Faculty of Resource Science and Technology

MORPHOLOGICAL IDENTIFICATION AND $(GTG)_5$-PCR FINGERPRINTING OF ENDOPHYTIC FUNGI ISOLATED FROM AKAR GAHARU

Lim Chai Fen

Bachelor of Science with Honours
(Resource Biotechnology)
2014

UNIVERSITI MALAYSIA SARAWAK

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Morphological Identification and $(GTG)_5$-PCR Fingerprinting of Endophytic Fungi isolated from Akar Gaharu

Lim Chai Fen

This project is submitted in partial fulfilment of the requirements for the degree of Bachelor of Science with Honours
(Resource Biotechnology)

Supervisor: Dr. Samuel Lihan

Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2014

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List of Abbreviations

cm	Centimeter
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
kg	Kilogram
$MgCl_2$	Magnesium chloride
ml	Milliliter
MYR	Malaysian Ringgit
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nm	Nanometer
PCR	Polymerase Chain Reaction
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
sp.	Species
TBE	Tris/Borate/EDTA
Tris	Trisaminomethane
U	Unit
˚C	Degree Celcius
µl	Microliter

List of Tables and Figures

Morphological Identification and $(GTG)_5$-PCR Fingerprinting of Endophytic Fungi isolated from Akar Gaharu

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ABSTRACT

Agarwood is a valuable incense produced from *Aquilaria* trees that are infected by fungi. The price of agarwood has increased dramatically throughout the years and its contribution to the economy is significant. However, the high demand of agarwood has exceeded its supply due to its high value and multiple functions. In order to overcome this problem, fungi can be used as an inoculant to induce agarwood formation. Akar gaharu is an aromatic tendril plant that produces inferior agarwood, which is also infected by fungi. In this study, the fungi species isolated from the akar gaharu were being compared to the ones commonly found in agarwood. The purposes of this project were to identify and group the fungi isolated from akar gaharu into different species. Morphological observation and molecular approaches which involve DNA extraction, PCR amplification and clustering analysis were used for characterization of the fungi. DNA fingerprinting method was carried out to group the fungi into different species. The identified fungi species were *Trichoderma* sp., *Penicillium* sp. *Beauveria* sp., *Mucor* sp. and *Phoma* sp. and they were grouped into two major clusters based on their genetic distance. Among the identified species, *Trichoderma* sp. and *Penicillium* sp. were considered to be related to agarwood formation. These fungal isolates can be utilized in formulating a microbial consortium to induce agarwood formation.

Key words: Agarwood, akar gaharu, DNA fingerprinting, endophytic fungi, morphological identification.

ABSTRAK

Gaharu adalah resin berbau harum yang dihasilkan dari pokok Aquilaria yang dijangkiti oleh fungi. Harga gaharu semakin meningkat di pasaran dan sumbangannya kepada ekonomi adalah penting. Permintaan gaharu di pasaran telah melebihi bekalannya kerana nilainya yang tinggi dan memiliki pelbagai fungsi. Untuk mengatasi masalah ini , fungi boleh digunakan sebagai inokulan untuk mendorong penghasilan gaharu. Akar gaharu merupakan sejenis tumbuhan aromatik yang menghasilkan gaharu gred rendah dan juga dijangkiti oleh fungi. Dalam kajian ini, spesis fungi didapati dari akar gaharu akan dibandingkan dengan spesis fungi yang biasa ditemui dalam gaharu. Tujuan projek ini adalah untuk mengenal pasti spesis fungi yang didapati dari akar gaharu dan membahagikan mereka kepada kumpulan spesis yang berbeza. Pemerhatian morfologi dan pendekatan molekul yang melibatkan pengekstrakan DNA, PCR dan analisis kelompok telah digunakan untuk menganalisis spesis fungi yang didapati. Kaedah fingerprinting DNA telah digunakan untuk membahagikan spesis fungi dalam kumpulan yang berbeza. Spesies fungi yang dikenalpasti dalam projek in adalah Trichoderma sp., Penicillium sp., Beauveria sp., Mucor sp. dan Phoma sp., mereka telah dibahagikan kepada dua kelompok utama berdasarkan jarak genetik. Trichoderma sp. dan Penicillium sp. adalah spesis yang boleh dikait rapat dengan pembentukan gaharu. Fungi yang didapati boleh digunakan untuk menghasilkan konsortium mikrob yang boleh mendorong pembentukan gaharu.

