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The effects of population size on genetic parameters and mating system of sandalwood in Gunung Sewu, Indonesia

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Abstract

We combined field observations with isoenzyme analysis to compare population demographic and its effects on genetic diversity and mating systems, among six populations of sandalwood in Gunung Sewu, Indonesia, during March to August 2015. This endangered economic-important species was originated from the southeastern parts of Indonesia, but is recently occured as new landraces in Gunung Sewu, Java island. The observed heterozygosity varied from H_o 0.184 to 0.385 in parents, and from H_o 0.083 to 0.348 in offspring levels, based on the degree of clonality and genetic base. Most of genetic variation is distributed within populations, and only 2.7% were presented among populations, that was indicated by the low D_{ST} and F_{ST} value (H_T 0.30; H_S 0.276; D_{ST} 2.4%; F_{ST} 7.98%). A dendrogram indicated a grouping of populations into three clusters. However, there were seemed to be no association between geographical and genetic distance. Genetic depletion occured due to (i) clonality events as result of heavy-exploitation and/or natural disturbance which induced root suckering, (ii) genetic drifts and bottleneck effects, (iii) the founder effects due to parental low diversity, and (iv) the alteration on mating systems to be more inbreeders. Some of the results confirmed a "reproductive assurance prediction" while some others were contradicting this. It seemed that genetic diversity and mating systems are not much affected by population size, but more by the parental heterozygosity and the degree of clonality. Our results emphasized the importance of populations' genetic base or parental genetic diversity to naturally maintain the genetic and evolutionary processes under equilibrium conditions.

Keywords: Gunung Sewu; genetic parameters; mating systems; population demographic; sandalwood

Introduction

The preservation of genetic diversity in endangered species is a main goal on designing conservation strategies (Pautasso, 2009). Many authors emphasized the importance of genetic studies with regard to demographic approaches. Genetic markers were widely used and, among these, some have conducted by allozyme markers. Many studies reported strong influences of geographical variation on population structures, and its effects on mating systems (Herlihy and Eckert, 2005; Arroyo *et al.*, 2006) and genetic diversity (Rao *et al.*, 2007; Bottin *et al.*, 2007; Dani *et al.*, 2011; Indrioko and Ratnaningrum, 2015). Generally, the small, isolated or fragmented populations might experienced gene flow limitation (Warburton *et al.*, 2000; Craft and Ashley, 2007) and mating systems alteration (Kelleher *et al.*,

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2005; Herlihy and Eckert, 2005; Arroyo *et al.*, 2006). This condition will in turn affect plant fitness (Torres *et al.*, 2003; Herlihy and Eckert, 2005), population viability (Schmidt *et al.*, 2009) and genetic structures (Craft and Ashley, 2007; Kettle *et al.*, 2007). Combined with the low population density or genetic base, this may reduce outcross-pollination, and particularly in self-compatible taxa, increase selfing (Herlihy and Eckert, 2005; Barrett *et al.*, 2006; Arroyo *et al.*, 2006). Furthermore, a decrease of outcrossing opportunities in small, isolated populations may conform to "reproductive assurance theory", the selection for alleles that increase autonomous-autogamous self-fertilization to maintain reproductive assurance (Herlihy and Eckert, 2005; Arroyo *et al.*, 2006; Barrett *et al.*, 2006). Selection for autonomous-autogamy in isolated populations will be represented by geographical variation on floral morphology, development, and/or physiology, that in turn enhance the level of selfing.

Loss of diversity has taken larger attention in sandalwood (*Santalum album* L., Santalaceae) genetical and ecological studies during last couple of decades (Brand, 1994; Warburton *et al.*, 2000; Angadi, 2003; Byrne *et al.*, 2003; Lhuillier *et al.*, 2006; Rao *et al.*, 2007; Bottin *et al.*, 2007; Dani *et al.*, 2011; Indrioko and Ratnaningrum, 2015). Australia, India, and Indonesia are among the main exporters of sandalwood's wood and oil (Angadi *et al.*, 1993; Rao *et al.*, 2007), but within the last three decades, this species has undergo significant degradation and habitat loss, and therefore classified as *vulnerable* species (IUCN, 2009), even *extinct in the wild* in most of its native in south-eastern parts of Indonesia (Anonymous, 2012; Indrioko and Ratnaningrum, 2015).

Many sandals species exhibited pattern of genetic differentiation between geographic regions, particularly when there are barriers for gene flow (Byrne *et al.*, 2003; Bottin *et al.*, 2007). Differences in geographical evolutionary history (Byrne *et al.*, 2003; Bottin *et al.*, 2007), as well as a history of disturbance (Warburton *et al.*, 2000; Lhuillier *et al.*, 2006; Shcmidt *et al.*, 2009; Dani *et al.*, 2011), may also affects various evolutionary forces that are resulting in different patterns of genetic structure. However, to date, only very limited studies have been conducted in correlating the geographical variation to the genetic diversity and mating systems of sandals group (Harbaugh and Baldwin, 2007; Rao *et al.*, 2007; Dani *et al.*, 2011). Such works were mostly conducted in Southern India (Sindhu-Vereendra and Anantha-Padmanabha, 1993; Brand, 1994; Suma and Balasundaran, 2003; Rao *et al.*, 2007; Dani *et al.*, 2011), Western Australia (Warburton *et al.*, 2000; Byrne *et al.*, 2003; Tamla *et al.*, 2012), insular Pacific (Lhuillier *et al.*, 2006), and New Caledonia (Bottin *et al.*, 2007) for more than couple of decades. However, only recently have such comparative analyses been extended to sandals of other regions. In Indonesia, that is believed as the center of origin of sandalwood (Brand, 1994; Angadi, 2003; Harbaugh and Baldwin, 2007), only very limited data of *Santalum album* were available from the southeastern (Brand, 1994; Herawan *et al.*, 2014; Indrioko and Ratnaningrum, 2015) and, particularly, central (Ratnaningrum and Indrioko, 2014; 2015) regions.

Despite significant degradation on its origin in southeastern parts of Indonesia, new landraces of sandalwood emerged in Gunung Sewu Area of Java island, the central part of Indonesian archipelago. Gunung Sewu is a mountainous limestone zone spanning three GeoAreas, comprising approximately 1300 km^2 of width (Simanjuntak, 2002). Preceding geological processes that started from about 1.8 million years ago –involving tectonic movements, volcanic activities and seawater erosion– has derived this area into various landscape structures that differed in altitude, elevation, soil characters, and microclimate conditions (Simanjuntak, 2002; Haryono and Suratman, 2010). The oldest herbarium speciment of sandalwood in Java islands (dated by year 1853) was collected from Imogiri District, and another specimen (dated by year 1960) was found in Nglipar District; both were part of Gunung Sewu area.

In this study, we compare population demographic variables and its effects on genetic parameters and mating systems, among geographically marked landraces of sandalwood along Gunungkidul GeoArea of Gunung Sewu. Here we combined field observations with biochemical marker-gene analysis to test two main hypotheses. (1) The small, isolated and/or fragmented populations will exhibit lower level of genetic diversity; and (2) As a result of selection conformed to "reproductive assurance theory", the populations with lower genetic diversity will exhibit higher self-fertilization in compared to the bigger and/or continuous populations.

