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Genetic Variation and Molecular Authentication of Selected *Aquilaria* Species from Natural Populations in Malaysia Using RAPD and SCAR Markers

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Abstract: *Aquilaria* (Thymelaeaceae) is an endangered agarwood-producing tropical tree that is endemic to the Indomalesia region. Molecular information on genetic diversity of *Aquilaria* is limited. The aims of this research were to study genetic diversity among three *Aquilaria* species, growing in natural and distant populations in Malaysia using RAPD markers and to develop SCAR markers for easy identification of *A. malaccensis*, the major agarwood producer. By analyzing 23 RAPD primers, a total of 368 bands were scored. Multi-populations Descriptive statistics revealed that 333 (90.49%) polymorphic bands were found at species level, where *A. malaccensis* had 107 (29.08%) bands, *A. hirta* had 56 (15.22%) and *Aquilaria* sp.1 had 11 (2.99%), for the percentage of polymorphic loci in a single population/species. Nei's unbiased measurement indicated moderate similarities among populations/species. Out of the 23 RAPD primers, three were found specific to *A. hirta* and one was specific to each *A. malaccensis* and *Aquilaria* sp.1. RAPD-based SCAR markers generated a total of five species-specific amplicons: three for *A. hirta*, one each for *A. malaccensis* and *Aquilaria* sp.1. SCAR markers for *A. malaccensis* were used to distinguish five other different *A. malaccensis* populations in Malaysia. SCAR markers for *A. malaccensis* tested in five other different *A. malaccensis* populations in Malaysia yielded positive and consistent results. The DNA fingerprints identified for each *Aquilaria* sp. will be useful for *Aquilaria* identification in natural population, young plantation and even at seedling and seed stages in the nursery, as it is rapid and cost-effective and does not rely on morphology.

Key words: Agarwood, *Aquilaria*, genetic variation, genetic distance, molecular markers

INTRODUCTION

Aquilaria, a genus from the family of Thymelaeaceae, is an endangered woody tree in Malaysia. It is endemic to the Indomalesia region (Mabberley, 2008). The tree is often harvested for its highly valuable fragrant wood called "gaharu" or "agarwood" which the tree produces upon pathological infection or mechanical wounding. Agarwood is highly prized in both local and foreign markets because of its usage in traditional medicines and other products (Barden *et al*., 2000; Naef, 2011). Five different *Aquilaria* species have been recorded in Malaysia, including *A. hirta* Ridl., *A. beccariana* Van. Tiegh., *A. rostrata* Ridl., *A. malaccensis* Lam. and *A. microcarpa* Baill. which are distributed in the Peninsular and East Malaysia (Chua, 2008). This endemic genus with restricted distributions has received concerns from foresters, biologists and naturalists who aim at conserving these species in the wild. Due to the uncertainty regarding the tree size or age that contain agarwood, harvesting process often involve indiscriminate felling of both infected and uninfected trees in the wild which in turn threaten the habitat of these trees (CITES, 2004). Overexploitation of the species in their natural environments is expected to be reduced by the development of massive *ex-situ* plantations combined with techniques able to induce agarwood production on young plants (Faridah-Hanum *et al*., 2009). Yet, the production and breeding of *Aquilaria* in the nursery require tools for the identification of seeds and seedlings. For *Aquilaria* in general, species recognition relies mainly on the leave, ovary, fruit and calyx characteristics (Tawan, 2004) and remains subjective if seedling distributions cannot be related to the position of an adult tree in the wild. At the nursery, possibilities for morphological identification are further degraded with changes in the expression of morphologies such as the leave size that is affected by variation of environmental

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conditions (personal observation). Sequence-based species identification using the plastid *trn*L-*trn*F intergenic spacer and nuclear ribosomal ITS regions have been reported for *Aquilaria* (Eurlings and Gravendeel, 2005; Kiet *et al*., 2005). However, in the context of production, resorting to DNA sequences or even Restriction Fragment Length Polymorphism (RFLP) (Jena *et al*., 2009; Ridgway *et al*., 2003) is much costlier than Random Amplified Polymorphic DNA (RAPD) and microsatellite DNA which in most cases, needs only standard PCR followed by simple agarose electrophoresis. Besides financial and technical constrains the choice of identification methods depends on the genetic resolution needed. Microsatellite markers or Simple Sequence Repeat (SSR) markers have for example been developed for populations studies of *A. sinensis* in China (Zhang *et al*., 2009) and *A. crassna* in Vietnam (Eurlings *et al*., 2010). The aptitude of SSR markers to reveal hyper variable DNA length polymorphism makes them ideal for detecting population's genotypic variability and for revealing segregations of alleles in breeding programs or wild population dynamics studies (Ouborg *et al*., 1999; Patra *et al*., 2011). However, in comparison to the design of RAPD primers, the high labor required for the development of SSR primers reduces the opportunities of finding adequate markers for cross-species amplifications. RAPD markers have a large coverage of the genome and can rapidly produce high number of polymorphisms. They have for example been used for the identification of grapevine, tomato and peach (Gokbayrak and Soylemezoglu, 2010; Kulkarni and Deshpande, 2006; Melgoza-Villagomez *et al*., 2009) and are consistent for cultivar registrations (Al-Saghir and Abdel Salam, 2011; Linos *et al*., 2002). Conversely, in some cases, fidelity of the RAPD as a genetic marker has been questioned since its reproducibility can be altered by small variation of DNA quality or primer and template concentration (Theerakulpisut *et al*., 2008). In order to overcome these limitations, SCAR (Sequence Characterized Amplified Regions) markers have been developed to contain longer primers from the sequence of a cloned RAPD fragment (Paran and Michelmore, 1993). In this study, RAPD technique was used to detect molecular polymorphisms and genetic variations among selected *Aquilaria* species in Malaysia. At the same time specific SCAR primers were designed and tested which allowed for a simple and rapid identification of these species at the nursery.

