BIODIVERSITAS
Volume 23, Number 9, September 2022
Pages: 4861-4871

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d230955

Characterization and identification of agarwood-producing plants (*Aquilaria* spp.) from North Aceh, Indonesia, based on morphological and molecular markers

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Manuscript received: 6 July 2022. Revision accepted: 17 September 2022.

Abstract. *Lukman, Dinarti D, Siregar UJ, Turjaman M, Sudarsono. 2022. Characterization and identification of agarwood-producing plants (*Aquilaria *spp.) from North Aceh, Indonesia, based on morphological and molecular markers. Biodiversitas 23: 4861-4871.* Agarwood is one of the non-timber export commodities important as raw materials for bioindustry. There are 26 agarwood-producing plant species in Indonesia belonging to three families (Thymelaeaceae, Euphorbiaceae, and Fabaceae). This study aims to identify the agarwood-producing plant species in North Aceh District, Aceh Province, Indonesia, based on their morphological and molecular characters. The evaluated morphological characteristics include the bark and leaf structures of endemic plants. Molecular characterization was carried out by sequencing of *mat*K, ITS, and *trn*L-*trn*F genes. The collected data were analyzed using the appropriate software, such as R software, BlastX, Geneious Prime and MEGA X. The evaluated 66 accessions were clustered into two major groups based on their morphology. Group 1 consisted of 10.61% of the evaluated accessions is identified as *Aquilaria beccariana*. Group 2 was further classified into two subclusters (Group 2a and 3b). Group 2a consisted of 10,61% of the accessions as *A. malaccensis* and Group 2b consisted of 78,79% of the accessions as *A. microcarpa*. All samples are identified as *A. malaccensis* based on the *mat*K DNA sequences. Meanwhile, Groups 1 are identified as *A. beccariana* and Group 2a and 2b as *A. microcarpa* based on the ITS sequences. Based on the *trn*L-*trn*F sequences, Groups 1 and 2a was identified as *A. malaccensis* and Group 2b as *A. microcarpa*.

Keywords: Aceh Province, Agarwood, *Aquilaria*, characterization, genetic diversity, identification

INTRODUCTION

Agarwood is a dark fragrance wood obtained from injured trees of several species in the *Aquilaria* and *Gyrinops* genera and of the Thymeleaceae family (Nguyen et al. 2014; Faizal et al. 2017; Liu et al. 2017; Siregar et al. 2017). The aromatic agarwood properties have been used as fixative material (Santoso 2015) and raw materials in many consumer products (Lee et al. 2016a). The agarwood has also been used in various religious events (Kang 2021) and illness treatments (i.e. as a sedative, carminative, and to relieve gastric problems, coughs, rheumatism, and high fever (Liu et al. 2017), antibacterial and acetylcholinesterase inhibitory activities (Li et al. 2015).

Kalra and Kaushik (2017) mentioned that more than 250 compounds were identified from agarwood. The five groups of agarwood comounds include sesquiterpene (cis-jasmone and aroma-dendrenepoxide); chromone (8-methoxy-2-(2-phenyl-ethyl) chromen-4-1, and the newly discovered chromone derivative, 7-[benzyloxy]-5-hydroxy-2-methyl-chromone; aromatic (benzyl-acetone, guaiacol, p-ethyl-guaiacol, phenol, syringaldehyde, vanillin, furfuryl alcohol, and furfural); fatty acid (palmitic acid, oleic acid, and lauric acid), and triterpene (squalene) groups (Nasution et al. 2019). Tiana et al. (2019) mentioned that more than 150 sesquiterpenoids have been identified from gaharu and are classified into eight main types: agarofuran, agarospirane, guaiane, eudesmane, eremophilane, cadinene, prezizaane, and acorane. The specific compounds of agarwood responsible for the distinctive scent are agarospirol, dehydrojinkoh-eremol, baimuxinal, selina-3,11-dien-9-ol, selina-3,11-dien-14-al, selina-3,11-dien -14-ol, selina-4,11-dien-14-al, guaia-1(10),11-dien-15-ol, dihydrokaranone, and guaia-1(10),11-dien-15-al (Hidayat et al. 2021).

The high demand for agarwood and the limited supply of natural agarwood products has made agarwood-producing countries unable to meet market needs (Hidayat et al. 2020). As an agarwood exporter, Indonesia can only contribute as much as 27% of 2018 worldwide demand, and Indonesia's contribution continues to decrease because it has become more challenging to obtain agarwood from

the natural forest (Hidayat et al. 2020). The agarwood from Indonesia is exported to Singapore and the Middle East, and they re-export the product to other countries (ASGARIN 2021).

The agarwood's high price has driven extensive agarwood-producing plant exploitation in the surrounding forest area and resulted in only a few surviving agarwood-producing trees due to non-selective logging (Kang 2021). To prevent over-exploitation, the Convention on the International Trade in Endangered Species of Wild Flora and Fauna (CITES) (CITES 2004) and the International Union for Conservation of Nature (IUCN) have included the endemic Indomalaysia *Aquilaria* as protected plant species (Lee et al. 2016a). The CITES has stated that all *Aquilaria malaccensis* Lam. species have been included in Appendix II since 1994 (CITES 2013).

In Indonesia, there have been at least 26 agarwood-producing plant species belonging to the three families, such as Thymelaeaceae (*Aquilaria* sp., *Gyrinops* sp., *Enkleia* sp., *Gonystylus* sp., and *Wikstroemia* sp.), Euphorbiacea (*Excoecaria* sp.) and Fabaceae (*Dalbergia* sp.) (Santoso 2015). There has been no published report of agarwood-producing plant species endemic to North Aceh, Indonesia. However, North Aceh local farmers have informed that the Sawang Sub-district, North Aceh District, Aceh Province, Indonesia, is one of the areas producing superior quality agarwood. For species conservation and commercial development, finding information on the agarwood-producing, North Aceh endemic plant species will be necessary.

Agarwood-producing plants have high species diversity. The species can be characterized and identified based on morphological and molecular markers (Parker and Helmstetter 2017). The collection of morphological character data is more straightforward and does not require modern technology, but morphological characters have limitations in distinguishing genetically similar individuals and require extensive observation of mature plants (Hariyati et al. 2013). Collecting the morphological characteristics are more straightforward and do not need modern technology, but morphological traits have limitations to differentiating genetically similar individuals and require extensive observation in mature plants (Hariyati et al. 2013). The negative side of morphological characterization includes limited ability to distinguish the diversity and the presence of environmental factor effects (Wang et al. 2010). Identification based on molecular markers is more accurate and generates more relevant data (Kress and Erickson 2012). The molecular marker identification could also be used to differentiate the genotype of each evaluated accession (Li et al. 2018), and it can potentially be generated using any plant tissue (Kumar et al. 2016; Lee et al. 2016a). DNA markers are independent of morphological characters, plant age, development, and environmental factors and can distinguish morphologically similar species (Lim 2015).

