International Journal of Scientific Research and Engineering Development-– Volume 4 Issue 6, Nov-Dec 2021

Available at www.ijsred.com

RESEARCH ARTICLE OPEN ACCESS

Representational Difference Analysis (RDA) for Identification of DNA Markers from *Aquilaria hirta*

Noorhidayah Md Nazir[1], Ab Rahim Mohd-Hairul[1]*, Tan Suat Hian[1], Zamakhsyary Mustapa[2]

[1]Faculty of Industrial Sciences and Technology, Universiti Malaysia Pahang, 26300 Gambang, Kuantan, Pahang, Malaysia
[2]Malaysian Timber Industry Board, Level 13-17, Menara PGRM, No 8, Jalan Pudu Ulu, 56100 Cheras, Kuala Lumpur, Malaysia
*Email:mhairul@ump.edu.my

----------**********----------

Abstract:

Aquilaria hirta is among potential *Aquilaria* spp. in tropical rainforests that produce high-quality resinous agarwood. In Malaysia, this plant has been domesticated and well planted in agarwood plantations to fulfill the demand from the fragrance industry. However, based on morphological and physiological observation, species identification is challenging due to their closely related characteristics. Driver DNA used in this study is the genomic DNA isolated from *A. hirta*. Meanwhile, selected tester DNA combines the genomic DNA isolated from *Aquilaria malaccensis*, *Aquilaria subintegra* and *Aquilaria crassna*. Representational difference analysis (RDA) used in this study is a subtraction approach that removes common sequences between the tester and driver DNA. Three RDA rounds successfully identified a total of ten RDA markers. BLASTn analysis showed six RDA sequences without a significant hit. Two RDA sequences shared 94.68% identity and the remaining two sequences hit an RDA marker from *Aquilaria malaccensis* and an mRNA of *Duriozibethinus*, respectively. Clustal W multiple alignments between the selected homologous sequences of *Aquilaria* genus show the difference in their nucleotide sequences. In the agarwood industry, choosing the correct species is very useful to maintain the quality and yield of agarwood produced by the next generations, either by conventional breeding or tissue culture micropropagation approaches.

Keywords **—*Aquilaria hirta*, Representational difference analysis, DNA tester, DNA driver, DNA marker.**

----------**********----------

I. INTRODUCTION

*Aquilaria hirta*Ridl. is naturally found in the tropical rain forest of Peninsular Malaysia, together with other local species, including *Aquilaria malaccensis*Lamk., *Aquilaria rostrata*Ridl., *Aquilaria beccariana* Van. Tiegh and *Aquilaria macrocarpa*Baill. [1]. The perfumery industry uses agarwood from these *Aquilaria* plants as an essential fragrance ingredient [2]. Wounded barks and the trunks of *Aquilaria* trees produce agarwood in the form of dark-coloured resin [3]. In their natural habitat deep in the forest, agarwood formation occurred naturally either by physical wounding, such as lightning and followed by microbial infection [4,5]. However, the agarwood formation by natural wounding takes a long time and is unsuitable to fulfill the high demand for agarwood from the perfumery industry [6]. Since hundreds of years ago, continuous deforestation for agarwood has led to the endangered *Aqualaria* plants in their natural habitats and finally being listed as endangered plant species [7,8].

Conservation of *Aquilaria* spp. in their natural habitats is critical to prevent the total loss of valuable species [6]. Interestingly, the continuous

 ©IJSRED:All Rights are Reserved

effort in these two decades by government agencies, local entrepreneurs, and individuals in the domestication of *Aquilaria* plants in resources countries enhanced the development of the agarwood industry, especially in South-East Asia [3,6]. Establishing large agarwood plantations is the best option to fulfill the high demand for agarwood from perfumery industries without disturbing the small number of remaining *Aquilaria* plants in the forest. However, agarwood production of the planted plants is challenging to optimize due to the complicated process of agarwood formation that only occurs after given physical stresses [3]. Wounding the grown *Aquilaria* plants with physical methods and inoculation with chemicals or biological agents leads to agarwood formation [9,10]. Extensive research into the relation of injured and inoculated *Aquilaria* plant defense mechanisms is interesting to explore for maintaining the agarwood's quality [11].

