IDENTIFICATION OF UNIQUE DNA SEQUENCES IN *Aquilaria malaccensis* AND *Aquilaria hirta* VIA GENOMIC REPRESENTATIONAL DIFFERENCE ANALYSIS (RDA)

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ABSTRACT

Aquilaria species that are listed among the endangered plant species are well known as the main agarwood producers in the world. Agarwood produced by *Aquilaria* spp. has been widely used in traditional medicine, incenses for religious rituals and also in perfumery industry. However, identification of the species in *Aquilaria* genus is very challenging due the changing of their morphological structures that are highly dependent on the changes of environmental conditions. Thus, this study was aimed to identify the unique DNA sequences that are available in the genome of *Aquilaria malaccensis* and *A. hirta* to be used as specific molecular markers for species identification purpose. Genomic Representational Difference Analysis (RDA) was applied by amplifying DNA sequences that are only available in the genome of interest species while eliminating DNA sequences that are redundant in both interest and non-interest genomes. A number of three rounds of RDA with different ratios of "tester" and "driver" DNA were carried out and finally, 15 unique DNA sequences were successfully isolated from the final round of RDA. The isolated sequences were sequenced and subjected to BLASTN analysis for identification and prediction of their functional role based on their similarity to homologous DNA sequences that are available in the NCBI GenBank database. From the BLASTN analysis, majority of the sequences did not match to any sequence that is available in the database. This might be due to the lacking of information discovered and reported on *Aquilaria* spp. in molecular biology as well as bioinformatics aspects. Based on the isolated sequences, different set of gene specific primers were designed specifically for each potential sequence. The primers were subsequently applied in PCR amplification of the unique DNA sequences directly from genomic DNA of selected *Aquilaria* spp. for confirmation of the presence of the DNA sequences uniquely in species of interest while absence in the other *Aquilaria* spp.. From the confirmation, 12 unique DNA sequences have shown their potential to be used as molecular markers in *A. hirta* while in *A. malaccensis*, there are only three identified potential sequences to be used as molecular markers. The isolated unique DNA sequences will be very useful for *Aquilaria* spp. identification that will benefit agarwood plantation industry in order to produce and maintain a good quality of agarwood for fragrance and perfumery industries.

ABSTRAK

Spesies *Aquilaria* adalah terkenal sebagai pengeluar utama minyak gaharu yang juga turut disenaraikan sebagai spesies tumbuhan yang kian terancam. Sesungguhnya gaharu yang dihasilkan oleh spesis *Aquilaria* telah lama digunakan didalam bidang perubatan tradisional, kemenyan untuk upacara keagamaan dan juga didalam industri pembuatan minyak wangi. Walaubagaimanapun, pengenalpastian spesies dalam genus *Aquilaria* adalah amat mencabar kerana struktur morfologinya yang bergantung kepada perubahan keadaan persekitaran. Oleh itu, kajian ini dilakukan adalah bertujuan untuk mengenalpasti jujukan siri DNA yang unik yang terdapat didalam genom *Aquilaria malaccensis* dan *Aquilaria hirta* untuk digunakan sebagai "marker" molekular bagi tujuan pengenalpastian spesis *Aquilaria* tertentu. "Representational Difference Analysis (RDA)" telah digunakan untuk memencilkan jujukan DNA yang hanya terdapat dalam genom spesies yang dikehendaki dan mennyahkan jujukan DNA yang juga wujud dalam spesies yang tidak dikehendaki. Tiga pusingan RDA dengan nisbah "tester" dan "driver" DNA yang berbeza telah dijalankan dan 15 jujukan DNA yang unik telah diasingkan melalui teknik ini. Jujukan DNA tersebut dijujukkan dan seterusnya dianalisis menggunakan program BLASTN untuk mengenalpasti fungsi jujukan DNA tersebut berdasarkan persamaan dengan jujukan DNA yang telah sedia ada di dalam pangkalan data NCBI GenBank. Hasil daripada kajian ini, majoriti jujukan DNA tersebut tidak mempunyai sebarang persamaan dengan mana-mana jujukan DNA yang terdapat dalam pangkalan data NCBI. Perkara ini berlaku adalah disebabkan oleh kurangnya penemuan dan kajian yang dijalankan keatas spesis *Aquilaria* dalam bidang biologi molekul dan 'bioinformatics'. Seterusnya, primer-primer yang spesifik telah direka khusus bagi setiap jujukan DNA yang telah dipencilkan. Primer-primer tersebut seterusnya digunakan didalam tindakbalas berantai (PCR) menggunakan DNA genome spesis-spesis *Aquilaria* sebagai acuan bagi pengesahan kewujudan jujukan DNA tersebut di dalam spesies yang dikehendaki tetapi tiada dalam spesies yang lain. Melalui pengesahan tersebut, 12 jujukan DNA unik telah menunjukkan potensi untuk digunakan sebagai "marker" molekular bagi *A. hirta* manakala, hanya tiga potensi jujukan DNA unik boleh digunakan sebagai "marker" molekular untuk *A. malaccensis*. Jujukan DNA unik tersebut adalah amat berguna untuk mengenalpasti spesies *Aquilaria* dan pastinya akan membantu industri perladangan gaharu bagi penghasilan dan pengekalan kualiti gaharu yang baik untuk industri wangian.