MATERIALS AND METHODS

Plant materials: For genetic diversity analysis and marker development, tree saplings were collected from three natural populations and grown in poly bags in the nursery under natural environment at the Faculty of Forestry, Universiti Putra Malaysia, Selangor, Malaysia. Young leaves from these saplings were collected for genomic DNA isolation. Sampling was conducted in 2009 to 2010 when young shoots became abundant. The three *Aquilaria* species were: 1) *A. hirta* from the Merchang Reserve Forest provided by the Forestry Department of Terengganu, 2) *A. malaccensis* from Karak, provided by the National Seed and Planting Material Procurement Centre at Lentang, Pahang and 3) *Aquilaria* sp.1 from the Botanical Research Centre of Sarawak Forestry Corporation, Semenggoh, Sarawak. Both *A. malaccensis* and *A. hirta* were represented by six individuals while *Aquilaria* sp.1 was represented by only two individuals because of its scarcity. There is a possibility that the individuals were from a half-sib family as they were collected from the vicinity of a single mother. For testing the specificity and consistency of the SCAR markers, individual *A. malaccensis* trees were collected from five natural populations in Peninsular Malaysia including from Bukit Bauk (Terengganu), Gua Musang (Kelantan), Jelebu (Negeri Sembilan), Jeli (Kelantan) and Sungai Udang (Melaka). Geographical location of each population is shown in Fig. 1.

RAPD assay: Plant genomic DNA was extracted from 2 g of young leaves and shoots using the DNeasy® Plant Mini Kit (Qiagen, Germany), according to the manufacturer's protocol, with minor modifications. The quantity and quality were determined by spectrophotometry (NanoPhotometer™, IMPLEN, Germany). For PCR amplifications, genomic DNA samples were diluted to 50 ng μL^{-1}. A total of 60 10-base RAPD primers (Eurofins MWG Operon, USA) from series A, B and C were initially screened for amplification. Twenty-three primers which generated clear and reproducible banding patterns were chosen for the final RAPD analysis. PCR amplification was carried out according to the method described by Williams *et al*. (1990), with minor modifications. The final reaction volume was 15 μL and contained: 0.67 x PCR buffer, 0.08 mM $MgCl_2$, 0.02 mM of each dNTPs, 1 U of Taq DNA polymerase (Invitrogen, USA), 0.5 mM of primer (Eurofins MWG Operon, USA) and 50 ng of genomic DNA template. Negative controls with distilled water instead of DNA were included in each run in order to verify the absence of contamination. DNA amplification was conducted in a MyCycler™ Thermalcycler (Bio-Rad, USA), programmed for 3 min at 94°C; 35 cycles for 54 sec at 94°C, 43 sec at 45°C and 2 min at 72°C, with a final 5 min extension at 72°C. Amplification products were separated using electrophoresis on 2% agarose gels in 1 X TAE buffer, stained in ethidium bromide (0.5 μg mL^{-1}) and