Kata kunci: *Gaharu, akar gaharu, fingerprinting DNA, fungi endofitik, permehatian morfologi.*

1.0 Introduction

Over the past decades, agarwood has been recognized as valuable incense because of its distinctive fragrance when it is burnt. Agarwood is also known as *gaharu*, *oud* or *oodh* which normally produced from infected *Aquilaria* trees, among the important species are *Aquilaria malaccensis, Aquilaria sinensis,* and *Aquilaria crassna*. Gratzfeld and Tan (2008) explained that agarwood is an aromatic resin produced by the infected tree to defend itself from the invasion of fungi, bacteria and insects. The uses of agarwood could be traced back from ancient time to the modern days, it is used for medicinal, religion and artistic purposes in various countries, and most importantly it is used to make high quality perfume (Snelder & Lasco, 2008).

Agarwood contributes to the economy of the countries that involve in harvesting and trading of agarwood, such as India, Indonesia, Malaysia, Thailand and Vietnam. From year 1995 to year 2005, Malaysian Customs reported that Malaysia had exported over 6 000 000 kg of agarwood to other countries (Lim & Anak, 2010). The price of the agarwood had increased dramatically from year to year, the price of premium grade agarwood was as low as MYR 4 per kg in 1880 and it had risen to MYR 10 000 to MYR 16 000 in 2013 (Lim & Anak, 2010; Nor-Azah et al., 2013). Due to its high values and the difficulty to synthesize high quality artificial agarwood, the demand for agarwood has increased and exceeded the supply. In order to overcome the depletion of this resource, various efforts have been taken to induce the production of agarwood such as the use of inoculant.

Fungi infections can be detected from the infected trees that produce agarwood. Fungus *Epicoccum granulatum* was isolated from an infected agarwood tree in 1952, *Menanotus flavoliven* was isolated from *Aquilaria* tree in 1976 by South China Botanical Garden and *Fusarium oxysporum* was used to induce agarwood in 2000 by Tamuli et al. (Tian et al.,

2013). Since then, experiments had been conducted to investigate the relationship of fungal attack and the ability of the plant to produce resin, scientists were also concerned that different fungus produced different inoculating effects.

Akar gaharu is a tendril plant that is aromatic but it is considered inferior compare to agarwood. Various fungi can also be found in akar gaharu, however limited research had been done to find the similarities of the fungi species found in agarwood and akar gaharu. In this project, morphological observation and molecular techniques which involve DNA extraction, PCR amplification and clustering analysis will be used for identification and characterization of the fungi. DNA fingerprinting methods will be carried out to group the fungi into different species using $(GTG)_5$ primer. The fungi species isolated from akar gaharu will be compared to the fungi commonly found in agarwood. If the fungi species isolated from akar gaharu are highly similar to the ones in agarwood, they may be useful source for the formulation of microbial consortium as inoculant to induce the agarwood formation in the future.

To overcome the problem of depletion of agarwood, it has been suggested that fungi can be used as an effective inoculant to induce its production. Therefore, the objectives of this project are (i) to study the morphological characteristics of the fungi isolated from akar gaharu, (ii) to identify different strains of fungi using DNA fingerprinting, and (iii) to group the fungi through clustering analysis.

2.0 Literature Review

2.1 Taxonomy

Agarwood producing trees are found in many countries such as China, India, Vietnam, Indonesia, Malaysia and Thailand (Mamat et al., 2010). Based on a report, 19 species of plants that can produce agarwood are found in Malaysia. The species are derived from five genera which are *Aquilaria, Gonystylus, Wikstroemia, Enkleia* and *Aetoxylon* (Lim & Anak, 2010). Among the five genera, *Aquilaria* sp. contributes the most in producing agarwood whereas other genera are thought to produce inferior agarwood.

2.2 History and Uses of Agarwood

Trade in agarwood had been recorded for more than 2000 years in the Middle East and East Asia (Lim & Anak, 2010). Traditionally, agarwood is used only for spiritual purpose. Today, there is a large range of agarwood products available and the uses of agarwood are seemingly endless. The agarwood chips are used to produce beads or sculptures, mainly for art and spiritual purposes. Besides that, agarwood can also be crushed into powder to make incense or traditional medicine. The medicine made from agarwood is used for pain and asthma relieves. Nevertheless, most of the agarwood are processed into oil through steam distillation to produce high quality fragrance and perfume. Akter et al. (2013) stated that agarwood is highly valued by the manufacturers of luxury perfume and fragrance such as Yves Saint Laurent and Dior. Certain products such as shampoo and soap also contain agarwood oil as the fragrance.

