ISOLATION AND IDENTIFICATION OF *Fusarium* spp. FROM AGARWOOD AND THEIR PATHOGENICITY TEST ON *Aquilaria subintegra*

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by

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BLAST	Basic Local Alignment Tool
bp	Base pairs
β-tubulin	Beta tubulin
$C_{17}H_{14}O_4$	5,6:7,8-diepoxy-2-(2-phenylethyl)-5,6,7,8-tetrahydrochromone
$C_{18}H_{16}O_5$	5,6:7,8-diepoxy-2-[2-(4-methoxyphenyl)ethyl]-5,6,7,8-tetrahydrochromone
$C_{18}H_{16}O_6$	5,6:7,8-diepoxy-2-[2-(3-hydroxy-4-methoxyphenyl)ethyl]-5,6,7,8-tetrahydrochromone
CITES	Convention on International Trade in Endangered Species
CLA	Carnation leaf agar
CREA	Creatine sucrose agar
CYA	Czapek yeast agar
CA-Kits	Cultivated agarwood kits
C-endophytes	Clavicipitaceous endophytes
ca	Calibration accuracy
DS	Disease severity
dNTP	Deoxynucleotide triphosphate
Enose	Electronic nose
FFSC	*Fusarium fujikuroi* species complex
FSSC	*Fusarium solani* species complex
GC-MS	Gas chromatography-mass spectrometric
½ PDA	Half-strength potato dextrose agar
H_2O_2	Hydrogen peroxide

IUCN	International Union for the Conservation of Nature
ITS	Internal transcribed spacer
MEA	Malt extract agar
MEGA	Molecular Evolutionary Genetics Analysis
ML	Maximum Likelihood
MeJA	Methyl jasmonate
$MgCl_2$	Magnesium chloride
NCBI	National Centre for Biotechnology Information
NUV	Near ultraviolet
NC-endophytes	Non-clavicipitaceous endophytes
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PVC	Polyvinyl chloride
psi	Pounds per square inch
rDNA	Ribosomal dioxyribonucleic acid
rpm	Revolutions per minute
SA	Salicylic acid
SSU	Small subunit
TEF1-α	Translation elongation factor 1-alpha
U	Unit
USD	United States dollars
V	Volt
WA	Water agar

LIST OF SYMBOLS

~	Approximately
°	Degree
°C	Degree Celsius
=	Equality
>	Greater than
<	Less than
≤	Less than or equal to
%	Percentage
±	Plus-minus
®	Registered
Σ	Summation
™	Trademark

PEMENCILAN DAN PENGECAMAN *Fusarium* spp. DARIPADA GAHARU DAN UJIAN KEPATOGENANNYA PADA *Aquilaria subintegra*

ABSTRAK

Gaharu merupakan damar hitam yang kebanyakannya dihasilkan oleh tumbuhan *Aquilaria* dari famili Thymelaeaceae. Gaharu digunakan secara meluas dalam industri kemenyan, perubatan dan minyak wangi di seluruh dunia. Pengeluaran gaharu sebenarnya adalah hasil daripada kecederaan dan/atau jangkitan mikrob, terutamanya kulat. Kajian ini dijalankan untuk memencilkan dan mengenal pasti kulat yang berasosiasi dengan pembentukan gaharu berdasarkan ciri morfologi dan molekul, serta untuk menilai keberkesanan kaedah inokulasi tiruan yang berbeza untuk mendorong pembentukan gaharu. Sejumlah 135 pencilan kulat diperoleh daripada sampel gaharu dari beberapa lokasi yang berbeza di sekitar Semenanjung Malaysia. Berdasarkan pengenalan morfologi, 13 genus kulat dikenal pasti di mana genus yang paling kerap dipencilkan adalah *Fusarium*, diikuti oleh *Aspergillus*, *Lasiodiplodia* dan *Colletotrichum*. Bergantung kepada genus kulat, jujukan DNA daripada tiga gen iaitu kawasan transkipsi dalaman (ITS), faktor pemanjangan translasi 1-alpha (*tef*1-α) dengan β-tubulin telah digunakan untuk menentukan identiti spesies kulat dengan lebih lanjut. Keputusan analisis molekul adalah berpadanan dengan pengecaman morfologi. Daripada kesemua genus kulat, spesies kulat yang paling pelbagai adalah *Fusarium* dengan lima spesies, diikuti oleh *Aspergillus* (4), *Colletotrichum* (2), *Trichoderma* (2) dan *Penicillium* (2). Keputusan ujian kepatogenan menunjukkan bahawa kesemua pencilan *Fusarium* yang di uji adalah patogenik terhadap pokok *A. subintegra* namun pada pelbagai tahap kevirulenan. *Fusarium solani* dianggap sebagai patogen utama berasosiasi dengan pembentukan

gaharu kerana keterukan penyakit (DS) dan bilangan pencilan paling virulen dicatatkan adalah yang tertinggi berbanding dengan spesies *Fusarium* yang lain. Tambahan pula, teknik suntikan mungkin kaedah inokulasi tiruan yang lebih baik untuk mendorong pembentukan gaharu berbanding menggunakan teknik batang buluh. Maklumat kajian ini menyumbang kepada pengetahuan tentang kehadiran kulat yang berasosiasi dengan pembentukan gaharu, serta pengetahuan untuk meningkatkan pengeluaran gaharu dalam industri perladangan melalui inokulasi tiruan.