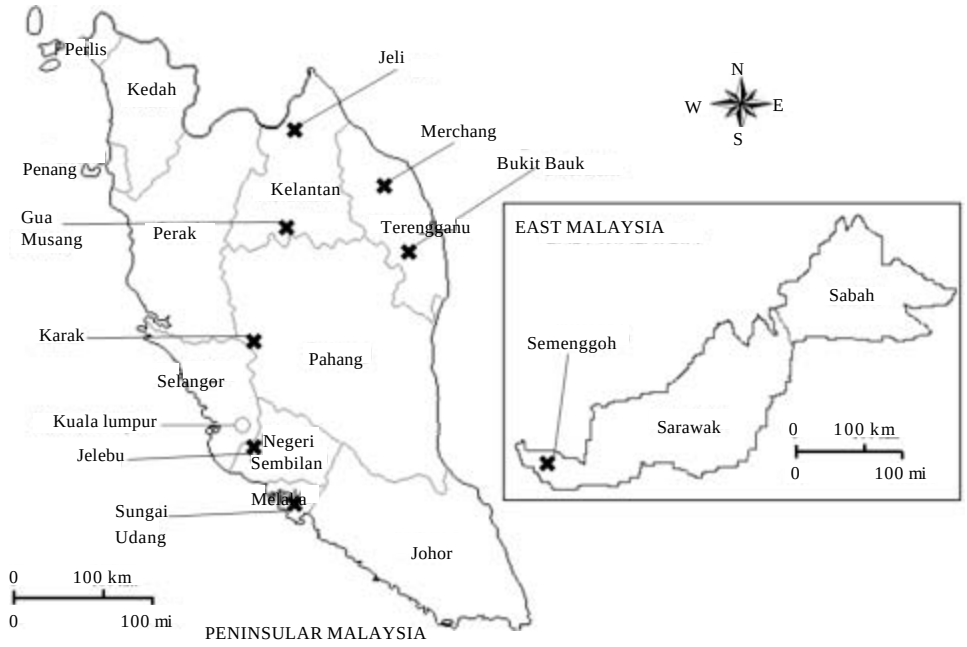

Fig. 1: Locations of *Aquilaria* populations in Malaysia, from which samples were collected. *Crosses* identify the populations which were assessed in the study

photographed under UV light (FlourChem™ Bio-imaging System, Alpha Innotech, USA). A 1 kbp DNA ladder (Fermentes, Canada) was included in the gels as a size reference. PCR amplification was repeated at least twice to show reproducibility of the results.

Statistical analysis: The RAPD profiles were scored visually and with the aid of the gel documentation system. Only clear and distinct RAPD bands were scored. Two binary matrices based on the RAPD banding patterns were created, where "1" was attributed to the presence of the targeted loci and "0" to the absence of a band. A similarity index was used to compare the banding patterns between the population/species. Nei's unbiased genetic distances (Nei, 1978) were calculated among populations using the POPGENE software (version 1.32) (Yeh *et al*., 1997). A dendrogram was constructed to show the genetic distance between different species in this study using the Unweighted Paired Group of Arithmetic Means Analysis (UPGMA) using the NTSYS-pc programme version 2.01e (Rohlf, 1990).

Cloning and sequencing of RAPD fragments: PCR was repeated three times for each DNA sample. Only reproducible bands below 1 kbp were considered for cloning. The species-specific amplicons were poked with pipette tips and dipped into freshly-prepared PCR mixture with the final reaction volume of 15 μL. All other components were as described above. The PCR mixture was re-amplified until a clear single band was observed. The specific band was excised from the ethidium bromide stained gel under UV light and purified using QIAquick Gel Extration Kit (QIAGEN®, Germany). The recovered DNA fragment was cloned into the pGEM® T-Easy vector (Promega®, USA). The ligation reaction contained 5 μL of 2X Rapid ligation buffer, 1 μL pGEM® T-Easy vector (50 ng), 3 μL PCR product and 1 μL T4 DNA Ligase (3 Weiss units μL^{-1}) in a total volume of 10 μL. The reaction was incubated overnight at 4°C. The ligation reaction was then transformed into *Escherichia coli* strain JM109 competent cells, by adding 25 μL recently thawed competent cells to a sterile 1.5 mL microcentrifuge tube containing 2 μL of the ligation reaction on ice. Recombinants were identified as white colonies on LB plates with X-gal and IPTG. Positive transformants were screened for gene insert using PCR method. Upon selection, the white colony was grown in 5 mL LB medium containing 50 μg mL^{-1} ampicillin and plasmid DNA was isolated from putative recombinants using QIAprep® Spin Miniprep Kit (QIAGEN, Germany), according to the manufacturer's protocol. The putative plasmid DNAs were sent for sequencing using T7 Promoter and SP6 universal primers.

Synthesis of SCAR primers: Based on the sequence of the cloned RAPD fragments, specific primers were designed to be used as SCAR primers. Each primer contained the original 10 bases of the RAPD primer plus another 10 bases from the flanking cloned fragment. The melting temperature, GC contents and secondary structures of each primer were verified using Gene Runner© V3.05 (http://www.generunner.net). Amplification of genomic DNA with the SCAR primers consisted of an initial hot start at 94°C, starting off with a 3 min pre-denaturation at 94°C, followed by 35 cycles of 54 sec denaturation at 94°C, 45 sec annealing at respective temperatures and 2 min extension at 72°C, with a final 5 min extension at 72°C. A series of annealing temperatures were utilized from 70 to 80°C in 1°C increments in the PCR reaction conditions in an effort to identify an annealing temperature that would yield a single and strongly amplified band of the expected size. Amplification products were electrophoretically resolved on 2.0% agarose gels with 100 bp DNA ladder (Fermentes, Canada), ethidium bromide stained and photographed under UV light using the FlourChem™ Bio-imaging System (Alpha Innotech, USA).