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LIST OF SYMBOLS

µg	Microgram
µl	Microliter
µM	Micromolar
g	Gram
kb	Kilo base
M	Molar
ml	Milliliter
mM	Milimolar
ng	Nanogram

LIST OF ABBREVIATIONS

ATP	Adenosine Tri-Phosphate
BLASTN	Basic Local Alignment Search Tool Nucleotide
bp	Base pair
BSA	Bovine serum albumin
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EPPS	N-(2-hydroxyethyl) piperazine-N-3-propanesulfonic acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria Bertani
MBN	Mungbean nuclease
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
pH	Power of hydrogen
RDA	Representational Difference Analysis
SSR	Short Sequence Repeat
TAE	Tris-acetate EDTA
TE	Tris-EDTA
tRNA	Transfer Ribonucleic acid
U	Unit
X-gal	5-bromo-4-chloro-3-indolyl-β-D galactoside

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Agarwood oil produced by *Aquilaria* spp. receives high demand from the entire world due to its usage in folk medicine, incense and perfumery industry (Näf, 2010). *A. malaccensis* is listed as an endangered species by the Malaysian government (Tajuddin and Yusoff, 2010). *Aquilaria (*Thymelaeaceae) is one of the most precious non-timber forest products (NTFP) due to the aromatic dark coloured resin produced in the bark and trunk of the species, predominantly in Malaysia. The resin produced by *Aquilaria* spp. is called as gaharu, agarwood or aloeswood depending on the regions (Zhang *et al*., 2008; Karimi *et al.,* 2011). The natural oil is produced from the fragrant resin of several *Aquilaria* species including *Aquilaria malaccensis* that is well distributed in Malaysia.

Naturally, to obtain agarwood, the bark of *Aquilaria* spp. is left to be infected by microbes. This natural mechanism is commonly occurred on infertile soil which triggered the entrance of insects in the bark (Wollenberg, 2001). In recent years, production of agarwood is triggered artificially by wounding or introducing microbes on the stem and bark of the trees. After the trees are infected or wounded, it requires a few years to generate the agarwood in the wounded or infected trees (Zhang *et al*., 2012). The fragrant resinous part (agarwood) is resulted from the accumulation of sesquiterpenoids and phenylethylchromone derivatives as defense mechanism against the stress given or counteracting infection by microorganisms (Ueda *et al*., 2006; Pripdeevech *et al*., 2011; Gao *et al*., 2012).