ISOLATION AND IDENTIFICATION OF *Fusarium* spp. FROM AGARWOOD AND THEIR PATHOGENICITY TEST ON *Aquilaria subintegra*

ABSTRACT

Agarwood is a dark resin which is mainly produced by *Aquilaria* plants from the family Thymelaeaceae. Agarwood is widely used in incense, medicinal and perfumery industries worldwide. Production of agarwood is actually the result of wounding and/or infection of microbes, particularly fungi. This study was conducted to isolate and identify fungi associated with agarwood formation based on morphological and molecular characteristics, as well as to evaluate the efficacy of different artificial inoculation methods in inducing agarwood formation. A total of 135 fungal isolates were obtained from agarwood samples from several different locations around Peninsular Malaysia. Based on morphological identification, 13 genera of fungi were identified whereby the most common genera were *Fusarium*, followed by *Aspergillus*, *Lasiodiplodia* and *Colletotrichum*. Depending on the fungal genera, DNA sequences of three genes namely internal transcribed spacer (ITS) region, translation elongation factor 1-alpha (*tef*1-α) and β-tubulin genes were applied to provide further confirmation of the fungal identities. Results of the molecular analysis have corresponded to the morphological identification. Out of all fungal genera, the most diverse fungal species were *Fusarium* with five species, followed by *Aspergillus* (4), *Colletotrichum* (2), *Trichoderma* (2) and *Penicillium* (2). Results of pathogenicity tests showed that all *Fusarium* isolates tested were pathogenic towards the *A. subintegra* trees but varying degrees of virulence. *Fusarium solani* was regarded as the main pathogen associated with agarwood formation as the disease severity (DS) and the number of highly virulent isolates recorded was the highest

compared to other *Fusarium* species. In addition, the injection technique may be the better artificial inoculation method to induce agarwood formation compared to the bamboo stick technique. The information of the present study contributes to the knowledge on the occurrence of fungi associated with agarwood formation, as well as the knowledge to improve the agarwood production in plantation industry through artificial inoculation.

CHAPTER 1

INTRODUCTION

Agarwood is a highly valued aromatic resin which is occasionally deposited in a few species of the Thymelaeaceae plant family, particularly the *Aquilaria* and *Gyrinops* trees (Gibson, 1977). *Aquilaria* sp. is fast growth tropical trees that are usually found in South and Southeast Asia, from the foothills of the Himalayas to the rainforests of Papua New Guinea (Barden *et al*., 2000). Fifteen species of the genus *Aquilaria* and several species that are primarily known to produce agarwood are *A. malaccensis*, *A. agallocha* and *A. secundaria* (Ng *et al*., 1997; Broad, 1995). In Malaysia, *A. malaccensis* is regarded as the best agarwood producer (Mohamed *et al*., 2010). These species is well distributed in Peninsular Malaysia but occurs at low density (Chua, 2008).

The unique aromatic scent of agarwood makes it an important trading commodity in cosmeceutical, perfumery, and incense industries as well as in the medical field. Recently, the high demand of agarwood in international trade has exceeded the production. *Aquilaria* trees were randomly cut down by unauthorized parties in order to find more agarwood and this resulted in a drastic decline in the natural *Aquilaria* population. Consequently, all species of *Aquilaria* including *A. malaccensis* have been listed in Appendix II of the Convention on International Trade in Endangered Species (CITES) due in the extensive harvesting. The vulnerable condition of *A. malaccensis* is considered a threat to the IUCN Red List Categories and has been included in The World List of Threatened Trees (Oldfield *et al*., 1998).

Agarwood is commonly assumed as a pathological product which is obtained after the host has been invaded by microbes, specifically fungi (Yuan *et al*., 1992). Formation of agarwood commonly occurs in the trunk and root of the infected *Aquilaria* trees. The response of the tree self-defense mechanism against the invasion of microbes is through a tylosis process, which results in the plant to produce a resin high in volatile organic compounds, which aids in suppressing or retarding the infection. The mass and density of the affected wood dramatically increase as the resin accumulate, changing the colour of the wood from pale beige to either dark brown or black in colour (Crous *et al*., 1996).

Other than natural infection by microorganisms, agarwood formation can also be artificially induced using various inoculation methods or treatments. Since the last few decades, numerous inoculation techniques have been developed and applied to the *Aquilaria* tree, which resulted in varying degrees of success (Ng *et al*., 1997). Examples of induction methods that are commonly practiced include the inoculation of inocula or sugar syrup on trees and insertion of metal or polyvinyl chloride (PVC) tubes on wounded parts of the tree (Blanchette, 2006). Theoretically, the application of these induction methods will cause the trees to respond to both physical and chemical defense mechanisms (Persoon, 2007).

The identification of fungal species which associated with the agarwood formation is essential. To date, there were only a few fungal species have been identified to be associated with agarwood formation, such as *Fusarium* species. *Fusarium* spp. was also regarded as the dominant species in previous studies by Mohamed *et al.* (2010), Cui *et al.* (2011) and also, Premalatha and Kalra (2013). In most cases, fungal identification and classification rely on the traditional analysis of

morphological features, such as macroscopic and microscopic characteristics (Larone, 2002). Watanabe (2002) described that morphological identification is commonly used to characterize fungal isolates into morphologically similar species and the naming of the new fungal species can be determined by comparison of the morphological features of the novel fungal species with the currently existing species.

However, the identification solely based on morphological criteria can be problematic as these characteristics may change considerably depending on the cultural conditions (Crous *et al*., 1992). There are several factors that affect the variation in colony morphology, such as the types of single carbon substrates (sugars and sugar alcohols) available in the growth medium, colony age, incubation temperature, light cycle and substrate type (Slepecky & Starmer, 2009). In order to overcome the limitation of morphological identification, the application of molecular methods is thought to be useful as it can help to distinguish the microorganism as well as the phylogeny relationship.