RESULTS

RAPD polymorphism: In this study, genetic variation was consistently detected using RAPDs in the three different *Aquilaria* species. RAPD analysis in 14 *Aquilaria* saplings yielded a total of 368 amplified reproducible bands from 23 random decamer primers Fig. 2 (a-w). The number of bands generated by a primer ranged from nine bands in OPA-20 and OPB-12, to 27 bands in OPA-18. The size of the amplified products varied from 375 to 4000 bp. All of the primers were found to be polymorphic and produced different percentages of polymorphisms. From the Multi-populations Descriptive Statistics, 333 (90.49%) polymorphic bands were found at the species level. The percentages of polymorphic loci for a single population/species were 29.08% in 107 polymorphic loci for *A. malaccensis*, 15.22% in 56 polymorphic loci for *A. hirta* and 2.99% in 11 polymorphic loci for *Aquilaria* sp.1.

Table 1: Nei's unbiased identity and distance based on RAPD markers

	A. malaccensis	*A. hirta*	*Aquilaria* sp.1
A. malaccensis	-	0.5780	0.5294
A. hirta	0.5481	-	0.5537
Aquilaria sp.1	0.6361	0.5911	-

Identity (above diagonal); Distance (below diagonal)

Genetic variation: Genetic similarity was estimated within and among populations using genetic identity. Through Nei (1978) unbiased measurement of identity, all three species in this study had considerable similarities to each other. When comparing Genetic Identity (I) (Table 1) between the three species, *A. malaccensis* shared the highest similarity (I = 0.5780) with *A. hirta*. By contrast, *Aquilaria* sp.1 had the lowest similarity value (I = 0.5294) when compared to *A. malaccensis* but a relatively higher value (I = 0.5537) when compared to *A. hirta*. A UPGMA dendogram was constructed based on Nei's genetic distance (Fig. 3). Similarly, the dendrogram revealed that *A. malaccensis* is more closely related to *A. hirta* while the *Aquilaria* sp.1 population is genetically distant from both (Fig. 3, Table 1).

Development of SCAR markers: Upon screening the 23 RAPD markers over all individuals, five distinct and reproducible bands were identified: ~950 bp from OPA02 (Fig. 2a) for *A. hirta*, ~635 bp from OPA05 (Fig. 2d) for *Aquilaria* sp.1, ~850 bp for OPA08 (Fig. 2f) for *A. hirta*, ~635 bp from OPA09 (Fig. 2g) for *A. malaccensis* and ~820 bp for OPB06 (Fig. 2o) for *A. hirta*. Five sets of SCAR primers were designed from the RAPD-derived sequences (Table 2). Primer specificity was evaluated using two saplings from each species. Using SCAR primer OPA05AS, amplification yielded a single distinct band for *Aquilaria* sp.1 at 637 bp (Fig. 4a). For *A. hirta*, three SCARs were developed; OPB06AH specifically amplified a band at 826 bp, OPA02AH at 955 bp and OPA08AH at 866 bp (Fig. 4b-d). In *A. malaccensis*, amplification using OPA09AM yielded a single band at 566 bp (Fig. 4e).

Table 2: Primer sequence, product length and annealing temperature of the SCAR primers for *Aquilaria* sp.1 (AS), *A. hirta* (AH) and *A. malaccensis* (AM)

SCAR primer designation	Annealing temperature (°C)	Size (bp)	SCAR primer sequences (5'-3')
OPA05AS	66	637	F (5'-AGGGGTCTTGCTTTCCATTA-3')
			R (5'-AGGGGTCTTGTAGTATTACA-3')
OPB06AH	70	826	F (5'-TGCTCTGCCCAACACCTTGG-3')
			R (5'-TGCTCTGCCCTATGGTTTAT-3')
OPA02AH	71	955	F (5'-TGCCGAGCTGCAAATAGAAT-3')
			R (5'-TGCCGAGCTGGGTATCGATG-3')
OPA08AH	71	866	F (5'-GTGACGTAGGACCCTGAGGA-3')
			R (5'-GTGACGTAGGCAAAAATGAA-3')
OPA09AM	68	566	F (5'-GGGTAACGCCACATTTCCAT-3')
			R (5'-GGGTAACGCCGGTTAAATAA-3')

