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# Research Article
# Genetic Diversity of Threatened *Aquilaria* sp. in Malaysia Using Inter Simple Sequence Repeat (ISSR) Markers

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## Abstract

**Background and Objective:** *Aquilaria* sp. (family Thymelaeaceae) or commonly known as gaharu, karas or agarwood is one of the threatened crops that have been listed in the Convention on International Trade in Endangered Species (CITES). Overexploitation in search of valuable agarwood or resinous wood has directly led to scarcity of *Aquilaria* sp. in the wild. The aim of this study was to investigate the genetic diversity among three *Aquilaria* sp. (*A. malaccensis* Lam., *A. subintegra* Ding Hou and *A. sinensis* (Lour.) Spreng.) using inter simple sequence repeat (ISSR) markers. **Materials and Methods:** Five annealing temperatures varying from 47-51°C were optimized. Out of 10 ISSR primers screened, 4 primers revealed clear and repeatable bands. **Results:** The clearest bands were observed at 50°C, which was later used for primer screening and PCR amplifications. The 4 primers (UBC 855, UBC 845, UBC 811 and UBC 809) yielded a total of 23 bands, of which 20 were polymorphic (87%). The UBC 809 and 845 showed 100% polymorphism with a total of 6 and 4 bands, respectively. The UBC 855 showed 6 polymorphic bands (75%) and UBC 811 showed 4 polymorphic bands (80%). Construction of UPGMA dendrogram has grouped all the 15 accessions of 3 *Aquilaria* sp. into 2 major clusters, where all *A. malaccensis* accessions were grouped into cluster I and a combination of *A. sinensis* and *A. subintegra* accessions were grouped into cluster II. **Conclusion:** Findings of this study provide basic information for conservation and utilization of the *Aquilaria* genetic resources.

Key words: *Aquilaria*, ISSR, genetic diversity, UPGMA, conservation, reproducible, resources

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

*Aquilaria* sp. (family Thymelaeaceae) is a tropical forest tree that is also known as gaharu, karas or agarwood. There are 25 *Aquilaria* species have been found in the countries including Bangladesh, Bhutan, India, Indonesia, Iran, Myanmar, Philippines, Thailand and Malaysia[1]. However, only 5 species (*A. beccariana* Teigh, *A. hirta* Ridl., *A. malaccensis*, *A. microcarpa* Baill. and *A. rostrata* Ridl.) can be found in the Peninsular Malaysia and three are introduced species (*A. crassna* Pierre ex Lecomte, *A. sinensis* and *A. subintegra*)[2]. *Aquilaria* sp. is currently listed in the Convention on International Trade in Endangered Species (CITES) Appendix II and classified as 'Vulnerable' to 'Critically Endangered' in the International Union for Conservation of Nature (IUCN[3]) Red List.

*Aquilaria* sp. has become beneficial and prominent for the production of agarwood or resinous wood[4]. The highly prized of this agarwood lead to the overexploitation of this species which directly led to scarcity of these trees in the wild[5]. Malaysia and Indonesia are the primary exporters of agarwood since agarwood received high demand from the local and international markets[6]. In Malaysia, *A. malaccensis* is the primary producer of agarwood and the high grade of gaharu can reach[7] up to RM 25 000/kg. In order to hinder the scarcity of this threatened species, several countries including Indonesia, India and China have initiated a plantation for *Aquilaria* sp. in order to preserve and conserve the species as well as to confront the demand of agarwood supply for industry[8,9].

According to Hamrick and Godt[10], a careful assay of genetic diversity is important to formulate effective conservation strategies for the threatened and endangered species. For *Aquilaria* sp., the main problem is that the identification of *Aquilaria* seedling either in the wild or nursery is quite challenging as the morphological features among the species in the genus are almost similar and could not be clearly distinguished among the species[7]. Hence, selection based on genetic information using molecular markers is extremely important, as it is more dependable and persistent[11]. The employment of PCR-based approaches for identification of plant genetic resource has been immensely used because of their simplicity and requirement for small quantity of DNA sample[12]. Previous studies reported that several PCR-based approaches have been piloted for identification of *Aquilaria* sp. such as those using microsatellite markers[13,14], random amplified Polymorphic DNA (RAPD)[15] and plastid *trn*L-*trn*F intergenic spacer sequences[16]. However, these studies had very limited coverage of existing *Aquilaria* populations and the information generated was not sufficient to practically inform any management plans[7]. Therefore, it is needed to understand the genetic variation within and between species as it can give information and can determine its adaptability for a long-term survival[17]. In this study, a molecular technique of inter simple sequence repeat (ISSR) has been performed to evaluate the genetic diversity of 3 *Aquilaria* sp. namely *A. malaccensis*, *A. sinensis* and *A. subintegra*.