In these two decades, research on the genetic markers on *Aquilaria* spp. was extensively studied by some researchers due to difficulty in species determination based on their morphological structures [12,13]. Previously, PCR-amplified sequences of the trn-L-trn-F intergenic spacer of selected *Aquilaria* spp. were used to construct a phylogenetic tree to investigate their genetic relationship [14]. Besides that, in *Aquilaria crassna*, four microsatellite markers have been developed as the starting point for forensic identification of agarwood traded materials [15]. Researchers from Forest Research Institute Malaysia (FRIM) reported 17 identified polymorphic microsatellite loci for *A. malaccensis* for the species' conservation strategy [16]. This paper focused on the molecular identification of unique DNA sequences of *A. hirta* isolated using the Representational Difference Analysis (RDA) approach. This DNA subtraction approach successfully eliminates the common DNA sequences shared between *A. hirta* and selected species. The discovery of all the unique DNA sequences of *A. hirta* will be helpful for species identification of Malaysian *Aquilaria* spp. for breeding, micropropagation and conservation purposes.

II. MATERIALS AND METHODS

A. Plant Materials

Leaves from four different *Aquilaria* spp. (*A. hirta*, *A. malaccensis*, *Aquilaria subintegra* and *Aquilaria crassna*) were collected from 4 years old mother plants in an agarwood orchard located at Sungai Merab, Bangi, Selangor, Malaysia. Each 0.2 g of collecting leaves was wrapped in aluminium foil and quick-frozen in liquid nitrogen before being stored in a -80 °C freezer for further use.

B. Preparation of Genomic DNA

Leaf samples stored at -80 ºC freezer were ground with liquid nitrogen using mortar and pestle until fine powder form. Genomic DNA from all four species was extracted from their leaves using the GeneAllExgene Plant SV mini kit (GeneAll, Korea). The genomic DNA samples were stored in a -20 °C freezer for subsequent RDA experiments.

C. Testers and Drivers Preparation

In this subtraction study (Table 1), tester DNA used was the genomic DNA extracted from *A. hirta*. In contrast, the driver DNA combines genomic DNA (1: 1: 1) from another three *Aquilaria* species (*A. malaccensis*, *A. subintegra* and *A. crassna*). Enzymatic digestion of all drivers and testers was done with *Bam*HI, *Hind*III and *Bgl*II in a preset 37 ℃ water bath incubator for 3 hours, followed by a purification with phenol: chloroform: isoamyl alcohol (25: 24: 1). A centrifugation step was performed at 12,000 rpm for 20 minutes to precipitate the purified digested DNA samples.

TABLE 1. SAMPLES' ARRANGEMENT IN RDA EXPERIMENT.

RDA set	Restriction Enzyme	Tester (gDNA)	Driver (combined gDNA)
1	*Bam*HI	*A.hirta*	*A.malaccensis*, *A.crassna* and *A. subintegra* (gDNA ratio 1: 1: 1)
2	*Hind*III	*A.hirta*	*A.malaccensis*, *A.crassna* and *A.subintegra* (gDNA ratio 1: 1: 1)
3	*Bgl*II	*A. hirta*	*A.malaccensis*, *A.crassna* and *A. subintegra* (gDNA ratio 1: 1: 1)

Subsequently, a centrifugation step applied to remove impurities were removed by using 80 % (v/v) ethanol to the precipitated DNA samples during the washing step. Another centrifugation step was carried out at 12,000 rpm for 20 minutes.

 ©IJSRED: All Rights are Reserved

Recovered DNA pellets were dissolved in 30 µl TE buffer by a thorough pipetting.

D. Representational difference analysis (RDA)

RDA experiments were applied to all the prepared drivers and testers by following the protocol previously described in oil palm [17] and orchids [18] with minor modifications. A total of three rounds of RDA (Table 2) was carried out with the respective ratios of the tester to the driver. The first round of RDA was carried out by enzymatic digestion of PCR-amplified testers and drivers, followed by a ligation step with J adaptors. Tester for each RDA set (30 ng) was subjected to hybridization with 3,000 ng of the respective driver. It was followed by a denaturation step at 95 °C for 5 minutes and a subsequent hybridization step at 67 °C for 20 hours. The successfully hybridized driver-tester sets were diluted with TE buffer to a final volume of 200 µl.

TABLE 2. RATIO TESTER TO DRIVER.

