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Genetic variation of agarwood producing tree (*Gyrinops versteegii*) from Pongkor, Manggarai District, Flores Island, Indonesia using ISSR molecular markers

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Abstract. *Irsyad AF, Rindyaastuti R, Yulistyarini T, Darmayanti AS, Daryono BS. 2020. Genetic variation of agarwood producing tree (Gyrinops versteegii) from Pongkor, Manggarai District, Flores Island, Indonesia using ISSR molecular markers. Biodiversitas 21: xxxx.* Agarwood is a black-colored tree wood that produces distinctive sap because of fungal infections which belong to Thymelaeaceae family (mainly *Aquilaria* and *Gyrinops*). Agarwood product is highly valuable that leading to over exploitations by the collectors. To develop the most effective and efficient conservation strategies, genetic information from these plants is required. The aims of this research are to determine the genetic variation and to confirm the species identity of agarwood producing tree (*Gyrinops versteegii* (Gilg.) Domke) population in Pongkor Community Forest, Pongkor, Manggarai District, Flores Island, East Nusa Tenggara. Information of the genetic variation, as well as the phenetic relatedness, were evaluated with inter-simple sequence repeat molecular marker (ISSR) using five primers; Ng2.01, Ng2.06, Ng3.01, Ng3.02, and UBC 855, with two other agarwood producing species as outgroup (*Aquilaria filaria* and *Gyrinops decipiens*). Amplified bands from all primers showed 55.17% polymorphic bands in *G. versteegii*. Genetic variation of *G. versteegii* identified with Nei's genetic diversity (h value) obtained at 0.218. Clustering analysis from UPGMA dendrograms formed three major clusters. Degree of similarity of *G. versteegii* based on the dendrograms obtained at 85.9% using SSM method. The results showed close phenetic relatedness between individuals and relatively high genetic variation of *G. versteegii*, however, imply the need for strictly maintenance of habitat preservation and larger population size.

Keywords: Agarwood, *Gyrinops versteegii*, genetic variation, ISSR, heterozygosity

INTRODUCTION

Agarwood is a black-colored tree wood that produces distinctive sap from various tree species that mostly belong to the *Aquilaria* genus. Agarwood is a part of sapwood which was infected by fungal microbes (usually *Phialophora parasitica*) that change the color of the agarwood producing tree from pale and brightly colored into dark and blackish (Akter et al. 2013; López-Samson and Page 2018). Agarwood has a very high economic value which is widely processed into perfumes and fragrances because of its distinctive aroma (Zich and Compton 2001; Turjaman et al. 2016). Moreover, agarwood producing trees could also be used as various medicines (Dahham et. 2016; Okugawa et al. 1993), an ingredient in sculpture, although it is rarely used in such way (Barden and Anak 2002). The quality of agarwood is determined by several factors, including the plant species, geographical location, parts of tree such as roots, stems, branches, the infection duration, as well as how it is harvested (López-Samson and Page 2018).

The high economic values of agarwood cause the producing tree to become vulnerable to exploitation, which was indicated by the decreasing product quality and quantity. The decline has caused the agarwood-producing trees are listed in Appendix II of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (CITES 2017). One of the listed species is *Gyrinops versteegii* (Gilg.) Domke, which is an agarwood-producing species originated from West Nusa Tenggara and East Nusa Tenggara regions, Indonesia (Sidiyasa 1986; Roemantyo and Partomiharjo 2010).

Decreasing population of agarwood producing trees can be overcome by conservation programs to maintain the existence of plant and its sustainability use for resinous products or medicines. In formulating the proper and effective conservation strategies, genetic information from related organisms is required (Sitepu et al. 2011; Turjaman and Hidayat 2017). Genetic identification is the first step to provide information as a reference in conservation management of organisms. This genetic identification is generally carried out molecularly using molecular marker technique.

One of molecular marker techniques included in the genetic study is Inter Simple Sequence Repeat (ISSR). This method amplifies the microsatellite, which is a sequence consisting of two to seven nitrogen bases in the DNA strand and repeatedly (Richard et al. 2008; Semagn et al.