## MATERIALS AND METHODS

**Study area:** This study was conducted for 10 months (September, 2018-June, 2019) in the Laboratory of Molecular Biology, Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin, Kampus Besut, Terengganu, Malaysia.

**Plant materials and DNA extraction:** A total of 15 accessions from three different *Aquilaria* species namely *A. malaccensis* (labelled as M), *A. subintegra* (labelled as S) and *A. sinensis* (labelled as SI) were used. Seedlings of *A. malaccensis* were collected from Forest Research Institute Malaysia (FRIM) Forest Reserve, Merchang, Terengganu; *A. subintegra* were collected from Alor Gajah, Melaka and *A. sinensis* were collected from Seri Kembangan, Selangor. All the seedlings were subsequently planted at Ladang Pasir Akar germplasm, Jertih, Terengganu. The genomic DNA was extracted from fresh leaves of each accession using DNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's protocol.

**ISSR-PCR conditions and gel electrophoresis:** The optimization of ISSR-PCR was performed varying in five different annealing temperatures (Ta) ranged from 47-51°C. Ten ISSR primers (Table 1, Eurofins, Genomics) were screened. The primers generating good amplification patterns were further used for PCR amplification of all the plant samples. The PCR amplifications were performed using New England Biolab's (NEB) kit in a final reaction volume 25 µL

Table 1: List of ISSR primers used in this study

| ISSR primers | Sequence 5'-3' | Melting temperature (°C) |
|---|---|---|
| UBC 855 | (AC) 8YT | 53.1 |
| UBC 845 | (CT) 8RG | 48.1 |
| UBC 808 | (AG) 9G | 51.2 |
| UBC 811 | (GA) 9A | 49.0 |
| UBC 815 | (CA) 8T | 50.1 |
| UBC 833 | (AT) 8YG | 28.1 |
| UBC 849 | (GT) 8YA | 51.4 |
| UBC 836 | (AG) 8YG | 50.5 |
| UBC 840 | (GA) 8YT | 47.4 |
| UBC 809 | (GA) 9T | 48.8 |

containing 2.5 µL of 10X buffer, 0.5 µL of 10 mM dNTPs, 1.0 µL of 10 µM each primer, 2.0 µL of approximately 30 ng $mL^{-1}$ DNA, 0.1 µL of Taq DNA polymerase and 18.9 µL of sterile distilled water. The PCR mix without DNA template was used as a negative control.

The PCR amplification was performed in a Thermal Cycler (Applied Biosystems™) with the following profile: An initial denaturation at 94°C for 5 min followed by 45 cycles of 94°C for 30 sec, 47-51°C for 45 sec, 72°C for 2 min and with a final extension of 72°C for 5 min. The amplified DNA fragments were separated using electrophoresis on 1% (w/v) horizontal agarose gel in 1 X TBE buffer. The electrophoresis was conducted for 1 h at a constant voltage of 90 V and the gel was photographed under an UV transilluminator. The 1 kb DNA ladder (Promega) was included in the gel as a size reference. The ISSR-PCR amplifications were repeated at least twice to ensure the band profiles were stable and reproducible.

**Data analysis:** Only clear and distinct ISSR bands were scored as presence (1) or absence (0). A binary ISSR data matrix was prepared for each species and used in the subsequent analysis.

Basic parameters such as the total number of bands, number of polymorphic bands and the percentage of polymorphic bands were manually calculated according to the equation[18]:

$$\text{Polymorphism (\%)} = \frac{\text{No. of polymorphic band}}{\text{Total No. of band}} \times 100$$

A dendrogram was constructed by unweighted pair group method using arithmetic averages (UPGMA)[19] and the genetic distances were calculated based on Nei[20] unbiased genetic distance. The analysis was performed by PAUP* software (version 4.0)[21].