In recent years, the internal transcribed spacer (ITS) region has been recognized as the universal barcoding marker for fungi which can differentiate fungal species from diverse genera as well as closely related species (Schoch *et al*., 2012). Besides the ITS region, there is a variety of protein-coding genes that had been validated for characterizing fungi at higher taxonomic levels, such as β-tubulin and translation elongation factor 1-alpha (*tef*1-α) sequences. Depending on the fungal genus, both sequences of ITS region and protein coding genes are widely used in the molecular characterization of fungi up to the species level.

Apart from that, pathogenicity test was also conducted on the *Aquilaria* trees to confirm the ability of fungal isolates to cause disease symptoms, or to induce the

symptom of agarwood formation. The pathogenicity test of the most prevalent fungal genus, namely *Fusarium*, was carried out on *Aquilaria* trees using injection and bamboo stick techniques, which are combinations of wounding methods and fungal treatments. Previously, *Fusarium* spp. have been proven pathogenic towards on the *Aquilaria* seedlings (Nurbaya *et al*., 2015), however, there is still a lack of study on the pathogenicity of *Fusarium* spp. on the older *Aquilaria* trees. In addition, all criteria of Koch's postulates should be fulfilled in order to validate the causative relationship between the fungal isolates and disease symptoms.

In conclusion, the identification of fungi associated with agarwood formation should be further clarified to find out the most potential agarwood inducer which in turn will improve the agarwood production in industry. At the same time, the pathogenicity of the particular fungi should be tested on *Aquilaria* trees to confirm their ability to cause disease symptoms. The specific objectives are highlighted in 1.1.

1.1 Objectives

The objectives of this study are:

a) To isolate and identify fungal species associated with agarwood formation in *Aquilaria* sp.
b) To evaluate the efficacy of different artificial inoculation methods to induce agarwood formation.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction of Agarwood

Agarwood is one of the most expensive raw materials in the world as it has many special usages in daily life as medicine, perfume and incense. This highly valued commodity is obtained from dark resinous heartwood of *Aquilaria* tree, which is the result of wounding or pathogenesis by microbes or insects on the bark of the tree stem (Novriyanti *et al*., 2010). Agarwood does not exist or produced in healthy plants. Only the injured or insect/microbe-attacked tissues of *Aquilaria* can form the resin in a natural forest (Ng *et al*., 1997). The heartwood of *Aquilaria* tree is relatively light and pale in colour, however, as a response to an infection, the tree produces a resin which changes it to very dense, dark and resin-embedded heartwood (Akter *et al*., 2013).

There are several different names given to agarwood under many different cultures. For examples, agarwood is locally known as ‘gaharu’ or ‘karas’ in Malaysia and Indonesia (Chua, 2008). In Thailand, it is known as ‘mai kritsana’, ‘akil’ in Tamil and ‘mai ketsana’ in Laos (Seng & Maodee, 2005). Meanwhile, in Hindi, it is known as ‘agar’, which originated from Sanskrit aguru (Pusey, 1885). Both agarwood and its resin extracts are called as ‘oud’ in Arabic (Burfield, 2005). In Europe, it was referred to as *Lignum Aquila* or eaglewood and *Lignum aloes* or aloeswood (Henry & Coke, 1903).Whereas, in Assamese it is known as sasi or sashi’ (Panda, 2009). Agarwood is called as ‘aguru’ by the Tibetan and ‘ghara’ in Papua New Guinea (Akter *et al*., 2013).

2.2 Genus *Aquilaria*

Aquilaria is an evergreen tree in the family Thymelaeaceae, which usually grows up to 40 m high and 60 cm in diameter. The bark of *Aquilaria* trees is commonly smooth and dark in coloured, while the inner part of the bark appears from white to creamish in coloured and soft. According to Chakrabarty *et al.* (1994), the wood is apparently light, soft and normally shows no distinction in colour between sapwood and heartwood. The tree is usually found in primary and secondary forests, which mostly grows at an altitude of a few meters above sea level to approximately 1000 m, and it grows best around 500 m in locations with average daily temperatures range from 20 to 22˚C (Keller & Sidiyasa, 1994; Wiriadinata, 1995). The tree species in genus *Aquilaria* mostly has adapted to live in various habitats, including those that are rocky, sandy or calcerous, well-drained slopes and land near swamps (Barden *et al*., 2000).

All members of the genus *Aquilaria* can produce agarwood upon natural or artificial wounding. The top leading agarwood-producing trees is *A. malaccensis*, which is mostly found in Malaysia, Indonesia and India. There are at least fifteen species of *Aquilaria* trees that are known to produce agarwood, including *A. subintegra*, *A. crassna*, *A. sinensis* and *A. beccariana* (Akter *et al*., 2013). Decay and wilt appearances are among several signs shown by the *Aquilaria* trees infected by natural inducers such as insects, parasites as well as microorganisms.

2.3 Chemical Constituents of Agarwood

In recent years, many researchers are interested in studying the chemical composition of agarwood. Some early scientific reports were by Tamuli *et al.* (2005), Bhuiyan *et al.* (2008), Azah *et al.* (2009), Tajuddin and Yusoff (2010), as well as

Pripdeevech *et al.* (2011). Results of previous studies have revealed that sesquiterpene components and its phenylethyl chromone derivatives are the major constituents of agarwood oil, which act as the main source of the fragrant. The composition of the compounds is useful to determine the quality of agarwood and its oil extracts (Ishihara *et al*., 1993). For example, a research in Japan has classified the quality of agarwood oil as either high or low based on peak area percentage, or the abundance of chemical compounds (Nurlaila *et al*., 2014).

