Evaluation of genetic diversity of Sandalwood (*Santalum album* Linn.) in Aceh, Indonesia, and it's essential oil characteristics

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ABSTRACT Intensive sandalwood utilization can have an impact on the quantity and quality of sandalwood in Aceh, Indonesia. In the long run, this practice can lead to a decrease in genetic resources. This study evaluates the genetic diversity of sandalwood populations in their natural habitat in Aceh. This study also assesses the genetic affinity between Aceh sandalwood and sandalwood from East Nusa Tenggara (NTT); and also its essential oil characteristics. This study provides information for the development of responsible sandalwood utilization policy. We collected leaf samples from five populations across four sites in Aceh and from one site in Maulafa, NTT. In Aceh, samples were collected from Kuta Malaka, Suka Makmur, Padang Tiji, and Muara Tiga. The evaluation of sandalwood genetic diversity was conducted using the *Random Amplified Polymorphic DNA* (RAPD) method with *Unweighted-Pair Group Method Arithmetic* (UPGMA) and *Numerical Taxonomy and Multivariate System* (NTSYS) programs. Sandalwood essential oil was extracted from the stems and roots of sandalwood trees from four different diameter classes using an eight-hour water and steam distillation method. This study examined the specific gravity and the concentration of santalol compounds in sandalwood oil. Santalol concentration were measured using a GC-MS method. Results showed that Suka Makmur population has the highest genetic diversity. Thus it has the potential to be developed for the center of sandalwood in Aceh. Sandalwood populations in Aceh have a genetic affinity with sandalwood populations in NTT with a level of genetic differences only 16%. This shows that sandalwood populations in Aceh likely originated from NTT. Characteristics of the sandalwood essential oil which met the ISO quality standards were derived from the tree diameter ≥ 15 cm with the proportions of heartwood were 48.53% on stems and 52.36% on roots. Therefore, we propose that that selection of sandalwood tree diameter can contribute to sandalwood utilization strategy for maintaining sandalwood quantity and quality.

Keywords: Aceh; essential oil; evaluation; genetic diversity; sandalwood

INTRODUCTION

Indonesia has a long history of sandalwood (*Santalum album*, Linn.) utilization, particularly in East Nusa Tenggara (NTT). Current information on sandalwood utilization in other areas in Indonesia are limited, especially in the western region, including Aceh. Sandalwood is known by the local community in Aceh as "Ceundana" or "Bak Cin," and the trees have been used by utilizing the logs for wardrobe or air freshener without any further processes (Nurochman et al., 2018). Aceh Statistical Agency reported that total production of sandalwood logs in Aceh was 357,124 tons from 2003 to 2015. This commercial use of sandalwood logs in Aceh makes it the only region that has sandalwood potential in Sumatera Island (Nurochman et al., 2018). Natural sandalwood populations in Aceh can be found in Aceh Besar and Pidie District. These areas are located near the Malacca Strait, which was the sandalwood trade route from Timor in the 17th century (Blusse and Veen, 2007; Fuller et al., 2011).

Natural sandalwood populations in Aceh provide important information on genetic diversity and the variation in chemical components of essential oils (Jones, 2008). The genetic diversity of sandalwood is used to determine kinship within populations and between populations to assess the origin of sandalwood presence in Aceh. Early methods to assess species diversity were based on morphological character markers. This method was recently updated by using molecular Deoxyribo Nucleic Acid (DNA) marking techniques. This technique has a high level of accuracy because it is not affected by the growth of individual sandalwood trees. Aminah et al. (2017) found that the genetic diversity of species within, and between, populations is correlated with the geographical distance between populations, so species with a wide distribution have high genetic diversity.

Sandalwood trees are also known as royal plants, because they have high economic value and are traded by kilogram units, making it easy to quickly generate cash (Jensen and Meilby, 2010). Sandalwood is used for essential oil production because of the distinctive aroma of santalol compounds (Brand et al., 2012; Page et al., 2012). ISO (2002) and Howes et al. (2004) reported that the standard for determining the quality of sandalwood essential oils is based on the concentration of santalol compounds (α-santalol and β-santalol). The concentration of santalol compounds in sandalwood trees varies between the roots and stems (Oyen and Dung, 1999) both in young sandalwood and mature trees based on their diameter (Brand et al., 2012).