Aquilaria spp. is predominantly found in both South Asian and Southeast Asian countries including Bhutan, Nepal, India, Myanmar, Malaysia, Indonesia, Thailand, Vietnam and Papua New Guinea. In Malaysia, there are five distinct *Aquilaria* spp. have been documented to be well distributed in the country which are *Aquilaria malaccensis* Lam., *Aquilaria hirta* Ridl., *Aquilaria beccariana* Van. Tiegh., *Aquilaria rostrata* Ridl., and *Aquilaria microcarpa* Baill.. All the species have been found to be scattered in both Peninsular and East Malaysia (Lee *et al*., 2011). Presently, Malaysia has been reported as one of the primary exporter of agarwood along with Indonesia (Soehartono and Newton, 2000; Uddin *et al*., 2008). In this industry, both agarwood and its derived products have received high demand from both local and international markets. Due to the high demand on agarwood, the resources have been declined drastically due to the uncontrolled harvesting and forest clearance (Zhang *et al*., 2009). Consequently, after the *Aquilaria* spp. has been categorized as vulnerable species, the agarwood collectors need to have legal license to harvest agarwood. As a result of the difficulty and hardness of finding agarwoods, the price of agarwood keeps increasing in this few years (He *et al*., 2005).

In order to preserve and prevent the scarcity of the species, several countries including Indonesia, Cambodia, Thailand, Vietnam, Laos, India and China have initiated *Aquilaria* spp. plantation to fulfill industrial demand and maintain agarwood supply for industry (Eurlings *et al*., 2010; Zhang *et al*., 2012). In *Aquilaria* planting research, shades and fertilizers have been found to play an important role in seedlings growth performance of *Aquilaria* species (Page and Awarau, 2011). From the study, they found that the girt and the height of seedlings are averagely increased after supplying the fertilizers and applying shades to the seedlings. This approach is quite simple and easy to be applied to *Aquilaria* seedlings either in greenhouse or nursery to maintain the *Aquilaria* plant development. Moreover, this planting approach helps to avoid the extinction of the species and as a result, the agarwood suppliers can still cater the agarwood even when the demand for agarwood is high.

Identification on *Aquilaria* spp. is quite difficult as the morphological structures among the species in the genus are almost similar and keep changing depending on environmental conditions. Previously, phylogenetic analysis has been carried out on

Aquilaria spp. based on the DNA sequences of the plastid *trn*L-*trn*-F spacer (Eurllings and Gravandeel, 2005). Unfortunately, the analysis was not able to differentiate between *A. malaccensis* and *A. beccariana* due to the close relatedness between both species. More recently, microsatellite or short sequence repeat (SSR) marker has been applied on nuclear genome of *A. crassna* collected from different geographic origin for DNA polymorphisms identification (Eurling *et al*., 2010). Currently, there is a paucity of data regarding the molecular markers of *A. malaccensis, A. hirta, A. beccariana* and A. *rostrata* that are available in Malaysia. However, some ongoing molecular works are carried out by researchers from research institutions and universities in Malaysia on *A. malaccensis* to identify the genes or DNA sequences that are specific to the species including SCAR-RAPD (Lee *et al*., 2011).

In this study we attempted to identify the unique DNA sequences in the genome of *A. malaccensis* and *A. hirta* using a different molecular approach, namely, Representational Different Analysis (RDA). RDA analysis is a promising technique for the identification of unique DNA sequences that are present in one species but absent in other species in the same genus of *Aquilaria*. The information derived from unique DNA sequences will be useful for rapid identification of the right species based on the amplified product of the unique DNA sequences in the tested plants using Polymerase Chain Reaction (PCR) approach.