2006). The ISSR method has several advantages, some of which are it does not require a sequence data in primary construction, the amount of DNA template needed is quite low because the analysis procedure only includes Polymerase Chain Reaction (PCR), and the process is randomly distributed throughout the genome (Godwin et al. 1997). Moreover, ISSR has been proven as successful method to evaluate intra-species genetic variation (Daryono et al. 2019). The genetic identification can provide information on species relatedness, population origin of the organism, and the estimation of the number of individuals in a population as the results. The genetic identification also assists in monitoring activities of endangered species (Allendorf et al. 2013). Molecular markers were widely used for genetic identification of agarwood producing species, such as *G. versteegii* from Papua using RAPD method (Siburian et al. 2017), *G. versteegii* from Lombok using RAPD markers (Widyatmoko et al. 2009), *Aquilaria malaccensis* using ISSR markers (Banu et al. 2015), natural *Aquilaria* populations in Peninsular Malaysia using ISSR markers (Lee et al. 2018a), selected *Aquilaria* species from Malaysia using RAPD and SCARS (Lee et al. 2011).

One of natural population of *G. versteegii* was recently discovered in Manggarai District, Flores Island in a small forest fragment of Pongkor Community Forest (Rindyastuti et al. 2019; Yulistyarini et al. 2019). A species that grow in small and fragmented population generally maintain lower level of genetic variation (Chung 2009; Li and Jin 2006). Genetic variation is the response of organisms in adapting to changed environment condition which leads to adaptation mechanisms. Genetic variation becomes the most important component for efficient species conservation (Frankham et al. 2002). The information on genetic diversity level and its distribution of species are very useful to design the conservation strategies for rare or endangered species (Widyatmoko 2011). On the other hand, Lee et al. (2018b) stated that information on genetic variation of agarwood-producing plants is still very deficient especially in the term of its species and habitat conservation. Therefore, it is necessary to establish the genetic research using molecular markers of related organisms to complete such information. The aims of this study are (i) to confirm the species identity of agarwood producing tree (*G. versteegii*) found in Pongkor, Manggarai District as well as the phenetic relatedness between individuals. (ii) to determine the genetic variation of agarwood producing tree in Pongkor Community Forest, Pongkor, Manggarai District, Flores Island, East Nusa Tenggara, Indonesia.

MATERIALS AND METHODS

Procedures

Sample collection and preparation

Fresh leaves from 12 *Gyrinops versteegii*'s seedling were collected from each sampling point in Pongkor Community Forest, Pongkor, Manggarai District, Flores Island, East Nusa Tenggara with the area of 17 Ha (Yulistyarini et al. 2019) (Figure 1). For the outgroups, plant samples were collected from the nursery of Purwodadi Botanic Garden, Research Centre for Plant Conservation and Botanic Gardens, Indonesian Institute of Sciences (LIPI), Purwodadi, Pasuruan, Indonesia. Samples were stored in a freezer with a temperature of-20^{o}C and removed when DNA isolation process was conducted.

DNA isolation and quantitative analysis

DNA was extracted from leaf samples using *Cetyl Trimethylammonium Bromide* (CTAB) method based on Doyle and Doyle (1987) protocol with modifications. DNA purity and concentration were checked quantitatively using Tecan Spark multiplate reader (Tecan, CH) with λ 260/280 nm ratio.

Figure 1. Sampling area of agarwood producing tree (*Gyrinops versteegii*) in Pongkor Community Forest, Pongkor, Manggarai District, Flores Island, East Nusa Tenggara, Indonesia (Sampling points marked with red dots)

Table 1. ISSR primers used for genetic analysis of agarwood producing tree (*Gyrinops versteegii*)

Primers	Sequence	Tm (°C)
Ng2.01	(AC)8B	56.1
Ng2.06	(CT)8D	53.6
Ng3.01	(ACA)5SS	47.6
Ng3.02	(AGA)5SS	53.6
UBC 855	(AC)8YT	56.1

DNA amplification with PCR-ISSR

Five ISSR primers were chosen for DNA amplification from Lee et al. (2018a) which have highest PIC value. Detailed information of the primer's sequence and melting temperature (Tm) are showed in Table 1.

