iaitu kompleks spesies *C.*

acutatum (n = 33) dan kompleks spesies *C. gloeosporioides* (n = 22) telah dikenalpasti berdasarkan jujukan ITS dan β-tubulin. Analisis filogenetik menunjukkan tiada pencilan yang dikenalpasti secara morfologi sebagai *C. acutatum* dan *C. gloeosporioides* daripada bintik daun dikelompokkan bersama strain epitype *C. acutatum* dan *C. gloeosporioides*. Jujukan ITS telah mengesahkan identiti pencilan yang telah dikenalpasti secara morfologi sebagai *P. mangiferae* (n = 12), *P. theae* (n = 8), *Phomopsis* sp. (n = 8), *Ph. glabrae* (n = 2), *L. theobromae* (n = 7), *G. mangiferae* (n = 5), *B. dothidea* (n = 3), *Cur. geniculata* (n = 4), *Cur. lunata* (n = 2), *N. sphaerica* (n = 5), *Nodulisporium* sp. (n = 1) dan *Cor. cassiicola* (n = 1). Analisis filogenetik menggunakan kawasan ITS menunjukkan pencilan dari spesies yang sama dikelompokkan dalam klad yang sama. Pengecaman secara molekul lima spesies *Fusarium*, iaitu *F. proliferatum* (n = 18), *F. semitectum* (n = 11), *F. mangiferae* (n = 3), *F. solani* (n = 2) dan *F. chlamydosporum* (n = 1) menggunakan jujukan TEF-1α dan keputusan analisis filogenetik menunjukkan bahawa pencilan dari spesies yang sama telah dikelompokkan dalam klad yang sama. Keputusan ujian kepatogenan menunjukkan 50 pencilan yang dipilih daripada setiap spesies adalah patogenik terhadap daun mangga kecuali *F. solani* dan *F. mangiferae*. Kajian ini menunjukkan pelbagai genus kulat berasiosiasi dengan bintik daun mangga.

CHARACTERIZATION OF FUNGI ASSOCIATED WITH LEAF SPOT OF MANGO (*Mangifera indica* L.)

ABSTRACT

Mango (*Mangifera indica* L.) is a popular fruit crop in Malaysia and is susceptible to leaf spot disease caused by diverse groups of Ascomycete and mitosporic fungi. The disease will interrupt photosynthesis and reduce the growth of mango trees. The present study was conducted to identify and characterize fungi associated with leaf spot of mango. Pathogenicity test was performed to determine the causal pathogen of the disease. Based on morphological identification, 264 fungal isolates were tentatively identified into 11 genera and 18 species, namely *Colletotrichum* (n = 93, *C. acutatum* and *C. gloeosporioides*), *Fusarium* (n = 90, *F. proliferatum, F. semitectum, F. mangiferae, F. solani* and *F. chlamydosporum*), *Pestalotiopsis* (n = 28, *P. theae* and *P. mangiferae)*, *Phomopsis* (n = 13, *Phomopsis* sp.), *Curvularia* (n = 12, *Cur. geniculata* and *Cur. lunata*), *Guignardia* (n = 9, *G. mangiferae*), *Lasiodiplodia* (n = 9, *L. theobromae*), *Nigrospora* (n = 5; *N. sphaerica*), *Botryosphaeria* (n = 3, *B. dothidea*), *Nodulisporium* (n = 1, *Nodulisporium* sp.) and *Corynespora* (n = 1, *Cor. cassiicola*). A total of 151 fungal isolates within the same species that showed similar morphological characteristics were chosen as representative isolates for molecular identification. Depending on the fungal genera, DNA sequencing and phylogenetic analysis of Internal Transcribed Spacer (ITS) region, β-tubulin and Translation Elongation Factor 1α (TEF-1α) genes were used for species confirmation. Two *Colletotrichum* species complex, *C. acutatum* species complex (n = 33) and *C. gloeosporioides* species complex (n = 22) were identified

based on ITS and β-tubulin sequences. Phylogenetic analysis showed that none of the isolates morphologically identified as *C. acutatum* and *C. gloeosporioides* from leaf spot of mango were grouped with *C. acutatum* and *C. gloeosporioides* epitype strains. ITS sequences confirmed the identity of morphologically identified *P. mangiferae* (n = 12), *P. theae* (n = 8), *Phomopsis* sp. (n = 8), *Ph. glabrae* (n = 2), *L. theobromae* (n = 7), *G. mangiferae* (n = 5), *B. dothidea* (n = 3), *Cur. geniculata* (n = 4), *Cur. lunata* (n = 2), *N. sphaerica* (n = 5), *Nodulisporium* sp. (n = 1) and *Cor. cassiicola* (n =1). Phylogenetic analysis using ITS region showed that the isolates from the same species were clustered in the same clade. Molecular identification of five *Fusarium* species, namely *F. proliferatum* (n = 18), *F. semitectum* (n = 11), *F. mangiferae* (n = 3), *F. solani* (n = 2) and *F. chlamydosporum* (n = 1) were done using TEF-1α sequences and the result of phylogenetic analysis showed that the isolates from the same species were grouped in the same clade. Results of pathogenicity test indicated that 50 selected isolates from each of the species were pathogenic towards mango leaves except *F. solani* and *F. mangiferae*. The present study showed that diverse groups of fungal genera were associated with leaf spot of mango.