The highest quality of agarwood oil was known to have a high resin content, which also means it is composed of various kinds of oxygenated sesquiterpenes and phenylethyl chromones derivatives (Ishihara *et al*., 1993). Sesquiterpenes in agarwood can be divided into several categories depending on their skeleton, such as agarofurans, agarospiranes, guaienes, eudesmanes, eremophilanes and prezizaanes (Chen *et al*., 2012). According to Ishihara *et al.* (1993), there are various guaiene and eudesmane sesquiterpenes that are found in high quality but most of them were not detected in low quality of agarwood oil. The β-agarofuran, α-agarofuran and 10-epi-γ-eudesmol were also listed as major compounds in high quality (Ishihara *et al*., 1993; Azah *et al*., 2009). Nonetheless, β-agarofuran is thought as the most important compound in high-quality agarwood as it contributed to the aromatic scent of agarwood oil (Pripdeevech *et al*., 2011).

Phenylethyl chromone or synonymously known as 2-(2-Phenylethyl)-4H-chromen-4-one derivatives are also among the major constituents in agarwood. These compounds can only be found in certain plant species, such as *Eremophila georgei*, *Bothriochloa ischaemum* (family Gramineae) and the agarwood, which originated from *Aquilaria* species (family Thymelaeaceae)

(Shu *et al*., 2005). The study by Yagura *et al.* (2005) had successfully isolated novel chromone derivatives from agarwood that produced by intentional wounding of *A. crassna* and *A. sinensis* trees. The three compounds were characterized as diepoxy tetrahydrochromones and has never been reported from natural agarwood product, for example, Oxidoagarochromone with a molecular formula of A: $C_{17}H_{14}O_4$; B: $C_{18}H_{16}O_5$; C: $C_{18}H_{16}O_6$ (Yagura *et al.*, 2005). To date, there are more than 40 phenylethyl chromone derivatives have been isolated from agarwood (Chen *et al*., 2012).

2.4 Hypotheses of Agarwood Formation

Typically, agarwood formation was influenced by several factors, either naturally by biotic or abiotic factors and by artificial induction (Sitepu *et al*., 2011). Biotic factors mostly referred to living components such as the association of microorganisms to cause infection on plants, invasion by insects or parasites, also by disturbance of animals and human. For abiotic factors, it includes the physical components such as wind, rain and lighting. Besides, agarwood can also be artificially induced by mechanical wounding, application of chemicals and microorganisms, particularly fungi. There are also three hypotheses associated with agarwood production, such as results of pathological, wounding/pathological and/or non-pathological processes (Ng. *et al.*, 1997).

Physical and chemical barriers are known as the major defense mechanisms developed by plants during the long process of evolution which had been used to obstruct pathogen invasion before or after the wound or microbe attack (Yang *et al.*, 1997). Agarwood was indicated as a plant phytoalexin which is consistently formed after the plant suffered from infection or invasion. Moreover, there are many plant natural terpene products that have been classified as phytoalexins with antimicrobial

or anti-insect activity (Banerjee *et al.*, 2006; Maldonado-Bonilla *et al*., 2008; Gershenzon & Dudareva, 2007). Thus, agarwood could be referred as the induced defense of agarwood-producing trees (Isnaini, 2004).

Previously, Yuan *et al.* (1992) has described that the healthy or undisturbed agarwood-producing trees will never produce fragrant sesquiterpenoid as its secondary metabolites. To ensure its survival, it is common for plants to synthesize and accumulate secondary metabolites in response to against agents of infection, physiological stimulus or stress (Goodman *et al*., 1986). Anatomic observation shows that the resin was only localized in the tissue adjacent to the wounded or pathogen-infected tissue (Nobuchi & Siripatanadilok, 1991). The resin is mainly included in the phloem strands which are the key pathway for the pathogen invasion (Rao & Dayal, 1992).

Theoretically, the defense mechanism developed by a tree against stress or disease was influenced by genetic factors (Novriyanti *et al*., 2010). Most of the chemical composition that is found in a tree is considered to have important roles which contribute to the defense system. As for *Aquilaria* spp., the recognition of chemical constituent of secondary metabolites was thought to be essential in order to select the potential susceptible tree for agarwood production. A few years ago, Novriyanti *et al.* (2010) assumed that the tree would respond well to the induction of agarwood causal agents if it is more susceptible, whereas the tree with less defense compounds is more preferable in artificial agarwood production.

2.5 Induction Methods of Agarwood

Most of the techniques used in agarwood induction involved the wounding of trees in a specific manner and some treatments applied to hasten the natural defense responses of the tree. According to Persoon (2007), wounding methods using blade or hammering of nails into the trunks have been used in the past, but the agarwood yielded from this treatment commonly did not reach the quality or standard desired by market demands. Nevertheless, Gunn *et al.* (2003) reported that the agarwood hunters in Papua New Guinea intentionally wounded the agarwood-producing tree in order to induce agarwood production and surprisingly succeeded to harvest grades B and C agarwood after three years of treatment. In an attempt to stimulate agarwood formation, the local people live in Northern Vietnam practically make holes in the trunks and main branches of *Aquilaria* trees (Gratzfeld & Tan, 2008). Subsequently, the agarwood can be harvested in minimal quantities if a regular chipping of the wounded parts is carried out continuously.