1.2 STATEMENT OF PROBLEM

Identification of *Aquilaria* spp. based on their physiological and morphological structures is very difficult to researchers and planters due to their almost similar structure of tissues and organs including stem, leaves, fruits and flowers (Lee *et al*., 2011). Besides that, the structures of those tissues or organs keep changing from time to time due to the changes of environmental conditions such as soil composition, forest diversity and climate changes. The morphological changes of *Aquilaria* spp. based on the changing of environmental conditions always confusing researchers and planters for identification and selection of the right species either for research or commercialization purpose. Moreover, identification of the right species is very important for selection of *Aquilaria* spp. to be planted in field either from seedlings or tissue-cultured plantlets for

maintaining the continuous supply of good quality agarwood to fulfill the demand by perfumery industry. Thus, identification of *Aquilaria* spp. through molecular approach by development of specific molecular markers is necessary for identification, confirmation and selection of the right *Aquilaria* spp. for conservation as well as commercialization of agarwood in fragrance, flavor and perfumery industries.

1.3 OBJECTIVES OF THE RESEARCH

The purposes/objectives of this study were;

1. To isolate unique DNA sequences of *Aquilaria malaccensis* and *A. hirta* via Representational Difference Analysis (RDA).
2. To identify and confirm the presence of unique DNA sequences in *A. malaccensis* and *A. hirta*.

1.4 SCOPE OF THE THESIS

1. Extraction of genomic DNA from selected *Aquilaria* spp. including *A. malaccensis, A. hirta, A. crassna* and *A. subintegra.*
2. Perform isolation of unique DNA sequences of *A. malaccensis* and *A. hirta* via a few rounds of Representational Difference Analysis (RDA).
3. Verification of the unique DNA sequences in *A. malaccensis* and *A. hirta* that are presence in species of interest while absence in non-interest species.

CHAPTER 2

LITERATURE REVIEW

2.1 NON-TIMBER FOREST PRODUCTS

Malaysia is a Southeast Asia country that has a very stunning tropical rain forest due to the richness of its biodiversity that contribute to the world non-timber forest products. Non-timber forest products such as tree nuts, mushroom, syrup, rubber, tree oils and resins are well known important sources in tropical forest besides timber forest products. According to Carolle *et al.* (2009), non-timber forest products played a big role during food shortage period many years back. Besides that, non-timber forest products have been reported as one of the main sources of domestic income for people who live in rural areas in Asia, Africa and some other developing countries to support their life. Non-timber forest products have been identified to contribute to domestic economy of rural population in three major ways. The first way of contribution is by fulfilling household basis and other necessity in term of energy and nutrition as well as medical and construction. While the second way is by acting as a safety-net in times of emergency and the third way is by giving regular earnings to them (Heubach *et al.*, 2011). Due to the high demand on non-timber forest products, the production and marketing of these products are very crucial with an approximate worth of USD 90 billion (Mahaparta *et al.*, 2005) and keep increasing from time to time.

According to Carolle *et al*. (2009), non-timber forest products are grouped into four categories which are; I) fruits and seeds, mainly fruit flesh, nuts and oil seed, II) plant exudates, resin and nectar, III) vegetative structure (bulb, leaves, stem, bud, barks, roots), and IV) small stem, poles and sticks for housing, fencing, and craft. Most of non-timber forest products have been reported to be collected from medicinal and aromatic plants (Heinen *et al.*, 2011). In these few years, concern on preservation of many plant species has been focused due to the high rate of deforestation and illegal logging (He *et al.*, 2005; Soehartono *et al*., 2001). Among the plants, *Aquilaria* spp. including *A. malaccensis*, *A. hirta*, *A. beccariana*, *A. subintegra*, *A. sinensis*, *A. crassna*, *A. microcarpa* and *A. rostrata* have been listed in the Appendix II of the Convention of International Trade in Endangered Species of Wild Fauna and Flora (CITES) since 2005 (Kumeta and Ito, 2011). Those *Aquilaria* spp. as the main agarwood source in the world are chosen for conservation to prevent the extinction of the species in tropical forest due to the uncontrolled exploitation that cause a serious loss to our tropical forest. After listed in the Appendix II of CITES, the international trading of valuable agarwood products from *Aquilaria* spp that may reach USD 10,000/kg has been strictly controlled by the government of related countries (He *et al*., 2005).

2.2 *AQUILARIA* SPP.