CHAPTER ONE

INTRODUCTION

Mango (*Mangifera indica* L.) is one of important fruit crops cultivated in Malaysia. The fruit crop is mainly cultivated in the northern states of Peninsular Malaysia due to favourable soil and climate conditions with heavy precipitation, high humidity and high temperature (Abdullah et al., 2011). Cultivated area and production of mango increased from 2013 to 2014 with 5 270 Ha and 16 625 Mt to 5 283 Ha and 17 709 Mt. In 2014, export of mango was estimated to be about 17 704 Mt, valued at RM 65 995.55 (Department of Agriculture, 2015).

Like any other crops, mango is also susceptible to diseases caused mainly by Ascomycetes and mitosporic fungi. One of the major diseases of mango is leaf spot, caused by the fungi from the genera *Colletotrichum*, *Alternaria*, *Cercospora*, *Corynespora*, *Curvularia*, *Cladosporium*, *Ascochyta*, *Pestalotiopsis* and *Botryodiplodia* (Agrios, 2005). Symptoms of leaf spot can vary depending on the fungal pathogen. The spot vary in size and shape but commonly begins with pinhead point's lesion and spread forming circular or irregular lesion with dry, brown or black raised centre. The infection of pathogen will cause chlorosis and necrosis on the leaf surface and thus reduce photosynthetic areas, which affects carbohydrate production as well as nutrient transportation to plant organs (Agrios, 2005). Consequently, the infection will reduce plant growth and fruit yield.

As many fungal genera can cause leaf spot disease, identification of the causal pathogen is important to initiate preventive or curative measures. For that

reason, accurate identification of fungal pathogens is necessary to determine appropriate disease control measures as well as to improve disease management. The most prevalent technique used to identify plant pathogens is by observing morphological characters. Morphological characteristic is commonly used for identification of fungi, which include macroscopic and microscopic characteristics such as colony colour and texture, pigmentation, growth diameter, the shape of conidia, arrangement of spore or conidia, conidiophore, presence of resistant structure such as chlamydospore and presence of fruiting bodies such as pycnidia and acervuli (Pitt and Hocking, 1985; Watanabe, 2002; Barnett and Hunter, 2006).

However, some characters within the same genus are very similar and difficult to distinguish based on morphological characteristics, thus insufficient to identify the isolate up to species level. Due to these limitations, molecular methods are used to assist in the identification process due to its high degree of specificity. One of the methods commonly used is DNA sequencing. DNA sequencing data can be used to identify and characterize fungal species, distinguish closely related species and provide information on phylogenetic relationships.

The most common region used for molecular identification of the fungi is ITS region which is a universal DNA barcode of fungi and widely used for species identification and genetic marker for phylogenies (Schoch et al., 2012). However, for some fungal genera, ITS does not always provide accurate species identification (Bruns, 2001). Therefore, protein coding genes such as β-tubulin and TEF-1α appear to be useful for species identification and phylogenetic analysis (O' Donnell et al., 1998a; Geiser et al., 2004). Phylogenetic analysis is very important to confirm the

species identity as well as to determine the genetic diversity of fungal species (Cannon et al., 2008).

After identification, pathogenicity test is carried out to test the pathogenic ability of fungal isolates which is be done by fulfilling Koch's postulates. By conducting the pathogenicity test, the degree of virulence of plant pathogenic fungi can be determined. Different isolates can show different levels of virulence or aggressiveness (Than et al., 2008). Furthermore, host range also can be determined through pathogenicity test in which some fungal species can infect more than one plant host, and one plant host can be infected by multiple fungal species (Agrios, 2005).

In Malaysia, fungal species associated with mango leaf spot is not well-documented and its pathogenicity has not been reported. Therefore, identification and characterization of fungi causing leaf spot disease of mango are important in order to protect the plant from further damage as the yield can be affected.

Therefore, the objectives of the present study were:

1) To identify fungi associated with leaf spot disease of mango based on morphological and molecular approaches.
2) To determine the phylogenetic relationship of fungal isolates by using ITS region, β-tubulin and TEF-1α genes.
3) To determine the pathogenicity of the fungi isolated from leaf spot disease of mango.

CHAPTER TWO

LITERATURE REVIEW

2.1 Mango

Mango is an important fruit crop in tropical and subtropical regions and was recorded as among the five most important fruit worldwide along with banana, apple, grape and orange (Food and Agriculture Organization Corporate Statistical Database, 2013). The fruit is very popular due to its wide range of adaptability, richness in variety, attractive colours, delicious taste, savoring smell, health benefits and also high nutritive value. Mango originated from Southern Asia, particularly from the areas of Eastern India, Burma and the Andaman Islands over 4000 years ago (Litz, 1997). Mango spread to the other parts of Asia and gradually become distributed around the world in the beginning of the 16th century (Morton, 1987). Nowadays, mango is commercially cultivated in more than 90 countries including the Philippines, Thailand, Malaysia, Burma, Indonesia and Sri Lanka (Rekhapriyadharshini, 2015).

Mango (*Mangifera indica* L.) belongs to the order Sapindales and family Anacardiaceae. The family contains over 600 species classified into 70 genera that include other cultivated species such as pistachio (*Pistacia vera* L.) or cashew (*Annacardium occidentale* L.). The genus *Mangifera* contains about 70 species that bear edible fruit including mango and other fruits with lower quality that are commonly referred to as wild mangos (Bally, 2006). Most of this genus can be found in tropical Asia and it is divided into two subgenera, namely *Limus* and *Mangifera* with several sections (Kostermans and Bompard, 1993).