RDA set	Restriction Enzyme	Ratio tester to driver		
		1st round RDA	2nd round RDA	3rd round RDA
1	*Bam*HI	1: 100	1: 1000	1: 100,000
2	*Hind*III	1: 100	1: 1000	1: 100,000
3	*Bgl*II	1: 100	1: 1000	1: 100,000

Ten microliters of each hybridized tester-driver set were PCR-amplified in 100 µl PCR reaction using PCR master mix (GeneAll, Korea). The PCR cycle was carried out by pre-denaturation at 94 °C for 3 minutes; 35 cycles of two-step PCR (denaturation at 94 °C for 1 min, annealing and extension at 72 °C for 2 min). The final extension was performed at 72 °C for 5 minutes. The PCR amplified DNA was then purified by phenol: chloroform: isoamylalcohol (25: 24: 1) extraction, followed by ethanol precipitation. The precipitated DNA pellets were dissolved in 40 µl of TE buffer. Mung Bean Nuclease (MBN) digestion removed single-stranded DNA from the tester-driver amplified products.

Second round RDA was performed in the tester to driver ratio of 1:1000 using the first round RDA products as drivers. The enzymatic restrictionreaction was performed with *Bam*HI, *Hind*III and *Bgl*II, respectively. Purified testers and drivers at this point were then ligated with subsequent adapters (N adapters). The steps were done in the same manner until MBN digestion. J adaptors were subsequently used in the third round of RDA with 1: 100,000 the ratio of the tester to the driver.

Final products from the third round RDA were subjected to agarose gel electrophoresis, ligation into pGEM-T Easy Vector (Promega, USA), transformation into DH5α *Escherichia coli* competent cells, plasmid DNA extraction and single-pass DNA sequencing using universal T7 and SP6 primers. The sequences were submitted to the NCBI GenBank and compared to their homologous sequences using the BLASTn tool.

III. RESULTS AND DISCUSSION

A. Isolated products from three rounds of RDA

In this study, RDA was chosen as a DNA subtraction approach to obtain unique DNA sequences in *A. hirta* compared to other species in the *Aquilaria* genus. This RDA experiment aimed to target DNA sequences that are not available or have low similarity to other *Aquilaria* species based on the use of genomic DNA in a 1: 1: 1 combined ratio of *A. malaccensis*, *A. crassna* and *A. subintegra*. Based on the RDA principle, several rounds of the subtraction process can identify DNA sequences that are only available in a genome (tester) but not available in another genome (driver). The success of this subtraction method could be due to the restriction site polymorphisms that do not allow hybridization between tester and driver, allowing the appearance of the non-hybridized product as subtracting sequence [17].

In this study, genomic DNA extracted from the leaves of *A. hirta* was chosen as tester DNA due to the target to isolate sequences that are unique for this species. For the tester, the combination of genomic DNA isolated from three *Aquilaria* species was used as a driver due to the target to eliminate common sequence in the genome of *A. malaccensis* (local Malaysian species) and imported species (*A. crassna* and *A. subintegra*). Unfortunately, the other Malaysian local species, including *A. rostrata*, *A. beccariana* and *A. microcarpa* were not chosen in this study due to the unavailability of the samples for the RDA experiment was conducted in our

 ©IJSRED: All Rights are Reserved

laboratory. In addition, all the tree species were not commercially planted in Malaysian agarwood plantations, besides the difficulty of identifying the species compared to *A. malaccensis* and *A. hirta*.

The RDA products observed from the first, second and third rounds are shown in Figure 1. In Figure 1A, the first round of RDA (Figure 1A), smears were obtained from each RDA reaction ranging from 500 bp to 10 kb. A similar smear pattern was obtained for both RDA set 2 and RDA set 3 initially digested with *Hind*III and *Bgl*II, respectively. This presence of smears instead of high intensity of amplified DNA is expected since the first round RDA focuses on preparing a 'tester' for the subsequence round of the RDA cycle. For RDA set 1 that was initially digested with *Bam*HI, an intense DNA band was observed in an estimated size of ~800bp with another two faint DNA bands. However, all the bands were not purified because the experiment targeted the final products from the third round RDA.

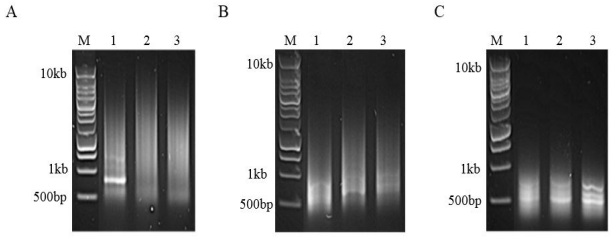

Figure 1: RDA products were obtained from first-round RDA (A), second-round RDA (B) and third-round RDA (C). Lanes M: 1kb DNA marker (Vivantis, Malaysia); Lanes 1: RDA set 1; Lanes 2: RDA set 2; Lanes 3: RDA set 3.