Plant species identification can be made by isolating and sequencing the PCR-generated DNA fragments of a short gene(s) from a standard genome (Hubert and Hanner 2015). The coding DNA sequences have been used for ecological surveys, taxa identification and medicinal plant sample confirmation (Xue and Li 2011). The *mat*K gene can identify gaharu species that encode the maturase K enzyme by secreting introns from the chloroplast genome (Tanaka and Ito 2019). Lee et al. (2016b) the matK gene can differentiate at the intra-species level and ITS and trnL-trnF can differentiate at the inter-species level. The ITS and *trn*L-*trn*F genes differentiate the intra-species levels (Lee et al. 2016b). The *mat*K, ITS and *trn*L-*trn*F DNA fragments have been used to characterize agarwood plant species in Malaysia (Lee et al. 2016b). The *mat*K gene has also been successfully used to characterize gaharu plants (Tanaka and Ito 2019). Characterization based on morphological and molecular characters could be used to identify endemic species of agarwood-producing plants found in North Aceh, Indonesia. This study aim to identify the agarwood-producing plant species in North Aceh District, Aceh Province, based on their morphological and molecular characters.

MATERIALS AND METHODS

The research was carried out in Sawang Sub-district, North Aceh District, Aceh Province, Indonesia (Figure 1), and the Plant Molecular Biology 1 Laboratory, Institut Pertanian Bogor, Bogor, West Java, Indonesia. Sampling locations range from 050.1743N to 05.07982N and 96.91976E to 9.934979E (GPS60 GARMIN data). Two activities were conducted, (1) Morphological characterization and identification of agarwood-producing plant species (Experiment I), and (2) Molecular identification of the species using sequence analysis of short gene(s) (i.e. *mat*K, ITS, and *trn*L-*trn*F genes (Experiment II).

Experiment I: Morphological characterization and identification

The samples of agarwood-producing plants were chosen based on agarwood formation (infected area). The sample plants exhibited such symptoms as yellow and falling leaves, small and thin branches, many broken and bumped branches, dry and brittle and easily pulled bark, and blackish-brown grooves on the trunk. Chips of the infected, black wood branch or stem parts produce a distinct fragrance. The selected agarwood-producing plants were morphologically characterized by clustering the plant group and identifying the species. The implementation method for qualitative data follows the procedure of (Susilo et al. 2014), and quantitative data uses the method of Wu et al. (2007) and Ellis et al. (2009). The quantitative parameters include leaf length, leaf width, petiole length, secondary leaf vein numbers, leaf angle, and the distance between the stalk and the widest leaf. The collected qualitative data include the leaf tip, base, edge shapes, color, leaf surface, and leaf vein structure shape. Data analysis was performed using R-studio.

Experiment II: Molecular identification

Molecular characterization was conducted to confirm and verify the species identity acquired based on

morphological characterization. The DNA fragments of the *mat*K, ITS, and *trn*L-*trn*F genes were PCR amplified using the total genome of the agarwood-producing plants as template DNA and the proper primers (Table 1). One accession representing each cluster generated from Experiment 1 was used as a sample in Experiment II. The DNA from leaf samples of agarwood-producing plants was isolated using the CTAB method (Gholibeigian 2021)

The *mat*K, ITS, and *trn*L-*trn*F gene fragments were sequenced and used to confirm the identity of the evaluated samples. The entire length of the *mat*K gene is 1533 bp (*Accession number* 04 *A. malaccensis*), while the ITS sequence is 791 bases (Accession number 22 *Aquilaria microcarpa* Baill.) and the *trn*L-*trn*F sequence is 964 bases. Accession number 43 *Aquilaria beccariana* Tiegh. out of the entire *mat*K gene sequences (1533 bp), the PCR amplification using *mat*K-specific primers generated 404 bp amplicon at the base position of 603 to 1184. The PCR amplification using ITS-specific primers generated a 646 bp amplicon at the base position of 19 to 665. Meanwhile, the *trn*L-*trn*F specific primers generated 487 bp amplicon starting from the base position of 487 to 958.

The *mat*K, ITS, and *trn*L-*trn*F gene fragments were PCR amplified in a Bio-rad T100 DNA Thermal Cycler using the following conditions: one cycle pre-denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, primer annealing at 65°C (*mat*K), 62.2°C (ITS), and 57.2°C (*trn*L-*trn*F) for 30 seconds, elongation at 72°C for 30 seconds, and final elongation at 72°C for 1 minute. The PCR products were then sent to Base Asia (Service (GENETIKA) cc.service@ptgenetika.com) for DNA sequencing. The BLAST analysis of nucleotide collections of the NCBI DNA Database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was conducted to determine the identity of the generated sequence data. Genetic distances estimation among identified sequences and phylogenetic tree construction were conducted using MEGA X software.