To date, there are many different *Aquilaria* species have been documented all around the world and five specific species which are *Aquilaria malaccensis* Lam., *A. hirta* Ridl., *A. beccariana* Van. Tiegh., *A. rostrata* Ridl. and *A. microcarpa* Baill. have been reported to be distributed in Malaysia and Indonesia tropical forests (Lee *et al*., 2011; Soehartono and Newton, 2000). Besides those five species, *Aquilaria crassna* Pierre ex Lecomte is found in Laos and Thailand (Chen *et al*., 2013), *Aquilaria sinensis* Gilg. can be found in China (Cheng *et al*., 2013; Jensen and Mailby, 2010) while *Aquilaria filaria* (Oken) Merr. is found in Indonesia, Malaysia, Papua New Guinea and Philippines (Turjaman *et al*., 2006). All those species are used for research purposes in these few years. Besides that, there are some other *Aquilaria* species that are not mentioned in any research papers because the species are difficult to be collected and maintained in glasshouse or they are already extinct.

In general, *Aquilaria* spp. have high economic value due to their roles as the main source of aromatic dark coloured resin (agarwood) that is produced in their barks and trunks (Jensen and Meilby, 2010; Turjaman *et al*., 2006). Currently, Malaysia is one of the primary suppliers for agarwood along with Indonesia (Soehartono and Newton, 2000; Uddin *et al*., 2008). In this industry, various form of agarwood received high demand from both local and international markets. According to Zhang *et al.* (2009), the resources of agarwood turn to decline in these years due to the uncontrolled harvesting and forest clearance.

In order to overcome these problems, there are several countries have started up *Aquilaria* spp. plantation including Indonesia, Cambodia, Thailand, Vietnam, Laos, India, and China (Zhang *et al*., 2012; Eurlings *et al*., 2010). In addition, Page and Awarau (2011) reported on the performance of *Aquilaria crassna* seedlings after applying shade and fertilizer. From their experiment, they found that the girt and the height of seedlings are averagely increased after supplying the fertilizer and shade to the seedlings and indirectly enhances the growth and survival of the species. This approach is quite simple and easy to be applied in greenhouse or nursery to maintain the species. This helps to avoid the species from extinction and as a result, the agarwood supplier can still cater the agarwood even when the demand for agarwood is high. Besides that, the seedling inoculated with arbuscular mycorrhizal fungi under greenhouse condition is another different way to increase the growth of *Aquilaria* species (Turjaman *et al*., 2006). The growth of the seedlings increases along with the growth of arbuscular mycorrhizal fungi. These successful approaches can be set as an example for those who are involved in agarwood industry to increase their income and help the species conservation.

2.2.1 Malaysian *Aquilaria* spp.

There are five different species of *Aquilaria* that can be found in Malaysia including *A. malaccensis* Lam., *A. hirta* Ridl., *A. beccariana* Van. Tiegh., *A. rostrata* Ridl. and *A. microcarpa* Baill (Lee *et al*., 2011). All the species are scattered all over the forest and local people known as '*Orang asli'* are the most reliable community to harvest agarwood in the forest because they are familiar with the areas. All of the five

species mentioned above can also be found in Indonesia since both Malaysia and Indonesia are located at the same region in the Southeast Asia and thus making Malaysia and Indonesia as the top producer of agarwood oil in the world (Soehartono and Newton, 2000).

In Malaysia, '*Orang asli'* harvested agarwood as their source of income and overexploitation of agarwood from the forest to fulfill the demand of agarwood oil and their related products all over the world causing the decrease of the species population towards extinction when no new plants are re-planted for future harvesting purpose (Howell *et al.*, 2010). According to Soehartono and Newton (2001), agarwood was previously harvested entirely from in natural forest before any agarwood plantation is initiated by several countries. In Malaysia, forest clearing is not permitted to protect and conserve our forest as natural source of biodiversity, but somehow local people are allowed to collect non-timber forest products (Howell *et al.*, 2010). This is a good activity for people who live in rural areas especially for '*Orang asli'* to raise their income for their survival. In the other word, there is a need to balance out the forest exploitation, forest conservation and rural development to ensure that both nature and human being equally gain advantages from it (Howell *et al.*, 2010).