In the subsequent second round RDA (Figure 1B), the smaller size of smears observed for all three sets of RDA. Meanwhile, in RDA set 1, a smaller smear size compared to RDA set 2 and 3 was observed. Interestingly, for both RDA sets 2 and 3, several faint DNA bands with the estimated size ~700-100 bp started to appear. In addition, several faint RDA products with smaller sizes compared to RDA set 2 and 3. However, none of the products from the second round RDA were selected for purification. The aim of the final product is expected in third round RDA as previously studied in fragrant orchids *Vanda* Mimi Palmer, *Vanda* Small Boy Leong and *Vandachostylis* Sri-Siam against non-fragrant orchids *Vanda* Tan Chay Yan [18].

In the third-round RDA (Figure 1C), three intense DNA bands were observed in RDA set 3 as successful output from the three rounds of RDA with *Bgl*II restriction enzymes used in all three cycles of subtraction. In addition, less intensity final RDA products were observed in both RDA set 2 (digestion with *Hind*III) and RDA set 1 (digestion with *Bam*HI). A total of ten final products (Figure 2) were successfully purified from agarose gel, cloned into pGEM-easy vector (Promega,USA) and transformed into DH5α *E. coli* competent cells. All the RDA products are 200-500 bp estimated size range. This short size of RDA product is expected because a similar size range is shown in orchids with 100-600 bp and oil palm with 158-291 bp [17,18].

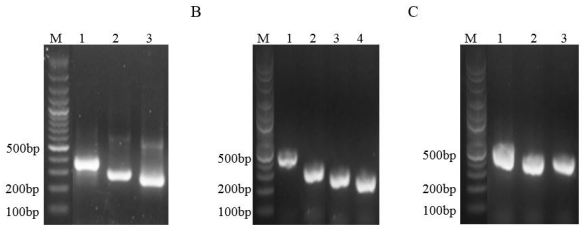

Figure 2: Final isolated RDA products from RDA set 1 (A), RDA set 2 (B) and RDA set 3 (C). Lanes M: 1kb GeneRuler™ DNA ladder (Fermentas, USA); Lanes 1, 2, 3 and 4: isolated RDA products.

B. Sequence analysis of RDA products from A. hirta

Elimination of common sequence available in *A. hirta* with all the testers (*A. malaccensis*, *A. crassna* and *A. subintegra*). After sequencing using SP6 and T7 primers, the size of the final RDA product that successfully subtracted out is 177-375 bp (Table 3). The size of the RDA products is slightly smaller than the visualized gel photo in Figure 2. In DNA sequencing analysis, removing the remaining sequence was conducted for J adaptors and *Bam*HI, *Hind*III and *Bgl*II restriction sites.

TABLE 3. SIZE AND ACCESSION NUMBER OF RDA MARKERS.

Marker	Size (bp)	Accession Number
RDA 11	361	KF208305
RDA 12	282	KF208306
RDA 13	245	KF208307
RDA 14	361	KF208308

 ©IJSRED: All Rights are Reserved

RDA 15	309	KF208309
RDA 16	210	KF208310
RDA 17	177	KF208311
RDA 18	375	KF208312
RDA 19	282	KF208313
RDA 20	269	KF208314

BLASTn analysis on all the ten isolated RDA products is shown in Table 4, named RDA 11 to RDA 20. The numbering is continuity from RDA 1 - RDA 10 (GenBank accession numbers: KF20895-208304) isolated from *A. malaccensis*, another species in the same genus. Homologous sequence analysis using the BLASTn tool shows six RDA markers (RDA 13, RDA 14, RDA 15, RDA 16, RDA 18 and RDA 20) did not hit to DNA sequences available in the NCBI GenBank.

TABLE 4. BLASTN ANALYSIS OF RDA MARKERS.