Intensive sandalwood utilization in Aceh is currently conducted without a tree selection policy, which may cause a decrease in the quantity and quality of sandalwood. In the long term, intensive utilization will lead to a decrease in genetic resources. This article analyzes the genetic diversity within, and between, sandalwood populations in Aceh; defines the level of genetic closeness with sandalwood from NTT to examine the origin of sandalwood in Aceh, and analyzes essential oil characteristics to develop policy recommendations and strategies for sandalwood utilization.

MATERIALS AND METHODS

Location of Study

To study genetic diversity, we collected sandalwood leaves from four populations in Aceh, including Kuta Malaka, Suka Makmur, Muara Tiga, and Padang Tiji. One population in Maulafa,

Kupang, NTT was used for comparison (see Figure 1). Sandalwood powder was made from the heartwood and sapwood of the stems and roots. The powder was refined with water and steam technique for producing essential oil. We intended to collect samples from different diameter classes at the four sites in Aceh; however, due to limited diameter distribution in each site, such classification cannot be done. In addition, prices of sandalwood trees were high.

Figure 1. Locations of sandalwood populations in Aceh and NTT.

Materials and Tools

In this study, we collected samples from the leaves, stems, and roots of sandalwood trees in their natural habitat. About 10-15 leaf samples were collected from each tree with three trees in each population. The proportion of heartwood on sandalwood stems and roots was calculated using a segmentation method. This method enables the analysis of cross-sectional images by partitioning them into several homogeneous characteristics. Image partitioning was done by grouping similar color characteristics. Diameter classes were grouped based on Indonesian Standard Number 01-5008.6/1999 on sandalwood by considering the common diameter utilized by the local community (BSN, 1999). Diameter classes and the average proportion of heartwood in stem and root sections are presented in Table 1. The selection of sandalwood trees to be harvested is based on the local knowledge: the barks of the stem have cracked or have dried leaves on the top of the canopy.

Table 1. Diameter classes of sandalwood and the average proportion of heartwood on stem and root sections.

Class	Diameter (cm)	Average proportion of heartwood (%)	
		Stem section	Root section
1	<5	0,00	0,00
2	5 - <10	31,04	38,54
3	10 - <15	36,73	39,48
4	≥15	48.53	52.36

Procedures

Samples of sandalwood leaves were inserted into a plastic clip containing silica gel with a ratio of 1:20 (n / n). DNA was extracted using a cetyl trimethyl ammonium bromide method (Dani et al., 2011; Arun Dev et al., 2014). DNA amplification was conducted based on the procedure by William et al. (1990) using five selected arbitrary Random Amplified Polymorphic DNA (RAPD) primers to produce sandalwood polymorphic bands.

Samples were collected from the heartwood and sapwood of stems and roots based on their diameter classes. These samples were mashed into powder and filtered using a size 20 mesh sieve. Based on the results of the preliminary test, one kilogram of sandalwood powder can produce essential oil after approximately eight hours by applying water and steam distillation techniques.

Data analysis

The analysis of *Polymerase Chain Reaction* (PCR) results using the RAPD method was scaled using a binary matrix which was shown in the presence of the bands in each DNA primer. For each genetic comparison, we recorded the data as 0 (zero) when no band was observed and 1 (one) when band presenced at the same locus position. Scoring was calculated using PYELPH 1.4 software. The results of RAPD binary matrices were analyzed using POPGENE software. A sandalwood dendogram was made using the Unweighted-Pair Group Method Arithmetic (UPGA) method and version 2.0 of the Numerical Taxonomy and Multivariate System (NTSYS) program.

The characteristics of sandalwood essential oil were examined by analyzing the concentration of santalol compounds and the specific gravity of the oil using ISO 3518 for the year 2002. The santalol compound was analyzed using gas chromatography and mass spectroscopy (GC-MS). According to Howes et al. (2004), this method is very helpful for authenticating and controlling the quality of sandalwood essential oil compared to gas chromatography (GC) methods. Two santalol compounds were used as determinants of Aceh sandalwood essential oil characteristics: α-santalol and β-santalol (ISO, 2002; Howes et al., 2004).