Materials and Methods

Population characteristics

Gunung Sewu consisted of more than ten sandalwood populations in the form of both planted and naturally regenerated stands. However, we only compared six populations which were representing distinctively different population structures in each of geographical zone. Research was conducted in one of population in the lowland of Middle Zone (Bleberan population, herein after refers to CP-Bleberan), two populations in the highland of North-western Zone (NWP-Nglanggeran and NWP-Sriten), two in the karst region of Southern Zone (SP-Botodayakan and SP-Petir), and one in the caves area of northern part (NP-Bejiharjo) of Gunung Sewu region, during March to August 2015. CP conformed to Central Population, NWP North-West Population, NP Northern Population, and SP Southern Population. These sites are spatially separated by more than 10 km, and even much more for the Bejiharjo site that was separated more than 30 km away from any others populations. These sites are at different altitude, experienced different climatic regimes and having clear ecological differences (Table 1; Figure 1).

In 2015, Gunung Sewu was announced as a new Global Geopark Network, along with eight other new sites throughout the world. Geographically, Gunung Sewu areas stretched 85 km west-east covers three GeoAreas of Gunungkidul (Yogyakarta), Wonogiri (Central Java), and Pacitan (East Java). Its north-south width varies between 10 and 29 km with approximate area of 1300 km^2 (Haryono and Suratman, 2010). Gunungkidul GeoArea itself is geographically derived into three zonations: (1) **Northern Zone,** the highland of Batur Agung at 300 m to 800 m asl; (2) **Middle Zone,** the lowland of Ledok Wonosari at 150 m to 300 m asl; and (3) **Southern Zone,** the Gunungsewu Karst at 0 m to 200 m asl. Recently, Gunung Sewu possessed two seasons. Rainy season occured during October to March and the dry season took place from April to September. As it is adjacent to Indian Ocean, Gunung Sewu areas are having two types of climate -- *Aw*, the semi-arid to arid type that are characterized by a long drought; and *Am* which is representing an intermediate condition between the tropical and sub-tropical climate (Simanjuntak, 2002; Haryono and Suratman, 2010).

Four groups of new sandalwood landraces were found in Gunungkidul GeoArea of Gunung Sewu (Table 1; Figure 1). **The first group** was the highland parts of North-western Zone, consisted of Nglanggeran (Patuk District) and Sriten (Nglipar District) populations. **The second group** was the lowland of Middle Zone, consisted of Bleberan (Playen District) and Ngingrong (Wonosari District) populations. This Middle Zone was also considered as the center of sandalwood population in Java Island, since along with these landraces, there were several *ex situ* conservation areas established in Playen District which were separated only by less than 10 km. These conservation areas, totally comprised more than 30 Ha of width, were established since 1967 and consisted of more than 40 provenances originated from Timor, East Nusa Tenggara and Java islands of Indonesia. **The third group** was the karst region of Southern Zone and were consisted of Semugih (Ponjong District), Pucanganom (Panggang District), and Petir, Semugih and Botodayakan (all were Rongkop District) populations. **The fourth group**, Bejiharjo

Table 1. Population structures and the habitat characteristics of study sites

Population, occupance, altitude, climatic types	Latitude	Landscape history and present habitat characteristics	Soil and rock units	Sandalwood history and present population characteristics
NWP-Nglanggeran; 79.3 ha; 710-750m asl; *Am* type	710m to 750m asl; 7^{0}50′28″ S to 110^{0}32′55′′E	A part of Nglanggeran Formation, Northern Zone of Gunung Sewu. Now existed as the mountainary landcapes, strong undulating, characterizing tropical mountain ecosystems.	Latosols with volcanic and sediment rocks, some with deeper solum.	Sandalwood was first documented in 1970's. Recently occured in groups of stands across the Nglanggeran mountain regions, in association with the tropical mountain vegetation. Habitat dominated by the association of naturally regenerated mahogany, *Gliricidea* sp, and several *Garcinia* and *Eugenia* families.
NWP-Sriten; 25 ha; 750-790m asl; *Am* type	750m to 890m asl; 7^{0}50′47″ S to 110^{0}38′57′′E	A part of Semilir Formation, Northern Zone of Gunung Sewu. Now existed as the highland landscapes, strong undulating, characterizing tropical mountain ecosystems. Isolated by mountainary physical barriers.	Latosols with volcanic and sediment rocks, mostly with deeper solum.	Sandalwood was first documented in 1960's. Recently covered three biggest hills in an association with the tropical highland vegetation. Habitat dominated by the association of naturally regenerated mahogany, *Gliricidea* sp, and several *Garcinia* and *Eugenia* families. Isolated by mountain physical barriers.
NP-Bejiharjo; 9.6 ha; 150-180m asl; *Aw* type	150m to 180m asl; 7^{0}55′35″ S to 110^{0}39′11″ E	A part of Sambipitu Formation, Northern Zone of Gunung Sewu. Now existed as the open dry-rocky hilly landscapes with caves and ground-rivers below. Representing the dryland ecosystems.	The association of red mediterrans and black grumosols with limestone rocks, mostly with the shallow solum	Sandalwood is a remnant of the 1970's planted stands. Fragmented due to heavy exploitation, urban and cave-tourism activities since 1990's. Now existed as a small-fragmented group of stands, dispersely occured in an open dry-rocky hills above the caves and ground-rivers. Sandalwood grew in an association with cajuputi and acacia regenerated from commercial plantation nearby. Younger sandal trees were largely derived from root suckers. Sites dominated by dryland herbs such as grasses and *Eupatorium* sp.
CP-Bleberan; 52.9 ha; 150-170m asl; intermediate between *Aw* and *Am* type	150m to 170m asl; 7^{0}50′8″ S to 110^{0}50′75″ E	A part of Wonosari Basin Formation, Middle Zone of Gunung Sewu. Now existed as the catchment area of the ancient subterranean Oya River at the lowland basin landscapes. Representing the tropical lowland ecosystems.	The association of red mediterrans and black grumosols with limestone rocks, mostly with the deeper solum.	Sandalwood was first documented in 1970's along the catchment area of the ancient subterranean Oya River, at the lowland basin of middle zone. Sandalwood dispersed widely along the riparian catchment area and nearby, in association with the tropical lowland forest vegetation which is consisted of more diverse vegetation including teak, mahogany, *Gliricidea* sp, *Schleicera* sp, cajuputi and acacia. Population is surrounded by several *ex situ* conservation areas which are sharing the same river.

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SP-Petir; 78 ha; 70-100m asl; *Aw* type	70m to 100m asl; 7^{0}50″60^0 S to 110^{0}60′15″ E	A part of Wonosari-Punung Karst Formation, Southern Zone of Gunung Sewu. Now existed as the karst hilly landscapes with open dry-rocky hills, strong undulating, characterizing the dry rocky-limestone ecosystems.	Latosols with limestone rocks. Solum is deeper at the basins, but very shallow at the limestone-rocky hills.	Sandalwood was first documented in 1960's in karst hilly areas, recently covering more than twenty open dry-rocky hills. Adult plants were mostly derived from root suckers; highly clonalized. In the open-undulating areas, sandalwood grew in association with dry rocky-limestone vegetation including acacia and cajuputi, but more dominated by shrubs and herbs such as grasses and *Eupatorium* sp.
SP-Botodayakan; 6.75 ha; 70-100m asl; *Aw* type	100m asl; 7^{0}50″10^0 S to 110^{0}52′30″ E	A part of Wonosari-Punung Karst Formation, Southern Zone of Gunung Sewu. Now existed as the karst landscapes with very dense teak forests, surrounded by the hilly slopes.	Latosols with limestone rocks; mostly with deeper solum, particularly at the basin and forests.	Surrounded by a very dense teak forest and was bordered by the inaccessible rocky-hills slopes as the physical barriers. Most of sandalwood grew in small groups in an association with dry-lowland forest habitat, shaded by dense teak canopies.