Marker	Top hits	Sequence coverage	E-value score	Identity (%)
RDA 11	RDA marker 1-sp6, KF208305.1 [*A.malaccensis*]	100%	$2e^{-150}$	94.18
RDA 12	RDA marker 19-sp6, KF208313.1 [*A.hirta*]	100%	$8e^{-119}$	94.68
RDA 13	No significant similarity	-	-	-
RDA 14	No significant similarity	-	-	-
RDA 15	No significant similarity	-	-	-
RDA 16	No significant similarity	-	-	-
RDA 17	transmembrane 9 superfamily member 7-like mRNA, XM_022869051.1 [*Duriozibethinus*]	94%	$2e^{-48}$	88.62
RDA 18	No significant similarity	-	-	-
RDA 19	RDA marker 12-sp6, KF208306.1 [*A.hirta*]	100%	$8e^{-119}$	94.68
RDA 20	No significant similarity	-	-	-

The no similarity hit of the RDA sequences to any sequence in the NCBI GenBank reflects their potential to be used as DNA markers for *A. hirta*. In the future, the markers could be used as molecular markers for validation of *A. hirta* mother plants for selective breeding, micropropagation and species conservation purposes.

The remaining for *A. hirta*'s RDA markers showed similarity to at least one homologous DNA sequence in the NCBI GenBank. RDA 11 marker shows top hit to RDA marker 1 from *A. malaccensis*with 94.18 % identity and followed by chromosome 2 mitochondrion, complete sequence from *Aquilaria sinensis* with 93.79 % identity. Figure 3 shows Clustal W multiple alignments between RDA 11 of *A. hirta* with RDA 1 from *A. malaccensis* and the genomic DNA sequence from *A. sinensis*. The analysis shows a missing region at positions 284 to 290 in *A. malaccensis* and *A. sinensis*. The additional region in the sequence of RDA 11 of *A. hirta* could be useful to be studied in the future for *A. hirta*'s confirmation study, validation of pure species and lineage tracking of *A. hirta*'s hybrids. Besides, in positions 244-255, a total of nine missing nucleotides in the genome of *A. sinensis*.

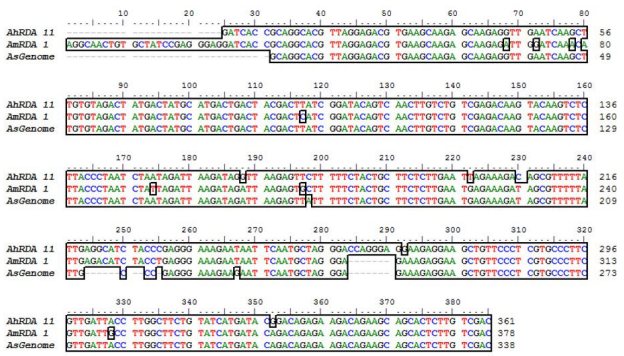

Figure 3: Clustal W multiple alignments between AhRDA 11 (RDA 11 from *Aquilaria hirta*), AmRDA 1 (RDA 1 from *Aquilaria malaccensis*: GeneBank Accession No. KF208295.1) and AsGenome (genomic sequence from the *Aquilaria sinensis* genome: GeneBank Accession No. MT318703.1).

Interestingly, in *A. malaccensis* all the nucleotides are available based on the sequences of RDA 1. This interesting DNA information is helpful in future studies on genetic polymorphisms between all species in *Aquilaria* genus. BLASTn analysis also shows that two isolated RDA sequences (RDA 12 and RDA 19) share 96.8% identity (Table 2). The difference in the sequences is shown in Figure 4.

 ©IJSRED: All Rights are Reserved

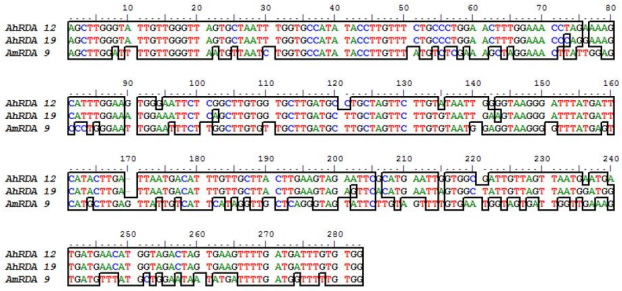

Figure 4: Clustal W multiple alignments between AhRDA 12 and AhRDA 19 from *Aquilaria hirta* with AmRDA 9, RDA markers from *Aquilaria malaccensis* (GenBank Accession No. KF208303.1)