Mango prefers a warm, frost-free climate with an optimum temperature range between 24-27°C and can withstand temperature as low as -39°C but only for few hours (Crane and Campbell, 1994). Malaysia has a uniform temperature around 27°C and this temperature lies within the optimum temperature range for mango growth. Rainfall requirements are 400-3600 mm with alternating wet and dry seasons. Rain, high humidity, heavy dew and fog during flowering and fruiting period contribute to the development of fungal diseases which cause huge crop losses (Ploetz and Prakash, 1997). Mango tree grows well in soil with pH ranging from 5.5 to 7.5 and are quite tolerant to alkaline condition. Mango tree requires deep soil to accommodate the extensive root system to encourage good growth (Orwa et al., 2009).

There is a wide variety of mangos in Malaysia especially in Peninsular Malaysia in which about 28 varieties have been recorded (Gulcin et al., 2004). Mango cultivars vary in size, shape, colour, flavor and fibre content. Common commercially planted mango cultivars include Harumanis (MA 128), Chok Anan (MA 224), Nam Dok Mai (MA 223), Golek (MA 165), Masmuda (MA 204) and Maha 65 (MA 165). Among the cultivar, Chok Anan is the most suitable for export market as it has a sweet taste and attractive color (Mirghani et al., 2009). The mango fruits can be eaten ripe or unripe depending on the variety or cultivar.

2.2 Plant Pathogenic Fungi

Fungi are small, eukaryotic, usually filamentous, spore-bearing microbe that lack chlorophyll with an estimated 1.5 million species (Hawksworth, 2001). Although this estimation is accepted, the actual number of fungal species is still

unclear. Schmit and Mueller (2006) estimated that there are at least 712 000 fungal species worldwide in which the estimation was based on the observed ratio between plant species diversity and fungal diversity in a certain area. Most of the 100 000 described species of fungi are associated with plants through interactions of symbiosis, parasitism, endophytism, and saprotrophy (Peršoh et al., 2012; Delaye et al., 2013; Hyde et al., 2013). As plant parasites, fungi can cause significant economic losses in agriculture, natural ecosystem and natural forestry as well as social implications (Fisher et al., 2012).

Fungi are called heterotrophs when they obtain nutrient from organic materials. Fungi which obtain nutrients from living plant tissues are called biotrophs while fungi which assimilate dead plant tissues are saprotrophs. Some fungi infect living host tissues and kill the host cells through the production of toxins or enzymes in order to obtain nutrients. These are called necrotrophs. Most biotrophic fungi have limited host ranges while necrotrophic fungi grow on a wide range of host, or specialized on restricted range of hosts (Carris et al., 2012).

Plant pathogenic fungi attack plant by using several methods such as mechanical force (use of special structure such as appressorium and haustorium to penetrate plant surface), chemicals (release of enzyme to degrade cell wall and membrane components), fungal toxin (secretion of poisonous metabolites) and growth regulators (produce hormone that cause abnormal plant growth) (Agrios, 2005).

Ascomycetes and Mitosporic fungi constitute the largest group of plant pathogenic fungi with approximately 33 000 species as described by Lu et al. (2003). These fungi are able to infect various plant parts such as leaves, stems, roots, fruits and flowers by causing local and general necrosis of plant tissues. Some can cause stunting of plant organ such such as leaf spots, blight, canker, dieback, root rot, damping off, basal stem rot, soft rot and dry rots, anthracnose and scab and some showed excessive enlargement or growth of plant parts such as clubroot, galls, warts, witches' broom and leaf curls. The survival of plant pathogenic fungi depends on the temperature and humidity of the environment. Most of them are spread from plant to another plant or different parts of the same plant by water, wind, insect, birds, animal and human (Agrios, 2005).

Figure 2.1 shows the disease cycle of Ascomycetes fungi. The disease cycle starts with the production of spores in a sac called an ascus (a). The leaves fall on the ground colonized by fungi and survival structure, pseudothecia (formed through sexual reproduction), to protect the fungi during extreme condition (b). The spores in an ascus are called ascospore, develop in the pseudothecia, become mature and released during wet condition and dispersed by wind and rain. Ascospores deposite and germinate on susceptible leaves or fruits in the presence of favourable condition such as suitable temperature and moisture. This stage is known as primary infection. The ascospores germinate and produced mycelium and eventually form lesions on the leaves or fruit surface. Mature mycelium produces conidia through asexual reproduction (c). These conidia are dispersed to other leaves or fruits mainly by rain and wind. When conidia are deposited on other leaves or fruits surfaces, they cause

new lesion that produce conidia and this is known as secondary infection (Walker, 2015).