(Karangmojo District), was the only population located in the northern part of Gunungkidul GeoArea.

Study species

Sandalwood is a long-lived, yearly flowering perennial found on semi arid and tropical region throughout south-eastern and middle parts of Indonesia. It is a shrub attaining a height of about 12 m and a girth of 10 to 30 cm, mainly vegetatively propagate naturally through root suckers (Warburton *et al.*, 2000; Lhuillier *et al.,* 2006; Dani *et al.,* 2011; Herawan *et al.,* 2014), particularly under marginal condition. Generally, sandalwoods in Indonesia flower twice a year: at the beginning of dry season on May to September, and at the beginning of rainy season on November to March; with 4 to 5 months of flowering period (Ratnaningrum and Indrioko 2014, 2015).

Sandalwood flowers arrange in inflorescences consisted of 20 to 40 small-red single flowers. Flowers pollinated by hymenopteran, lepidopteran, and dipteran insects (Sindhu-Vereendra and Anantha-Padmanabha ,1996; Suma and Balasundaran, 2003; Ratnaningrum and Indrioko, 2014). Studies on *Santalum* spp. indicated highly outcrossing rate and self-incompatibility (Rao *et al.,* 2007; Dani *et al.,* 2011; Indrioko and Ratnaningrum, 2015). However, partially self-compatibility has been reported for *S. accuminatum* (Warburton *et al.,* 2000) and *S. album* (Suma and Balasundaran, 2003; Ratnaningrum and Indrioko, 2014), particularly under isolated condition. Previous study showed evidence of dichogamy in which pollen mature 2 days before stigma receptivity (Ratnaningrum and Indrioko ,2014). Santalum has strong ability to produce root suckers, the vegetatively propagated sprouting emerged from the roots. Clonality event occurred when most of off-springs in population were derived from a genetically identical individuals (Warburton *et al.,* 2000; Lhuillier *et al.* 2006; Dani *et al.,* 2011). Previous researches documented pollen limitation due to less pollen production (Sindhu-Vereendra and Anantha-Padmanabha, 1996; Tamla *et al.,* 2012) and male sterility (Warburton *et al.,* 2000). Sandalwood's sexual reproductive success was also very low, ranging from

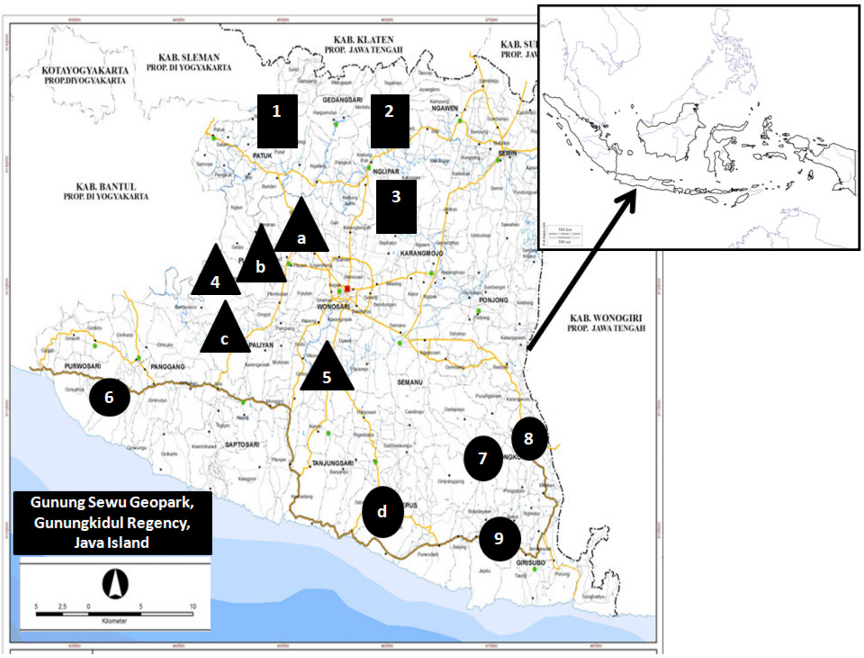

Figure 1. Study sites: sandalwood populations in the Gunung Sewu Geopark, Gunungkidul Regency, Java island. **The first group** (North-western Zone): Nglanggeran (1) and Sriten (2); **the second group** (Middle Zone): Bunder (a), Wanagama (b), Banyusoco (c), Bleberan (4), and Ngingrong (5); **the third group** (Southern Zone): Pucanganom (6), Petir (7), Semugih (8), Botodayakan (9) and Tepus (d); and the fourth group (Northern Zone): Bejiharjo (3) populations. The arabic numbers represents natural landraces, while the alphabetic fonts represents *ex situ* conservation areas. Within each of Gunung Sewu zone, sandalwood populations are marked by squares (North-western and Northern Zone), triangles (Middle Zone) and circles (Southern Zone) shapes, respectively. Study was carried out on **Nglanggeran (1), Sriten (2), Bejiharjo (3), Bleberan (4), Petir (7)** and **Botodayakan (8)** populations which were representing each of geographical zones.

0.03% in natural population to less than 20% in *ex situ* plantation (Ratnaningrum and Indrioko, 2014).

Population structures measurements

Populations were defined as spatially discrete clusters of plants, separated from others by at least 500 m (Herlihy and Eckert, 2005). We measured effective population size (N_e) as the number of flowering individuals divided by the total number of reproductive (adult) individuals (Frankham *et al.*, 2002). Population density conformed to the number of single individuals per ha (Applegate *et al.*, 1990). We made measurements in 2014 to determine the population width and size with modified methods of Warburton *et al.* (2000) and Applegate *et al.* (1990). Visible separated stands were regarded as individual, whereas stem separated by less than 10 cm (Warburton *et al.*, 2000) and/or shared the same root systems (Applegate *et al.*, 1990) were regarded as part of the same individual. Individual maturity determined by the presence of flowering (Warburton *et al.*, 2000).

Allozyme analysis

Genetic diversity was measured spatially across six populations along geographical gradients in Gunung Sewu, and temporally between two generations of parents and offsprings. To measure genetic diversity of parents, we sampled juvenile leaves from randomly chosen flowered individuals (N = 99 at Bleberan, 50 at Nglanggeran, 116 at Bejiharjo, 55 at Petir, 83 at Sriten and 29 at Botodayakan). To quantify the genetic diversity of the offsprings, we germinated the bulked seeds collected from each populations (N = 205 at Bleberan, 39 at Nglanggeran, 23 at Bejiharjo, 52 at Petir, 26 at Sriten and 50 at Botodayakan) and sampled the plumulae. Samples were wrapped, frozen in ice packs and taken to the laboratory for allozyme extraction and electrophoresis. Allozyme analysis conducted at 6 putative loci following a similar analysis previously performed for the *ex situ* plantations (Indrioko and Ratnaningrum, 2015). Previous study gained three enzymes, shikimate dehydrogenase (E.C. 1.1.1.25.), esterase (E.C. 3.1.1.) and diaphorase (E.C. 2.6.4.3.) which observed to be polymorphic. Zymogram phenotypes that were interpretable were found for only six loci, shikimate dehydrogenase *Skd-1*, esterase *Est-1*, *Est-2*, and *Est-3*, and diaphorase *Dia-1* and *Dia-2*. Gel electrophoresis procedures was conducted with vertical polyacrilamide gel electrophoresis procedure following David-Ornstein method (Seido, 1993). The leaves were homogenized in modified extraction buffer and centrifuged at 15,000 rpm for 15 min at 4 °C. The supernatant was loaded onto polyacrylamide vertical slab (Sigma Inc., USA) gels and electrophoresed at 4 °C at 220 V and 200 mA current for about 3 h. After electrophoresis, the gels were stained using staining solution of each enzyme system. Three enzyme systems, shikimate dehydrogenase (E.C. 1.1.1.25.), esterase (E.C. 3.1.1.) and diaphorase (E.C. 2.6.4.3.) were stained and the allozyme gels were genetically interpreted.