2.3 Market of Agarwood

The price of agarwood ranges from a few dollars per kilo for lowest-grade agarwood to over thirty thousand US dollars for first-grade agarwood (Akter et al., 2013). The price of

the agarwood chips are determined by the amount of resin contained in the chips. When it is processed into oil, the top-grade agarwood can be sold at USD 30000 (Mamat et al., 2010). The main reason for the price of agarwood increasing dramatically over the years is its high demand that had exceeded its supply. Besides that, agarwood cannot be synthesized artificially.

2.4 Inoculant for Agarwood

Inoculant is used to inoculate the formation of agarwood. As agarwood takes a long time to be produced naturally, artificial inoculant is needed to accelerate the process. According to Suharti et al. (2011), inoculant can be injected to the tree when it is five years old and has a stem diameter of 15 cm. The inoculant will wound the tree and thus stimulate its resin production. Tian et al. (2013) conducted a method called pinholes-infusion to inoculate different species of fungi into non-resinous part of the *Aquilaria sinensis*. Resin was formed around the holes of the trees after one to two years of inoculation.

2.5 Endophytic Fungi

Endophytic fungi are fungi that live within the plant tissue without causing any symptoms of disease to the host (Yoo & Eom, 2012). Besides that, they may give benefits to their host by producing antimicrobial substances, thus promoting the plant growth and reduce disease caused by other pathogens or environmental stress. Research had been conducted to investigate the antimicrobial activity of endophytic fungi from *Aquilaria sinensis*, the results obtained were positive for certain endophytic fungi such as *Fusarium solani* showed antimicrobial activity to *B. subtilis* and *S. aureus* (Cui et al., 2011). Another research on antimicrobial activity also reported that *Fusarium* spp. showed the highest antimicrobial activity among the fungi isolated from Aquilaria sinensis (Gong & Guo,

2008). Agarwood is formed by agarwood tree as a defense act to the infection of the fungi, therefore it is significant to study the endophytic fungi species isolated from the agarwood.

2.6 Morphological Identification of Fungi

Morphological approach to study fungi species were used in many researches. Besides having a deep knowledge about the morphological characteristics of various fungi species, a good slide preparation method is also important in morphological identification. Wijedasa and Liyanapathirana (2012) stated that slide culture was the most widely used slide-preparation method. The disruption of the fungal structures is minimized when the fungi is grown directly on the slide. However, the removal of the cover slip for staining may cause changes in the arrangement of the fungal structures and this method is time-consuming compared to the other techniques such as cello-tape method.

Harris (2000) introduced a modified cello-tape method for fungal slide preparation. This method used frosted tape instead of clear tape as it is readily available in the market and it is thin and flat material that can be torn easily. It was reported that using two sticks to handle the tape during the procedure caused less destruction to the fungal features. High-quality mount can be obtained as the frosted tape is thinner than cover slip. Woo et al. (2010) developed a novel method for slide preparation known as agar block smear preparation. This method is better than the cello-tape method as it preserves the fungal structures such as the conidiophores and conidia in certain species. Another advantage of this method is that it allows the structure of fungi embedded in the agar to be examined.

2.7 Biochemical Techniques for Identification of Fungi

Traditionally, identification of fungi is done by observing the morphology of the fungi under the microscope. However, the determination of fungi species through the

macromorphology and micromorphology requires a deep knowledge of mycology. One of the biochemical techniques is protein-based techniques. The analysis is done using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for identification of wood decay fungi at specific levels (Nicolotti et al., 2010). Biochemical techniques are not considered as effective way to identify fungi because a large amount of fungal tissues and pure fungal cultures are needed in this technique. Moreover, environment factors can influence the accuracy of protein profiles and the procedure can be time consuming.

2.8 Molecular Techniques for Identification of Fungi

2.8.1 Random Amplified Polymorphic DNA (RAPD) Fingerprinting

RAPD is one of the most popular techniques used for DNA fingerprinting of fungi. Ciancio and Mukerji (2008) stated that RAPD utilizes short primers that represent the genomic sequence. RAPD fingerprinting scans the whole genome randomly to detect and amplify uni-dominant single nucleotide DNA that prevents primer annealing at amplification locus. The amplified products are separated using agarose gel and ethidium bromide for staining. The primer hybridizes to homologous sequences on DNA strands and *Taq* polymerase is used for amplification. The process is continued with the synthesis of amplicon sequence fragments. RAPD fingerprint can identify fungi without any prior information of the target DNA and the whole process can be conducted at relatively low cost. However, the limitation of reproducibility is one of the problems in using RAPD that causes difficulty in developing a common database (Ciancio & Mukerji, 2008; Nicolotti et al., 2010; Soll, 2000).