RESULTS AND DISCUSSION

Genetic diversity of sandalwood

We obtained five out of 30 primers that produced clear polymorphisms for further RAPD analysis, including OPB-16, OPP-19, OPP-09, OPB-08, and OPP-15. The selected primers had 10 nucleotide bases resulting from good and stable amplification of DNA fragments. This is consistent with Waugh and Powell (1992), who stated that primers used for amplification of plant DNA should generally have a length of 9-10 nucleotides with random acid-base sequences. Figure 2 shows polymorphic bands, which are sandalwood genome DNA amplification results in each primer, which shows the informative character of genetic kinship in each allele. The polymorphic bands of each primer show a different pattern; thus, the relationship of each sandalwood DNA sample can be determined by looking at the presence of the band on each allele. Sandalwood DNA fragments from amplification with an unclear band pattern

showed a recessive genotype (aa), whereas fragments with clear bands showed dominant genotypes (AA and Aa). The percentage of sandalwood DNA polymorphic band values in each population can be seen in Table 2.

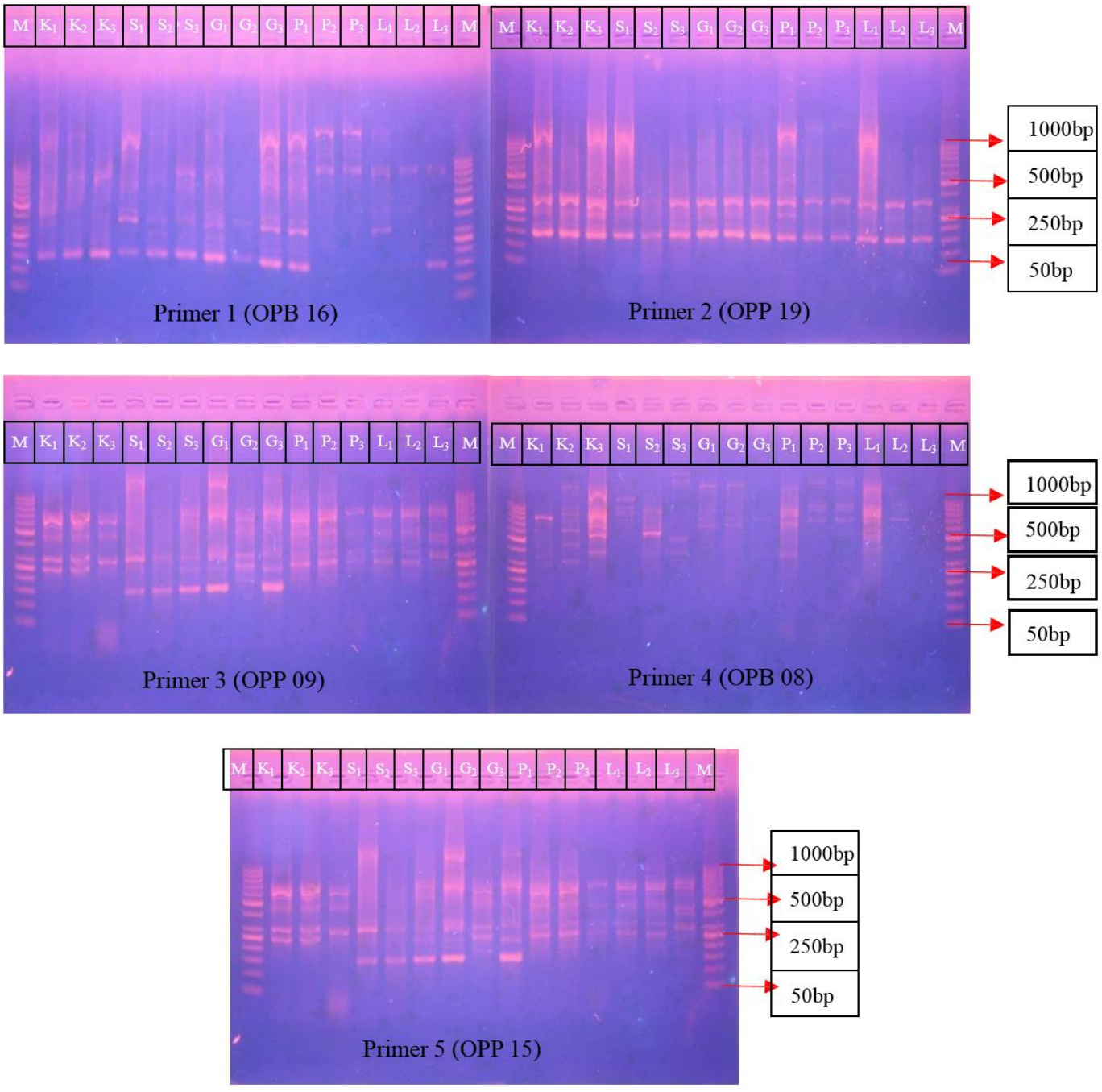


Figure 2. Pattern of sandalwood polymorphic band using 5 DNA primers.
Where: M: DNA Marker (50 bp ladder Promega). K: Maulafa, S: Kuta Malaka, G: Suka Makmur, P: Padang Tiji, L: Muara Tiga