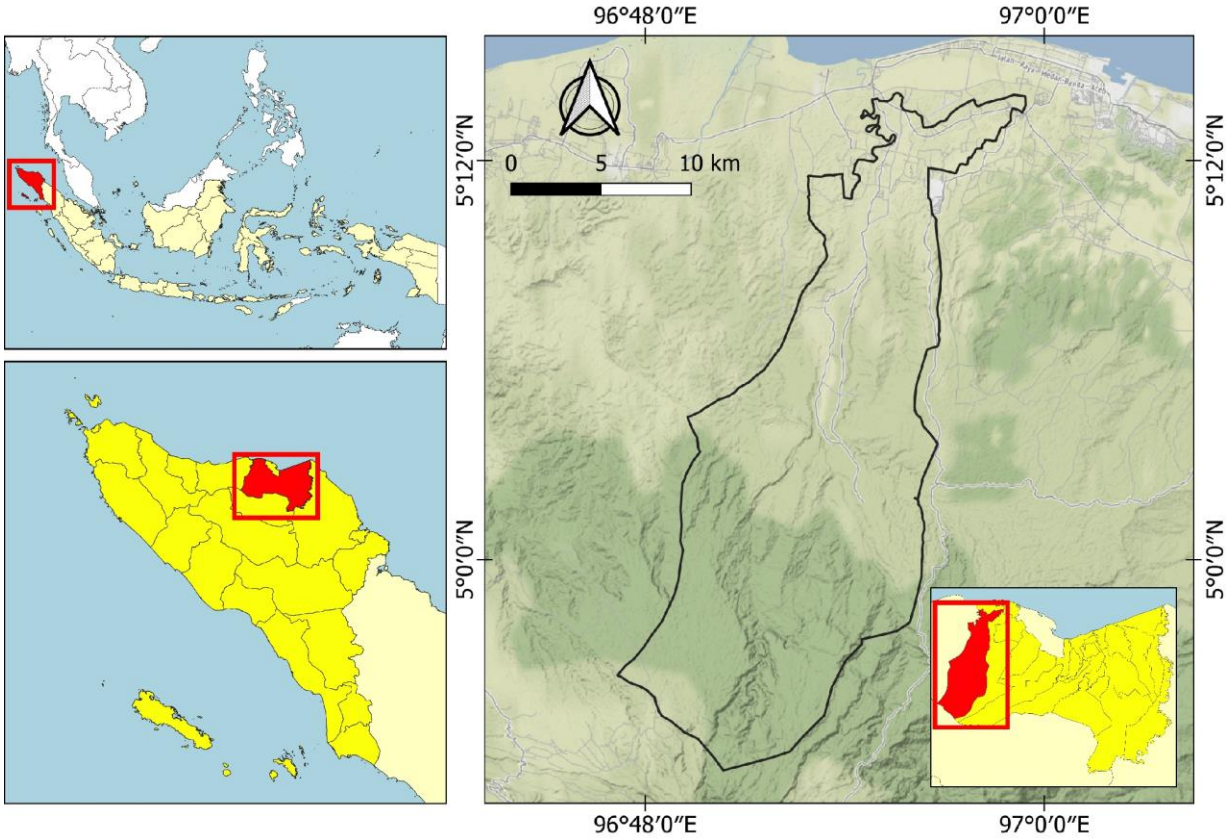


Figure 1. Study location map of agarwood producing (*Aquilaria* spp.) in Sawang Sub-district, North Aceh District, Aceh, Indonesia

Table 1. Gene-specific primer sequences used to PCR amplify the *mat*K, ITS, and *trn*L-*trn*F gene fragments and determine the identity of the evaluated agarwood-producing plants

Primer	Primer	Primer sequence (5'-3')	Number of bases	TM (°C)	Reference
*mat*K	D1	GCAATCTTTCTTGAACGGATCT	22	65	Tanaka and Ito (2019)
	D2	AATCGACCCAAGTTGGCTTA	20	65	
ITS	ITS92	AAGGTTTCCGTAGGTGAAC	19	62.2	Lee et al. (2016b)
	ITS75	TATGCTTAAACTCAGCGGG	19	62.2	
*trn*L-*trn*F	E	GGTTCAAGTCCCTCTATCCC	20	57.2	Lee et al. (2016b)
	F	ATTTGAACTGGTGACACGAG	20	57.2	

RESULTS AND DISCUSSION

Morphological characterization and identification

A Survey of agarwood-producing plants in the Pantee Pisang Hamlet, Teupin Reusep Village, North Aceh District, Aceh Province, Indonesia (Figure 1), based on the presence of agarwood (infected area) formation in the branch or stem, found as many as 66 plant accessions. Morphological character assessment conducted among the 66 accessions showed the presence of varied morphological characters (i.e. their stem, leaf, fruit, and seed characters). Representative morphological character variations evaluated among the agarwood-producing plants are presented in Figure 2. Moreover, the morphological data analysis results using R-software for the whole samples showed they were clustered into two major groups (Groups 1 and 2). Furthermore, the accessions belonging to Group 2 were further divided into sub-clusters (sub-cluster 2a and 2b). The Group 1 consisted of seven accessions (10.6%), namely: accession 42, 43, 52, 53, 55, 60, and 64. Group 2a also comprised seven accessions (10.6%), namely accession 21, 22, 23, 24, 25, 26, and 27. Group 2b comprised the other 52 accessions (78.8%) (Figure 3).