## RESULTS AND DISCUSSION

**Annealing temperature and primer screening:** In order to obtain an efficient identification, conservation and utilization of the threatened species of *Aquilaria,* extensive research on PCR-based markers is needed. Among all dominant markers, ISSR have its advantages with higher polymorphism of bands, lower cost and have been widely used for the genetic diversity studies of various crop plants[22-24]. The adjustment of annealing temperature is known to have a great impact on the richness and legibility of DNA banding profile. The higher temperature

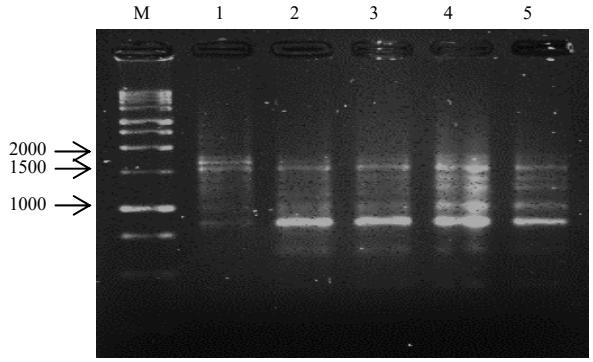


Fig. 1: Effects of annealing temperature on ISSR-PCR amplification (UBC 808 using sample *A. subintegra*)
Lane M: 1 kb marker, Lane 1: 47°C, Lane 2: 48°C, Lane 3: 49°C, Lane 4: 50°C, Lane 5: 51°C

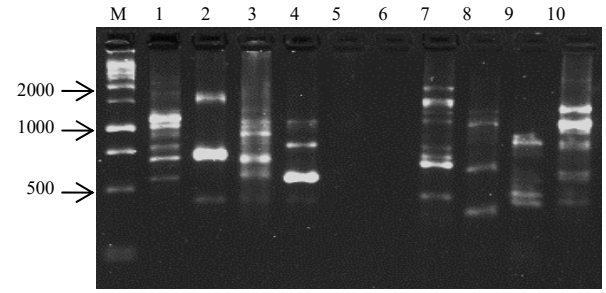


Fig. 2: ISSR-PCR band profiles generated by ten primers using sample *A. sinensis*
Lane M: 1 kb marker, Lane 1: UBC 855, Lane 2: UBC 845, Lane 3: UBC 808, Lane 4: UBC 811, Lane 5: UBC 815, Lane 6: UBC 833, Lane 7: UBC 849, Lane 8: UBC 836, Lane 9: UBC 840, Lane 10: UBC 809

is favoured for large fragments amplification whilst the lower temperature is favoured for short fragments amplification[25]. According to Bornet and Branchard[12], annealing temperature (Ta) is primer specific and always superior to melting temperature (Tm) because of the need of high stringency to facilitate annealing of primers. They reported that Ta values of 45-50°C were optimal for ISSR amplification with different primers. In the present study, the optimization of Ta varying from 47-51°C was achieved by performing a gradient PCR. Using the primer UBC 808 and DNA sample of *A. subintegra*, the clearest and intensive bands were observed at an optimal annealing temperature of 50°C (Fig. 1), which was later used for primer screening. Out of 10 primers screened, 4 primers (UBC 855, UBC 845, UBC 811 and UBC 809) produced the clearest, intensive and reproducible bands, whilst the primers UBC 815 and UBC 833 gave no amplified bands on the tested sample of *A. sinensis* (Fig. 2).