At each of the allozyme locus, the frequency of each allele and the genotype were counted. For each locus the number of heterozygote genotype were counted and expressed as percent observed heterozygosity (H_o). The observed heterozygosity was then pooled and averaged over all loci to determine the percent observed heterozygosity for a population. The expected heterozygosity (H_e) for each locus and over all loci for each population in Hardy–Weinberg equilibrium was counted following $H_e = 1 - \Sigma\, p_i^2$, where p_i refers to the allele frequency. Fixation index, the deviation from expected frequencies under Hardy–Weinberg equilibrium, was measured following the formula: $F_{IS} = 1 - H_o / H_e$. Genetic diversity between population (D_{ST}) was calculated by reducing the value of total heterozygosity (H_T) to heterozygosity within population (H_S). The mean proportion of total gene diversity at polymorphic loci due to differences between populations (G_{ST}) was measured by dividing D_{ST} to H_T (Nei, 1987). Interpopulational relationships were established by computing the unbiased genetic identity and distance coefficients (Nei, 1987) for all possible pairwise comparisons. Based on the Nei's genetic distances between pair-wise populations, cluster analysis was performed following UPGMA algorithm and a dendrogram was then constructed. To measure the mating systems, the outcrossing (*t*) and selfing rate (*s*) was calculated according to Dudash and Fester (2001) following the formula, $t = (1 - F_{IS}) / (1 + F_{IS})$, while $s = 1 - t$.

Results

The structure of populations

There was variation in population occupance, size and density among sites (Table 2). The small, isolated populations in NWP-Sriten and SP-Botodayakan (26 and only 6.75 ha of width) were sparser (12.7 and 22.4 adult individuals per ha) than bigger populations. The biggest population in SP-Petir (78 ha) exhibit highest level of plant density and population size (117.9 adult individuals per ha, and *Ne* 50.9%).

However, in contrary, a fragmented population in NP-Bejiharjo (only 9.6 ha) performed high level of plant density (51.7 adult individuals per ha). In contrary, a bigger population area in NWP-Nglanggeran (79.4 ha) was much sparser (only 14.4 adult individuals per ha) than some of smaller populations. A CP-Bleberan, the most central population that is located in the center of species' range, performed intermediate level of population width and density (52.9 ha with 34.7 adult individuals per ha).

Table 2. Population demographic characteristics, number of individuals, and genetic parameters measurements on six sandalwood populations in Gunungkidul GeoArea of Gunung Sewu

H_e = Hardy-Weinberg expected panmictic heterozigosity, H_o = observed heterozigosity, F_{is} = mean fixation index over all loci, N_{ep} = effective population size.

* Number in parentheses is the number of sampled individuals

H_S (heterozygosity within population) = 0.276, H_T (total heterozygosity) = 0.30, D_{ST} (genetic diversity between population) = 2.40, G_{ST} (proportion of genetic diversity between population to total genetic diversity) = 7.98

Population	Population characteristics		Number of individuals			Population size (N_{ep})
	Occupance (ha)	Density (indiv/ha)	Adult*	Flowered	Seedling*	
CP-Bleberan	52.89	34.68	1834 (99)	129	364 (205)	0.070
NP-Bejiharjo	9.59	51.72	496 (116)	92	124 (23)	0.185
NWP1-Nglang.	79.,37	14.43	1145 (50)	141	39 (39)	0.123
NWP2-Sriten	26.00	12.69	330 (119)	52	40 (26)	0.158
SP1-Petir	77.96	117.88	9190 (55)	4680	655 (52)	0.509
SP2-Botodykn.	6.75	22.37	151 (29)	26	72 (50)	0.172

Population	Genetic parameters						Mating parameters			
	H_o		H_e		Multilocus F_{IS}		Outcrossing rate (*t*)		Selfing rate (*S*)	
	Parent	Offspring	Parent	Offspring	Parent	Offspring	Parent	Offspring	Parent	Offspring
CP-Bleberan	0.294	0.333	0.282	0.308	-0.042	-0.079	1	1	0.001	0
NP-Bejiharjo	0.202	0.083	0.284	0.184	0.289	0.551	0.984	0.321	0.016	0.679
NWP1-Nglang.	0.261	0.284	0.269	0.247	0.029	-0.149	0.944	1	0.056	0
NWP2-Sriten	0.317	0.348	0.308	0.296	-0.031	-0.175	1	1	0	0
SP1-Petir	0.184	0.141	0.252	0.262	0.271	0.464	0.573	0.366	0.427	0.634
SP2-Botodykn.	0.385	0.310	0.319	0.304	-0.209	-0.018	0.791	1	0.209	0

The big and most dense population in SP-Petir showed highest population size (*Ne* 50.9%). However, in contrary, a most central population CP-Bleberan exhibit the lowest population size (*Ne* only 7%) compared to those of smaller, isolated or fragmented populations in NP-Bejiharjo, NWP-Sriten, and SP-Botodayakan (*Ne* 18.5%, 15.8% and 17.2%).

Genetic diversity and mating system parameters

It was predicted that as a result of selection for reproductive assurance in small, isolated populations, plants in fragmented populations will exhibit lower genetic diversity, higher levels of self-fertilization and higher parental inbreeding coefficients than continuous populations. Contrary to prediction, some of small and/or isolated populations tended to have higher level of genetic diversity, lower selfing-rate and lower parental inbreeding coefficients (Table 2).

Using isoenzyme markers, the genetic diversity of total of six sandalwood populations in Gunungkidul GeoArea of Gunung Sewu, consisted of a total of 863 individuals (N = 468 at adult level and 395 at offspring level), was assessed. Genetic diversity analysis over three different polymorphic isoenzymes (EST, DIA and SKD) corresponding to six putative loci (*Est-1, Est-2* and *Est-3* loci; *Dia-1* and *Dia-2* loci; and *Skd-1* locus) with a total of 15 alleles were assessed in this study. Six loci showed considerable polymorphism (*Est-1, Est-2, Est-3, Dia-1, Dia-2, and Skd-1*) in all of the populations except SP-Petir in which *Dia-1* observed to be monomorphic. Most alleles were shared among populations, but significant difference in allele frequency was detected for *Est-3* (Table 2, see Appendix 1 for the details).

Despite having the largest size, SP-Petir population exhibited lower level of observed heterozygosity for both level (H_o 0.184 for parental trees and 0.141 for offsprings). The population also tended to perform high level of selfing-rate (*S* 0.427 at the parent level and was extremely increased to 0.634 at the offsprings). The positive parental inbreeding coefficient (F_{is} 0.271 for parental trees and 0.464 for offspring) indicating strong heterozygote deficiency and high level of parental inbreeding, probably due to the founder effects of a low parental diversity. All polymorphic loci deviated significantly and positively from Hardy Weinberg equilibrium, indicated a very strong self-fertilization.

The dense population of NP-Bejiharjo, which also previously reported to experience fragmentation and clonality, performed lowest level of heterozygosity (H_o 0.202 for parental trees and only 0.083 for offsprings), highest level of selfing-rate (*S* only 0.016 for parental trees but was extremely increased to 0.679 for offsprings) and parental inbreeding coefficient (F_{is} 0.289 for parental trees and 0.551 for offspring), respectively. A very strong selfing rate was indicated by all polymorphic loci which were deviated significantly and positively from Hardy Weinberg equilibrium.