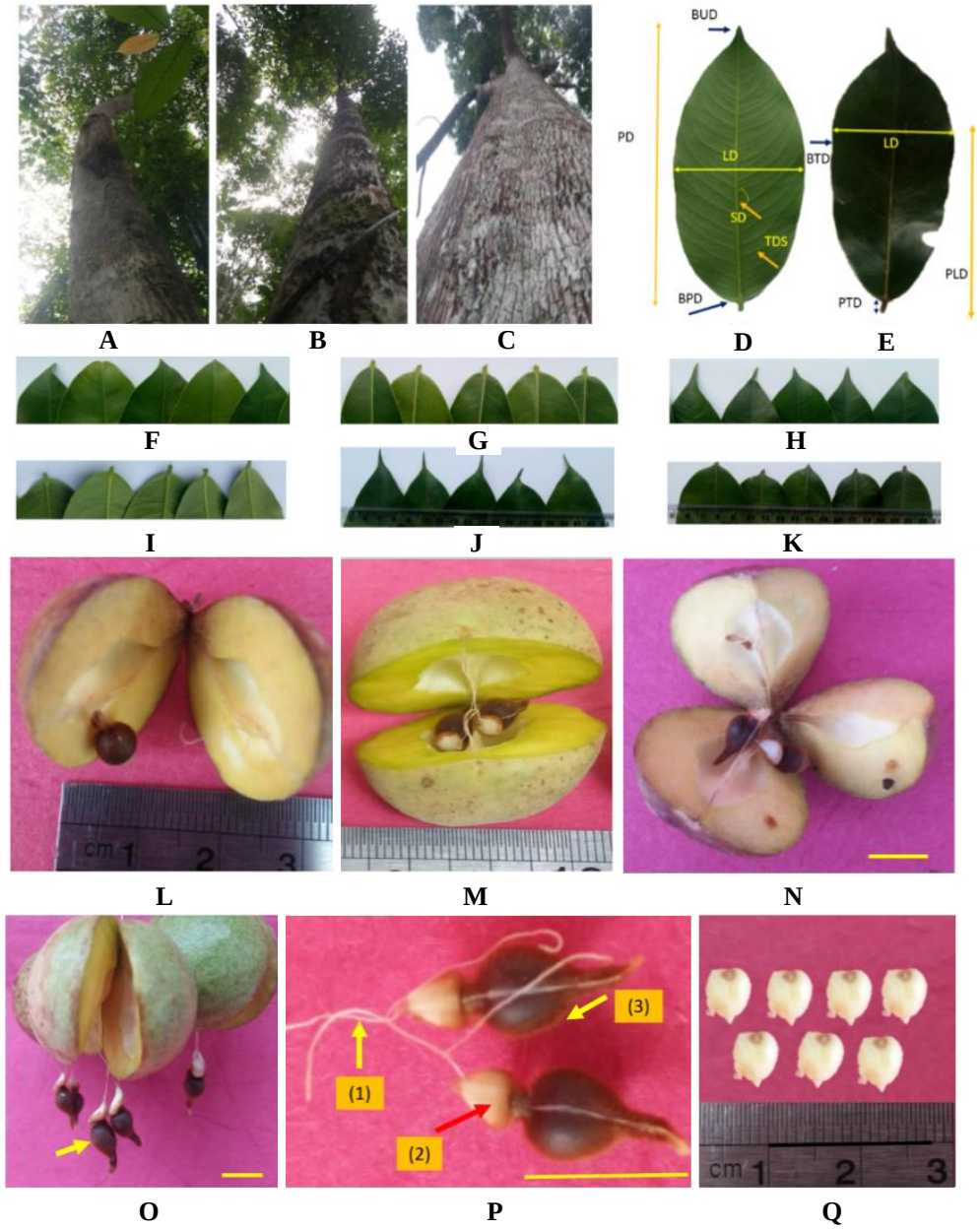


Figure 2. Morphological characteristics of agarwood-producing plants: The bark characteristics of (A) *Aquilaria beccariana*) (B) *A. malaccensis*, and (C) *A. Microcarpa*; (D) lower and E) upper leaf surfaces; leaf tip and leaf base of (F and G) *A. beccariana*, (H and I) *A. malaccensis*, and (J and K) *A. Microcapa*; fruits with (L) one, (M) two, and (N) three seeds; (O) seeds connected to the fruit, (P) whole seeds, and (Q) fertilized seeds with seed coats. PD: leaf length, BUD: leaf tip shape, LD: leaf width, SD: leaf angle, TDS: secondary leaf veins, BPD: leaf base shape, BTD: leaf margin shape, PTD: petiole length, PLD: leaf length, bar = 1 cm

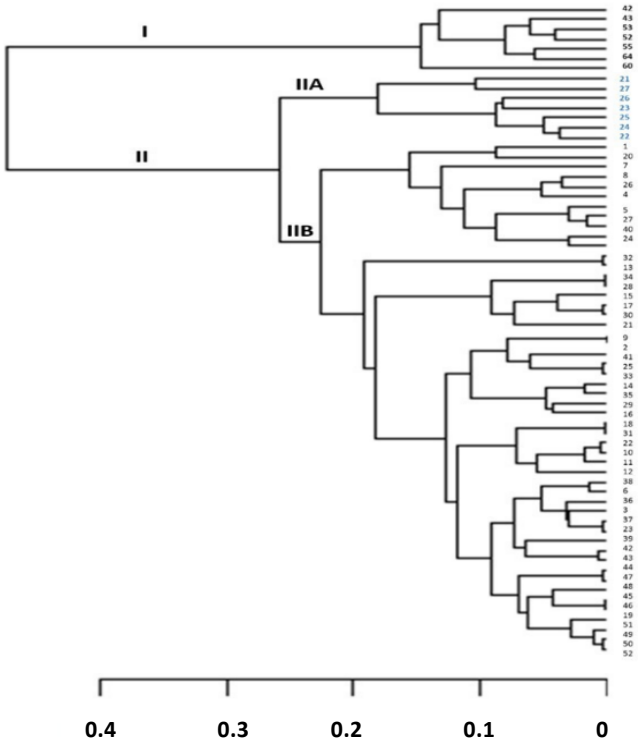


Figure 3. Results of cluster analysis results of 66 agarwood-producing plant accessions based on morphological characters

The morphological characters of Group 1 accessions include the elongated leaf type, elongated and broad leaf, with leaf length of 16.4-17.0 cm and leaf width of 5.8-6.4 cm; leaf tip shape is tapered to pointed with aspect ratio value >1. The leaf base shape is blunt, and the leaf edge shape is slightly wavy; the leaf color is green and thin, and the leaf surface is smooth. The petiole of Group 1 accessions is short (0.14-0.24 cm), the secondary leaf bone holder is not parallel and irregular, the secondary leaf bone numbers are between 16.2-18.8, and the secondary leaf bone shape is visible from the underside of the leaf. The leaf angle is 53-59 degrees, and the distance from the leaf base to the largest leaf width is between 5.82-8.08 cm. Wu et al. (2007) mentioned that a leaf with an AR (aspect ratio) value <1 is categorized as a broad leaf type, and AR>1 is categorized as an elongated leaf type.

The stem of Group 1 accessions is perpendicular, and the stem height is 15 meters. There was no large tree belonging to this Group 1 accession in the surveyed areas, and no plant was at the flowering and fruiting stages during the observation. The stem of the Group 1 accessions is grey-brown, has a thin bark, and the bark is grooved on the outside, fibrous and robust (Figure 2A). Based on their morphological characteristics, accessions belonging to the Group 1 are the member of *A. becccariana* species. The morphologies for Group 1 accessions match the characteristics of *A. beccariana* as described by Susilo et al. (2014).

The morphological characteristics of Group 2a accessions include elongated and broad leaf characters; pointed leaf tips to tail with a blunt leaf base; slightly wavy leaf edges, green leaf colour, thin leaf shape, smooth leaf surface, misaligned and irregular secondary leaf bone position, and visible shape of the leaf bone from the leaf underside. The leaf length (7.92-12.04 cm), leaf width (3.54-4.96 cm), leaf aspect ratio >1, short petiole (0.1-0.5 cm), secondary leaf bone numbers (13-20 pairs), 50 degrees leaf angle, and leaf base distance to leaf width ranged from 3.4-4.92 cm. The stem is perpendicular, the bark is whitish and striped, the skin is smooth and has thin cracks, and the stem height at the time of the survey was 20 meters (Figure 2B). Based on their morphological characteristics, accessions belonging to Group 2a are members of the *A. malaccensis* species. The morphologies for Group 2a accessions match the characteristics of *A. malaccensis*, as Susilo et al. (2014) described.