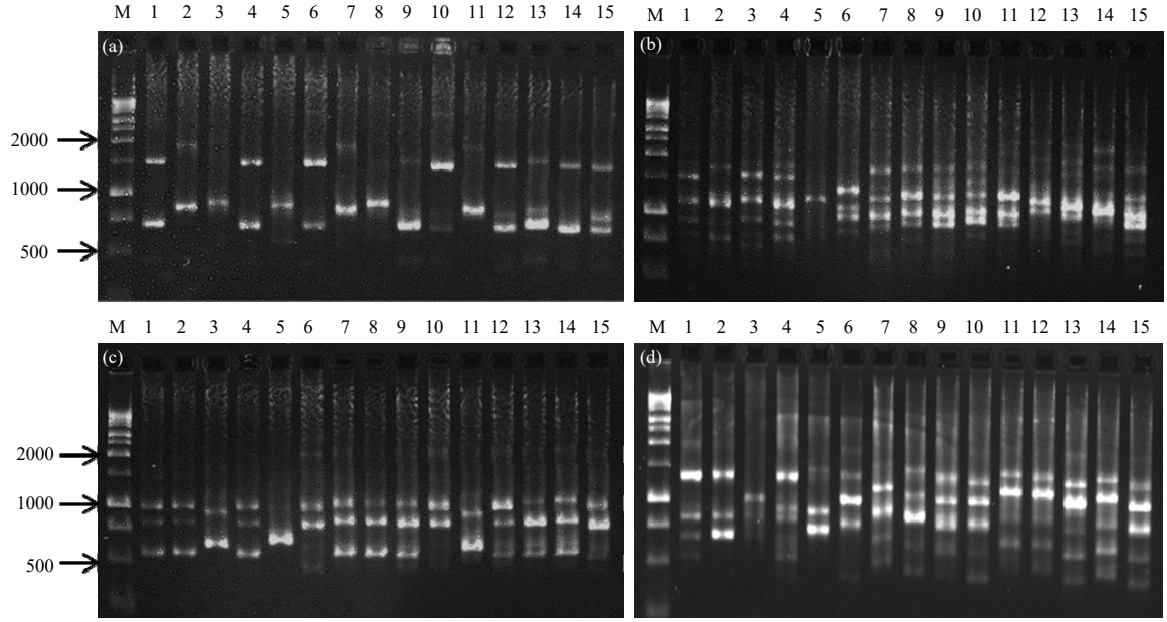


Fig. 3(a-d): PCR amplifications of 4 selected primers, (a) UBC 855, (b) UBC 845, (c) UBC 811 and (d) UBC 809

Lane M: 1 kb marker, Lane 1: *A. malaccensis* accession M1, Lane 2: *A. malaccensis* accession M3, Lane 3: *A. malaccensis* accession M5, Lane 4: *A. malaccensis* accession M7, Lane 5: *A. malaccensis* accession M8, Lane 6: *A. subintegra* accession S2, Lane 7: *A. subintegra* accession S4, Lane 8: *A. subintegra* accession S5, Lane 9: *A. subintegra* accession S7, Lane 10: *A. subintegra* accession S9, lane 11: *A. sinensis* accession SI 1, Lane 12: *A. sinensis* accession SI 3, Lane 13: *A. sinensis* accession SI 5, Lane 14: *A. sinensis* accession SI 6, Lane 15: *A. sinensis* accession SI 8

Table 2: Percentage of polymorphic band obtained from selected primers

| Primers | No. of polymorphic band | No. of monomorphic band | Total band produced | Polymorphism (%) |
|---|---|---|---|---|
| UBC 855 | 6 | 2 | 8 | 75 |
| UBC 845 | 4 | 0 | 4 | 100 |
| UBC 811 | 4 | 1 | 5 | 80 |
| UBC 809 | 6 | 0 | 6 | 100 |

**ISSR polymorphism and genetic diversity:** The 4 selected primers (UBC 855, UBC 845, UBC 811 and UBC 809) were subsequently used for genetic diversity analysis of three *Aquilaria* sp. A total of 23 bands were amplified, of which 20 bands were polymorphic (87%) and the remaining bands were monomorphic (13%). The percentage of polymorphism for each primer varied from 75-100% (Table 2). The ISSR-PCR amplification by UBC 855 resulted in 8 polymorphic bands ranging from 625-2000 bp, UBC 845 produced 4 bands ranging from 750-1750 bp, UBC 809 produced 6 bands ranging from 500-1500 bp, whilst amplification by UBC 811 resulted in 5 bands ranging from 500-2000 bp (Fig. 3). The primer UBC 855 produced the highest number of bands but the lowest percentage of polymorphism (75%). On the other hand, both primers UBC 845 and 809 showed 100% polymorphism and similar result is also reported by Banu *et al.*[26]. Variable banding patterns were observed in three *Aquilaria* sp., however, no species-specific band was produced by any primer at any band range.

