BIOTIC STRESS PROTEIN MARKERS OF *AQUILARIA SP.* FOR GAHARU SPECIES IDENTIFICATION IN MALAYSIA

PENANDA *AQUILARIA SP.* BERDASARKAN PROTEIN BIOTIC STRESS DALAM IDENTIFIKASI SPESIS GAHARU DI MALAYSIA

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Abstract

Gaharu trees (*Aquilaria*) is in danger of extinction in the wild due to illegal logging. Its resin (Gaharu) is used for the production of highly valued incense throughout Asia. In *Aquilaria* sp. systemic induction of defense genes in response to mechanical wounding in nature is regulated by an 18-amino-acid peptide signal protein called systemin. This protein is produced in response to the natural stress at the vicinity of the wound and is also influenced by its genetic background. As the protein can be differentiated by its locality, the protein expressed is also found to be significantly different which, in turn, can be used for identification of this plant species. In this work, *A. malaccensis* and *A. hirta* were evaluated based on the targeted genes related to systemin. Targeted gene refers to specific sequence in genomic DNA. Sequence mining from public databases is part of the crucial process in getting the specific genes. The sequences will go through alignment step to identify conserved region prior to primer design. The primers were used in Polymerase Chain Reaction (PCR) techniques to amplify the conserved regions. It was found that both samples can be differentiated. This would be useful for plant breeders, trader and planter in ensuring authentic planting materials. This paper will describe the use of targeted genes primers as markers in identifying the *Aquilaria* species.

Abstrak

Pokok gaharu(*Aquilaria*) adalah dalam bahaya kepupusan dalam hutan akibat pembalakan haram. Damar(Gaharu) digunakan untuk pengeluaran kemenyan yang sangat bernilai di seluruh Asia. Induksi sistemik gen pertahanan pada pokok gaharu adalah sebagai tindak balas kepada kecederaan mekanikal yang sifatnya dikawal oleh protein dikenali sebagai protein systemin yang terdiri daripada 18asid amino.Protein ini dihasilkan sebagai tindakbalas kepada tekanan semulajadi di lokasi kecederaan dan ini juga mempunyai pengaruh genetik. Perbezaan di dalam penghasilan protein ini menjurus kepada perbezaan untuk mengenal pasti spesis pokok tersebut. Di dalam kajian ini, A.malaccensis dan A.hirta dinilai berdasarkan kepada gen sasaran yang berkaitan dengan protein systemin. Gen yang disasarkan merujuk kepada urutan tertentu dalam DNA genomik. Pencarian urutan dari pangkalan data adalah sebahagian daripada proses penting dalam mengenalpasti gen ini. Penjajaran pada peringkat DNA akan dilakukan untuk mengenal pasti rantau terpelihara sebelum reka bentuk pencetus dapat dilakukan. Pencentus akan digunakan dalam Teknik Tindakan Berantai Polimerase(PCR) untuk menggandakan jujukan pada kawasan terpelihara tersebut. Didapati *A. malacensis* dan *A. hirta* boleh dibezakan. Perbezaan ini berguna untuk pembiakbakaan tumbuhan, peniaga dan penanam dalam menentusahkan spesifikasi spesis yang digunakan.Kertas ini akan menerangkan penggunaan pencetus disasar sebagai penanda dalam mengenal pasti spesis *Aquilaria*.

Keywords: Gaharu, systemin, PCR, targeted genes

Introduction

A molecular marker, also refers to as a genetic marker, is a particular sequence of deoxyribonucleic acid, or DNA, that is identifiable within the context of an entire genome. Some molecular markers can only be identified by examining the sequence of genetic information while others can be identified by visual examination of an organism. There are many different types of molecular markers of varying lengths and configurations that can be used for many different purposes. Some occur naturally while others are specifically developed by researchers to mark the positions of certain DNA sequences. These markers serve many purposes in biological research and breeding programs including those of an academic nature and seedling propagationfor commercialization.

Commercial plantation of Agarwood or Gaharu or *Aquilaria sp.*in Malaysia has been carried out and is becoming a big industry for resin production. Planters will buy a purportedly good quality seedling from a nursery without knowing if the *Aquilaria* species actually do produce the quality resin at their maturity as claimed. With markers, planters will get more information about the seedlings that they buy. Screening of *Aquilaria* species is also important in controlling illegal trade and provides reliable quality of gaharu resin.

Aquilaria is the most expensive wood in the world which is well known for its gaharu resin with its distinctive fragrance. It has been used for medicinal, aromatic and religious purposes worldwide. There are 25 species of *Aquilaria* species over the world, commonly found in many tropical countries, from India to Indonesia (Barden et al., 2000) and Malaysia has at least 5 species including *A. malaccensis* and *A. hirta*. *A. malaccensis* is known as the first gaharu-producing species and the most common *Aquilaria* species found in Peninsular Malaysia and Sabah (Whitmore 1973).As with *A. malaccensis*, *A. hirta* is also known as agarwood-producing species in Malaysia, and well distributed in Peninsular Malaysia.