PCR mixture in this research consist of 12,5 µL Bioline 2x MyTaq HS Red Mix (Bioline, UK), 2 µL 25 ng/µL for each primers, 2 µL 10 ng/µL DNA samples, 8.5 µL ddH_2O for each reaction (total 25 µL per mix). PCR running process was started with initial denaturation at 94°C for 10 minutes, denaturation at 94°C for 1 minute, annealing at specific temperature based on Tm for each primer at 1 minute, each process was repeated for 40 cycles. PCR was running then continued by elongation at 72°C for 2 minutes, and followed by post elongation at 72°C for 10 minutes.

Results of PCR product were analyzed using 2% agarose gel electrophoresis with fluorosafe DNA Stain (1st BASE, MY) and Thermoscientific GenePlus DNA ladder (Thermo Fisher Scientific, US) 100 bp at 50v for 60 minutes. The results were visualized with GelDoc.

Data analysis

DNA bands from electrophoresis were converted into matrix of binary data. Present bands were scored as '1', while absent bands in locus were scored as '0'. Basic parameters as number of locus, monomorphic bands, polymorphic bands, and percentage of polymorphic bands were calculated manually from the matrix. Heterozygosity (h value) based on Nei (1973) from *G. versteegii* was obtained from the matrix analysis using GenAlEx 6.503. Heterozygosity value was performed using the parameters of %P (the number or percentage of polymorphic loci), He (expected heterozygosity) and uHe (unbiased expected heterozygosity).

Another software used to show phenetic relatedness between individuals was MVSP 3.1. The software of MVSP constructed Unweighted Pair-Group Method with Arithmetic means (UPGMA) based dendrogram, using simple matching coefficient (SSM) clustering analysis.

RESULTS AND DISCUSSION

Species characteristics and identification

The *Gyrinops* genus is distinguished based on the characteristics of its flowers and leaves (Zich and Compton 2001). The species of *Gyrinops* in this study is *G. versteegii*, which is often exploited in the West Nusa Tenggara and East Nusa Tenggara regions. *G. versteegii* has oval-shaped leaves, 5-20 cm x 1.5-5 cm, with a short stem that measured around 3-5 mm. The base of the leaf is peg-shaped, the tip of the leaf is pointed out with 12-16 pairs of leaf veins, and the surface of the lower leaves is dull green and the upper part is bright green and shiny. The dry leaves on the lower surface are yellowish-brown, while the upper part of the dry leaves is reddish-brown. The corolla is salverform with the stamen number equal to the petals (i.e. 5) (Hou 1960; Susilo et al. 2014). Based on the field observation, *G. versteegii* found in Pongkor Community Forest has membranaceous leaves with a bright green color on the upper surface, with 16 pairs of leaf veins, greenish-brown stems, as well as an entire leaf margin. The flowers have tube shaped corolla, with 5 petals and 5 stamens (Yulistyarini et al. 2019).

DNA isolation and quantitative analyses

DNA was extracted from 12 leaf samples of *G. versteegii*, 2 leaf samples of *Gyrinops decipiens*, and 2 leaf samples of *Aquilaria filaria*. DNA concentration and purity from each sample were analyzed quantitatively using Tecan Spark multiplate reader with λ 260/280 nm ratio. Table 2 shows the result of DNA quantitative analysis. The sample of *G. versteegii* is denoted by the code 'RDS,' while the outgroup *Gyrinops decipiens* sample is denoted by the 'Gd' code, and outgroup *A. filaria* was denoted by the code of 'Af'.