Surprisingly, both of smallest and most sparse populations in NWP-Sriten and SP-Botodayakan performed highest level of heterozygosity, both at parental (H_o 0.317 for NWP-Sriten and 0.385 for SP-Botodayakan) and offspring (H_o 0.348 for NWP-Sriten and 0.310 for SP-Botodayakan) levels. Parental inbreeding coefficient at both parental (F_{is} -0.031 for Sriten and -0.209 for Botodayakan) and offspring (F_{is} -0.175 for Sriten and -0.180 for Botodayakan) levels, indicated clear evidence of the excess of heterozygosity. Both populations also performed high level of outcrossing (t 0.791 to 1) with a very low selfing rate (0 to 0.209). All polymorphic loci in both populations deviated significantly and negatively from Hardy Weinberg equilibrium, indicated very strong outcross-mating within population.

In spite of its low population size (*Ne* 7%), the higher genetically diverse population CP-Bleberan (H_o 0.294 at parent and 0.333 at offspring level) tended to be more outcrosser. This population possessed high value of *t* (1.00 at both parent and offspring levels), a very low value of *s* (0.001) and negative F_{IS} (-0.042 at parent and -0.079 at offspring levels). All polymorphic loci showed no significant departure from Hardy Weinberg equilibrium, indicated a preference to random mating within population.

The widest but most sparse population NWP-Nglanggeran, which exhibit moderate level of population size (*Ne* 12.3%), was do so for genetic diversity (H_o 0.261 for parental trees and 0.284 for offspring) and parental inbreeding coefficient (F_{is} 0.029 for parental trees and -0.149 for offspring), respectively. The parental inbreeding coefficient which was close to zero (F_{IS} parent 0.029) indicated random mating at the previous generation, which was tended to be more outcrosser at the next generation (F_{IS} offspring -0.231). This preference to be more outcrosser was also indicated by high level of outcrossing (*t* 0.944 at parent and 1.000 at offspring level) and low level of selfing rate (*s* only 0.056). All polymorphic loci at the parent level showed no significant departure from Hardy Weinberg equilibrium, indicated a preference to random mating within population. However, preference to be more outcrosser, indicated by strong departure from Hardy Weinberg equilibrium, was detected at the offspring level.

This research assumed that to maintain reproductive assurance, selfing taxa will occur in isolated population to enhance selfing ability. A high level of parental selfing-rate, which was extremely increased at the offspring level, was surprisingly observed in the biggest population SP-Petir. It might occured due to inbreeding as a result of the founder effects, since the parent trees exhibited lowest level of diversity (H_o 0.184) compared to other populations. The positive and strongly deviated parental inbreeding coefficient indicated clear evidence of genetic

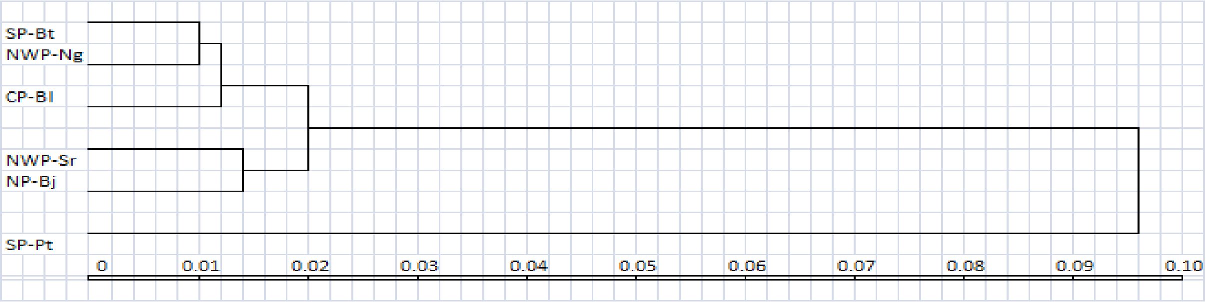

Figure 2. Dendrogram analysis for six sandalwood populations in Gunungkidul GeoArea of Gunung Sewu

loss across generation due to high level of parental inbreeding. A very strong selfing rate, particularly at the offsprings, was also observed in the dense population of NP-Bejiharjo. This population was previously reported to experience fragmentation and clonality, resulting in a very low level heterozygosity and highly-inbreeder population.

Genetic distance

Based on the Nei's genetic distance, a dendrogram clustering of sandalwood populations in Gunung Sewu was constructed (Figure 2). The dendrogram indicated a grouping of populations into three clusters. However, there were seemed to be no association between geographical and genetic distance. Dendrogram clearly showed evidence that the populations was not clustered based on their geographical sites of occurence, but more by their similarity on allele frequency and distribution. This finding implied that populations in the same cluster shared a high portion of same alleles and genotypes, and therefore might be indicated as a close relative. In general, the more center, un-isolated and bigger populations allowed gene flow to occur among sites, and therefore they shared same alleles and possessed lower genetic differentiation.

Despite its small and sparse population, CP-Bleberan, the most central population that is located in the middle of several *ex situ* conservation areas and share the same river, was tended to experience seed dispersals through the stream flow. Some of seed deposits were observed along the river catchment areas in this study. Therefore, this population performed lower level of genetic distance (*D* 0.02 in average) and higher level of genetic diversity (H_o 0.294 at the parental and H_o 0.333 at the offspring levels).

As it was previously reported, NP-Bejiharjo ---that is within the same cluster to NWP-Sriten--- was established at 1970's using the unknown sources collected from Nglipar Dsitrict, a site that is recently recognized as the same district of NWP-Sriten population. This evidence may explain the closest distance of the two populations (*D* 0.013).

Other cluster consisted of three populations: NWP-Nglanggeran, SP-Botodayakan and CP-Bleberan, that were geographically different and each was separated more than 30 km away in distance. Especially for the highland of NWP-Nglanggeran and lowland-karst area of SP-Botodayakan, which are separated along north to south gradient of more than 60 km away, and occupied significantly different geographical, altitudinal and ecological features, the genetic distance is surprisingly very close (*D* 0.011). These two populations largely shared similar allele frequencies and distributions at the parental levels, performed similar parental genetic diversity, and experienced similar mating patterns and offspring recruitments. Another population, a lowland-riparian side of CP-Bleberan is also within the same cluster, and this may attributed to the central position and continuous pattern of the population which allow larger gene flow. Oppositely, SP-Petir population seemed to be genetically farther apart from the rest of sandal populations in Gunung Sewu (*D* ranged from 0.096 to 0.118). Hence this population remains isolated and cannot be linked to any other groups based on genetical markers due to difference on genetic and mating parameters.

Discussion

Genetic diversity and mating system parameters

This study has indicated that sandalwood populations in Gunung Sewu, particularly those which experienced heavy exploitation and/or grew on marginal-rocky condition, existed as genotypically-identical parent trees, and recruited individuals mostly by root suckers. This may reflect the history of disturbance and fragmentation of the populations due to harvesting and urban activities, that coupled with disrupted gene flow and possibly genetic drift. The observed heterozygosity in this study varied significantly from H_o 0.184 to 0.385 in parental trees, and from H_o 0.083 to 0.348 in the offsprings levels. Some of the results confirmed a "reproductive assurance prediction", in which population with lower genetic base and/or lower heterozygosity were reported to be a highly inbreeder. However, the departure from a "reproductive assurance prediction" was also observed, as some of small and/or isolated populations tended to have higher level of genetic diversity, lower selfing-rate and lower parental inbreeding coefficients. It seemed that genetic diversity and mating systems are not much affected by population size, but more by the heterozygosity of parents (genetic base) and the degree of clonality in population.