Young rapidly growing *Aquilaria* trees in a plantation or any tree grown in forests can be treated successfully to produce agarwood. Research carried out by Pojanagaroon and Kaewrak (2005) have successfully applied four different mechanical methods to promote the agarwood production; by making a hole with screws, gouging with chisels, bark removal with hatchets and puncturing with nails. These methods have been reported to be influenced by seasonal changes, in which the rainy season accelerated the agarwood formation faster than dry seasons. Other than that, a wounding method such as filling any pieces of wood or porcelain can be done towards the wounded tree. By doing this, the wound will be kept opened and it was found that this method was more dependable in stimulating the agarwood formation (Gratzfeld & Tan, 2008).

Production of agarwood using a coppice system was commonly practiced by the native Penan tribe of Indonesian Borneo (Donovan & Puri, 2004). Traditionally, these methods only aim to excise the resinous wood parts instead of chopping down the whole tree. By wounding the trees alternately by using the coppicing technique, it can help to promote the agarwood production and supply a continuous yield. However, the agarwood formed from coppice system mostly is of low quality and can only be harvested in small quantities (Donovan & Puri, 2004). To improve agarwood formation, various methods have been developed to produce agarwood or the major compounds through establishing stress conditions via cutting, cauterizing, or exogenous application of the elicitors related to plant defensive response, including methyl jasmonate (MeJA), hydrogen peroxide (H_2O_2) or salicylic acid (SA) (Ito *et al.,* 2005; Kumeta & Ito, 2010; Wijitphan, 2009).

Another classic example is the partial-trunk-pruning and the burning-chisel-drilling methods which are often used by Chinese farmers in recent decades. Low quantity of agarwood yield was produced by these methods, yet it requires a long time for agarwood formation to take place (Liu *et al*., 2013). Fungal inoculation method was also introduced as the agarwood formation usually resulted from plant defense mechanism against fungal invasion. According to Akter *et al.* (2013), the inoculation of *Torula* sp. on *A. agallocha* was successful and it was found to accelerate the agarwood formation. Besides, a combination of 2 % sugar solution, methyl jasmonate and fungal species, such *Acremonium* sp. was reported to produce agarwood of quality class IV.

In recent years, some relatively new and efficient methods called cultivated agarwood kits (CA-Kits) have been developed in Vietnam by Blanchette from the

University Minnesota (Blanchette & Beek, 2009). The basis of this method is to drill holes in the tree trunks and keep the wounded area open by inserting a small piece of plastic pipe, and followed by inoculation of different chemical media into the wound. Theoretically, this treatment can accelerate the resin formation as it caused the tree to respond on both physical and chemical defense mechanism (Akter *et al*., 2013). With the advantage of easy evaluation of discoloration area, this novel finding symbolized a great enhancement of agarwood production compared to the conventional physical wounding methods (Liu *et al.*, 2013).

2.6 Fungi Association in Agarwood Formation

Much of the early research on *Aquilaria* research focused on identifying the specific fungus that is responsible or associated with agarwood formation. There are several different fungal species described to be associated with agarwood production and other species were found to exhibit pathogenesis by developing a symptom of the disease. Results of scientific research by Wiriadinata (1995), Santoso (1996), Soehartono and Mardiastuti (1997), and Anonymous (1998) had successfully identified several genera and species of fungi that are commonly associated with the agarwood production, namely *Aspergillus* spp., *Botryodyplodia* spp., *Diplodia* spp., *Penicillium* spp., *Pythium* spp., *Fusarium bulbiferum*, *F. laterium*, *F. oxysporum* and *F. solani*.

Other than that, fungi genera that are species specific plant pathogens such as *Acremonium* sp., *Botryodiplodia* sp., *Cytosphaera* sp., *Fusarium* spp., *Libertella* sp., *Pythium* sp., *Penicillium* sp., *Rhizoctonia* sp., *Scytalidium* sp., *Thielaviopsis* sp. and *Trichoderma* sp. could also be found from the agarwood samples (Sumarna & Santoso, 2002). Most of the early researchers agreed that it is improbable that a

single, specific fungal species can be associated with the agarwood formation since there are more than 20 varieties or genetic strains of fungus that have been identified in the presence of agarwood (Gibson, 1977; Rao & Dayal, 1992; Tamuli *et al*., 2000; Tabata *et al*., 2003). However, the study by Santoso *et al*. (2006) reported that the results on purification of agarwood-synthesizing fungi were presumably dominated by the species of *Fusarium*.

Numerous reports were available on the association of fungi in the formation of agarwood. For instance, Yuan *et al.* (1992) reported that agarwood is a pathological symptom that is produced when the fungus invaded the host tree. According to Ng *et al.* (1997), it was estimated that almost 10% of the agarwood occurrence on the trees are naturally infected by the fungus. Since 1938, a few workers have studied the agarwood formation and reported that agarwood zones to be associated with mold and decay fungi (Tamuli *et al*., 2000; Mitra & Gogoi, 2001). In addition, results of recent studies by Mohamed *et al.* (2010), Cui *et al.* (2011), as well as Premalatha and Kalra (2013) showed that the fungal species identified from agarwood samples of different *Aquilaria* sp. were diverse.

The wounded *Aquilaria* trees are prone to the invasion by insects and microorganisms, particularly fungi. There is a high potential of fungal spore dispersal by wind, water, or insects from reservoirs in the soil or diseased tissues in the same or neighboring trees (Ivory & Speight, 1993). Wounding provides entry for a variety of pathogens and allowed the proliferation of certain fungi in the host tissue (Mohamed *et al*., 2010). This exacerbates the stress situation and caused the tree to produce more resin containing aromatic terpenes (Mohamed *et al*., 2010). As for *Aquilaria* trees, a

previous research study deduced that the range of fungal colonists decreased as the resin content increased (Donovan & Puri, 2004).