The DNA concentration was obtained in the range of 120.44-1286.51 ng/µL, while the purity ratio λ 260/280 nm showed a range of 1.50-1.92. A great DNA purity ratio for molecular analysis is in the range of 1.8-2.0. A purity ratio below 1.8 can be caused by protein contamination. As for the ratio above 2.0, it is assumed that there is RNA content and phenol, chloroform and urea contamination (Sambrook and Russel 1989). The results of this research, which has 6 RDS samples, including RDS 1, RDS 4, RDS 7, RDS 8, RDS 9, and Af 2, have ratios in the range mentioned above. The other samples have a ratio under 1.8, but slightly different from Gd 2 that has the lowest ratio. This indicates the presence of protein contamination in the sample. This can be overcome by optimizing pellet washed with alcohol, especially when the centrifugation process takes place, as well as adding *polyvinylpyrrolidone* (PVP) power on DNA isolation as an antioxidant to bind contaminant compounds (Azhari and Mohamad 2013). The results of DNA samples that have been analyzed quantitatively need to be further tested by running the PCR to determine the quality of the bands formed.

DNA amplification using PCR-ISSR

Five PCR primers were selected for amplification of the DNA of agarwood producing trees based on the research by Lee et al. (2018a) for the *Aquilaria* genus. Primers were selected based on reproducibility and the high value of polymorphism information content (PIC). The results of DNA amplification using each primer are shown in Figure 3.

Table 2. Species, sample codes and quantitative analysis results of agarwood producing tree including ingroup of *Gyrinops versteegii* and outgroups.

Species	Samples	DNA concentration (ng/µL)	DNA purity (λ 260/280)
G. versteegii	RDS 1	244.90	1.87
	RDS 2	287.75	1.72
	RDS 3	311.54	1.72
	RDS 4	258.89	1.89
	RDS 5	271.55	1.71
	RDS 6	382.26	1.67
	RDS 7	174.35	1.92
	RDS 8	195.60	1.88
	RDS 9	373.73	1.80
	RDS 10	215.39	1.78
	RDS 11	311.08	1.79
	RDS 12	120.44	1.63
G. decipiens	Gd 1	1286.51	1.55
	Gd 2	863.22	1.50
A. filaria	Af 1	272.44	1.67
	Af 2	213.37	1.81

The results of DNA amplification in all primers produced clear and reproducible bands in all samples of *G. versteegii* with a total band of 58 with the polymorphic bands percentage of Ng2.01 primers, Ng2.06, Ng3.01, Ng3.02, and UBC 855 respectively at 69.23%, 50%, 64.29%, 40%, and 54.55% (Mean: 55.61%) (Table 3). Overall, all primers showed high genetic information, because the polymorphic percentage showed a value of ≥ 50%. The only primer that showed a polymorphic percentage value of less than 50% was Ng3.02, implying that Ng3.02 was not optimally used in the analysis of genetic variation, particularly in *G. versteegii*. Low polymorphic percentage can be caused by similar species from a similar population, therefore the genetic variation obtained will be relatively low.

Clustering analysis

The results of clustering analysis with UPGMA divide 16 samples consist of *G. versteegii*, *G. decipiens*, and *A. filaria* into 2 main clusters (Cluster A and B) using SSM method. Dendrogram was shown in Figure 4.

Based on the dendrogram, samples of Af 2 and Af 1 form sub-clusters at 72.5% similarity index, and Gd 2 and Gd 1 which form sub-clusters in the 87.9% similarity index, which groups on the similarity index of 59.9%, forming cluster A. Cluster B represents all 12 RDS samples clustered on the 85.9% similarity index, therefore, the similarity index between samples was obtained at a value of ≥85.9%. Clusters A and B later grouped on the similarity index of 50.1%.

Heterozygosity

DNA band scoring data was used for the analysis of heterozygosity (h value) based on Nei (1973). The higher heterozygosity in a population, the higher the genetic diversity of plant species. The results of heterozygosity analysis on *G. versteegii* in Pongkor Community Forest obtained % P (the number or percentage of polymorphic loci) at 56.18% and the h value in parameters of He (expected heterozygosity) at 0.218 and uHe (unbiased expected heterozygosity) at 0.227.