In addition, both sequences are aligned together with RDA 9 (GenBank accession number: KF208303) from *A. malaccensis* with 88.62% identity to RDA 12 and RDA 19. Meanwhile, RDA 17 show 88.62% identity to transmembrane 9 superfamily member 7-like mRNA of *Duriozibethinus* (GenBank accession number: XM_022869051.1), 86.83% identity to transmembrane 9 superfamily member 7-like mRNA of *Hibiscus syriacus* (GenBank Accession number: XM_039149886.1) and 85.63% identity to transmembrane 9 superfamily member 7 mRNA of *Gossypium hirsutum* (GenBank Accession number: XM_016832970.1). The percent identity shared by RDA 17 of *A. hirta* and all the three species is expected due to the complete genome of all the three plant species *Duriozibethinus*, *Hibiscus syriacus* and *Gossypium hirsutum*. This might suggest the location of RDA 17 in transmembrane 9 superfamily member 7 mRNA of *A. hirta*. However, confirmation could only be done when the genome of *A. hirta* is fully sequenced in the future. The current available next-generation sequencing (NGS) with affordable cost could allow an extensive *A. hirta*'s genome sequencing.

BLASTx analysis of all the RDA sequences is shown in Table 5. Five RDA markers show sequence homology to known functional proteins with identity 44.12 % to 94.83 %. Meanwhile, the remaining five RDA sequences hit to amino acid sequences of any protein. Proteins works on *Aquilaria species* are still minimal even though there are many reports on the fragrance constituents of agarwood produced by *Aquilaria* species.

TABLE 5. BLASTX ANALYSIS OF RDA MARKERS.

Marker	Top hits	Sequence coverage	E-value score	Identity (%)
RDA 11	No significant similarity	-	-	-
RDA 12	uncharacterized protein isoform X2 [*Arachis ipaensis*]	36%	1.5	44.12
RDA 13	No significant similarity	-	-	-
RDA 14	No significant similarity	-	-	-
RDA 15	related to DEAD/DEAH box helicase [*Ramularia collo-cygni*]	100%	$3e^{-47}$	91.26
RDA 16	glutathione s-transferase like protein [*Zymoseptoria brevis*]	68%	$1e^{-05}$	86.36
RDA 17	Transmembrane nine 7 isoform 4 [*Theobroma cacao*]	98%	2e-30	94.83
RDA 18	No significant similarity	-	-	-
RDA 19	No significant similarity	-	-	-
RDA 20	Retrovirus-related Pol polyprotein from transposon RE1 [*Vitis vinifera*]	99%	$6e^{-33}$	61.80

Based on our sequence search of the complete genome sequence of *Aquilaria* species that are available in the NCBI GenBank, only *A. sinensis* (GenBank accession: VZPZ00000000.1) and *Aquilaria agallochum* (GenBank accession: JMHV00000000.1) complete genomes are publicly available. Unavailability of the complete genomes of other *Aqualaria* species could be due to the incomplete genome sequencing, challenging working with the plants or the data on genomic DNA of each species is not publicly released due to their high commercial value in the agarwood industry.

 ©IJSRED: All Rights are Reserved

IV. CONCLUSIONS

This work reports the output of three round RDA cycles involving driver (*A. hirta*) and tester (combination of genomic DNA from other three *Aquilaria* species which are *A. malaccensis*, *A. crassna* and *A. subintegra*). From the RDA experiment, a total of ten potential RDA markers in size range of 177-375 bp were successfully identified. BLASTn analysis showed six sequences without significant similarity to any DNA sequences available in the NCBI GenBank. Two RDA markers shared 94.68 % identity (RDA 12 and RDA 19). An RDA marker (RDA 11) hit to another RDA marker from *A. malaccensis*. Another RDA marker (RDA 17) hit superfamily member 7-like mRNA of *Duriozibethinus*.

V. ACKNOWLEDGEMENT

The authors would like to thank the Ministry of Higher Education, Malaysia for Exploratory Research Grant Scheme (ERGS) RDU110601 that supported this exploratory research.

REFERENCES

[1] Lee, S.Y. and Mohamed R. (2016) in Agarwood, tropical forestry: The origin and domestication of *Aquilaria*, an important agarwood-producing genus (Mohamed, R., ed.) Springer, Singapore, pp. 1-20.