The results of genetic analysis from each sandalwood sample from four populations in Aceh and one population in NTT are presented in Table 2. The genetic diversity (He) of the sampled populations was quite diverse, ranging from 0.146 to 0.221. Aceh samples had higher genetic diversity than the population in NTT. The Suka Makmur population had the highest genetic diversity. Poerba and Martanti (2008) reported that high genetic diversity of a species populations could be inferred as its likelihood as the center of the species diversity. Thus, we suggest that Suka Makmur is one of the genetic diversity centers or tree breeding for sandalwood trees in Aceh. The genetic diversity in this study was also higher than three sandalwood populations in Mare Island, New Caledonia with genetic diversity ranging from 0.030 to 0.140 (Bottin et al., 2005). Higher values of genetic diversity indicate that sandalwood trees in the population were exposed to cross-pollination between sandalwood trees assisted by wind and pollinators (Bottin et al., 2005; Poerba et al., 2007). Pither et al. (2003) found that tropical plants, including sandalwood, are capable of cross-pollination that can cause genetic transfer between individual trees and result in high genetic diversity in the population. The genetic diversity of sandalwood trees can differ within a population due to several factors, including the reproduction system, the history of origin, and the geographic distribution (Botin et al., 2005).

Table 2. Sandalwood genetic diversity in four populations in Aceh and one population in NTT.

No	Population	N	PLP (%)	Na	Ne	He	I
1	Kuta Malaka (Aceh)	3	42.31	1.423	1.235	0.146	0.223
2	Suka Makmur (Aceh)	3	57.69	1.577	1.382	0.221	0.327
3	Padang Tiji (Aceh)	3	58.97	1.589	1.367	0.217	0.326
4	Muara Tiga (Aceh)	3	48.72	1.487	1.269	0.167	0.256
5	Maulafa (NTT)	3	38.46	1.384	1.238	0.142	0.212
Average			49.23	1.492	1.298	0.178	

Where: N: Number of samples, PLP: Percentage of polymorphic band, Na: Number of alleles observed, Ne: effective alleles count, He: Expected diversity value, I: Shannon's diversity index

The average value of Aceh sandalwood genetic diversity (He) was smaller than the genetic diversity of sandalwood populations in Western Australia (He: 0.210) and in Fiji and Tonga (He: 0.490) (Byrne et al., 2003, Huish, 2009). This is presumably due to geographical or spatial distance and characteristics of sandalwood population differences areas as external factors that influence sandalwood genetic diversity in the population. Byrne et al. (2003) analyzed sandalwood genetic diversity in Australian states with large geographical areas and different regional characteristics. Huish (2009) examined sandalwood genetic diversity of Fiji and Tonga where both countries are separated by the different islands.

The geographical distance of a species has a positive correlation with its genetic distance, so the further the geographical distance of sandalwood distribution, the higher the genetic diversity (Alpert et al., 1993). Furthermore, Stankiewicz et al. (2001) reported that genetic distance between populations is correlated with the geographical distance between populations, depending on natural and artificial factors involved in shaping the population's genetics.

Data and information about sandalwood genetic diversity in natural habitats are very important to conserve and cultivate sandalwood. Jones (2008) found that genetic diversity among sandalwood populations is correlated with the variation in the chemical components of essential oils in each population The results of the study showed that sandalwood genetic diversity was low (He = 0.047) and the variety of essential oil components was also low (Jones 2008). This is in line with Bottin et al. (2007), who reported that the level of genetic diversity of *Santalum austrocaledonicum* has a positive correlation with the chemical components of essential oil. Furthermore, Finkeldey and Hattemer (2007) suggested that higher sandalwood genetic diversity tends to have higher plant reproduction and higher adaption to their environment. Table 3 shows a comparison of average genetic diversity of Aceh populations and NTT populations with previous studies in Indonesia.