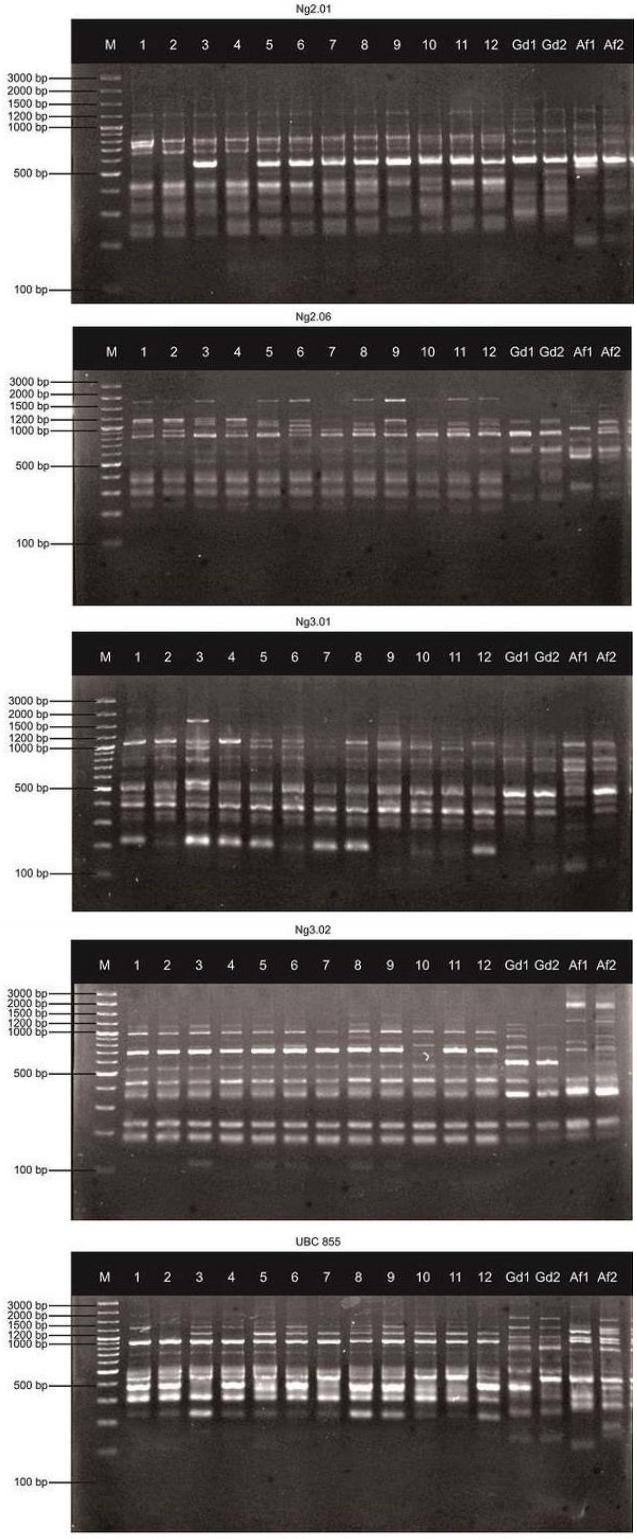

Figure 3. DNA amplification results of 12 samples of *Gyrinops versteegii* using 5 primers, from top to bottom: Ng2.01, Ng2.06, Ng3.01, Ng3.02, and UBC 855 primer. PCR products were separated by 2% agarose gel at 50v for 60 minutes.

Table 3. The number of DNA strands, monomorphic DNA, polymorphic DNA, PIC value and percentage of polymorphic allele of each primer.