The fungi may either develop as endophytes or plant pathogens. Endophytes can be defined as microorganisms which invade the living plant tissue and cause asymptomatic infections within the plant host (Wilson, 1995). Endophytes are usually found in vascular plant species including medicinal plants, and have been isolated from diverse plant tissues, including bark, leaves, wood and seeds (Huang *et al*., 2009; Tejesvi *et al*., 2009). The microorganism may establish endosymbiotic relationship with the host plant, by improving and promoting plant growth as well as help to reduce disease symptoms caused by pathogens or environmental stress (Giordano *et al.*, 2009; Nimnoi & Pongslip, 2009).

According to Rodriguez *et al.* (2009), there are two major groups of endophytes that can be distinguished according to the phylogeny and life history traits, such as clavicipitaceous (C-endophytes) and non-clavicipitaceous (NC-endophytes). All members of C-endophytes group are restricted to certain grass species. Meanwhile NC-endophytes have a broad range of both nonvascular and vascular host plant species (Rodriguez *et al.*, 2009). However, recent studies suggest that members of the NC-endophytes group should be separated into three subgroups according to host range, tissue colonized, transmission, *in planta* colonization, biodiversity, and fitness benefits conferred to hosts (Rodriguez *et al*. 2009; Purahong & Hyde, 2011). Other than that, the endophytic fungi could also become pathogenic under certain environmental condition and are referred as latent pathogens (Photita *et al*., 2004).

Moreover, endophytic fungi are typically found closely related to plant pathogens (Delaye *et al*., 2013). Many endophytes have been regarded as dormant pathogens (Photita *et al*., 2004) and mutations at a single locus can change a fungus from a pathogenic to a non-pathogenic lifestyle, with no effect on host specificity (Eaton *et al.,* 2011). Fungal pathogens can broadly be classified into two groups, namely the biotrophs and necrotrophs (Jones & Dangl, 2006). Biotrophic pathogens are parasites that have evolved the means to grow within living plant cells without stimulating plant defense mechanisms (Mendgen & Hahn, 2002). In other words, biotrophic pathogens are capable of spreading rapidly throughout the plant tissues, yet in the meantime, they divert the nutrients from the living plant to fuel its own growth at the expense of plant productivity (Delaye *et al*., 2013). By contrast, the necrotrophic pathogens use toxins and depolymerizing enzymes to kill and degrade plant cells and consumed the end products (Oliver & Ipcho, 2004).

Overall, there are several characteristics that can be used to distinguish the endophytes and plant pathogenic fungi, such as host range, presence or absence of haustoria, secretion of lytic enzymes and toxin production. The summary of major characteristics of life histories of plant-infecting fungi is shown in Table 2.1.

2.7 Genus *Fusarium*

Fusarium is one of the widely known phytopathogenic fungi that showed a significant increase in their virulence and importance in causing plant disease in the tropics (Salleh, 2007). Other than that, some *Fusarium* species also known as a mycotoxin producer that can affect human and animals of which in turn lead to the economic loses. The diversity of *Fusarium* species were influenced by climate and

Table 2.1 Major characteristic of life histories of plant-infecting fungi (Delaye *et al*., 2013)

Characteristics	Endophyte		Pathogen	
	Clavicipitaceous	Non-clavipitaceous	Necrotroph	Biotroph
Host range	Mostly specialists (grasses)	Many generalists	Many generalists	Mostly specialists
Haustorium	Absent	Absent	Absent	Present
Secretion of lytic enzymes	[a]Limited	[a]Limited	Yes	[a]Limited
Toxin production	Yes	Yes	Yes	No
Host hypersensitive response	Suppressed	Suppressed	Promoted	Suppressed
General growth	Systemic	Local and systemic	Local	Local and systemic
Hyphal growth	Intercellular	Intercellular	Intracellular	Intracellular
Sexuality	Asexual	Asexual and sexual	Sexual	Sexual
Effects on host reproduction	Stopped and reduced	Unaffected	Reduced	Stopped or reduced

[a]Does not result in tissue maceration

this factor can be used to predict the diseases caused by *Fusarium* species. The genus *Fusarium* existed as saprophytic, endophytic and parasitic fungi that distributed worldwide (Leslie & Summerell, 2006). It is broadly distributed in soil, plant debris, aerial plant parts and other organic substrates from tropical and other regions, such as temperate, desert and Arctic environment. The other issues regarding the genus *Fusarium* were further described in the following subsection.

2.7.1 Taxonomy of *Fusarium*

The genus *Fusarium* belongs to the Ascomycota phylum, Ascomycetes class, Hypocreales order (Leslie, 1995), while the teleomorphs of *Fusarium* species are mostly classified in the genus *Gibberella*, and for a smaller number of species, *Haemanectria* and *Albonectria* genera (Moretti, 2009). The generic concept of *Fusarium* was first diagnosed by Link (Booth, 1971), based on the primary character such as the distinctive canoe- or banana-shaped conidia that well known associated with this genus (Leslie & Summerell, 2006).

Following the diagnosis of Link, much of the early research on *Fusarium* focused on the diagnosis, identification and enumeration of taxa that caused plant diseases. There were numerous species, numerous proposed characters, and multiple culture media that combined to make strain identification and species diagnosis very complicated (Leslie & Summerell, 2006). Several documented taxonomic systems that have been proposed regarding the genus *Fusarium* (Table 2.2), such as Wollenweber and Reinking (1935), followed by Snyder and Hansen (1940, 1941, 1945), Raillo (1950), Gordon (1952), Bilai (1955), Booth (1971), Gerlach and Nirenberg (1982), Nelson *et al.* (1983) and also, Leslie and Summerell (2006).