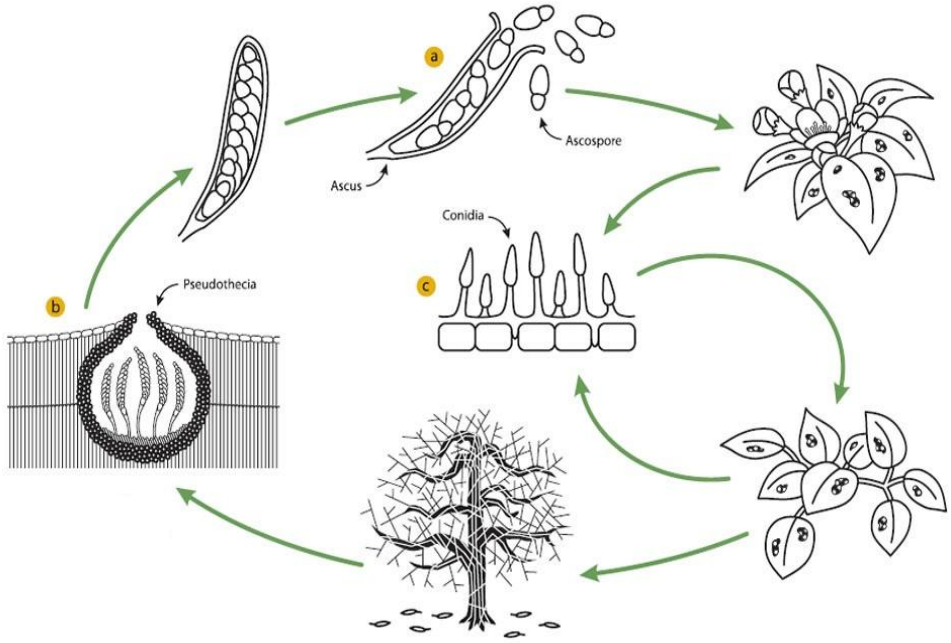

Figure 2.1: Disease cycle of Ascomycetes fungi described by Walker (2015)

2.3 Foliar Diseases

There are many types of foliar diseases caused by different types of plant pathogenic fungi. The diseases are common, but do not seriously affect the trees, except those that cause defoliation (Tainter and Baker, 1996). Some of the most common Ascomycetes causing foliar disease include *Cochliobolus*, *Blumeriella, Magnaporthe*, *Microcyclus*, *Mycosphaerella* and *Pyrenophora* while the most common Mitosporic fungi causing foliar diseases in a variety of plants are *Alternaria, Ascochyta, Cercospora, Cladosporium, Phyllosticta, Pyricularia, Septoria* and *Stemphylium* (Agrios, 2005).

Foliar pathogens are highly dependent on weather for infection. Temperatures between 20°C to 30°C with 100% relative humidity favour the foliar disease development (Paul and Munkvold, 2005). Usually, foliar pathogens destruct the plants by killing the plant tissue and cause plant stress. The pathogen invade the host plants through natural openings such as stomata, hydathodes and through wounds caused by mechanical damage, pruning, harvesting and insects. Most foliar pathogens penetrate mesophyll and parenchyma cells of leaves by direct penetration using haustoria to obtain carbon and nutrients (Agrios, 2005). Haustoria are specialized feeding organ of fungal pathogen which enter plant host cells in order to obtain and absorb food or nutrient (Szabo and Bushnell, 2001).

Leaf spot is one of the most common foliar diseases and is characterized by a small lesion on the leaf. Some leaf spot disease have specialized names according to the type of pathogen that cause the spot such as Alternaria leaf spot, Septoria leaf spot, Cercospora leaf spot and Curvularia leaf spot. Some of the diseases are named based on the effect of the disease to the leaves for instance, black spot, anthracnose, downy spot or white mold, ink spot, leaf blister and tar spot. Leaf spot may be varying in size, shape and colour depending on the stage of the spot development and specific pathogen that involved. The symptoms of leaf spot may start with a small water-soaked lesion. The lesion turns to yellow, grey, reddish-brown, brown or black and may be surrounded with different colours of halo or ring. Sometimes, fungal-fruiting bodies such as pycnidia, acervuli, and perithecia may appear as dots in the centre of the spot. Fungi that commonly caused leaf spot diseases include the species from genera *Alternaria*, *Cercospora*, *Corynespora*, *Cylindrosporium*, *Guignardia*,

Gloeosporium, *Marssonina*, *Mycosphaerella*, *Phyllosticta*, *Septoria*, *Taphrina* and *Venturia* (Pataky, 1998).

The combination of numerous spots present on leaves is called blight or blotch. Blight results in general and rapid killing of leaves. The disease also reduces the quality of leaves. Initial symptom can be observed commonly on young leaf. The symptom of the infected leaves are characterized by circular to irregular with grayish to brown in colour, usually surrounded by yellow halo. Later, the lesions expand and turn to dark brown and the leaves eventually died (Agrios, 2005). Examples of leaf blight and the causal pathogen are *Cochliobolus heterostrophus* causing maize leaf blight (Mubeen et al., 2015) and *Alternaria triticina* causing blight of wheat (Perello and Sisterna, 2006).

Another common leaf spot disease is anthracnose which is characterized by small, circular and oval-shaped necrotic lesion with red to purple colour, often surrounded by a yellow halo with 2 to 5 mm in diameter (Berner and Cavin, 2011). Necrotic lesions expand rapidly and cover the entire foliar surface in the presence of high moisture and humidity (Rios et al., 2015). Commonly, *Colletotrichum* species are the causal pathogen of leaf anthracnose.

Other foliar diseases that cause major damage to leaves include sooty mould and blast diseases. Sooty mould coat leaves superficially with black mycelia, which reduce photosynthesis activity of the host plants (Chomnunti et al., 2014). Insect excrete honeydew to facilitate the growth of the fungi that cover the surface of the leaves and encourage the multiplication of sooty mould fungi (Jouraeva et al., 2006).

Blast disease is caused by *Pyricularia* (teleomorph: *Magnaporthe*) is known to be the most serious foliar disease of rice (Namai, 2011). The disease is generally considered the most important disease of rice worldwide due to its widespread distribution. The symptoms of blast begin as a small grey necrotic lesion with brown halo to large elliptical lesions with a grey necrotic centre and brown or grey halo (Piotti et al., 2005).