According to Ibrahim *et al*. (2011), *A. malaccensis* is regularly consumed for treating fever, asthma, pain and rheumatism. There are many research activities have been carried out on Malaysian *Aquilaria* species. Each part of the species is used for chemical compounds isolation, analysis and characterization to discover the uses and the benefits of the species in general. The study and curiosity on the species are never stopped and researchers are still investigating on the metabolism involved in the species via chemical and biological approaches (Tajuddin *et al*., 2013).

In the past, people believed that oils extracted from *Aquilaria* spp. were able to cure various types of cancer diseases (He *at al*., 2005; Cui *et al*., 2011). Thus, a few experimental works have been carried out to investigate the anti-cancer properties of agarwood essential oils through scientific approaches (Ibrahim *et al*., 2011). Ibrahim *et al*. (2011) have found the presence of a very low concentration of cytotoxic properties in agarwood oil of *A. malaccensis*. Because of that, they subsequently came up a larger

scale extraction of agarwood oil via supercritical fluid extraction (SFE) method and followed by specific separation and fractionation steps to obtain higher concentration of the cytotoxic properties from the agarwood oil (Ibrahim *et al*., 2011). Furthermore, the agarwood oil was tested for its anticancer activities against human colon cancer cell lines. As a result, the extracted agarwood oil was successfully inhibited 99% of the cancer cell growth proving that *Aquilaria* species have potential to treat cancer (Ibrahim *et al*., 2011).

Biochemical analysis on Malaysian agarwood oils via gas chromatography TOF-MS has shown that the major constituents of the oils are sesquiterpene compounds, which contribute to the fragrance of agarwood (Tajuddin *et al*., 2013). In addition, there is another interesting study on the effect of burnt agarwood smoke on rat whereby after exposing the burnt agarwood smoke, no effect has been identified on the rats suggesting that agarwood smoke might give no effect to human. However, some symptoms of heart attack and depression might occur and further studies with specific parameters must be carried out although no significant observation was obtained (Karimi *et al*. (2011).

Theoretically, agarwood is formed after the plants response to a treatment which act as a signal to trigger the defense mechanism and one of the causes is by fungal infection. Premalatha and Kalra (2013) have identified and isolated some of the fungi from healthy and unhealthy tree whereby agarwood is produced inside the tree. The fungi species of *Alternaria*, *Curvularia*, *Fusarium, Trichoderma*are are commonly found from healthy tree and there are some fungi species might not be inoculated during the process. This is due to the reason that the fungi might not grow under the controlled condition or they come from the slow growth fungi species. On the other hand, the infected bark becomes the habitat of various types of fungi because the infected area is an entry spot of microbes. Among identified fungi species that infect *Aquilaria* spp. to cause agarwood formation are *Aspergillus* sp., *Botryodiplodia* sp., *Diplodia* sp., *Fusarium bulbiferum, Fusarium laterium, Fusarium oxysporum, Penicillium* sp., *Pythium* sp. and *Trichoderma* sp. (Premalatha and Kalra, 2013). In addition, a *Preussia* sp. was first time to be isolated from agarwood sample which indicate the community that colonized in agarwood are totally different from the healthy wood tissues (Premalatha and Kalra, 2013).

In these recent years, scientists are trying to find ways to produce agarwood artificially due the high demand from its consumers. Even so, the process takes time because they need to understand the mechanism of agarwood formation (Kenmotsu *et al*., 2011). This is a good approach since it was difficult to obtain agarwood from the tree species due to a longer time needed for agarwood formation after the tree is wounded. Besides that, Okudera and Ito (2009) had successfully induced *Aquilaria* species calli and cell suspension cultures to produce sesquiterpenoid (α-guaiene, α-humulene, δ-guaiene) which are the fragrant compounds in agarwood that are induced by the presence of methyl jasmonate to regulate the defense responses against wounding and bacterial attack (Kenmotsu *et al*., 2013).