Previous isoenzyme-based studies on Wanagama *ex situ* conservation ---located at only 2 km away from the NP-Bleberan population--- showed relatively higher level of genetic diversity, particularly in stand consisted of many sources (seven provenances of Timor, Nusa Tenggara Timur and Java islands; H_o 0.30 to 0.46). Whilst, another stand which was constructed from only single provenance of Timor exhibit lower level of diversity (H_o 0.21 to 0.38). Stands with wider genetic base indicated higher outcrossing rate (F_{IS} -0.33), and the diversity of offsprings observed to be higher than their parents (H_o 0.38 to 0.51) (Indrioko and Ratnaningrum, 2015). Another *ex situ* conservation area in Watusipat, that was located at only 3 km away from NP-Bleberan population, was also exhibit higher level of diversity (17 provenances of Timor and Nusa Tenggara Timur islands; H_o 0.32 to 0.39; Rimbawanto *et al.,* 2006). On various sandal natural provenances in peninsular India, heterozygosity varied from H_o 0.18 to 0.5 (Angadi *et al.,* 1993); H_o 0.13 (Suma and Balasundaran, 2003); and H_o 0.26 to 0.38 (Rao *et al.,* 2007), based on the population size, genetic base and the degree of clonality. Sandalwood samples that were recently collected from their origin in Nusa Tenggara Timur in 2014, showed very low level of heterozygosity (H_o 0.03 to 0.09; Indrioko dan Ratnaningrum, 2015), indicating a rapid depletion on genetic diversity.

The total gene diversity (H_T 0.30) value in this study was similar to those of *S. lanceolatum* in Victoria (H_T 0.25; Warburton *et al.,* 2000) and *S. album* in India (H_T 0.31; Brand, 1994), while those of Timor was somehow lower (H_T 0.14; Brand, 1994). Within only 20 years later in 2014, extreme reduction reported on gene diversity of remnant natural population in Timor and East Nusa Tenggara (H_T 0.01 to 0.03), while the *ex situ* and rehabilitated stands of the same areas performed higher level of diversity (H_T 0.21 to 0.24) (Indrioko and Ratnaningrum, 2015).

For comparison, studies on other sandals species also reported similar influences of population size and/or genetic base to genetic diversity. RFLP-based study on *S. spicatum* across geographical range of their populations in Western Australia showed that isolated populations are less diverse than bigger populations, but in average the populations exhibit moderate level of diversity (H_o 0.21; H_T 0.23; F_{IS} -0.026; Byrne *et al.,* 2003). Microsatellite-based study on *S. insulare* in insular Pacific reported that significant excess of homozygosity was only observed within isolated islands (H_o 0.28; F_{IS} 0.12; 58% level of clonalities) and not in mainland (H_o 0.49; F_{IS} -0.07) (Lhuillier *et al.,* 2006). The microsatellite-based total genetic diversity in their study (H_T 0.22; Byrne *et al.,* 2003) was also observed to be significantly lower than those of *S. australocaledonium* which occupied undisturbed and bigger population size in New Caledonia (H_T 0.84; Bottin *et al.,* 2007). RAPD-based analysis on five isolated and clonalized populations of *S. lanceolatum* in south-eastern Victoria, Australia reported a very low heterozygosity (H_S 0.06; Warburton *et al.,* 2000).

Current patterns of gene dynamics and genetic structure may also be significantly affected by historical pattern of mating and gene flow, that may varied among sites due to the differences of evolutionary history (Lhuillier *et al.,* 2006; Bottin *et al.,* 2007). Populations that have existed to suitable undisturbed conditions for a long enough period of time may maintain its genetical processes in equilibrium. In contrary, populations that have subjected to disturbed historical events, such as exploitation, fragmentation or isolation, will not be expected to exhibit such an equilibrium. For example, the lowest level of parental observed heterozygosity (H_o 0.184) in the biggest SP-Petir population showed clear evidence of the founder effects. The high value of *S* and positive F_{IS} strongly indicated high parental inbreeding within population. Combined with the effect of clonality events, geitonogamy tended to dominate mating systems in this population. However, this population has had subjected to such a condition which favoured inbreeding for a long time period since 1960's (Vice Head of Petir District, personal communication), and therefore seemed to be adapted to its new mating pattern. It was reflected in the ability to maintain a relatively high level of seed production without any considerable inbreeding depression.

The dense population of NP-Bejiharjo also performed highest level of selfing rate within population. However, in this population, the evolutionary history which resulted in the change of genetic diversity and mating systems occured under a different scenario. Previously this population seemed to have higher level of genetic diversity at the first generation (H_o 0.202 in parental trees), and therefore the founder effects were not considerably affected genetic processes within population. Later at 1980's the heavy exploitation began, up to the big riots in 1998's, followed by habitat fragmentation due to eco-tourisms and urban activities that was started in 2000's, resulting in a very intensive clonality that in turn significantly increased self-mating and reduced genetic diversity. These relatively short time period is not sufficient enough for the population to adapt to the new population structure and disrupted genetical processes. Hence, NP-Bejiharjo population had not been occupied for sufficient enough period to allow equilibrium, and therefore behaved differently from the SP-Petir population. Previous study in NP-Bejiharjo indicated a sexual reproductive failure that is considered as a result of inbreeding depression. Limited sexual reproduction observed following flowering and pollination, resulting in a very poor seedling recruitments (Indrioko and Ratnaningrum, 2015). Extreme heterozygosity depletion (H_o declined from 0.202 in parental trees to only 0.083 in offsprings) and highly missing alleles at the offspring level indicated disrupted gene flow and possibly genetic drift.

Several factors may be responsible for the reduction of heterozygosity, particularly at the offspring level, in some of populations. **The first reason** for the diversity depletion is the clonality events in which many of the remnant populations consisted of vegetatively propagated individuals of single stem by root suckers. Up to 15 m horizontal roots expanding from a single mother tree was observed in NP-Bejiharjo and SP-Petir populations, a condition that was similar to those observed in Australia, Polynesian archipelagos and India, in which the 20 m horizontal roots of *S. acuminatum* (Warburton *et al.,* 2000), 10 m in *S. insulare* (Lhuillier *et al.,* 2006), and 7 to 10 m in *S. album* (Applegate *et al.,* 1990) have also been documented. Mating between genotypically identical clones favour high level of geitonogamy which may increase the homozygosity. Two factors affected the extensive clonality in sandals were (1) the overexploitation which induced root suckering from the cut-off trees (Warburton *et al.,* 2000; Indrioko and Ratnaningrum, 2015), and (2) the natural strategy in responding to environmental stresses (Lhuillier *et al.,* 2006; Bottin *et al.,* 2007). This clonality events has also been reported in other sandals populations such as *S. album* in some of clonalized populations in Gunungkidul (Indrioko and Ratnaningrum, 2015); *S. lanceolatum* (Warburton *et al.,* 2000) and *S. spicatum* (Byrne *et al.,* 2003) in Australia, *S. insulare* in the insular Pacific (Lhuillier *et al.,* 2006), *S. album* in peninsular (Rao *et al.,* 2007) and Southern India (Dani *et al.,* 2011), and *S. austrocaledonicum* in New Caledonia (Bottin *et al.,* 2007).