2.8.2 Restriction Fragment Length Polymorphism (RFLP) Fingerprinting

RFLP is based on the involvement of restriction enzymes to digest the pathogen DNA, separation of the DNA fragments through gel electrophoresis. Polymorphisms are used to distinguish the different species of fungi. PCR-RFLP was used by Tian et al. (2013) to identify various fungi from the agarwood tree such as *Fusarium* sp., *Chaetomium* sp., *Penicilium* sp. and *Pestalotiopsis* sp. Mirhendi et al. (2001) used PCR-RFLP to identify *Candida* sp., *Cryptococcus neoformans, Fusarium solani* and *Aspergillus famigatus*. Drenth et al. used PCR primers that are specific to *Phytophthota* to obtain specific restriction pattern of 27 different species of *Phytophthora* (Capote et al., 2012). PCR-RFLP is an effective and reproducible method to identify fungi at different species level, it is a better approach to be used compare to RAPD fingerprinting if the DNA are extracted from samples that might contain other microorganisms to obtain a more reliable result (Nicolotti et al., 2010).

2.9 Potential Fungi Species to be formulated as Microbial Consortium

Different fungi have different infection level to induce the formation of Agarwood. The main criterion for a good microbial consortium is the fungi used for the formulation are obtained from different strains and species. According to Panda (2009), the fungi that were isolated in the conducted research on agarwood were *Aspergillus* sp., *Penicillum* sp., and *Fusarium* sp. Tian et al. (2013) found *Fusarium* sp., *Trichoderma* sp., *Penicillium* sp. along with less common species such as *Botryospaeria* sp., *Cylindrocladium* sp. and *C. gloeosporoides* involved in the formation of agarwood. Santoso et al. (2011) stated that *Fusarium s*p. had the most infection value to induce large amount of agarwood. However, it was thought that the agarwood formation is most probably induced by the interaction of several fungi instead of a single fungus (Tian et al., 2013).

3.0 Materials and Methods

3.1 Materials

Potato Dextrose Agar, Potato Dextrose Broth, Tris-HCl, EDTA, NaCl, SDS, Potassium acetate, Isopropyl ethanol, PCR master mix, Agarose powder, TBE buffer, 1 kb DNA ladder, 70 % ethanol, 30 % glycerol, Distilled water, Household-chlorine bleach, Lactophenol cotton blue, 1.5 ml eppendorf tube, 2 ml cryo-vial, Scalpel.

3.2 Source of Samples

Akar gaharu taken from the jungle in Kota Samarahan, Sarawak were used in this study. The wood samples were collected from the plant on March 8, 2014. The darken part of the tree branch was chosen as it indicated injury of the plant by fungi and other microorganisms (refer to Figure 5 in Appendix).

Another source of the endophytic fungi was the fungal isolates available in the laboratory, Department of Molecular Biology in UNIMAS. There were 16 fungal isolates available and the fungi were previously isolated from akar gaharu in the jungle near Kota Samarahan, Sarawak (Lawrence, 2013).

3.3 Preparation of Culture Media

Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) were prepared and stored in the petri dish and the conical flask respectively. To prepare the medium, the powder was weighed and suspended in 1000 ml of purified water according to the manufacturer's instructions (refer to Table 1).

Table 1: Types and weight of Potato Dextrose powder used for 1 L media preparation.

Powder	Weight
Merck Millipore™ Potato Dextrose Agar	39 g
Merck Millipore™ Potato Dextrose Broth	24 g

The mixture was heated with frequent agitation and boiled for 1 minute to completely dissolve the medium. It was autoclaved at 121 ˚C for 15 minutes. The media were then poured into the petri dishes and conical flasks and left to cool at room temperature in the laminar flow hood. The color of the prepared medium was light beige.

3.4 Isolation of Fungi

To isolate the fungi within the akar gaharu, samples were initially surface sterilized by a protocol modified from Stone et al. (2004). This is to make sure that the fungi isolated are from the akar gaharu, but not from the environment. Briefly, the infected wood samples were cut into six 5 mm x 5 mm pieces with sterile razor blade and another four same-sized-pieces were cut from uninfected part of the samples to be used as controls. The wood chips were treated with house-hold chlorine bleach (NaOCl) diluted in water to 5 % concentration for 5 minutes, rinsed with distilled water for 3 to 5 times and dried on sterilized filter paper.

The dried wood chips were placed on PDA and incubated for 5 days at room temperature in the incubator. Different shape and color of the colonies were observed on the plates after incubation, pure cultures of each colony type were isolated and sub-cultured onto fresh PDA plates and incubated for 7 days at room temperature. The sub-culturing step was repeated until pure colonies were obtained in each plate (Ibrahim & Rahma, 2009).