Table 3. Average of genetic diversity based on the analysis of Nei (1978) using RAPD markers.

Species	Population	Ht	He	Gst	Dst	Nm	Source
Santalum album	Maulafa, Kuta Malaka, Suka Makmur, Padang Tiji, Muara Tiga	0.281	0.178	0.363	0.103	0.875	Primary Data
Santalum album	Watusipat	0.331	0.317	0.044	0.014	-	Haryjanto (2009)
	West Timor	-	0.391	-	-	-	Rimbawanto et al. (2006)

Where: Ht = Genetic diversity in all populations; He = genetic diversity in the population; Dst: Genetic diversity between populations; Gst = genetic differentiation; Nm = genetic flow.

Genetic diversity of sandalwood in the population (He) shows different responses of individual trees to the environment (Finkeldey and Hattemer, 2007). The genetic diversity of the populations in our study site was lower than that in Western Timor and Watusipat Gunung Kidul. This indicates that outcrossing genetic transfer between individual sandalwood trees at the study site was lower than outcrossing at West Timor and Watusipat Gunung Kidul populations. The differences in genetic diversity among populations are due to the occurrence of gene transfer and natural selection at the population level (Bawa, 1998). Sandalwood genetic diversity can be identified based on morphological variation

and differences in growth parameters, including height and diameter of stands that are the same age (USDA, 1990; Sumardi et al., 2014). The proximity of four sandalwood populations in Aceh and in NTT is presented in a dendogram (Figure 3).

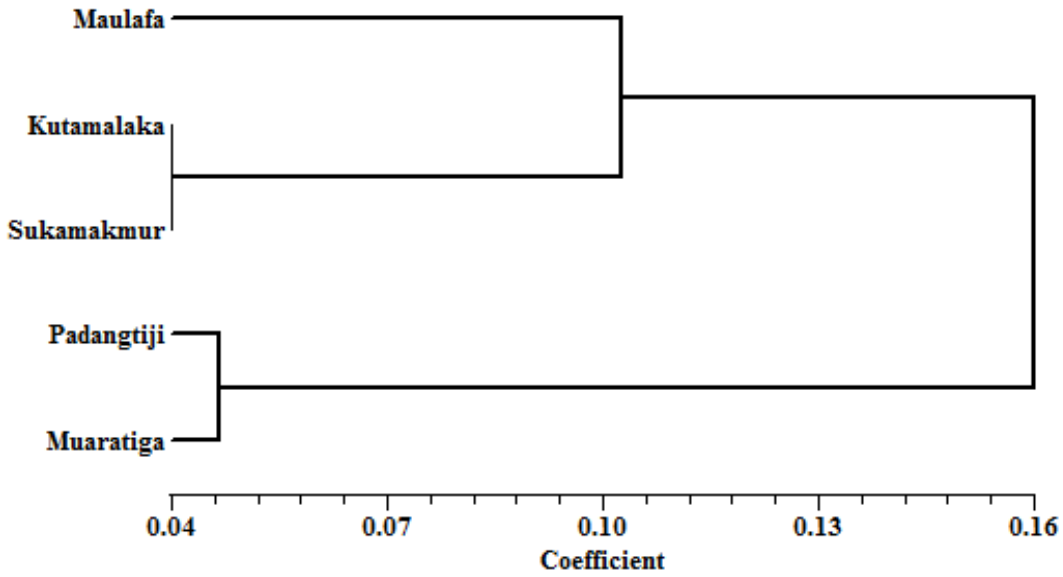


Figure 3. Dendogram of sandalwood population in Aceh and NTT

Figure 3 illustrates that sandalwood populations in Aceh and NTT separated into two main groups. The first group consists of two sub-groups, including the Maulafa (NTT) population, which stands alone, and Kuta Malaka and Suka Makmur populations. The second group consisted of Padang Tiji and Muara Tiga populations. This indicates that the populations of Kuta Malaka and Suka Makmur are genetically similar, as are the Padang Tiji and Muara Tiga populations. Sandalwood populations that have similar genetic levels are thought to have a kinship or genetic proximity. In general, the grouping of sandalwood populations in Aceh and NTT relates to its geographical distance. We obtained this result as the RAPD markers that we used can display DNA variations both in DNA that encodes protein sequences (coding regions) and DNA that do not encode protein sequences (non-coding regions). Therefore, it can provide good genetic diversity information.