The second reason for the offsprings diversity depletion is correlated to the genetic drifts and bottleneck effects, which was indicated by the rare and missing alleles in this study. A bottleneck effect may favours genetic drift and inbreeding depression resulting in an increase of rare and missing alleles (Byrne *et al.,* 2003; Bottin *et al.,* 2007; Ratnaningrum and Indrioko, 2015), significant reduction on genetic diversity (Lhuillier *et al.,* 2006) and lower reproductive fitness (Warburton *et al.,* 2000; Tamla *et al.,* 2012). **The third reason** is related to the founder effects due to a low diversity of parental trees, which may favours high level of geitonogamy (Lhuillier *et al.,* 2006; Craft and Ashley, 2007). **The fourth reason** is related to the alteration on mating systems to be more inbreeder, particularly in populations which subjected to the low parental genetic base, strongly clonalized (Warburton *et al.,* 2000; Lhuillier *et al.,* 2006; Dani *et al.,* 2011) or isolated condition (Herlihy and Eckert, 2005; Arroyo *et al.,* 2006). Significant heterozygosity deficits, represented by a significantly positive F_{IS} which was indicating highly inbreeding and strong departures from Hardy Weinberg equilibrium, were observed in some populations in this study. This also conformed the "reproductive assurance theory" (Herlihy and Eckert, 2005; Arroyo *et al.,* 2006), in which selfing taxa occur in the population subjected to inbreeding, to enhance the selfing ability. In this study, both of strongly clonalized populations in NP-Bejiharjo and SP-Petir confirmed this theory.

The more accessible populations were also more subjected to disturbance for human activities, as was reported in NP-Bejiharjo site which is located at the dry rocky hills on the top of cave eco-tourism area. Higher preference to regenerate offsprings vegetatively in populations threatened by human activities has also been reported in other clonal sandals species such as *Santalum lanceolatum* in Australia (Warburton *et al.,* 2000), *S. insulare* in the insular Pacific (Lhuillier *et al.,* 2006) and *S. austrocaledonicum* in New Caledonia (Bottin *et al.,* 2007), and another species of perennial herb *Geum urbanum* (Schmidt *et al.,* 2009) in Europe, that were subjected to disturbance due to grazing, urbanization and land-cultivation activities.

Both highland populations in NWP-Nglanggeran and NWP-Sriten were less disturbed by human impacts. However, some extent of vegetatively propagated offsprings were still found. These root suckers mostly emerged from the wounded roots, particularly under hazardous natural disturbance such as land slides, or on the dry, hard and rocky soils with shallow solum. In these highland sites, the preference to perform clonal rather than sexual reproduction existed due to their strategy to survive in responding to harsh environmental stresses and in the absence of regeneration by seeds (Lhuillier *et al.,* 2006).

Correlation between geographical and genetic distance

Many studies on the effects of geographical structures to genetic diversity reported isolation and genetic differentiation by distance (Lhuillier *et al.,* 2006; Bottin *et al.,* 2007; Pautasso, 2009; Schmidt *et al.,* 2009), and it was considered that the presence or absence of isolation by distance may be related to the pattern and dynamics of distribution and by the species' life history traits. In general, high genetic differentiation among populations is often associated with heavy seeds (Pautasso, 2009). In contrary, the wind pollinated and/or the long-distance bird-dispersal seeds, as of sandals, are often showed no or low association between genetic and geographic distance (Warburton *et al.,* 2000). Naturally, seeds of sandalwood dispersed by birds to more than 5 kilometers in distance (Applegate *et al.,* 1990; Warburton *et al.,* 2000; Bottin *et al.,* 2007; Ratnaningrum and Indrioko, 2014), although some extent of dispersal by stream river flow was also observed. Five bird species, *puyuh pepekoh* (*Coturnix chinensis*), *madu sriganti* (*Nectariana jugularis*), *cekakak sungai* (*Todirhampus chloris*), *kutilang* (*Pycnonotus aurigaster*) and *tekukur* (*Stretopelia chinensis*), reported as the dispersal agents of sandalwood in Gunung Sewu (Ratnaningrum and Indrioko, 2014). This agents were also known to break seed chemical-dormancy and facilitate seed germination by their ingestion. This may explain the relatively high seedling recruitments in populations which provide suitable environments for bird activities, such as SP-Botodayakan and NWP-Sriten which are surrounded by dense woody stands.

Most of the sandalwood genetic variation in this study is distributed within populations, and only 2.7% of the observed diversity presented among populations, that was indicated by the low D_{ST} and F_{ST} value (H_T 0.30; H_S 0.276; D_{ST} 2.4%; F_{ST} 7.98%). A dendrogram constructed in this study indicated a grouping of populations into three clusters. However, there were seemed to be no association between geographical and genetic distance, as some of populations were clustered within a group of different geographical region. Dendrogram clearly showed evidence that the populations was not clustered based on their geographical sites of occurence, but more by their similarity on allele frequencies and distribution. In general, the more center, un-isolated and bigger populations allowed gene flow to occur among sites, and therefore they shared the same alleles and possessed lower genetic differentiation.

Similar result also reported with *S. insulare* in Pacific islands (Lhuillier *et al.,* 2006) where the dendrogram did not clearly separate the three archipelagos. Some of populations in the same cluster were quite consistent with the geographic position of the populations, but in other side, some highland and lowland populations were found in the same cluster due to the similarity of genetic structures. Other similar result also reported for a long-lived, monoecious tree species *Quercus macrocarpa* in the north eastern U.S. (Craft and Ashley, 2007), in which the savanna landscape barriers did not restrict gene flow, and therefore the differentiation among populations were low and were not correlated to distance (F_{ST} 0.027).

For comparison, Timor and Indian *S. album* populations had a mean genetic distance (*D*) of 0.447; which is very large compared to intra-specific distances in other species. In contrast, a very low genetic distance was observed in 16 populations of *Quercus ilex* along Mediterranean gradients (*D* only 0.023). The average of *D* between western and eastern populations of *Acacia albida* in Africa was 0.273, and they were considered as different races (Brand, 1994). Hence, the large genetic distance between SP-Petir and other populations (*D* ranged from 0.096 to 0.118) in this study indicated that this population is genetically different and therefore should be considered, at least, as a separate races.

The continuous population, as reported with CP-Bleberan in this study, generally performed lower level of genetic distance. This was also reported in *S. spicatum* in their connected populations along Western Australia (*D* ranged from 0.006 to 0.073; Byrne *et al.,* 2003). While for *S. australocaledonium* in New Caledonia (Bottin *et al.,* 2007), the populations were strongly differentiated by the combination effects of marked genetic drift, limited gene flow, and natural selection due to contrasted environment condition on the different geographical regions.

RAPD-based study on three small and fragmented populations of *S. album* in Southern India reported highly genetic structuring due to a restricted gene flow (F_{ST} 54.9%; Dani *et al.,* 2011), a same case to those occurred on five isolated populations of *S. lanceolatum* in south-eastern Victoria, which performed high level of differentiation among populations (D_{ST} 19%; F_{ST} 76%; Warburton *et al.,* 2000). Ten provenances of *S. album* in India and two in Timor performed lower level of genetic differentiation (F_{ST} 28.6% and 17.2%, respectively; Brand, 1994). In contrary, the more un-isolated and/or bigger populations allowed gene flow to occur among sites, and therefore they shared the same alleles and possessed lower genetic differentiation. The D_{ST} measured in this research was 2.4% with F_{ST} 7.98%, indicated that most of genetic diversity was maintained within population, although some of diversity was gained between population. This result was similar to those observed on the bigger and continuous populations of *S. album* in Peninsular India, where a highly gene flow was occurred and only 3.4% of total genetic variability was due to differences among population (F_{ST} 3.4%; Rao *et al.,* 2007); in contrast to their counterparts in small, fragmented populations in Southern India which possessed highly genetic structuring due to a restricted gene flow (F_{ST} 54.9%; Dani et al. 2011). Similarly, *S. spicatum* in Western Australia, in which their undisturbed and bigger populations could maintained their equilibrium and facilitated gene flow among populations, was reported to have a low value of F_{ST} (8.3%) and genetic distance (*D* 0.031) (Byrne *et al.,* 2003).