The morphological characters of Group 2b accessions include broad and elongated leaf shape, with leaf length of 7.8-13.0 cm, and leaf width of 3.58-5.36 cm, leaf tip shape pointed to tail with leaf aspect ratio >1, the shape of the leaf base shape is blunt, the leaf edge shape is slightly wavy and thin, the leaf color is green, the leaf surface is smooth, and the petiole is short (0.1-0.8 cm). The position of the second leaf bones is not parallel and irregular. The number of secondary leaf bones is between 13-20 pairs. The leaf vein shape is visible from the underside of the leaves, the leaf angles ranging from 44.40-53.40 degrees. The distance from the leaf base to the largest width ranges from 3.14 to 5.26 cm.

Based on their morphological characters, the Group 2b accessions are *A. microcarpa*. The *A. microcarpa* accession characteristics include erect stems and more than 40 meters of tree height (Figure 2C). The skin of *A. microcarpa* is grey-brown and butted. There are cracks on the outer skin and slight hard bumps of 0.5-1.0 cm. The protrusion on the stem means physically protecting plants from friction to prevent skin damage. Dry skin and many indentations along the trunk or branches are characteristics of gaharu-producing plants (Lee and Mohamed 2016).

Flowers, fruit, and seeds were only found in Group 2b accessions (*A. microcarpa*) and were absent from accessions belonging to either Groups 1 or 2a. Therefore, flower and fruit morphological description could only be done for the Group 2b accessions. The flowers of the Group 2b accessions are umbrella-shaped, light green, with yellowish-white stamens, white petals, yellow crowns, and 5-12 flower branches. Flowers appear at the tip of the twig and under the leaf axil. The flower stalk length is 5-8 mm, and the flower length is 3-4 mm.

The fruit shape is oval, the fruit tip is blunt, and the flesh is thick and short-stemmed with light green fruit skin colour and white to yellowish inside fruit flesh colour. Fruit stalk length is 5-8 mm, fruit length is 27-29 mm, and fruit diameter is 25-26 mm. The fruit flesh consists of 2-3 locules, although they are generally only two (Figures 2L-N). The seed number is between 1-2, and two seeds are usually found in fruits having three locules. The mature fruits would split open and see the hanging seeds. Eventually, the seeds would fall to the ground (Figure 2O). The seed is blackish brown, with oval (ovoid) and white

cotyledons. The seed length is 13 mm, the width of 4.5 mm, the average weight of 0.04-0.06 g, and the 100 seed weight of 4.78 grams. The representative photographs of the detailed fruit and seed morphologies are presented in Figure 2L-Q.

The elongated shape, comprehensive and tailed seeds, dense downy hair, and lightweight are characteristics of plants with wide seed distribution (Susilo 2014). Seed dispersal occurs through wind, animals, and surface water flow. Wind, water, and animals are the primary means of seed dispersal (Soomers et al. 2013; Traveset and Perez 2018). Agarwood seeds are recalcitrant seeds (Hou 1960). Recalcitrant seeds have high water content, cannot be stored for long, are easily hydrated, cannot tolerate drying and are sensitive to low temperatures (Berjak and Pammenter 2013; Pammenter and Berjak (2014). Slight morphological characteristic variation among accessions within Group 1, Group 2a and Group 2b are observed. The slight variations are probably due to environmental factors, such as population density, soil fertility and water availability. Morphological variation among accessions within a species is quite common. Hartati and Sudarsono (2014) showed differences in F1 accessions of *Jatropha* due to cross-pollination. Identification based on morphological characteristics is easy to do. Therefore, molecular analysis should be used to confirm that morphological identification.

Based on the morphological evaluation, at least three groups of agarwood-producing plants are found in North Aceh District, Aceh Province, Indonesia, namely *A. beccariana* (10.6%), *A. malaccensis* (10.6%), and *A. microcarpa* (78.8%). Therefore, these data confirm local farmers' reports that the surveyed location is an agarwood-producing area. However, no other agarwood-producing plant species, such as *A. filaria*, *A. hirta*, and *A. inclea*, were identified, indicating they might have been extinct due to overexploitation. The seed-producing tree is not observed in two out of three identified species. Such data also indicated over-exploitation of the natural population due to agarwood harvesting. Therefore, these data also suggest the need to conserve the agarwood-producing plant population in the surveyed area. The finding in this evaluation further supports Lee et al. (2018), who have stated that agarwood-producing plants of Aquilaria spp. are primarily found in the western part of Indonesia, while *Gyrinops* spp. is found in the eastern part of Indonesia.

Tan et al. (2019) stated that the agarwood exploitation harmed the existence of *Aquilaria* and *Gyrinops* spp. wild populations. Therefore, CITES included *Aquilaria* and *Gyrinops* species as endangered and protected species in 2004 (CITES 2004; Lee et al. 2016a). The threat of extinction for agarwood-producing plants and the availability of good quality and sustainable agricultural production of agarwood must be alleviated through mass-scale cultivation, either individually or in plantations of high-quality agarwood-producing plant species. The export quota for Indonesian natural agarwood species of *A. malaccensis* (CITES Appendix II) for 2018 is set at 151,725 kg; such quota was a 15% decrease from 2017 (KLHK 2018).

As the largest agarwood producer in the world, Indonesia has great potential and opportunity for mass agarwood production (Suharti 2014). As agarwood-producing plants are endemic to Indonesia, many species producing high-quality agarwood are available in Indonesia (Turjaman and Hidayat 2017). Based on the results of this study, *A. malaccensis* could be recommended for mass cultivation since it is in the highest demand by consumers. The high-quality agarwood has sesquiterpene compounds, such as jinkoh-eremol and epi-γ-eudesmol, produced only by the *A. malaccensis* species (Hashim et al. 2016). The presence of specific compounds determining agarwood quality should be used as one of the quality control standards for agarwood in the future (Lee et al. 2016a,b)

The *mat*K, ITS and *trn*L-*trn*F DNA Amplification

The PCR amplification using the *mat*K-specific primers and the DNA template of accession no. 04, 22 and 43 yielded a 500 bp DNA fragment. The PCR amplification using the ITS-specific primers yielded 680 bp, and the *trn*L-*trn*F-specific primers yielded a 470 bp DNA fragment. Representative gel photographed of the PCR amplified DNA fragment agarose gel electrophoresis was presented in Figure 4A. The sizes of the PCR amplified products using the proper primers were as expected (Figure 4A). The representative DNA sequencing results of the PCR amplified ITS DNA fragment was presented in Figure 4B.