Based on the dendogram above, we suggest that sandalwood populations in Aceh have a genetic kinship with sandalwood populations in NTT (Maulafa population) with a genetic diversity coefficient of 16%. This indicates that sandalwood stands in Aceh and NTT have a similar genetic origin. Thus, we propose that sandalwood populations inAceh originated from sandalwood populations in NTT. Our assumption is supported by the geographical location of Aceh, which is located adjacent to the Malacca Strait, which was the center of sandalwood trade from NTT to China and India in the 17th century (Blusse and Veen, 2007; Fuller et al., 2011). In addition, there are similarities

in the characteristics of sandalwood habitats in Aceh and NTT, including dry land dominated by grasses and shrubs with an average temperature of 24°C to 32°C. The two sites also contain low levels of organic C, N Total, and P, along with a neutral acidity level (Nurochman et al., 2018). Furthermore, Loveless (1992) and Alpert et al. (1993) found that despite similar population characteristics, due to the wide geographical or spatial distance between populations, it can cause variations in genetic distance within the population. The proximity of genetic and geographical distance of sandalwood populations in Aceh and NTT are presented in Table 4.

Table 4. Genetic and geographical distance of sandalwood populations in Aceh and NTT

Population	Kuta Malaka	Suka Makmur	Padang Tiji	Muara Tiga	Maulafa
Kuta Malaka	****	5 G	64 G	70 G	5 188 G
Suka Makmur	0.0435 g	****	68 G	74 G	5 192 G
Padang Tiji	0.1967 g	0.1188 g	****	27 G	5 157 G
Muara Tiga	0.2137 g	0.1486 g	0.0498 g	****	5 181 G
Maulafa (NTT)	0.1121 g	0.0943 g	0.1004 g	0.1683 g	****

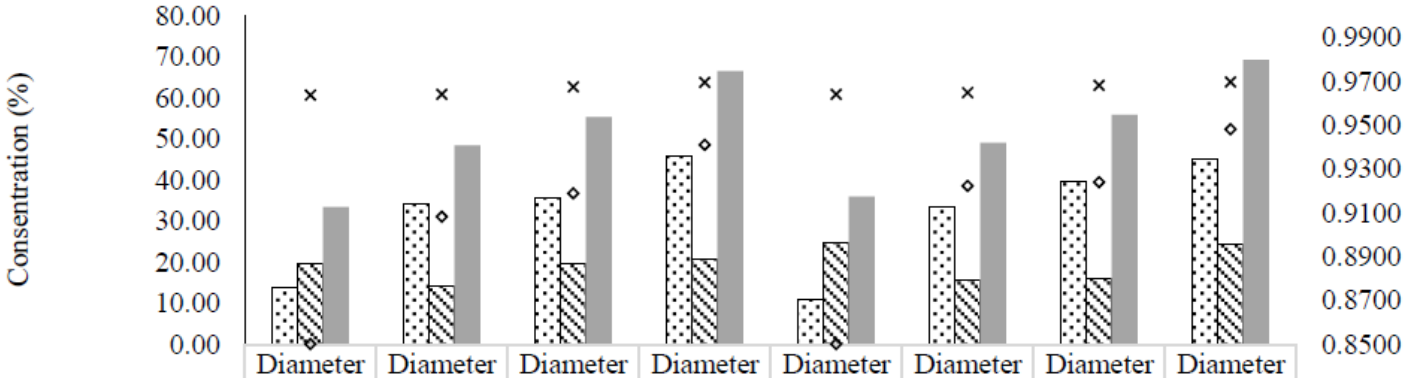
Where: (g) = genetic distance between populations; (G) = geographical distance between populations (km).

Our data suggest that the closest genetic distance was between Kuta Malaka and Suka Makmur populations (0.0435) and between Padang Tiji and Muara Tiga populations (0.0498). The results also showed that the Kuta Malaka sandalwood population has close genetic origin to the Suka Makmur population, while sandalwood in Padang Tiji is closely related to Muara Tiga populations. This is possible because of the geographical proximity between these populations. In contrast, the farthest sandalwood genetic distance is between Kuta Malaka and Muara Tiga (0.2137), and Kuta Malaka and Padang Tiji populations (0.1967) due to the wide distance between the populations. This study found a positive correlation between the geographical distance of sandalwood populations and their genetic distance. This is consistent with previous findings by Alpert et al. (1993) on strawberry plants in the United States and Aminah et al. (2017) on *Pongamia* plants in Indonesia that the species with a wide distribution (far geographical distance) will have high genetic diversity. Loveless (1992) reported that in addition to geographical distance, other factors influence genetic distances, including regeneration, reproduction, seed dispersal, and fertility rates.