Primer	Primer Sequence 5'-3'	Number of DNA strands	Number of DNA monomorphic	Number of DNA polymorphic	PIC value	Percentage of polymorphic alleles (%)
Ng2.01	(AC)8B	13	4	9	0.46	69.23
Ng2.06	(CT)8D	10	5	5	0.47	50
Ng3.01	(ACA)5SS	14	5	9	0.47	64.29
Ng3.02	(AGA)5SS	10	6	4	0.47	40
UBC 855	(AC)8YT	11	5	6	0.45	54.55
Mean						55.61

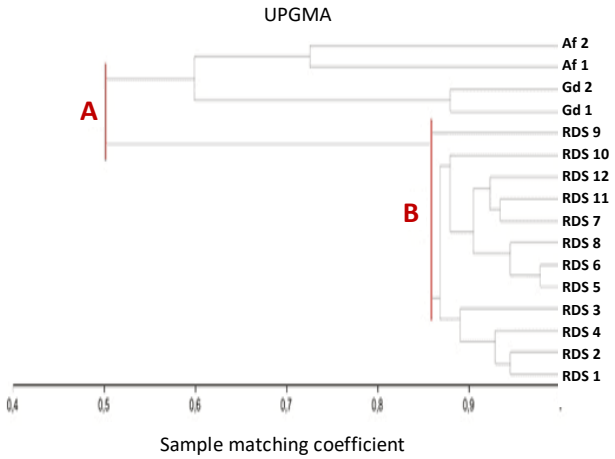

Figure 4. UPGMA clustering analysis of agarwood producing trees using SSM method. The code of RDS is for ingroup *Gyrinops versteegii*, Af and Gd are for outgroup (*A. filaria* and *Gyrinops decipiens*)

Discussion

Species identification and phenetic relatedness

According to the principle of taxospecies, if the similarity index reaches ≥70%, the grouped individuals will be assumed as one species. The greater the value of the similarity index, the closer the phenetic relatedness of species studied (Habich 2001). The result showed that the population found in the forest were properly identified as *G. versteegii*, a different species from *Aquilaria* and other *Gyrinops* species which probably distributed in Flores Island. Twelve samples of *G. versteegii* based on the SSM method, belong to the similar species because they met these taxospecies principles. Moreover, all *G. versteegii* individuals in Pongkor Community Forest population were assumed to have a very close phenetic relatedness.

Agarwood producing trees including five species i.e. *Gyrinops versteegii, G. ledermannii G. decipiens, G. podocarpus, G. caudate,* and *G. salicifolia* (natural habitat in Indonesia and Papua New Guinea) (Roemantyo and Partominharjo 2010; Zich and Compton 2001) and *G. walla* (natural habitat in Sri Lanka) (Schun and Cordell 1985). These species was still taxonomically intricated (Turjaman and Hidayat 2017; Roemantyo and Partomihardjo 2010), especially for two most important genera of agarwood producing tree i.e. *Aquilaria* and *Gyrinops*. These two genera are able to distinguish based on the number of stamen (Hou 1960). In *Aquilaria*, the number of stamens is twice the number of petals (i.e. 10), while *Gyrinops* have equal numbers of stamens as petals (i.e. 5). However, the classification basis is debatable because it only uses single character (Zinc and Compton 2001). Paraphyletic taxonomical classification based on the DNA bands using molecular methods (Lee et al. 2018b) and morphological characteristics were established (Wangiyana 2019), therefore some experts noted the need of merging the genus of *Aquilaria* and *Gyrinops* as one evolutionary unit (Lee at al. 2018b). Genetic evidence from this study showed lower variation and similarity between plants of *Aquilaria* and *Gyrinops*. Based on this result, *A. filaria* is grouping with *Gyrinops decipiens* and *G. versteegii* on the similarity index below 70% (50.1%), indicating high variations among three species based on the taxospecies principle. The result of this study is quite different from other previous studies which showed high similarity between *Aquilaria* and *Gyrinops* plants. The study using broader samples of two genera, combining other taxonomical evidence such as morphology, anatomy, etc. is needed to construct reliable taxonomic classification (Lee et al. 2018b).