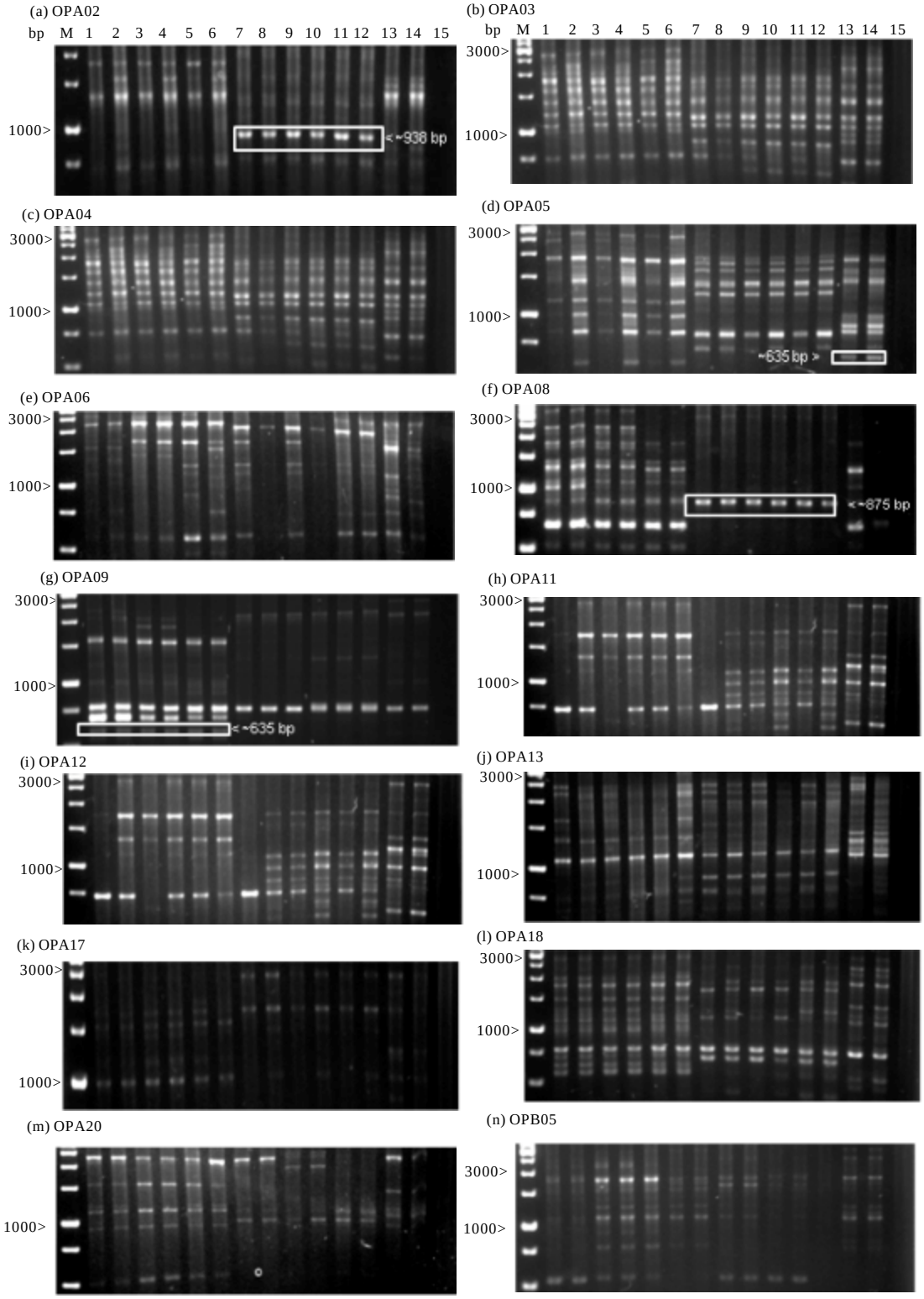

Fig. 2: Continued

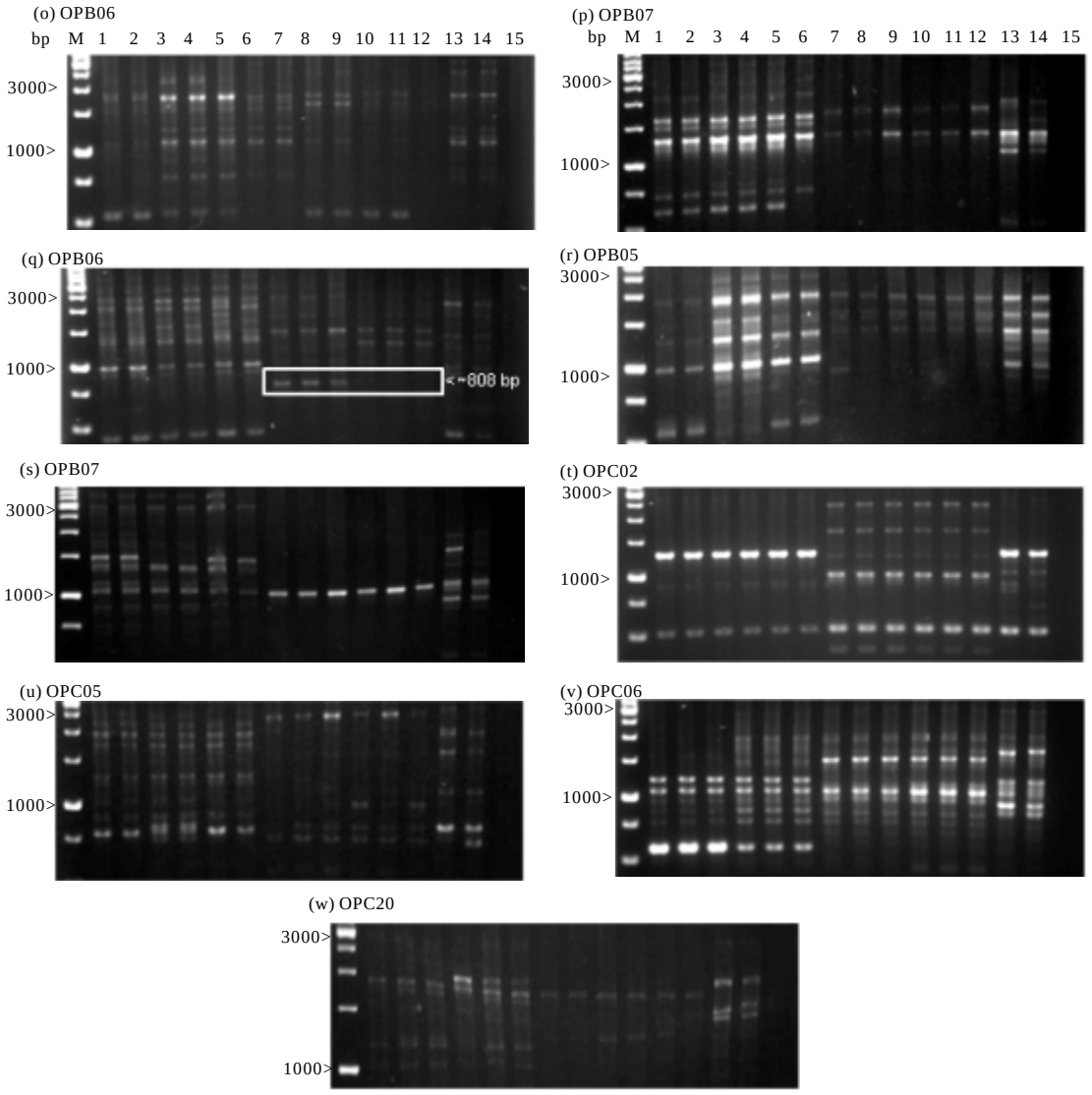

Fig. 2 (a-w): RAPD profiles of *Aquilaria* species using Operon 10-mer random primers. *Aquilaria malaccensis* (Karak) (Lane: 1-6), *A. hirta* (Merchang) (Lane: 7-12), *Aquilaria* sp.1(Semenggoh) (Lane: 13-14) and a negative control (Lane: 15). a to w are agarose electrophoresis of the respective RAPD primers. M represents a 1 kb DNA ladder. Arrowheads and boxes indicate the species-specific RAPD amplicons used in SCAR marker development

Specificity of SCAR marker: To examine the specificity and validity of the SCAR marker, a PCR verification test was performed on *A. malaccensis* from various populations. This particular species was selected because it is widely distributed in Peninsular Malaysia if compared to the other two species. Population of *A. hirta* is limited to a few locations in Peninsular Malaysia and many