Up to this moment, identification of Malaysian *Aquilaria* spp. is quite difficult due to the morphological structures among the species in the genus are almost similar. In the old days, species identification was depended primarily on their morphological characteristics including leaves, fruits, ovul and sepal. Unfortunately, this method is not reliable due to the changes of their morphological expression that depending on environmental conditions. According to Lee *et al.* (2011), *Aquilaria* spp. take several years to get into flowering stage and fruit development. Thus, to differentiate between species, identification using morphological structures is quite difficult (Kumeta and Ito, 2011). Therefore, molecular approach is another promising option to understand more about the species.

2.2.2 Morphology of *Aquilaria* spp.

Agarwood is a valuable resin produced by *Aquilaria* species. Thus, studying *Aquilaria* spp. is essential due to their economic value and the fact that these species are close to extinction (Tajuddin and Yusoff, 2010). In other words, research on *Aquilaria* spp. must be continuously carried out especially on species identification as well as mechanisms of agarwood biosynthesis. However, the information reported in previous researches on taxonomy and morphology of *Aquilaria* spp. is still incomplete because not all *Aquilaria* spp. morphological structures were recorded in depth. The limited availability of the species is one of the limitations to continue the study (Lee *et al*.,

2011). Due to that reason, the main report on Thymelaeaceae morphology by Ding Hou (1960) is still used as important reference and guidance of the species until now.

In general, as stated by Ding Hou (1960), *Aquilaria* spp. is a non-timber tree which can be found in rain forest. The leaves of *Aquilaria* spp. are curved in shape and their positions on the branches are arranged alternately from each other. Besides that, the leaves are also appeared to have many veins that extend towards the leaf margin from the center. While inflorescences of the species are identified growing on the leaf axil that is located at the space between leaf stalk and stem or on internodes and rarely grown on the tree trunk. After the beginning of flowering stage, three months is taken for seeds of *Aquilaria* spp. to be fully developed (Soehartono and Newton, 2001). The main characteristics of *Aquilaria* flowers are pentamerous (5-merous), have pedicel (stalk for individual flower in inflorescent) and the floral tube that is in long cylindrical (tubular) shape with fine short hairs outside. While the calyx which is a collective number of sepals in a flower, is usually shorter than the tube as shown in Figure 2.1. In addition, number of stamen (anther and filament) in a flower is double compared to the calyx. The stamens that rise from the tube usually have the same length, swollen at the upper part and connected to the anther of the flower (Ding Hou, 1960).

The fruits of *Aquilaria* spp. are in capsule shaped or oblong, have rough or smooth surface, or slightly compressed laterally and have a very tough or woody ovary wall. Each fruit of the species contains two or one seed in it. The seed is tail-like shape and it dangle out from the capsule of the end of funicle (stalk attaches seed to ovary wall) and it has a thick cotyledon. Soehartono and Newton (2001) observed that smaller trees like *A. malaccensis* and *A. microcarpa* generate more seeds compared to bigger *Aquilaria* species.

Based on Ding Hou (1960) findings, *A. malaccensis* can be found in India, Burma, Malaysia, Indonesia and Philippines. The tree can grow about 40 m to 60 m. The tree bark is whitish in colour and the surface is smooth. The branches are pale brown, slender and hairless. The leaf resembling paper textures and tough, hairless, shining on both surfaces, the shape varied from oval to oblong ranging from 7.5 cm to 12 cm and the veins are very distinct. The inflorescent of *Aquilaria* spp. grow on the