[2] Ali, N.A.M., Jin, C.B, Jamil, M. (2016) in Essential oils in food preservation, flavor and safety: Agarwood (*Aquilaria malaccensis*) oils (Preedy, R., ed.) Academic Press, San Diego, pp. 173-180.

[3] Chong, S.P., Osman, M.F., Bahari, N., Nuri, E.A., Zakaria, R. and Abdul-Rahim, K. (2015) Agarwood inducement technology: A method for producing oil grade agarwood in cultivated *Aquilaria malaccensis*Lamk. J. Agrobiotech. 6, 1-16.

[4] Naef, R. (2011) The volatile and semi-volatile constituents of agarwood, the infected heartwood of *Aquilaria* species: A review. Flavour and Fragrance J. 26 (2), 73-87.

[5] Azzarina, A.B., Mohamed, R., Siah, C.H. and Wong, M.T. (2017) Temporal expression of a putative homogentisatesolanesyltransferase cDNA in wounded *Aquilaria malaccensis*, an endangered tropical tree. Pertanika J. Trop. Agric. Sci. 40 (3), 351-366.

[6] Rasool, S. and Mohamed, R. in Agarwood, tropical forestry: Understanding agarwood formation and its challenges (Mohamed, R., ed.) Springer, Singapore, pp. 39-56.

[7] CITES (2013). Sixteenth Meeting of the Conference of the Parties Regarding of Amendment of Appendices and Inclusion of Species in Appendix II. Geneva: CITES Secretariat.

[8] Karlinasari, L. and Nandika, D. (2016) in agarwood, tropical forestry: Acoustic-based technology for agarwood detection in *Aquilaria* trees (Mohamed, R., ed.) Springer, Singapore, pp. 137-148.

[9] Pojanagaroon, S. and Kaewrak, C. (2005) Mechanical methods to stimulate aloes wood formation in *Aquilaria crassna* Pierre ex H Lec (Kritsana) trees. Acta Hort. 676, 161-66.

[10] Nobuchi, T. and MohdHamami, S. (2008) The formation of wood in tropical trees: a challenge from the perspective of functional wood anatomy, PenerbitUniversiti Putra Malaysia, Serdang.

[11] Turjaman, M., Hidayat, A. and Santoso, E. (2016) in Agarwood, tropical forestry: Development of agarwood induction technology using endophytic fungi (Mohamed, R., ed.) Springer, Singapore, pp. 57-72.

[12] Lee, S.Y., Weber, J. and Mohamed, R. (2011) Genetic variation and molecular authentication of selected *Aquilaria* species from natural populations in Malaysia using RAPD and SCAR markers. Asian J. Plant Sci. 10 (3), 202-211.

[13] Toruan-Mathius, N., Rahmawati, D. and Anidah (2009) Genetic variations among *Aquilaria* species and *Gyrinopsversteegii* using amplified fragment length polymorphism markers. Biotropia 16 (2), 88-95.

[14] Eurlings, M.C.M. and Gravendeel, B. (2005). TrnL-trnF sequence data imply paraphyly of *Aquilaria* and *Gyrinops* (Thymelaeaceae) and provide new perspectives for agarwood identification. Plant Syst. Evol. 254, 1-12.

[15] Eurlings, M.C.M., Heveling van Beek, H. and Gravendeel, B. (2009) Polymorphic microsatellites for forensic identification of agarwood (*Aquilaria crassna*). Forensic Sci. Int. 197, 30-34.

[16] Tnah, L.H., Lee, C.T., Lee, S.L., Ng, K.K.S., Ng, C.H., Nurul-Farhanah, Z., Lau, K.H. and Chua, L.S.L (2012) Isolation and characterization of microsatellite markers for an important tropical tree, *Aquilaria malaccensis*(Thymelaeaceae). Am. J. Bot. 99 (11), 431-433.

[17] Sarpan, N., Ooi, S.E., Ong-Abdullah, M., Ho, C.L., Chin, C.F. and Namasivayam, P. (2013) Representational difference analysis (RDA) for the identification of DNA markers associated with tissue culture amenity in oil palm. J. Oil Palm Res. 25 (3), 305-313.

[18] Mohd-Aiman, B., Mohd-Hairul, A.R. and Hian, T.S. (2015) Identification of fragrance-related transcripts from selected orchids using cDNA representational difference analysis (cDNA-RDA) approach. Int. J. Interdiscip. Multidiscip. Stud. 2 (4), 198-207.

 ©IJSRED: All Rights are Reserved