The identification sequences of PCR amplified DNA fragments generated by either *mat*K, ITS, or *trn*L-*trn*F specific primers and gaharu-producing plant DNAs using BLAST analysis confirmed the identity of the PCR amplified DNA. The BLAST analysis of the nucleotide sequences to all nucleotide accessions in the NCBI DNA Database resulted in at least 86.17% sequence identity to *mat*K, ITS, or *trn*L-*trn*F sequences of *Aquilaria* spp. (Table 2).

Based on the *mat*K sequences, the sample accession no. 43 (representative accession of Group 1) showed 99.8% sequence identity to several *A. malaccensis*. Sample accession no. 22 (representative accession of Group 2a) showed 99.8% sequence identity to *A. malaccensis* and some accessions of *A. microcarpa*. Meanwhile, sample accession no. 4 (representative accession of Group 2b) showed 99.5% sequence identity to either *A. malaccensis* or *A. hirta* (Table 2).

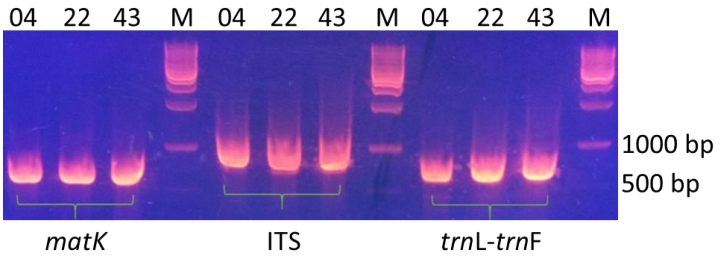
Based on the ITS sequences, the sample accession no. 43 (representative accession of Group 1) showed 86.1% sequence identity to either *A. beccariana* or *A. malaccensis* and 87.5% to *A. hirta*. Sample accession no. 22 (representative accession of Group 2a) showed 98.9 to 99.1% sequence identity to accessions of *A. microcarpa*. Meanwhile, sample accession no. 4 (representative accession of Group 2b) showed 99.1 to 99.2% sequence identity to *A. microcarpa* (Table 2).

Based on the *trn*L-*trn*F sequences, the sample accession no. 43 (representative accession of Group 1) showed 98.7 to 99.1% sequence identity to *A. microcarpa*. Sample accession no. 22 (representative accession of Group 2a) showed 98.2 sequence identity to accessions of *A. malaccensis*. Meanwhile, sample accession no. 4

(representative accession of Group 2b) showed 98.1 to 98.5% sequence identity to *A. microcarpa* and 97.6% to *G. verteegi* (Table 2).

The NCBI nucleotide accessions showing at least 85% sequence identity for *mat*K, ITS and *trn*F-*trn*L fragments using the BLAST analysis output were selected (Table 3) and used to construct the phylogenetic tree. The phylogenetic tree construction results using the Neighbor-Joining method were presented in Figure 5A for *mat*K, Figure 5B for ITS and Figure 5C for *trn*L-*trn*F sequences.

The level of identity among the query sequences to accessions in the NCBI DNA Database could be used to figure out the species identity (Daniels et al. 2013; Nugraha et al. 2014). The sequence identity in Table 2 above is more significant than 86% (i.e. for *mat*K, ITS and *trn*L-*trn*F for accession no. 04, 22 and 43). However, not all three DNA sequences are equally informative for differentiating at the species level. Such results are like those reported by Tallei and Kolondam (2015) in their inability to show *Myristica fragrans* intra-species variations based on the *mat*K gene. However, accession no. 43 (Group 1) was named *A. beccariana*, and accessions no. 4 (Group 2a) and no. 22 (Group 2b) were named as the same species of *A. microcarpa* based on their ITS DNA sequences. Identification using the *trn*L-*trn*F gene showed accession no. 4 from Group 2b and no. 43 from Group 1 was *A. microcarpa*, while accession no. 22 from group 2b was *A. malaccensis*. Therefore, the ITS and the *trn*L-*trn*F DNA sequences are more informative for differentiating the evaluated accessions at the species level than *mat*K DNA sequences. Rangkuti et al. (2021) stated that using the ITS1 DNA sequences was more suitable for differentiating the accessions of rattan plants.



A

CCCTGCCGGGGTCGCGATGCGCGCTATGATTACATCGGGGTTGTCCACGAAACACACCTCAAGGGCATGATGC
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CGAGGGTGGGGGGGCTACAATGCGTGACACCCAGGCAGACGTGCCCTCGGCCCAAAGGCTTGGGGCGCAAC
TTGCGTTCAAATACTCGATGGTTCACGGGATTCTGCAATTCACACCAAGTATCGCATTTCGCTACGTTCTTCATCG
ATGCAAGAGCCGAGATATCCGTTGCCGGGAGTCATTTTAGATTTCATTTCAACGCTTGATCCCCAACACCCCCAT
TTCTGGGTGCATGCGGCAAACGTATTATGTGATCAAGTTCCTTGGCGCAGTCCACGCCGGGGTTTGGTTATGAC
GGCCGCAAGGGCCAACCGAAGAGGGAATGGGGCGTAACGACACAATTCCAACTCGGCACATTGTTATTAACG
TTCACGGGTCGTGCTGCTGTGTAGGAATCGACAATGATCCTTCCGCAGGTTCACCTACGGGAAAACCTTAAA

B

Figure 4. A. Gel photograph of the generated PCR amplified DNA fragments using *mat*K, ITS and *trn*L-*trn*F specific primers for agarwood-producing plants, sample no. 04, 22 and 43. B. The representative ITS DNA sequences were isolated from a sample belonging to Group 2b. accession no. 04

Table 2. The BLAST analysis results in the nucleotide collection of the NCBI DNA Database using the PCR amplified of either *mat*K, ITS, or *trn*L-*trn*F DNA from agarwood-producing plants found in North Aceh, Aceh Province, Indonesia, as query sequences