Foliage disease is of concern because photosynthesis is reduced due to the reduction of the photosynthetic area of the plant. Severe infected leaves will cause degeneration of chloroplast and can lead to total defoliation of the crop. In some foliar diseases, photosynthesis is reduced because the toxins produced by the foliar pathogen inhibit some of the enzymes that are involved in photosynthesis (Agrios, 2005).

2.4 Diseases of Mango

Like many other crops, mango is also attacked by a number of diseases at all stages of its development. Almost every part of the plant including stem, branch, twig, root, leaf, petiole, flower and fruit are affected by various pathogens. Diseases that commonly infect mango by plant pathogenic fungi including fruit rot, dieback, powdery mildew, anthracnose, scab, blotch, stem bleeding, wilt, leaf spots, canker and malformation (Akhtar and Alam, 2002; Haggag, 2002).

Anthracnose is the most common and wide spread disease associated with mango in all mango growing countries (Ploetz and Prakash, 1997; Freeman et al., 1998; Arauz, 2000). The disease incidence of mango anthracnose can reach almost

100% under very humid conditions and cause 30-60% yield losses on mango cultivars (Arauz, 2000; Chowdhury and Rahim, 2009). Five *Colletotrichum* species have been reported as causal pathogens of mango anthracnose, namely *C. asianum*, *C. fructicola*, *C. tropicale*, *C. karstii* and *C. dianesei* (Lima et al., 2013). Anthracnose symptoms commonly occur on leaves, twigs, petioles, flower clusters (panicles), and fruits. On leaves, anthracnose infections start as small, angular, brown to black spots that can enlarge to form extensive dead areas. The fruits affected by anthracnose are characterized by sunken, prominent, dark brown to black decay and may drop from trees prematurely (Nelson, 2008).

Mango malformation is also one of the most serious diseases of mango. After the first report of the disease in India in 1891, the disease has been distributed to other mango growing countries worldwide. The symptoms of this disease are characterized by abnormal development of vegetative shoots and panicles (Krishnan et al., 2009). Several species of *Fusarium* such as *F. subglutinans*, *F. moniliforme*, *F. sterilihyphosum*, *F. mangiferae* and *F. proliferatum* have been reported to be associated with mango malformation (Marasas et al., 2006; Nik et al., 2013; Joshi et al., 2014).

Powdery mildew affecting almost all mango cultivars and is widely distributed in Asia, Middle East, Africa, the Americas and Australia (Nasir et al., 2014). The occurrence of powdery mildew on mango is attributed to an obligate fungus, *Pseudoidium anacardii* formerly known as *Oidium mangiferae.* The infected young leaves and inflorescences are covered with white mycelia appearing as powdery. Young infected leaves fall prematurely if the underside of the leaf is

covered with the mycelia and mature infected leaves develop purplish brown spots. Infected fruits are often malformed and off-colored. Symptoms of dieback may also occur (Singh et al., 2000).

Lasiodiplodia theobromae (synonym: *Botryodiplodia theobromae*) is responsible for mango dieback. The fungus has been reported as a pathogen associated with mango dieback in Egypt (Ismail et al., 2012). Besides *L. theobromae*, *F. decemcellulare* has also been reported as a causal pathogen of mango dieback in China with the symptom appeared as large irregular brown colour on petiole and twigs (Qi et al., 2013). In severe infection, all the bark of petioles and twigs turn black causing vascular necrosis. Defoliation occurs which gives a scorch appearance.

Mango scab caused by *Elsinoe mangiferae*, infects the leaves, fruits, twigs, panicles and blossoms. Scab appears as blotches on the bark of stem and spot on mango fruit. On fruits, the lesion formed differred in size and colour depending on the age of the plant while on leaves, the spots are smaller and the surface is covered with velvety texture. Severe attacks cause crinkling and distortion of the leaf, followed by premature shedding (Conde et al., 2007).

Besides fungal diseases, mango is also infected with bacteria. A study by Pitkethley et al. (2006) showed that *Xanthomonas campestris* pv. *mangiferaindicae* was the causal pathogen of bacterial canker, leaf spot, black spot, mango blight and bacterial black spot. The bacteria attacked leaves, twig, branches, inflorescence and fruits. On leaves, the diseases first appear as small spot water soaked with irregular to angular raised lesions with or without a yellow halo. With age, the lesions enlarge

or coalesce to form irregular necrotic cankerous patches while on twig and stem, the lesions are black and cracked. The bacteria attack the leaves through the stomata and wound on the leaves (Stovold and Dirou, 2004).

2.5 Morphological Identification of Plant Pathogenic Fungi

Naming and classifying of fungus include three main processes which are describing and grouping, storage of information, and prediction of phylogenetic relationships of the isolates (Talbot, 1971). The identification process starts with comparing unknown species with known species, naming the species and determining relationships among the identified species (Shenoy et al., 2007). Seifert and Rossman (2010) stated that identification of a species is an important part of fungal systematic in which correct identification of a species leads to understanding of its correct biological function including ecological roles, physiological and biochemical properties and its risks or benefits to plant and animal as well as to formulate strategies for controlling plant diseases to initiate preventive or control methods.