Table 2.2 The significant development of genus *Fusarium* taxonomic systems

Year	Event
1809	Link originally described the genus *Fusarium* as *Fusisporium*
1935	Wollenweber and Reinking had documented of *Fusarium* species in 'Die Fusarien' with around a thousand *Fusarium* species described, often with a different species described for every host. The sub-generic system of *Fusarium* species was based on 16 sections, 65 species and 77 sub specific varieties and forms.
1940s	Snyder and Hansen reduced the number of species within the genus to nine, and demonstrated that only by using cultures derived from a single spore could species identifications be made reliably.
1950	Raillo proposed a taxonomic system based on the shape of the macroconidia, and the presence of microconidia and chlamydospores.
1952	Gordon developed a pragmatic approach to *Fusarium* taxonomy that followed ideas of Wollenweber and Reinking philosophy with some suggestion of Snyder and Hansen systems, and included teleomorph characters in his descriptions.
1955	Bilai published a book 'Fusarii' and recommended combining some of the Wollenweber and Reinking sections in section Liseola with Elegans and Gibbosum and Discolor.
1971	Booth published a monograph, 'The Genus *Fusarium*' and also followed Wollenweber and Reinking's approach and especially focused on morphology of conidia bearing cells.
1982	Gerlach and Nirenberg published a monograph of *Fusarium* species, 'The Genus *Fusarium*: A pictorial Atlas' and recognized a large number of distinct species.
1983	Nelson, Toussoun and Marasas produced an identification manual of *Fusarium*, '*Fusarium* species: An Illustrated Manual for Identification'. This monumental book contains isolation and cultural methods, variations of identification and pigment, colour and black-white photographs, synoptic keys and information about 46 Fusarium species.
2006	Leslie and Summerell published 'The *Fusarium* Laboratory Manual' which contains about 70 species descriptions. This publication contains over 474 comprehensive collections of photographs and figures, proposed new media, *Fusarium* culture identification checklist, flow chart of identification through sexual crosses and more information about nucleic acid analyses.

In recent years, the taxonomy of *Fusarium* species has been further organized. Previously, the nomenclature of *Fusarium* species is known in several names under their teleomorph stage, such as *Gibberella*, *Nectria* or *Haemonectria* (Leslie & Summerell, 2006). In 2011, the International Code of Nomenclature for algae, fungi and plants (ICNafp) has decided to change the dual nomenclature of anamorph and teleomorph into 'one fungus one name' system (Geiser *et al*., 2013; Aoki *et al*., 2014). Thus, the teleomorph names were replaced by the asexual names of *Fusarium* to standardize the nomenclature as well as to prevent any confusion in naming the fungi.

2.7.2 *Fusarium* as Plant Pathogen

Fusarium is commonly known as a major plant pathogenic fungi which caused various types of diseases such as bakanae disease, damping off, dieback, stem splitting, vascular wilting, canker and rot on economically important crops (Leslie & Summerell, 2006; Burgess *et al*., 2008). Genus *Fusarium* are widely distributed in natural soil, cultivated soil, organic materials, persist as chlamydospores or as hyphae in plant residues, and even some species can produce airborne conidia that colonized plant parts (Booth, 1971; Burgess *et al*., 1994; Leslie & Summerell, 2006). The ability of *Fusarium* to grow in diverse range of substrates and climates also increases their chances to infect the plant host worldwide.

The probability of *Fusarium* to cause infection in host plant is influenced by several factors including environmental conditions, physiology of the host and spore condition among others (Marin *et al*., 1995; Doohan *et al*., 2003). Other than that, temperature and humidity conditions are also believed to be determinants in the infection process, development and dissemination as well as mycotoxin producing

ability of *Fusarium* (Dilkin *et al*., 2002; Etcheverry *et al*., 2002; Murillo-Williams & Munkvold, 2008). According to Yates and Jaworski (2000), the infection process of *Fusarium* also relied on the physiological status of both host plant and fungus. In addition, the germination rate of *Fusarium* conidia was affected by the spore density, which in turn influenced the disease development (Reid, 1995).

Depending on the biotic and abiotic factors surrounding the plants, the infected plants may either showed disease symptoms or remain symptomless (Wilke *et al*., 2007). In most cases, the fungal hyphae play a significant role in host attack and infection due to their ability to produce enzymes that degrade host cell walls. For instance, *F. culmorum* hyphae were noted to penetrate different parts of wheat spikelets and sometimes via stomata (Kang & Buchenauer, 2000). Other than that, *F. graminearum* have been reported to cause infection on floral organs of wheat, of which the infection began with the formation of foot structures, lobate appresoria and infection cushions (Boenisch & Schafer, 2011).

2.8 Fungal Identification

Several different concepts have been introduced to define the fungal species. One of the classical approaches is the phenotypic concept, of which the fungi were identified based on the morphological and physiological features such as sexual and asexual state (Taverna *et al*., 2013). A variety of biochemical and physiological methods for fungal identification have been also devised (Guarro *et al*., 1999; Lusta *et al*., 2003). However, these approaches have several limitations such as laborious, time-consuming and provide insufficient taxonomic resolution (Lusta *et al*., 2003). Hence, an alternative approach using DNA-based methods is used to overcome these problems. The basic DNA sequences of an organism are insensitive to short-term

environmental change and thus should provide a more stable alternative for strain identification (Soll, 2000; Lusta *et al*., 2003). Further explanation regarding the morphological and molecular approach is described in 2.8.1 and 2.8.2.