of them are inaccessible or unknown while *Aquilaria* sp. 1 is exclusively confined to the State of Sarawak, in East Malaysia. Genomic DNA was extracted from two individuals representing five different *A. malaccensis* populations including Bukit Bauk, Gua Musang, Jelebu, Jeli and Sungai Udang. Control trees representing *Aquilaria* sp.1 from Semenggoh, *A. hirta* from Merchang and *A. malaccensis* from Karak were also included in the testing (Fig. 1). Using OPA09AM primer in PCR, all the *A. malaccensis* individuals from various geographical locations yielded the expected specific band, similar to the positive control, *A. malaccensis* from Karak (Fig. 5). As expected no bands

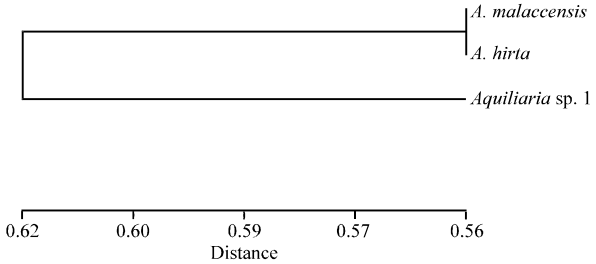

Fig. 3: Dendrogram of selected *Aquilaria* species in Malaysia, constructed using UPGMA analysis based on Nei's Genetic Distance calculated from 23 RAPD markers. Scale is the genetic distance measurement