Ascomycetes and mitosporic fungi are usually identified based on morphological characteristics such as cultural, microscopic and physiological characteristics (Watanabe, 2002). Cultural characteristics include colony texture (cottony, velvety and powdery), colony colour and colony elevation (flat, thick, raised and elevated). The microscopic characteristics commonly used for fungal identification are conidiophore (size, branching pattern, stipe, ornamentation, septate or non-septate) and conidia (shape and size, ornamentation, septate or non-septate, solitary or born in chains). Survival structures have also been used to identify and

characterize plant pathogenic fungi. The survival structures include chlamydospore, pycnidia, sclerotia and sporodochia (Watanabe, 2002). Some of the physiological characteristics used for identification are growth temperatures and growth diameters.

Most fungi reproduce asexually and sexually producing different types of spores. Asexual reproduction through mitosis produces asexual spore such as conidia (borne free), sporangiospores (produced in a sac called sporangium) and zoospores (motile spore). Other mechanisms of asexual reproduction are fragmentation and budding. These asexual spores are used in morphological identification of Ascomycete and mitosporic fungi (Alexopoulos et al., 1996; Agrios, 2005).

Fungi reproduce sexually when conditions are unfavourable. Sexual reproduction produces sexual spore through meiosis. Examples of sexual spores are ascospores and basidiospores produced by Ascomycetes and Basidiomycetes, respectively. Both types of sexual spores are commonly used for morphological identification of Ascomycete and Basidiomycete (Alexopoulos et al., 1996).

The term anamorph and teleomorph are used to represent asexual and sexual reproduction, respectively. In older classification, both teleomorph and anamorph characters are used in morphological identification and is known as dual nomenclature. In dual fungal nomenclature, anamorphic and teleomorphic stages have different species names. For example, *Calonectria morganii* is the sexual state of *Cylindrodadium scoparium* and *Botryosphaeria rhodina* is the sexual state of *Lasiodiplodia theobromae* (Wingfield et al., 2012). However, redundancy in naming

the fungal species using dual nomenclature has created confusion among plant pathologists and fungal taxonomists (Crous and Groenewald, 2005).

To solve the complication and confusion of dual nomenclature system, a concept known as ‘one fungus one name’ was introduced. In this concept, one fungus can only have one name of which the anamorph and teleomorph stage can serve as the correct name of a particular fungal species. This means that all valid names suggested for any species, regardless of what stage they are, can be implemented as the correct name for that particular species. The selection of the species name will consider the priority of the stage represented fungus. In this concept, the change of species names as well as rejection of the names must take into consideration of the existing type cultures (Hawksworth, 2011).

Morphological characteristics are not sufficient to define the identity of many fungal isolates due to variation caused by environmental conditions such as temperature, illumination and humidity (Weir et al., 2012). These limitations have led to the use of molecular approaches to improve the accuracy and reliability of fungal identification. However, molecular approaches should not be a replacement for morphological identification. Molecular approaches are useful and helpful in the case of identification of species in species complexes and cryptic species (Bickford et al., 2007).

2.6 Molecular Identification of Plant Pathogenic Fungi

Molecular method can provide detailed understanding of systematics, taxonomy and ecology of plant pathogenic fungi due to its high degree of specificity

and sensitivity (Maharachchikumbura et al., 2012; Manamgoda et al., 2012; Woudenberg et al., 2013). PCR-based methods such as amplified-fragment length polymorphism (AFLP), random-amplified polymorphism DNA (RAPD) and DNA sequencing are among the molecular methods used for identification and characterization. Molecular data especially from DNA sequence analysis have been widely applied in taxonomic, classification, phylogenetic inference, species delimination and identification of plant pathogenic fungi (Hibbett et al., 2007; Nilsson et al., 2011; Hibbett and Taylor, 2013).

DNA barcoding is an identification approach using a short genetic marker for rapid identification and characterization of plant pathogenic fungi. The main criteria for selection of any gene or region as DNA barcode are the target sequence should be identical among the individual's fungal isolates of the same species. The gene or region should have high conserved priming sites for reliable DNA amplifications and sequencing. The data from DNA sequencing must be phylogenetically informative and short enough to have low processing costs (Valentini et al., 2009). Applying DNA barcodes can also reveal cryptic species or species that are difficult to distinguish based on morphology and thus, contributes to a precise and accurate identification. For fungi, ITS region of the nuclear ribosomal DNA (rDNA) was chosen as the most appropriate gene for DNA barcoding of true fungi (Schoch et al., 2012).

The ITS is a non-coding region comprised of ITS1 and ITS2 separated by 5.8S gene and located between small-subunit (SSU) 18S and large-subunit (LSU) 28S of nuclear rDNA repeat unit **(Figure 2.2)**. The ITS become the most popular

genetic marker for fungal identification due to the availability of universal primers (White et al., 1990; Gardes and Bruns, 1993), multi-copy structure of ITS in the genome increase the amplification efficiency even from small amount of DNA samples, the relatively limited length of the ITS region allowing easy amplification and sequencing (Seifert, 2009) and its good resolution power leading to species discrimination in most fungal taxa due to high evolutionary rates (Schoch et al., 2012). Many different universal primers have been designed to amplify the ITS region and the most common are ITS1, ITS2, ITS3, ITS4 and ITS5 (White et al., 1990). For example, leaf spot pathogen such as *Cercospora zeina*, *C. gloeosporioides*, *P. microspora*, *Alternaria simsimi* have been identified using ITS region sequence (Meisel et al., 2009; Rojas et al., 2010; Choi et al., 2014).