Group (Accession no.)	Species ID based on morphological characters	Species ID based on fragment sequences (% identity)		
		***mat*K**	**ITS**	***trn*L-*trn*F**
Group 1 (accession no. 43)	*Aquilaria beccariana*	*A. malaccensis* (99.77%)	*A.beccariana* (86.17%)	*A. microcarpa* (99.14%)
		A. microcarpa (99.77%)	*A. malaccensis* (86.17%)	*A. malaccensis* (99.13%)
		A. microcarpa (99.77%)	*A.hirta* (87.48%)	*A. malaccensis* (98.71%)
		A. malaccensis (99.77%)	*A.hirta* (87.48%)	*A. malaccensis* (98.71%)
		A. malaccensis (99.77%)	*A.hirta* (87.48%)	*A. malaccensis* (98.71%)
Group 2a (acc. 22)	*A. malaccensis*	*A. malaccensis* (99.77%)	*A.microcarpa* (99.08%)	*A. malaccensis* (98.24%)
		A. microcarpa (99.77%)	*A.microcarpa* (99.08%)	*A. malaccensis* (98.24%)
		A. microcarpa (99.77%)	*A.microcarpa* (99.08%)	*A. malaccensis* (98.24%)
		A. malaccensis (99.77%)	*A.microcarpa* (99.08%)	*A. malaccensis* (98.24%)
		A. malaccensis (99.77%)	*A.microcarpa* (98.92%)	*A. malaccensis* (98.24%)
Group 2b (acc. 4)	*A. microcarpa*	*A. malaccensis* (99.54%)	*A. microcarpa* (99.23%)	*A. microcarpa* (98.48%)
		A. malaccensis (99.54%)	*A. microcarpa* (99.23%)	*A. microcarpa* (98.05%)
		A. hirta (99.54%)	*A. microcarpa* (99.23%)	*G.verteegi* (97.63%)
		A. hirta (99.54%)	*A. microcarpa* (99.23%)	*G. verteegi* (97.63%)
		A. hirta (99.54%)	*A. microcarpa* (99.08%)	*G. verteegi* (97.63%)

Table 3. List of accessions available in the National Center for Biotechnology Institute (NCBI) DNA Database and the species names for the *mat*K, ITS, and *trn*L-*trn*F nucleotide sequences used in the phylogenetic analysis

NCBI acc.	Species name	NCBI acc.	Species name
***mat*K gene (13 NCBI database accessions):**			
KU244197	*Aquilaria microcarpa*	MF443404	*G. walla*
KU244193	*A. malaccensis*	FJ572802	*A. beccariana*
LC467518	*A. crassna*	LC383998	*A. malaccensis*
MH603237	*A. malaccensis*	MH6032339	*A. subintegra*
LC461818	*A. crassna*	LC467517	*A. crassna*
MF443403	*Gyrinops caudata*	MH603229	*A. crassna*
MW118101	*A. sinensis*		
ITS sequences (23 NCBI Database accessions):			
KX024768.1	*A. malaccensis*	MH594325.1	*G. yunanensis*
KX024766.1	*A. malaccensis*	MH134137.1	*A. agallochum*
MH134145.1	*A. rugosa*	KY817983.1	*A. yunnanensis*
KY817985.1	*A. yunnanensis*	KY817977.1	*A. sinensis*
KT364483.1	*A. subintegra*	MH594318.1	*A. crassna*
KT364482.1	*A. rugosa*	MH134141.1	*A. hirta*
KT364479.1	*A. hirta*	KY817966.1	*A. hirta*
KT364480.1	*A. malaccensis*	KU244090.1	*A. malaccensis*
MH594314.1	*A. malaccensis*	KT364477.1	*A. beccariana*
KT779118.1	*A. microcarpa*	MH134143.1	*A. microcarpa*
KT364481.1	*A. microcarpa*	KT779116.1	*A. beccariana*
KU244093.1	*A. microcarpa*		
***trn*L-*trn*F gene (11 NCBI Database accessions):**			
KU244050.1	*A. subintegra*	KY927209.1	*A. crassna*
KU244034.1	*A. hirta*	KY927248.1	*A. sinensis*
KT726326.1	*G. verteegii*	KT726319.1	*A. beccariana*
KT726321.1	*A. malaccensis*	KY927223.1	*A. malaccensis*
LC467509.1	*A. malaccensis*	LC467511.1	*A. microcarpa*
LC467512.1	*A. microcarpa*		

Note: The accessions were downloaded from NCBI DNA Database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) based on the BLAST output analysis

Cluster analysis based on the *mat*K sequences among evaluated accessions in this study and the available accessions in the NCBI database showed that the three agarwood-producing plants from Indonesia were in one cluster, and they are closely related to NCBI accessions labelled as *A. beccariana*, *A. malaccensis*, *G. walla*, and *A. microcarpa*. According to Lee et al. (2016a), *A. malaccensis*, *A. beccariana*, and *G. wala* should be in different clusters. Similar results were also seen in the clustering of *A. subintegra* (MH603237), *A. crassna* (LC46818, LC467517 and MH603229), *G. caudata* (MF443403), and *A. sinensis* (MW18101) which belong to the same group. However, the *G. caudata* is species of a different family than *A. subintegra*, *A. crassna*, and *A. sinensis*.

Cluster analysis using ITS fragment showed accession no. 4 (Group 2b) and no. 22 (Group 2a) are closely related to *A. microcarpa*, while accession no. 43 (Group 1) is closely related to *A. beccariana*. Meanwhile, clustering using the *trn*L-*trn*F fragment showed accession no. 4 (Group 2b) and no. 43 (Group 1) are closely related to *A. microcarpa*, and accession no. 22 (group 2a) is closely related to *A. malacensis*, which shows that the *trn*L-*trn*F gene fragment can differentiate among *A. beccariana* and other species, but not between the *A. microcarpa* and the *A. malaccensis*.