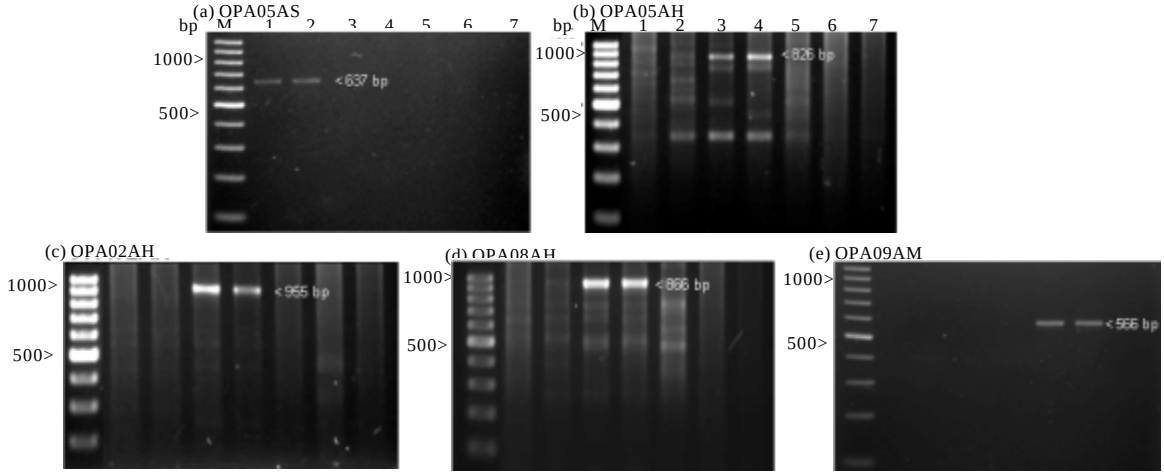

Fig. 4 (a-e): Marker profiles of *Aquilaria* species generated by specific SCAR primers. *Aquilaria* sp.1 (Semenggoh) (Lane: 1-2), *A. hirta* (Merchang) (Lane: 3-4), *A. malaccensis* (Karak) (Lane: 5-6) and a negative control (Lane: 7). a to e are agarose electrophoresis of the respective SCAR primers. M represents a 100 bp DNA ladder. Arrowheads indicate the expected size of species-specific SCAR marker for each *Aquilaria* species

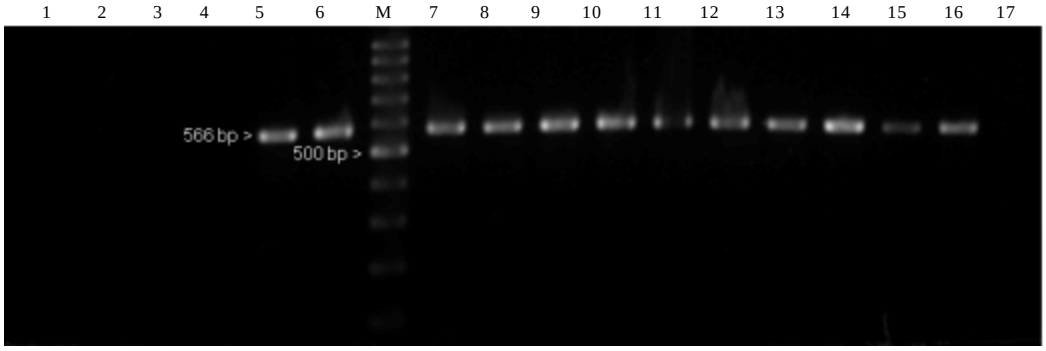

Fig. 5: Marker profiles of *Aquilaria* species generated by specific SCAR primers OPA09AM developed for *A. malaccensis*. *Aquilaria* sp.1 (Semenggoh) (Lanes: 1-2), *A. hirta* (Merchang) (Lanes: 3-4), *A. malaccensis* (Karak) (Lanes: 5-6), *A. malaccensis* (Bukit Bauk) (Lanes: 7-8), *A. malaccensis* (Jelebu) (Lanes: 9-10), *A. malaccensis* (Jeli) (Lanes: 11-12), *A. malaccensis* (Gua Musang) (Lanes: 13-14), *A. malaccensis* (Sungai Udang) (Lanes: 15-16) and a negative control (Lane: 17). *M* represents a 100 bp DNA ladder. A single band of 566 bp was observed in all lanes containing *A. malaccensis* samples

were amplified from individuals representing *A. hirta* and *Aquilaria* sp.1.