These two species have a good potential for producing gaharu with *A. malaccensis* producing gaharu of the higher value. This made *A. malaccensis* is included in *The World List of Threatened Trees* (Oldfield *et al*., 1998). Morphologically, both species have some different characteristics which can be used to distinguish these two varieties. *A. malaccensis* has leaves with smooth surface, whilst that of *A. hirta* has hairy undersurface. In addition, *A. hirta* has white spots distributed all over its trunk.

DNA fingerprinting can be used in species identification and to understand the genetic variations in plant species. Thus far, the identification of *Aquilaria* sp. is done by observing the anatomy and morphology of the trees. There are some previous works on the genetic differentiation and variation among *Aquilaria sp* using RAPD and SCAR markers and between *Aquilaria* sp. and another specie, *Gyrinops*, using AFLP markers (Nurita et al., 2009, Shiou et al., 2011). By using RAPD markers, Shiou et al. (2011) found some particular bands which were specific only for *A. hirta*, *A. malaccensis* and *Aquilaria sp.*, respectively. These findings are useful for *Aquilaria* identification in natural population and nursery.

According to Shiou et al. (2011) RAPD markers is one of the useful PCR-based techniques to identify the *Aquilaria sp.* It has a large coverage of the genome and can rapidly produce a high number of polymorphism. However, a primary drawback to RAPD markers is that they are dominant and do not permit the scoring of heterozygous individuals. Thus, it is necessary to prepare many closely linked markers to ensure reliable comparisons among plant populations.

Gaharu resin is formed by pathological process in the stem caused by injury. This injury will activate polypeptide signals which function as defense genes, similar and originally found in tomato leaves at very low concentrations (Pearce et al., 1991). As in tomato, *Aquilaria* sp. systemic induction of these defense genes is regulated by an 18-amino-acid peptide signal called systemin. It is synthesized from a 200 amino acid pro-protein known as prosystemin that is encoded in 11 exons. The cDNA and gene encoding the signaling molecule of systemin have been isolated and characterized (McGurl et al., 1992).

Based on several recent studies, it can be assumed that systemin can be transported through a plant by applying it onto the site of the wound thus making it a mobile signal for wound response. In tomato, systemin is released from the damaged cells at the site of wounding and systemically activates the expression of over 20 defense-related genes although it is also believed that systemin can also be found in the tubers which are related to major storage protein. However, according to Jacinto *et al.* (1997), prosystemin expression in tomato leaves is restricted to the vascular bundles consistent with a role for this polypeptide in long distance signaling.

Studies in systemin related to the production of gaharu in *Aquilaria sp.* is still in its infancy. In this research, systemin primers designed based on conserved regions in tomato biotic genes were used to identify the *A. malaccensis* and *A.hirta* for DNA fingerprinting markers.

Material and Methods

Plant materials

Leaves from *A. malaccensis* and *A. hirta* species were obtained from Malaysian Nuclear Agency. The samples were selected from different locations based on standard morphological characterizations.

DNA isolation and quantification

The DNA was extracted from fresh leaves by using CTAB extraction method. Fresh leaves were ground to powder in liquid nitrogen using mortar and pestle, then transferred to a 50 mL falcon tube with 10 mL of CTAB buffer [3% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mMTris-HCl, pH 8.0, and 0.2% (v/v) β–mercaptoethanol. The homogenate sample was incubated at 60.0°C for 1 hour, extracted with an equal volume of chloroform:isoamyl alcohol (24:1 v/v) and supernatant was collected after centrifugation at 5,000 rpm for 10 min. DNA was then precipitated from aqueous phase by mixing with an equal volume of isopropanol. After centrifugation at 5,000 rpm for 5 min, DNA pellet was collected and washed with 70% (v/v) ethanol, air-dried and resuspended in TE buffer (10 mMTris-HCl, pH 8.0, and 0.1 mM EDTA). DNA quantifications were performed by using *Nanodrop Spectrophotometer* at 260/280nm and its quality checked by separation in electrophoresis gel (1%) at 70V for 1 h. The resuspended DNA was then diluted in TE buffer to 50 ng/µL prior to use for polymerase chain reaction (PCR).

Primers selection and design

16 primers were designed and optimized, based on prosystemin protein sequence derived from *Lycopersicon esculentum* (Accession no: M84801; Protein ID AAA34184.1). The protein is a precursor for systemic wound signal and related to systemin gene.