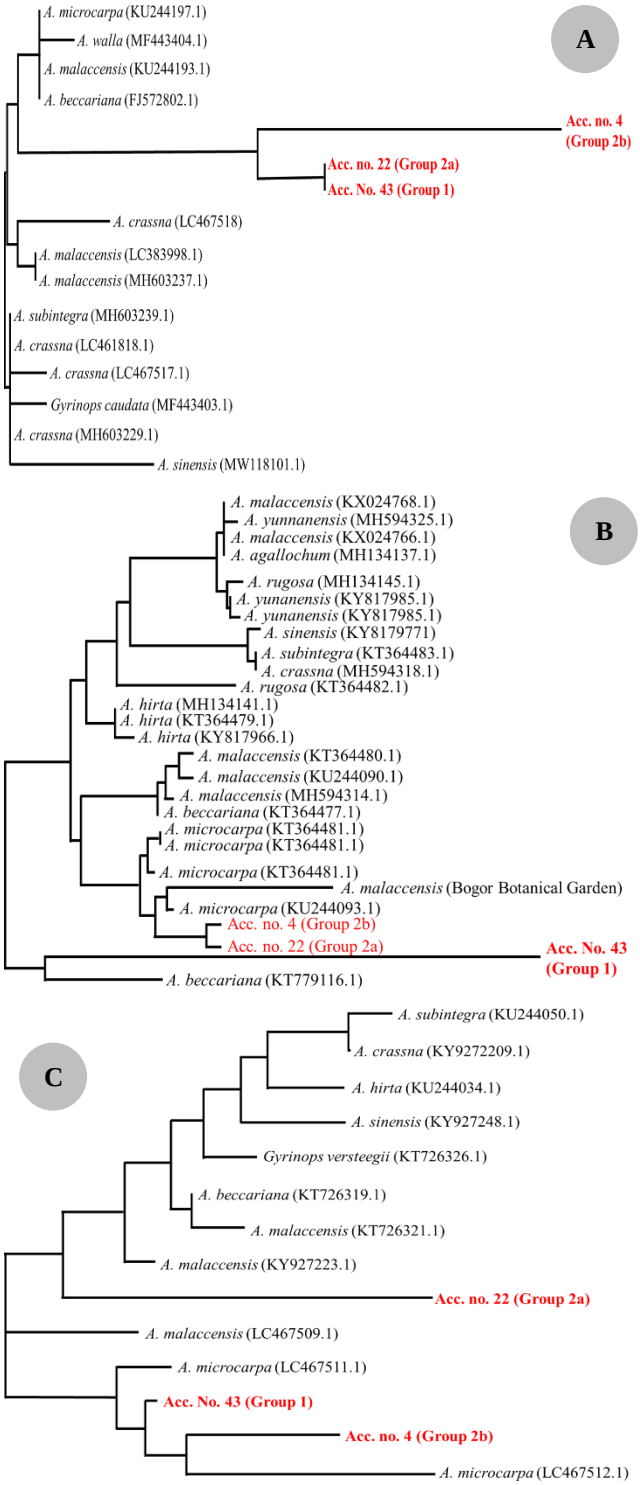

Figure 5. Results of the phylogenetic tree construction using the Neighbor-Joining method based on (A) *mat*K, (B) ITS and (C) *trn*L-*trn*F nucleotide sequences among agarwood-producing plant accession no. 43 (Group 1), no. 22 (Group 2a), no. 4 (Group 2b) from Aceh Province, Indonesia and accessions are available in the NCBI DNA Database. Phylogenetic analysis was constructed using MEGA X software. Accessions with red labels are samples of unknown species sequenced in this study. Accessions no. 43 as the representative sample of Group 1, no. 22 of Group 2a, and no. 4 of Group 2b of the cluster analysis based on morphological characteristics. Meanwhile, accessions with black labels are the reference sequences from NCBI DNA Database

Our result showed species identification results based on morphological characters were close to those based on ITS sequences. The *trn*L-*trn*F and ITS identification showed that *A. microcarpa* and *A. malaccensis* are more closely related than other species. However, identification based on the *mat*K sequences is less informative for identifying the agarwood-producing plant from Aceh, Indonesia, at the species levels. The existence of discrepancies in the data in Figures 3. Outgroups is considered an error in distinguishing species. The same thing was also conveyed by (Lee et al. 2022), who stated that it was necessary to revise the taxonomy of Aquilaria and Gyrinops to distinguish between intra and interspecies.

The marker sensitivity is determined by the ability of the markers used to classify presumed accessions of different species into different phylogenetic groups. Therefore, the more sensitive the marker, the more informative the cluster analysis results (Songa et al. 2022). Combining several markers for phylogenetic analysis will increase the ability to identify different species (Tanaka and Ito 2019) and (Kang 2021). The problem is that markers might not always be used to differentiate within the species level. Tanaka and Ito (2019) reported that combining some markers for identifying agarwood-producing plants could only amplify the phylogenetic results but are still unable to differentiate among accessions of the same species.

The DNA sequences have been used for species identification, and DNA fragments or short sequences (i.e. the conserved regions) were used to figure out the identity of unknown accessions (Wang et al. 2016). The conserved DNA sequences are the ITS sequence, tRNA-L, and others. The longer the amplified sequence, the more sensitive the identification results of the query sequences (Songa et al. 2022). In this study, the ITS gene, which contains relatively more complete sequences than the *mat*K and *trn*L-*trn*F, showed more informative results.

The less informative of the *mat*K and *trn*L-*trn*F genes in identifying the plant species studied in the North Aceh District is partially due to the limited availability of reference sequences in the NCBI DNA database. Tanaka and Ito (2019) stated that the query sequence identification and characterization partially depended on the availability of database sequences at NCBI. The more reference data sequence availability in the NCBI database, the correct identification of the evaluated query sequences. Tallei and Kolondam (2015) stated that the *mat*K gene could not differentiate intra- and inter-species in nutmeg (*Myristica fragrans*). Although several studies using the *mat*K, ITS, and *trn*L-*trn*F genes can distinguish agarwood-producing plants at the species level (Tanaka and Ito 2019), there were some conflicting results (Roslim 2019). Conflicting results may partially be contributed by the PCR amplified region of the target genes and the availability of enough numbers of informative reference sequences in the NCBI DNA database. It is also advisable to update the *Aquilaria* taxonomy to ensure that the available data are correct and the proper reference sequences are available in the database. Lee et al. (2018) stated that the taxonomy of *Aquilaria* from Indonesia is still based on old data and has not been revised, so the proper identity of the reported species from Indonesia is uncertain.

This study concluded that the agarwood-producing plant species in North Aceh District, Aceh Province, Indonesia belong to the *Aquilaria* spp. Leaf morphology characters differentiate the accessions into *A. beccariana* (Group 1), *A. malaccensis* (Group 2a), and *A. microcarpa* (Group 2b). Validation using the DNA sequences of the *mat*K, ITS and *trn*L-*trn*F fragment showed that ITS is more informative than the other two genes. The finding of this study may be used as diagnostic tools for identifying and conservating agarwood-producing plants and selecting high-quality and quantity agarwood-producing plants in the future. The inability to find seed-producing trees for the two evaluated species showed there was over-harvesting of agarwood from the two species. Therefore, various measures should be implemented to support the sustainable development of agarwood-producing plants in the areas, such as by cultivating the endemic agarwood-producing plant species.

ACKNOWLEDGMENTS

Postgraduate Scholarship Program supported part of this work, the Ministry of Research, Technology, and Higher Education, Republic of Indonesia and the Dissertation Research Support Program, Human Resources Development Agency – Aceh, Indonesia, Grant No.57/S3/2021 to LU. The authors acknowledge the support of Plant Molecular Biology Laboratory, Institut Pertanian Bogor, Bogor, West Java, Indonesia, by providing the necessary equipment to conduct the molecular analysis activities.

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