3.5 Sub-culture of Fungi

The 16 fungal isolates available in the laboratory, Department of Molecular Biology in UNIMAS were sub-cultured from solid to solid using a scalpel. A cube of the fungal mycelium was removed from the agar slant and transferred to fresh PDA plate. It was placed on the center of the agar plate. The fungi were incubated for 7 days at room temperature in the incubator.

Fungi which did not grow on the PDA were sub-cultured using a solid to liquid method. Two to three cubes of fungal mycelia were removed from the agar slant and transferred to PDB in the conical flask and incubated for 7 days at room temperature on the shaker.

3.6 Morphological Observation

To examine the fungi under the microscope, the slide preparation was done using the slide culture method modified from Wijedasa and Liyanapathirana (2012). PDA was cut into agar blocks of size 15 mm x 15 mm and placed on the glass slide in a petri dish. Each fungal isolates to be identified were transferred and inoculated onto the agar block using a sterile toothpick by touching the center of the agar block. After inoculation, a sterile cover slip was placed on top of the agar block. The petri dish with the glass slide was partially sealed with parafilm and incubated at room temperature for 3 days.

After incubation, the cover slip was carefully removed from the agar block using sterile forceps. A clean glass slide added with 1 to 2 drops of lactophenol cotton blue (LPCB) was prepared and the cover slip from the slide culture was placed on top of the LPCB. Subsequently, the slide was examined under the compound microscope (Olympus BX51, Japan). The hyphae, conidia, conidiophores and spores of the fungi were the focuses of the microscope observation for fungi identification, the magnification of the microscope was

set from x100 to x1000 accordingly to obtain clearer image of the fungi. The photos of the fungi were captured by the digital camera attached on the microscope (Olympus DP72, Japan) and saved in the computer connected to the compound microscope.

3.7 $(GTG)_5$-PCR Fingerprinting

3.7.1 DNA Extraction

The fungal genomic DNA was extracted from the isolated fungi using a rapid mini-preparation of fungal DNA method developed by Liu et al. (2000). A 1.5 ml eppendorf tube containing 500 µl of lysis buffer comprised of 400mM Tris-HCl (pH 8.0), 60 mM EDTA (pH 8.0), 150mM NaCl, 1 % sodium dodecyl sulfate (SDS) and 115 µl distilled water was prepared. A sterile toothpick was used to transfer a small lump of fungal mycelium into the tube and left for 10 minutes at room temperature in the incubator. The tube was vortex and spun at 13000 rpm for 1 minute after 150 µl of potassium acetate was added. The supernatant was transferred to a new eppendorf tube and centrifuged again as in the previous step. After centrifuge, the supernatant was transferred again to another 1.5 ml eppendorf tube and added with an equal volume of isopropyl ethanol. The tube was spun at 12000 rpm for 2 minutes and the supernatant was discarded. The DNA pellet obtained was washed in 300 µl of 70 % ethanol. The supernatant was discarded after the pellet is spun at 10000 rpm for 1 minute. The DNA pellet was air-dried and dissolved in 50 µl of 1x Tris-EDTA (TE) buffer (Liu et al., 2000). The extracted fungal DNA was stored at -20 ˚C prior to PCR amplification.

3.7.2 Polymerase Chain Reaction (PCR) Amplification

The oligonucleotide $(GTG)_5$ was used as a single primer in this PCR-based fingerprinting method. The PCR assay was performed with a 25 µl reaction mixture consisting of Tris-HCl buffer, $MgCl_2$, dNTP, *Taq* DNA polymerase, distilled water, primer and DNA with respective volumes as shown in Table 2 below.

Table 2: Volume of each component in PCR reaction mixture.

PCR Master Mix	**Volume (µl) [x1 Reaction]**
Buffer	5.0
$MgCl_2$	3.0
dNTP	0.8
$(GTG)_5$ primer	1.0
ddH_2O	9.9
DNA	5.0
Taq Polymerase	0.3

Thirty cycles of amplification were performed in thermal cycler (Little Genius TC25/H, Bioer, China). After pre-denaturation of DNA at 95 ˚C for 2 minutes, each cycle consisted of a denaturation step at 95 ˚C for 1 minute, an annealing step at 50 ˚C for 1 minute, an extension step at 72 ˚C for 1 minute. A final extension step at 72 ˚C for 5 minutes was carried out following the last cycle (Matsheka et al., 2006). After PCR amplification, the samples were stored at -20 ˚C until used.

3.7.3 Agarose Gel Electrophoresis (AGE)

The PCR product was electrophoresed for 80 minutes at 90 V 400mA in a 0.5 % agarose gel in 1x TBE buffer. A 1 kb DNA ladder (Promega, USA) was loaded in the first lane of