Table 3 showed the genetic distances based on the similarity coefficients of Nei[20] between all the *Aquilaria* accessions. The UPGMA dendrogram showed 2 major clusters, I and II (Fig. 4). Cluster I consisted of all *A. malaccensis* accessions. Cluster II consisted of combination of *A. subintegra* and *A. sinensis* accessions. The major cluster I formed 2 sub clusters, IA and IB. The sub cluster IA was then divided into 2 sub clusters, $IA_1$ and $IA_2$. Two accessions of *A. malaccensis* (M1 and M7) were clustered in $IA_1$ while another two accessions of *A. malaccensis* (M3 and M5) were clustered in $IA_2$. The sub cluster IB consisted of one accession of *A. malaccensis* (M8). Major cluster II composed of two sub clusters, IIA and IIB. The sub cluster IIA was further divided into 2 sub clusters, $IIA_1$ and $IIA_2$. Three *A. subintegra* accessions (S2, S9 and S7) were clustered in the sub cluster $IIA_1$ while two *A. subintegra* accessions (S4 and S5) clustered in the sub cluster $IIA_2$. The sub cluster IIB consisted of all *A. sinensis*. An accession of *A. sinensis* (SI 1) was clustered in sub cluster $IIB_1$ followed by the four accessions of *A. sinensis* (SI 3, SI 6, SI 8 and SI 5) which were clustered in $IIB_2$.

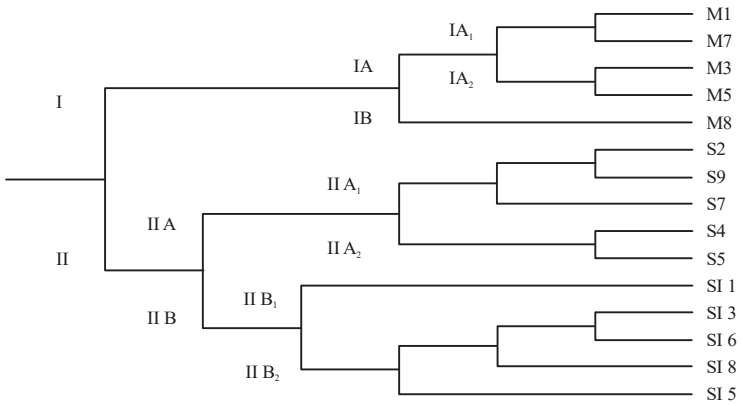


Fig. 4: Dendrogram of 15 accessions of *A. malaccensis* (labelled as M), *A. subintegra* (labelled as S) and *A. sinensis* (labelled as SI) based on ISSR data

Table 3: Genetic distance matrix of three *Aquilaria* sp. based on 23 bands generated by 4 primers used, based on Nei[20] coefficients

| Strain | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | - | | | | | | | | | | | | | | |
| 2 | 0.2174 | - | | | | | | | | | | | | | |
| 3 | 0.3913 | 0.1739 | - | | | | | | | | | | | | |
| 4 | 0.0000 | 0.2174 | 0.3913 | - | | | | | | | | | | | |
| 5 | 0.4783 | 0.3478 | 0.3478 | 0.4783 | - | | | | | | | | | | |
| 6 | 0.3478 | 0.5652 | 0.5652 | 0.3478 | 0.5652 | - | | | | | | | | | |
| 7 | 0.4783 | 0.3478 | 0.4348 | 0.4783 | 0.5217 | 0.2173 | - | | | | | | | | |
| 8 | 0.4348 | 0.3044 | 0.3913 | 0.4348 | 0.4783 | 0.2609 | 0.0434 | - | | | | | | | |
| 9 | 0.2609 | 0.4783 | 0.5652 | 0.2609 | 0.5652 | 0.0867 | 0.2174 | 0.1734 | - | | | | | | |
| 10 | 0.3478 | 0.5652 | 0.5652 | 0.3478 | 0.5652 | 0.0000 | 0.2174 | 0.2609 | 0.0870 | - | | | | | |
| 11 | 0.5652 | 0.4348 | 0.4348 | 0.5652 | 0.5217 | 0.4783 | 0.2609 | 0.2174 | 0.3913 | 0.4783 | - | | | | |
| 12 | 0.4348 | 0.5652 | 0.5652 | 0.4348 | 0.6522 | 0.2609 | 0.3044 | 0.3478 | 0.2609 | 0.2609 | 0.2174 | - | | | |
| 13 | 0.3913 | 0.5217 | 0.5217 | 0.3913 | 0.6087 | 0.3044 | 0.3478 | 0.3044 | 0.2174 | 0.3044 | 0.1739 | 0.0435 | - | | |
| 14 | 0.4348 | 0.5652 | 0.5652 | 0.4348 | 0.6522 | 0.2609 | 0.3043 | 0.3478 | 0.2609 | 0.2069 | 0.2174 | 0.0000 | 0.0435 | - | |
| 15 | 0.4348 | 0.5652 | 0.5652 | 0.4348 | 0.6522 | 0.2609 | 0.3043 | 0.3478 | 0.2609 | 0.2069 | 0.2174 | 0.0000 | 0.0435 | 0.000 | - |