Sandalwood oil characteristics

Sandalwood oil is an essential oil obtained from refining sandalwood powder. Arun Dev et al. (2014) described the unique characteristics of the santalol compounds in sandalwood oils, which makes the distinctive fragrance difficult to replicate with other plants or synthetic oils. Santalol are dominant compounds

contained in sandalwood oil and are used as main standards for determining oil quality (Rao et al., 2016). Fahlbusch et al. (2012) reported that α-santalol and β-santalol compounds are responsible for the unique fragrance of sandalwood oil. Evaluation of sandalwood oil quality by analyzing α-santalol and β- santalol concentrations has performed better result than compared to 90% w/w of free alcohol analysis or known as Total Santalol 90% (Howes et al., 2004). This is due to its ability to assist in authentication and control of oil quality to be more accurate, effective, and efficient. Therefore, santalol compounds (α-santalol and β-santalol) and specific gravity have been used as the standard to determine sandalwood oil quality (ISO, 2002). Characteristics of santalol compounds, heartwood ratios, and the specific gravity of Aceh sandalwood oil are presented in Figure 4.



	Stem				Root			
	Diameter class 1	Diameter class 2	Diameter class 3	Diameter class 4	Diameter class 1	Diameter class 2	Diameter class 3	Diameter class 4
⊡ α-santalol	14.00	34.04	35.73	45.83	11.10	33.52	39.70	44.96
⊠ β-santalol	19.47	14.39	19.68	20.77	24.87	15.56	16.16	24.36
■ Total	33.47	48.43	55.41	66.60	35.97	49.08	55.86	69.32
⋄ Heartwood ratio	0.00	31.04	36.73	48.53	0.00	38.54	39.48	52.36
● Yield	0.108	0.123	0.204	0.551	0.111	0.189	0.272	0.730
× Specific gravity	0.9636	0.9641	0.9673	0.9695	0.9641	0.9648	0.9681	0.9696

Figure 4. Characteristics of Santalol compound, heartwood ratio, and specific gravity of sandalwood oils in each diameter of stems and roots.

Higher proportions of heartwood ratio and oil yield rate were observed from the larger diameter classes (Figure 4). Larger diameter trees will produce a higher proportion of heartwood, which will increase the yield of sandalwood oil. Kumar et al. (2011) found that the diameter of sandalwood has a positive correlation to the proportion of sandalwood heartwood (R^2 = 39.80%). He also reported a positive correlation between the tree diameter and the yield of sandalwood oil (R^2= 0.90%). This relationship can be explained as larger stems and roots were commonly observed from an older tree with a greater proportion of heartwood and

higher oil yield. In addition, Haryjanto et al. (2017) found that the diameter and circumference of sandalwood trees also have a positive correlation with the concentration of α-santalol and β- santalol compounds with regression coefficients of 7.85% and 21.48%, respectively. However, both correlations were weak.

We also observed that sandalwood roots tend to produce a higher yield of essential oils and santalol concentrations (α-santalol and β-santalol) compared to the stems (Figure 4). This is due to the higher proportion of heartwood in roots compared to the stems. Similar findings were also reported by previous studies conducted by Subasinghe et al. (2013) and Ariyanti and Asbur (2018). The highest concentration of santalol compounds was found in roots, followed by the buttress and stems (Mc Comb, 2009; Oyen and Dung, 1999). This is in line with Sindhu et al. (2010) who found that the concentration of santalol compounds (α-santalol and β-santalol) in the lower section of sandalwood trees was higher than in the upper section. In addition, sandalwood trees with a high proportion of heartwood also accumulate high concentrations of santalol compounds (Jones, 2008).