DISCUSSION

In this study, genetic variation was consistently detected using RAPDs in three *Aquilaria* species. Genetic identity values revealed considerable similarities among *A. malaccensis*, *A. hirta* and *Aquilaria* sp.1. The larger genetic distance observed between the later and *A. malaccensis* and *A. hirta* might be explained by the species geographic distributions. Sample for *Aquilaria* sp.1 originated from Sarawak of the East Malaysia region (Borneo Island) while samples for both *A. malaccensis* and *A. hirta* were collected from the Peninsular Malaysia region. Geographical variability is one of the common drivers that can generate high level of polymorphisms between populations, as environment differences such as the weather, air moisture content, soil nutrient, forest diversity and others can be different for both East and Peninsular Malaysia forests. Such phenomena was also supported by Josiah *et al.* (2008) who stated that geographical isolation may restrict gene flow, causing pollen and seed dispersal to be limited within the population and hence reduces the gene variability of the population. It is also plausible that the apparent correlation between genetic and geographic distance suggests that *Aquilaria* sp.1 might have been geographically isolated from *A. malaccensis* and *A. hirta* at an earlier time.

The use of RAPD permits screening through a large number of individuals in a relatively short time, providing easily-defined molecular information of a population. In addition, preliminary studies or initial works are not required before RAPD screening (Josiah *et al.*, 2008; Sun and Wong, 2001; Williams *et al.*, 1990). As *Aquilaria* had limited genetic information, RAPD allowed a quick start-off for this genus. In several cases, RAPD profiles revealed intra specific polymorphism within individuals collected in a same area. For example, within *Aquilaria* sp.1 from Sarawak, a clear band at 750 bp was detected in lane 14 but not in lane 13 (Fig. 2q). For identification at the species level, the dominance of RAPD markers usually does not allow to distinguish between marker/marker homozygotes and marker/null heterozygotes (Lynch and Milligan, 1994; Sunnucks, 2000). The required homozygocity of a species-specific marker can be assumed when the marker is consistently revealed for different populations. This assumption was strongly supported for *A. malaccensis* for which the retained RAPD and corresponding SCAR markers were consistently amplified from six samples collected in a same area and five samples collected across Peninsular Malaysia. For *Aquilaria* sp.1 and *A. hirta*, SCAR marker homozygocity and species specificity have only been tested on a reduced number of samples collected locally.

The reproducibility of RAPD results has often been questioned due to its sensitivity to parameters and the quality of the DNA template (Semagn *et al.*, 2006). This limitation can be overcome by designing SCAR markers which, due to longer primer sequences and higher annealing temperatures are less sensitive to uncontrolled variations of PCR conditions (Jiang *et al.*, 2009). Furthermore, in the context of quality control at the nursery, the detection of a single presence/absence signal allowed by SCARs is straight forward in comparison to the interpretation of RAPD patterns or microsatellite band lengths that may change according to intra-specific polymorphism. However, with the absence of complementary amplification patterns, false negatives may go undetected in the presence of PCR-inhibitory substances. If no false negative could be revealed during the present work, future changes in the selection of plant material and DNA extraction methods need to be thoroughly tested for their reliability before being used at the nursery. Additionally, other markers such as those based on the plastid *trn*L-*trn*F intergenic spacer and nuclear ribosomal ITS regions should be used to provide supplementary evidence in case of unexpected negative results.

In comparison to *Aquilaria* sp.1 and *A. hirta*, *A. malaccensis* is the most widely distributed species and is found available in all the states in Peninsular Malaysia, except Perlis. The species also supplies the bulk of agarwood traded locally and internationally. Using OPA09AM marker, a distinct band was amplified in all *A. malaccensis* samples collected across Malaysia but not in the other two species, leading to its possible use as control marker for fast and efficient identification of *A. malaccensis*. Besides being an efficient source of polymorphism for the design of species-specific SCAR, RAPD profiles have potential for use in identification of markers linked to genes or Quantitative Trait Loci (QTL) for selective breeding purposes (Collard *et al.*, 2005; Verhaegen *et al.*, 1997). For such application, the presence of polymorphism within the selected RAPD band sequence would allow the design of co-dominant SCAR markers (Paran and Michelmore, 1993). Yet, in the field of conservation biology, co-dominant markers such as microsatellites are usually preferred to RAPD since they are more informative for the estimation of the impact of genetic drift on genetic variation, the level of inbreeding within populations and gene flows between populations (Lynch and Milligan, 1994). Nevertheless, for a less

known tropical tree like *Aquilaria*, RAPD and SCAR provide effective and cheap identification tool, suffice for species verification.

CONCLUSION

Previously, morphological characters have been used to describe the many different species in *Aquilaria*, however, species identification remained subtle as many of the characters are prone to environment conditions. In this study, RAPD markers were used for the estimation of genetic diversity in three *Aquilaria* species native to Malaysia. RAPD was found to be a powerful tool in assessing the genetic diversity amongst the different species and SCAR markers derived from RAPDs could serve as an efficient tool for the identification of specific *Aquilaria* species and could be used in breeding program.

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