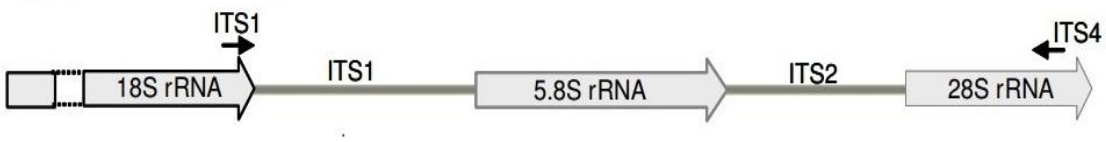

Figure 2.2: Schematic diagram indicating ITS regions and 5.8S ribosomal RNA flanked by small and large subunit ribosomal RNA and the location of the universal primers. Source: White et al. (1990).

Although ITS region has been used in phylogenetic analysis, several leaf spot pathogen such as *Botryosphaeria, Colletrotrichum, Diaporthe, Pestalotiopsis* and *Phyllosticta,* the ITS region provide minor variation within isolates of the same species (Hyde et al., 2014). Hence, protein coding gene has been introduced as an alternative marker such as β-tubulin, TEF-1α, actin (ACT) and calmodulin (CAL) (Glass and Donaldson, 1995; Geiser et al., 2004; Mulè et al., 2004; Gherbawy and Voigt, 2010). Most protein coding genes contain introns, which are highly variable,

making them an attractive target for species identification as well as phylogenetic analysis. However, the choice of protein coding gene depends on the fungal genera. Manamgoda et al. (2012) suggested glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is suitable genetic marker for identification of species in the genus *Bipolaris* and combined sequences of ITS, TEF and GPDH can resolve almost all species of *Bipolaris*.

β-tubulin is the primary constituent of microtubules and abundant in eukaryotic cells (Einax and Voigt, 2003; Glass and Donaldson, 1995) **(Figure 2.3)**. The β-tubulin gene sequences were more phylogenetic information than the SSU rRNA gene (O' Donnell et al., 1998b). Therefore, it has been reported that β-tubulin gene is an ideal marker to analyze phylogenetic relationship and complex species groups (Begerow et al., 2004). In a study by Hyde et al. (2014), partial β-tubulin gene sequences was suggested to be used as an alternative phylogenetic marker for several plant pathogenic fungi including leaf spot pathogen such as *Botrytis*, *Colletotrichum*, *Diaporthe*, *Diplodia*, *Botryosphaeria*, *Pestalotiopsis* and *Phyllosticta.* The recommendation of this gene is to resolve the genus up to species level as well as for species delineation.

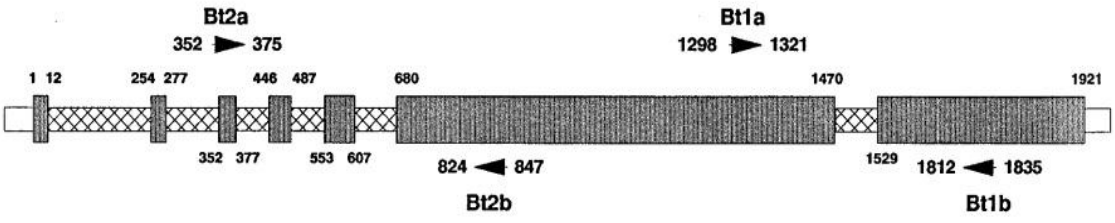

Figure 2.3: Schematic diagram of β-tubulin gene region and the locations of Bt2a and Bt2b primer set. Source: Glass and Donaldson (1995).

The translation elongation factor 1α (TEF-1α) gene has also been proposed as an alternative marker for several fungal genera **(Figure 2.4)**. This gene plays an important role in the translation process of eukaryotic cells as it encodes the essential part of the protein translation machinery and also have phylogenetic utility especially in diverse group of plant pathogenic fungi especially *Fusarium* species (Geiser et al., 2004). The TEF-1α gene was also recommended as a genetic marker both for identification and phylogenetic analysis of several leaf spot fungal genera including *Diaporthe, Diplodia, Pestalotiopsis, Phyllosticta, Lasiodiplodia* and *Neofusicoccum* and most frequently used for identification of *Fusarium* species (Hyde et al., 2014).

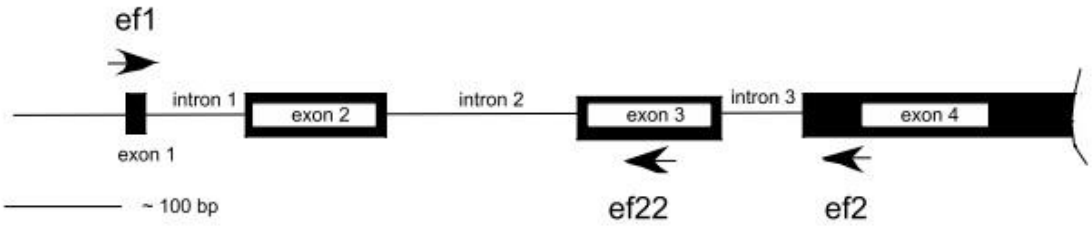

Figure 2.4: Map TEF-1α gene region showing the position of the primers. Source: Geiser et al. (2004)

Phylogenetic species concept is widely used in systematics and taxonomy of plant pathogenic fungi. Phylogenetic relationships helps to characterize unrelated species with similar morphological characteristics and has been applied to recognize species as well as to resolve species in species complexes such as *F. solani* species complex, *Cladosporium herbarium* species complex and *Phoma exigua* species complex (Geiser et al., 2004; Schubert et al., 2007; Aveskamp et al., 2010).