We also observed that specific gravity was higher for trees with large diameters (Figure 4). The lowest and highest specific gravity of sandalwood oil was obtained from diameter classes 1 and 4, respectively. Nugraheni et al. (2016) found that the concentration of molecular fractions in essential oils relates strongly with heavier specific gravity. Specific gravity is influenced by the chemical composition of the oil fraction. The main components of sandalwood oil from Aceh were α- santalol, β-santalol, cis-lanceol, and z-α- trans bergamotol with a molecular weight of 220 ($C_{15}H_{24}O$) with ring bond and double bond as presented in Figure 5. Our results showed that the specific gravity of sandalwood oil will increase with a higher concentration of santalol compounds. Guenther (1948) reported that essential oils with long molecular chains and double bond structures would have heavier specific gravity.

Sandalwood oil in Aceh that meets the quality comes from stems and roots with a class 4 diameter with α-santalol concentration ranging from 44% to 45%. For β-santalol compound, sandalwood oil that meets quality comes from diameter class 3 and 4 for both sections with concentration ranged from 16% to 24%. The specific gravity of the oils that meet the quality was from diameter class 3 for the root section and diameter class 4 for both sections with oil density ranging from 0.9681 g/cm^3 to 0.9696 g/cm^3. Based on this evaluation, we suggest that the quality of Aceh sandalwood oil that meets the requirements of the ISO standard (2002) comes from the diameter class 4 only, which is sandalwood trees with diameter ≥ 15 cm and proportion of heartwood about 48.53% in the stem section and 52.36% in the root section. The characteristics of sandalwood oil from diameter class 4 also meet the recommendations from Howes et al. (2004) concerning quality standards for *Santalum album*, which is traded internationally and must be derived from mature sandalwood trees containing ≥ 43% α-santalol and ≥ 18% β-santalol. This study shows that the sandalwood trees with large diameters and high proportions of heartwood will produce a higher yield of oil that meets quality standards. Kumar et al. (2011) found that there was a positive

correlation between the diameter of sandalwood stems and the formation of heartwood, thus producing high quality sandalwood oil.

CONCLUSION

Suka Makmur population has the highest sandalwood genetic diversity; thus, it has the potential to be developed as the center of sandalwood diversity in Aceh. Sandalwood in Aceh has a genetic affinity with sandalwood in NTT (genetic diversity coefficient of 16%). This indicates that the origin of Aceh sandalwood is likely from NTT. Sandalwood oil was produced by using an eight-hour water and steam distillation technique. Oil yield rate ranged from 0.108% to 0.730%. Sandalwood oil that meets the ISO quality standard was extracted from trees with diameter ≥ 15 cm with heartwood proportions of 48.53% and 52.36%. Based on this result, we propose that selection of mature trees with diameter ≥15 cm with heartwood proportion ≥ 48.53% can be used as the strategy of sandalwood tree utilization.

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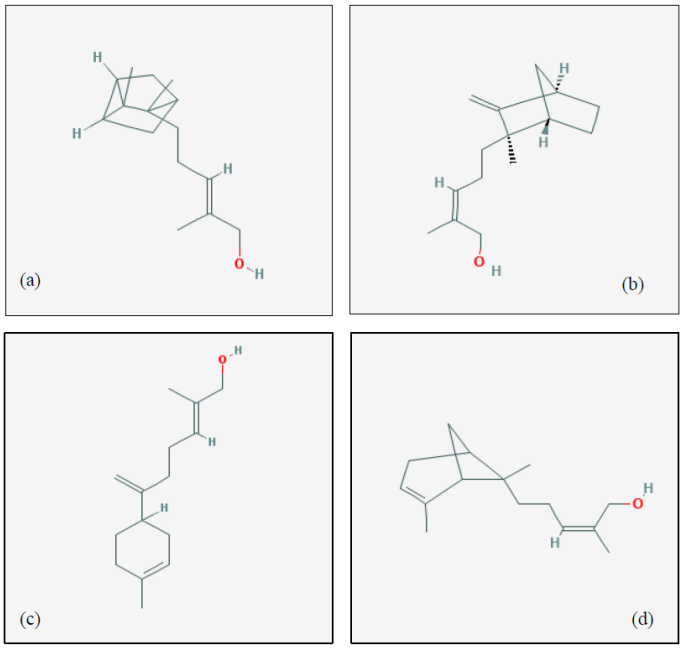

Figure 5. Stereoisomer molecular structures of the main components of sandalwood Oil ($C_{15}H_{24}O$) with molecular weight of 220.

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