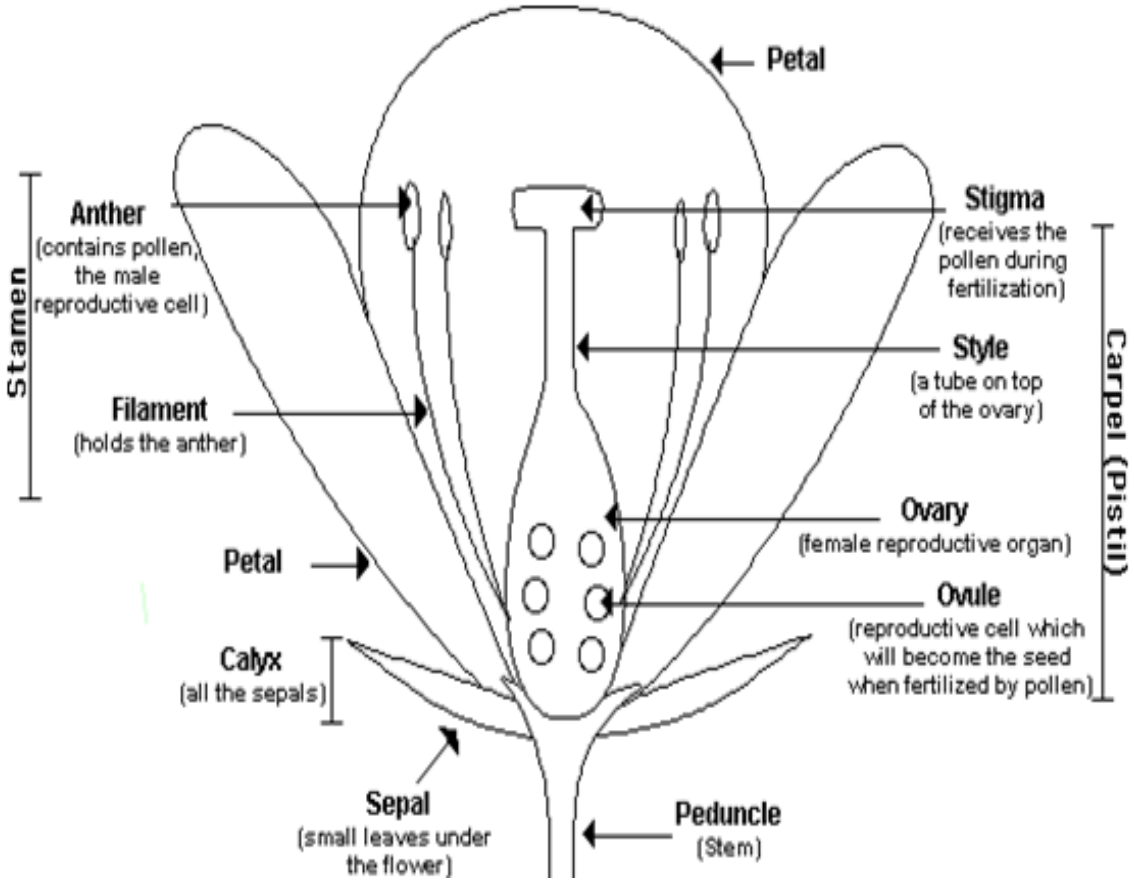

Figure 2.1: The basic flower morphological structure.

Source: Enchantedlearning.com

Figure 2.2: The morphology diagram of *Aquilaria* species; (a) leaves and inflorescent attaches to the stem of *Aquilaria beccariana*; (b) open flower of *Aquilaria beccariana*; (c) fruit emerging from top of floral tube with one seed dangling of *Aquilaria beccariana*; (d) open flower of *Aquilaria brachyantha*; (e) open flower of *Aquilaria hirta*; (f) dehisced fruit with seed of *Aquilaria hirta*; (g) dehisced fruit with seed of *Aquilaria microcarpa*; (h) dehisced fruit with seed of *Aquilaria malaccensis*; (i) dehisced fruit with seed emerging from lateral slit of floral tube of *Aquilaria cumingiana*.

Source: Ding Hou (1960)