2.8.1 Morphological Approach

Fungal species or the taxa are commonly named on the basis of morphological features (Watanabe, 2002). A morphological concept is a classical approach used in fungal identifications based on their macro- and microscopic features. Identification of the fungi species is commonly based on mycelia (colour, size and shape) and morphological characteristics (morphology, conidia size and morphology conidiophore) (Pitt & Hocking, 2009). According to Glass and Donaldson (1995), the identification of filamentous fungi can be done using cultural morphologies including colony and color characteristics on specific culture media, the size, shape, and development of sexual and asexual spores and spore-forming structures, and/or physiological characteristics such as the ability to utilize various compounds as nitrogen and carbon sources.

Microscopic features such as shape, size, and ornamentation of conidia, as well as the branching pattern and stipe ornamentation of conidiophores, may also guide in identification of fungal species. For instance, different morphological characteristics used in *Fusarium* sp. identification, such as types of spore: macroconidia, microconidia and chlamydospores. Leslie and Summerell (2006) described that the shape of conidia varies from club form, reniform, obovoid, pyriform, napiform, globose and fusiform. In contrast, for *Aspergillus* sp., ascospore sizes and morphology, particularly the often diagnostic ornamentation (roughening, rims, wings, and furrows) are important for identifying species (Samson *et al.*, 2014).

2.8.2 Molecular Approach

Fungi have historically been identified and classified using morphological characteristics, such as sexual structure. However, fungi, especially microscopic forms, often have few useful morphological features and show pronounced morphological variability (Burnett, 2003). Moreover, an increasing number of morphologically indistinguishable (cryptic) species have recently been described by Grunig *et al*. (2007). Accordingly, the use of morphology for fungal identification and classification purpose can be severely biased. In the past decade, the ability to identify species at the molecular level has changed our understanding of the species concept for different groups of fungi (Hibbett *et al*., 2007). Particularly, molecular phylogenetic approach avoids subjectivity in determining the limits of a species by relying on the concordance of more than one gene genealogy and have been approved as well suited for teleomorphic and anamorphic fungi (Taylor *et al*., 2000).

Nowadays, a researcher can quickly and accurately identify all fungal species by using targeted amplification of specific regions of the fungal genome via the polymerase chain reaction (PCR) (Horton & Burns, 2011). A large variety of primers currently were created for the amplification of different regions of fungal DNA (White *et al*., 1990). Target regions for sequence-based approaches ideally would show sufficient conservation among fungi to allow cross-species amplification. It also allows robust discrimination between closely related species (Borman *et al*., 2008).

The internal transcribed spacer (ITS) region has been proposed as the primary fungal barcode marker for fungal species identification (Schoch *et al*., 2012). The ITS region is relatively short (500-800 bp) and has been successfully used in

molecular characterization studies in fungi (White *et al*., 1990). The ITS regions (Figure 2.1) comprising ITS1, 5.8S and ITS2 can be easily amplified by universal primers in the conserved flanking regions of 18S and 28S (Begerow *et al*., 2010). In addition, the multicopy nature of the rDNA repeat makes the ITS region easy to amplify from small, dilute or highly degraded DNA samples (Gardes & Bruns, 1993). For example, differences in ITS sequences have been used to develop PCR-based assays for the detection of many phytopathogenic fungal species in host plants without previous isolation of the fungi (O'Brien *et al.*, 2009).

However, there are some limitations in using the sequences from ITS regions of certain fungal species. Seifert *et al.* (1995) reported that some species within the genera *Penicillium* and *Sclerotinia* showed little genetic variation (< 0.5%) or no variation within the sequences of the ITS regions. O'Donnell and Cigelnik (1997) also reported that many fusaria within the *Gibberella* clade possess non-orthologous copies of ITS2, which can lead to incorrect phylogenetic inferences. Therefore, the usage of sequences from ITS region seems to be unreliable for the identification of these species.

The marker of choice for species-level phylogenetics in fungi is the intron-rich portions of protein coding genes (Geiser, 2003). According to Bhargava *et al*. (2012), there are several types of protein coding gene that can be used as an alternative marker in fungal identification, namely actin, β-tubulin, calmodulin and translation elongation factor 1-alpha (TEF1-α). These gene regions tend to evolve at a rate higher

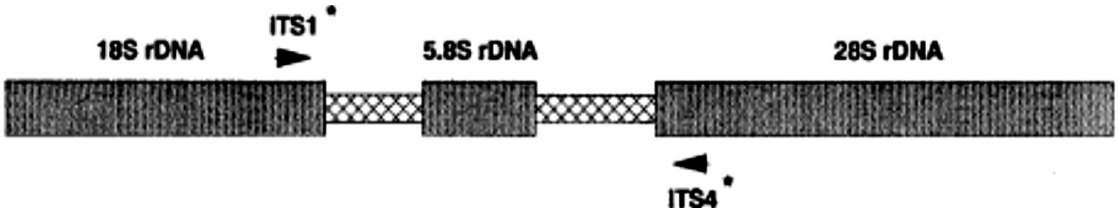

Figure 2.1 Schematic diagram indicating *Neurospora crassa* ITS regions and the locations of ITS1 and ITS4 primer set. Shaded boxes denote the 18S, 5.8S and 28S ribosomal DNA respectively. Cross-hatched boxes denote the ITS1 and ITS2 region. Arrows are identified as the labeled primers and denote the position of the primers (Glass & Donaldson, 1995)