PCR amplification

PCR amplifications were performed in a total volume of 25 µl containing 3 µl of genomic DNA, 10 µM of each primer, 200µM, 1X PCR buffer, 25mM $MgCl_2$ and 0.1UTaq DNA polymerase (Promega, USA). PCR was initiated by a denaturation step at 95°C for 3 min, followed by 30 cycles of 95°C for 1 min, 41°C for 30 sec, 72°C for 50sec and a final extension at 72°C for 3 min. PCR was performed on MyCycler (Bio-Rad, USA). Amplification products were resolved by 1.5% agarose gel electrophoresis in TBE buffer, stained with 2 µl(10mg/ml) ethidium bromide and visualized under UV illumination.

Results and Discussion

Genetic variations may lead to physiological or behavioral problems of genetic origin, such as malformed physical structure, poor biochemical balance, improper organ formation and function, altered social behavior, and susceptibility to disease (Chai 1976). However, the variations may also provide a survival trend to plant species in adapting to new environmental stress. Thus, specific primer is important in understanding and detecting the changes which can dictate the survival of the plant species.

Primers labels as DSYF8 and DSYR6 were found to be the best marker to distinguish between *A. malaccensis* and *A. hirta*. Fig. 1 shows the amplified PCR product for both species, consisting of 500bp and 300bp. *A. hirta* has an extra product of approximately 600bp in size compared to *A. malaccensis.* This could be used to discriminate between the two species.

For further analyses, the 500bp and 300bp bands for both species and the 650bp band for *A. hirta* will have to be cut to be further purified for sequencing analyses. The sequences obtained from these bands are expected to be useful in designing new primers.

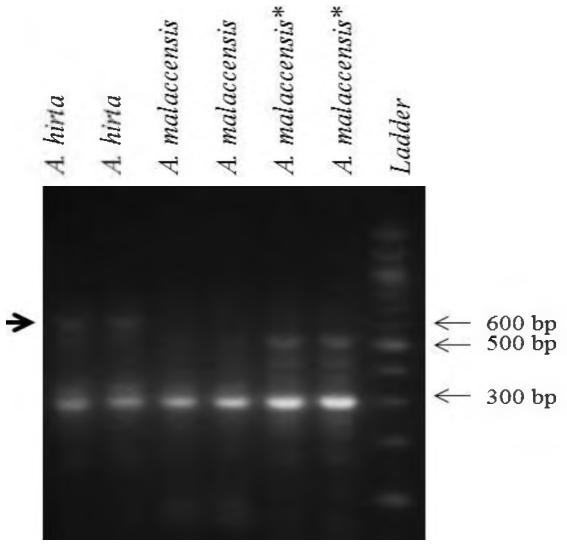

Fig. 1: PCR amplification using primer set of DSYF8 and DYSR6 showed an extra band size, approximately 600bp for *Aquilaria hirta*. From left; 1-2: *A. hirta*, 3-6: *A. malaccensis*. Wild type samples are indicated by asterisk (*) Ladder: 100bp ladder. L: 100bp ladder.

The patterns obtained from the *A. malaccensis* and *A. hirta* indicated that it is possible to identify these two species based on DNA polymorphisms. The variations in pattern revealed that both species have unique copy number in the biotic response related to the protein systemin. Further, it is believed that the Gaharu resin subsequently produced would be different in terms of its quality, density, odour, quantity and colour. Understanding the interaction between the environment and biotic response at genetic level are important keys to the advancement of the Gaharu industries. The genetic variation is important because it provides the "raw material" for natural selection.

Aquilaria species are scattered in the Asian region. Other than *Aquilaria, Gyrinops* species are also known to produce resin. Although these two species can be distinguished, the close genus in *Aquilaria* such as *A. microcarpa, A.beccariana, A. subintegra, A. crasna* are relatively difficult to differentiate. Better understanding of the species is important for future plant improvement and in producing higher quality resin.

It is suggested that more samples be screened, with more primers related to the biotic stress developed, optimised and tested in order to understand the diversity and variations of *Aquilaria* species and further determine the uniqueness of genotypes of different geographical locations. An increased number of correctly identified plant samples derived from both the wild and cultivated plants, should be made available in order to validate the accuracy of the results obtained. In addition, more molecular markers need to be screened and integrated in similar work like this in order to get a more complete overview of the genetic variation of agarwood producing species.

Conclusions

This study showed the development of a molecular based identification tool for *Aquilaria sp*. The results obtained showed that biotic stress protein which is related to systemin genes can be used to identify the unique genotypes of plants grown at different locations. PCR amplification of partial genes derived from conserved regions in tomato was found to be -suitably used as a DNA fingerprinting for *Aquilaria sp.* With more screening of these markers and the availabilityof sufficient plant samples, cultivated and wild agarwood could be differentiated in the future using DNA techniques. The DNA fingerprinting for *Aquilaria sp.* can also benefit its natural population, young developing plants and seedlings in the nursery as the PCR-based technique is rapid, cost effective, robust and reproducible and does not rely on morphology characteristics to accurately identify the species. This information, however, has limitations in controlling illegal trade, since the limited DNA sequence database of *Aquilaria sp* needs to be enlarged first.

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