Phylogenetic species concept utilizes DNA sequence of single or multiple genes to develop phylogenetic tree in which the isolates that are grouped in the same

clades are regarded as the same species (Taylor et al., 2000). However, for some plant pathogenic fungi, a single gene analysis has certain limitation. For example, ITS region is not sufficient to delineate species in species complex or cryptic species of the genus *Colletotrichum.* Cai et al. (2009) analyzed 343 ITS sequences of species in *C. gloeosporioides* species complex but only 14% of those sequences showed an agreement with the epitype of *C. gloeosporioides*. Therefore, multigene analysis is important for certain species. By using multiple gene such as ACT, CAL and GAPDH, the species in *C. gloeosporioides* species complex can be resolved (Weir et al., 2012).

2.7 Pathogenicity Test

Pathogenicity is used to describe the ability of a fungal species or isolate to cause disease on a plant host (Agrios, 2005). Fungal isolates produce visible symptoms has to be proven to be pathogenic. Pathogenicity is usually assess using inoculation experiments of which it is performed according to Koch's postulate. Procedures to carry out Koch's postulates are the symptoms expressed by the infected plants are described and the second step is the suspected fungal isolate is isolated and grown as pure culture. The pure culture is then used to inoculate healthy plant of the same cultivar. If the inoculated plant show similar disease symptoms, then the fungal isolates must be re-isolated (Agrios, 2005).

The degree of pathogenicity by the same or different fungal pathogens to cause disease symptoms is referred to as virulence or aggressiveness. The fungal isolates may differ in term of their degree of virulence on a particular plant host. Virulence is usually related to the capability of the pathogen to proliferate in the host

in which a virulent pathogen is defined as the ability of the pathogen to cause severe disease (Casadevall and Pirofski, 2001).

Pathogenicity test is also used to determine host range of plant pathogenic fungi. Some fungal species may be able to infect a large number of plant hosts, whereas other pathogens may able to infect only a few plant hosts (Agrios, 2005). For example, *Phomopsis* species is one of the plant pathogenic fungi causing dieback, canker, leaf spot and blight of wide ranges of hosts such as soybean, sunflower, almond and peach (Udayanga et al., 2011; Gomes et al., 2013) while powdery mildew fungi typically have a very restricted host range. For example, *Erysiphe polygoni* causes powdery mildew of peas, *E. cichoracearum* infect cucurbits and *Blumeria graminis* infect cereals and grasses (Heffer et al., 2006).

In pathogenicity testing, several factors need to be considered including temperature, relative humidity, inoculum loading, nutrient and growing conditions. Suitable temperature to facilitate fungal growth is between 20ºC to 30ºC (Moore and Six, 2015). Humidity is important for disease development and spread of the infection as it encourages fungal sporulation (Rath, 2000). A healthy plant load with higher inoculum level produces disease symptoms within a short time (Sugha et al., 2002). Fungi require certain nutrients for growth in which the amount of nutrients taken depend on the ability of the pathogens to obtain the nutrient from the plant host. Certain fungi produce haustoria to absorb water and minerals from the plant host. Meanwhile, light intensity can also influence the growth of pathogens (Agrios, 2005).

The level of pathogenicity can be influenced by interaction in time between susceptible host plant, a virulent pathogen and favourable environmental conditions. If one of these three components changes, it affects the degree of severity of the plant host. For example, the host plant can be changed by growing disease-resistant varieties. The pathogens can be removed by cultural practice such as tilling residue and rotating crops so that pathogens do not survive on the same crop, controlling insects that carry pathogens to plants, or using fungicides to kill the pathogens. The environment can be managed so that it is less favourable for disease, such as by changing row spacing of the crops and draining excess water from low areas (Agrios, 2005).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample Collection

Mango leaf showing symptoms of leaf spot disease were collected during a series of sampling in mango farm in Kampung Perlis, Balik Pulau, Pulau Pinang; MARDI Bukit Tangga, Kedah and several residential areas in Perlis from Disember 2012 to March. The leaf samples were randomly collected and brought back to the laboratory for fungal isolation. Fungal isolates were given a code based on their location and mango variety **(Table 3.1)**. For example, isolate BPC93, 'BP' denotes the location (**B**alik **P**ulau), 'C' represents the variety of mango tree (**C**hok Anan) and **'93'** is the number of the isolate. Several typical symptoms of mango leaf spot were observed such as dark brown, yellow, grey, red or black spots. Some spots are raised, shiny and others had droped out leaving ragged holes and some were marked with light and dark concentric halos. Numerous spots develop yellow, reddish brown to black colour, increased in size and merge into large, angular to irregular dead areas **(Plates 3.1A, B, C & D).**

Table 3.1: The coding system for fungal isolates recovered from mango leaf spot

Location	Location code	Variety	Variety code
Balik Pulau, Pulau Pinang	**BP**	**Chok Anan**	**C**/CA
Bukit Tangga, Kedah	BT	Nam Dok Mai	N
Mata Ayer, Perlis	MA	Harumanis	H
Batu Pahat, Perlis	BP	Telur	T
Beseri, Perlis	B	Nang Klangwan	M
Pauh, Perlis	P		