Genetic variation of Gyrinops versteegii

A population can be considered to have a high genetic diversity value if it has a high percentage of polymorphism and high expected heterozygosity value (Frankam et al. 2002). DNA band scoring data was used for the analysis of polymorphism and heterozygosity (h value) based on Nei (1973). Low heterozygosity value indicates scarcity of genetic variation of organisms in the wild population, therefore it serves as an important parameter in determining conservation strategies of plant species (Siburian et al. 2017). Many previous studies classified the value of heterozygosity more than 0.20 for common plant families, especially rare species as high (Hamrick and Godt 1996). Nei's genetic diversity of endangered and endemic species with limited distribution range is not expected to be high, because, the relationships between population size, plant fitness, and genetic diversity are generally significantly positive (Leimu et al. 2006). Many studies reported relatively high genetic diversity of rare species such as in *Tuberaria major* (0.197) (Trindade et al. 2012), rare and endemic orchids of *Vanda foetida* (0.1999 and 0.1778) (Rindyastuti et al. 2015) and rare medicinal plant *Magnolia*

officinalis (0.342) (Yu et al. 2011), vulnerable woody species from Sulawesi *Diospyros celebica* (0.28) (Widyatmoko et al. 2011).

The results of polymorphism percentage and heterozygosity analysis on *G. versteegii* in Pongkor Community Forest obtained at 56.18% and h value in the parameters of He at 0.218 and uHe 0.227. The polymorphism percentage was classified as moderate, because other previous study obtained %P at more than 70% (Rindyastuti et al. 2015; Yu et al. 2011). Compared to other endangered and endemic species, the h value of *G. versteegii* obtained in this study is high. Other research results by Siburian et al. (2017) revealed that the heterozygosity values of *G. versteegii* in Kebar, West Papua, and Manokwari, West Papua were obtained at 0.2944 and 0.2357, respectively. This value is slightly greater than heterozygosity of *G. versteegii* in Pongkor Community Forest. However, it can be assumed that the genetic variation of *G. versteegii* in Pongkor Community Forest is still within the common range of the species. Compared to h value of other agarwood producing tree, h value obtained from this study was higher than h value of *A. malaccensis* from Assam, India which was obtained at 0.14 (Banu et al. 2015). Nonetheless, the research of Singh et al. (2015) showed that other agarwood-producing species, *A. malaccensis* had heterozygosity values in the range of 0.597-0.811. This shows that the heterozygosity value of *G. versteegii* is relatively high however could be increased to the level of higher plant fitness (> 0.30) by endorsing reliable conservation programs.

Habitat fragmentation negatively affects genetic variation and plant fitness (Leimu et al. 2006). The result of this study showed that *G. versteegii* in Pongkor which growing upon the remnant forest fragment in the small area of 17 Ha should be a priority in the conservation programs because although it has relatively high genetic diversity, the natural population is threatened by environmental changes, habitat distraction by human and other forest disturbances. Beside establishing habitat preservations, conservation strategy could be established by maintaining and increasing population size of *G. versteegii* in Pongkor Community Forest through plant propagation and reintroduction (Falk and Holsinger 1991). The plants of *G. versteegii* from Pongkor Community Forest could be recommended as the source of species propagation and reintroduction, however, needed to increase its genetic variation using cross-pollination among individual within-population or inter-population both naturally and artificially. Cross-pollination supports the plants to have random mating within-population which could maintain genetic variation of plant species. Moreover, collecting seed and transferring seedling from other populations could also increase the gene flow among population of *G. versteegii* in Flores Island.

In conclusion, the plant samples in this population were properly identified as *G. versteegii* and have very close phenetic relatedness among the individual, indicating that there are no significant gene differences. All primers showed relatively high genetic variation of *G. versteegii* in Pongkor Community Forest population. Heterozygosity values were relatively high, slightly different from the common range of the species but lower compared to those other species. Genetic variation of *G. versteegii* which growing in smaller populations implying the need for maintaining larger population size to obtain high genetic diversity and species fitness.

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AFI was mentioned as first author for mainly doing the research thesis and wrote the manuscript. RR as a team leader of the CITES project facilitated the funding, developed the research concept and wrote the discussion. TY and ASD validated the field data and discussion, especially for conservation implication. BSD wrote and verified research method, scientific framework, and finding.

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