1: *A. malaccensis* accession M1, 2: *A. malaccensis* accession M3, 3: *A. malaccensis* accession M5, 4: *A. malaccensis* accession M7, 5: *A. malaccensis* accession M8, 6: *A. subintegra* accession S2, 7: *A. subintegra* accession S4, 8: *A. subintegra* accession S5, 9: *A. subintegra* accession S7, 10: *A. subintegra* accession S9, 11: *A. sinensis* accession SI 1, 12: *A. sinensis* accession SI 3, 13: *A. sinensis* accession SI 5, 14: *A. sinensis* accession SI 6, 15: *A. sinensis* accession SI 8

*Aquilaria malaccensis* was genetically distant from *A. subintegra* and *A. sinensis*. This study was in agreement with Lee *et al.*[17] where all *A. malaccensis* were clustered into the same cluster, in which it can strictly distinguishable from *A. hirta* and A. *rostrata* collected from the Peninsular Malaysia. The larger genetic distance observed between the *A. malaccensis* with *A. subintegra* and *A. sinensis* might be explained by the species geographic distributions where both *A. subintegra* and *A. sinensis* were the introduced species to Malaysia, while *A. malaccensis* was native to Malaysia. Geographical variability is one of the prevalent drivers that can develop high level of polymorphisms between populations, as environment differences such as the climate, moisture content, soil nutrient, forest diversity and others can be different for both East and Peninsular Malaysia forests[7]. Such phenomena was supported by Josiah *et al.*[27], who stated that geographical isolation may reduce gene flow, causing pollen and seed dispersal to be limited within the population and hence, reduces the gene variability of the population.

Assessment of genetic diversity and relationships within and between crop species and their wild relatives is vital not only for basic studies of evolution but also for informed utilization and protection of plant genetic resources[28-31]. In addition, a thorough knowledge on the genetic diversity of a crop is crucial for selection of parental material that maximizes genetic variability[32]. Among various PCR-based markers, ISSR markers have been recommended for assessment of genetic diversity because the ISSR markers were highly polymorphic and were successfully used to elucidate the patterns of genetic variation in plants at population, species as well as genus level[17,26,33].

## CONCLUSION

This study indicates that ISSR markers could provide reproducible and specific tools to study genetic diversity among three *Aquilaria* sp. namely *A. malaccensis*, *A. sinensis* and *A. subintegra*. Four ISSR makers (UBC 855, UBC 845, UBC 811 and UBC 809) are found to be suitable for polymorphism assessment in *A. malaccensis*, the main producer of agarwood in Malaysia. Although no species-specific band was produced, ISSR markers are successfully used to elucidate the levels and patterns of genetic variation among *Aquilaria* species. Such information is important to guide conservation and management efforts of this threatened species. The presence of species-specific band will be of great value for the development of species-specific marker. Further studies using more markers such as RAPD and RFLP are required.

## SIGNIFICANCE STATEMENT

This study discovered the potential use of ISSR markers to investigate the genetic diversity among three threatened *Aquilaria* sp. namely *A. malaccensis*, *A. sinensis* and *A. subintegra*. The findings of this study can be beneficial for the formulation of conservation strategies and utilization of *Aquilaria* genetic resources. This study will help the researchers to uncover the critical areas of genetic variation among *Aquilaria* sp. that many researchers were not able to explore. Thus a new theory using ISSR markers describing the genetic variation and polymorphism in *A. malaccensis*, the main producer of agarwood in Malaysia is now available.

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