However, it seemed that physical barriers did not restrict gene flow in some of isolated populations in this study. Both of populations isolated by highland geographical barriers, NWP-Nglanggeran and NWP-Sriten, behaved similarly on allele distribution and mating patterns, and shared a large portion of same genotypes. In contrary, despite their larger and denser population, the two clonalized populations of NP-Bejiharjo and SP-Petir experienced mating barriers due to low genetic diversity that lead to geitonogamy and reproductive failure, resulting in different patterns of allele frequencies and distribution.

This research clearly showed that although the genetic diversity of parental trees in the fragmented vs. continuous populations did not significantly differ, the next generation performed significant differences on the level of genetic diversity and outcross-mating, based on the parental diversity and/or the history of population disturbance. This condition was considered to be the effects of (i) the evolutionary of non-random mating in fragmented areas (Barrett *et al.,* 2005; Pautasso, 2009) as reported in NP-Bejiharjo population of this study, and for comparison, the *Quercus petraea* and *Q. robur* in Ireland (Kelleher *et al.,* 2005); (ii) lack of recruitments in the later generations (Warburton *et al.,* 2000; Lhuillier *et al.,* 2006) as reported in almost all of, and particularly, the most disturbed populations in this study, with comparison to *Colubrina oppositifolia* and *Alphitonia ponderosa* in Hawaiian islands (Kwon and Morden, 2002); (iii) loss of rare alleles in the next generations in population which undergo high self-mating due to low parental genetic base (Warburton *et al.,* 2000; Lhuillier *et al.,* 2006), as reported with SP-Petir, and for comparison, *Acacia nemorosa* and *A. columnaris* in New Caledonia (Kettle *et al.,* 2007); and (iv) continuous bottlenecks during evolutionary history and of current heavy logging (Pautasso, 2009), as reported in the most heavily-harvested and disturbed populations SP-Petir and NP-Bejiharjo, with comparison to the most endangered conifers *Pinus squamata* in tropical Yunnan, China (Zhang *et al*., 2005).

Implications and recommendations for designing the conservation strategy

Our results emphasized the importance of populations' genetic base or parental genetic diversity to naturally maintain the genetic and evolutionary processes of sandalwood population under equilibrium conditions. Hence, to define a conservation strategy, our main recommendation focused in maintaining those processes within each population, but with different strategy according to parental genetic base and population size. Population with wider genetic base, higher rate of heterozygosity and/or higher outcrossing rate may be expected to naturally maintain such processes under equilibrium, as long as the individuals in population prefer to regenerate sexually instead of vegetatively (Bottin *et al.,* 2007).

In order to increase the genetic diversity, and to prevent inbreeding depression due to mating between relatives, we recommend to facilitate natural outcross-mating by pollination and pollinators management (Torres *et al.,* 2003). Many self-incompatible species, as reported in sandalwood in this study, were pollinator limited and had very low seedling recruitment. In this effort, flowering stimulation, providing artificial rewards, and building artificial nests for pollinators should be any of consideration. Furthermore, since habitat fragmentation is not only posing a threat to endangered species due to gene flow restriction, but also due to its negative effects on genetic diversity and reproductive traits, it is recommended to maintain landscape connectivity by preserving and establishing natural corridors, for they may serve as refuges also for pollinating insects (Pautasso, 2009).

To facilitate gene flow and seed dispersals, it was also recommended to reintroduce and protect the dispersal agents (Lhuillier *et al.,* 2006). The high-diversity and highly-outcrossed populations could also be considered as the "hot-spots" of genetic variability which might be projected for *in situ* conservation (Rao *et al.,* 2007) and the genetic resources for any of rehabilitation and researches activities (Dani *et al.,* 2011).

Regeneration of populations through seedling recruitment is important, particularly for the small, fragmented and/or isolated populations. Efforts should also be focused on genetic infusion with a genetically-diversed and highly-outcrossers sources in order to enhance the genetic base (Byrne *et al.,* 2003). Genetic infusion by introducing different genotypes into the existing remnant may also be an option, but some risks of outbreeding depression should be deeply considered (Warburton *et al.,* 2000; Bottin *et al.,* 2007). Seed for regeneration should preferred to be sourced from local areas rather than alien provenances to prevent disturbance on the genetic equilibrium that has been maintained within population (Byrne *et al.,* 2003), to lower the risk of outbreeding depression (Warburton *et al.,* 2000; Bottin *et al.,* 2007), and to maintain local adaptation that contribute to the adaptive diversity (Bottin *et al.,* 2007).

Moreover, the immediate restoration of population size should also be applied with a recruitment of local highly-outcrossers sources. Some of researchers also proposed to conserve the clonalized and small sandals populations in *ex situ* conservation (Rao *et al.,* ,2007) and seed orchards (Lhuillier *et al.,* 2006). In addition, efforts should also be made to establish new plantations consisted of more genetically-diversed sources (Warburton *et al.,* 2000). Some pattern of differentiation indicated that germplasms collection should be sampled across the range of populations to ensure adequate representation of the variation (Byrne *et al.,* 2003). And finally, the development of conservation strategy should be integrated to the regional and national conservation program along with the conservation management under geopark scheme.

Conclusions

The observed heterozygosity in this study varied significantly from H_o 0.184 to 0.385 in parental trees, and from H_o 0.083 to 0.348 in the offsprings levels, based on the degree of clonality and the parental genetic base. Most of genetic variation is distributed within populations, and only 2.7% of the observed diversity presented among populations, that was indicated by the low D_{ST} and F_{ST} value (H_T 0.30; H_S 0.276; D_{ST} 2.4%; F_{ST} 7.98%). A dendrogram indicated a grouping of populations into three clusters. However, there were seemed to be no association between geographical and genetic distance, as some of populations were clustered within a group of different geographical region. In general, the more center, un-isolated and bigger populations allowed gene flow to occur among sites, and therefore they shared the same alleles and possessed lower genetic differentiation. Genetic depletion occured due to (i) clonality events as result of heavy-exploitation and/or natural disturbance which induced root suckering, (ii) genetic drifts and bottleneck effects, (iii) the founder effects due to parental low diversity, and (iv) the alteration on mating systems to be more inbreeders.

Some of the results confirmed a "reproductive assurance prediction", in which population with lower genetic base and/or lower heterozygosity were reported to be a highly inbreeder. However, the departure from a "reproductive assurance prediction" was also observed, as some of small and/or isolated populations tended to have higher level of genetic diversity, lower selfing-rate and lower parental inbreeding coefficients. It seemed that genetic diversity and mating systems are not much affected by population size, but more by the heterozygosity of parents (genetic base) and the degree of clonality in population. Our results emphasized the importance of populations' genetic base or parental genetic diversity to naturally maintain the genetic and evolutionary processes under equilibrium conditions.

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Appendix

		Allele Frequency					
Loci	Allele	CP-Blb	NP-Bjh	NWP-Ngl	NWP-Srt	SP-Ptr	SP-Btd
Est-1	a	0.184	0.054	0.043	0.211	0.029	0.038
	b	0.816	0.946	0.957	0.789	0.971	0.962
Est-2	a	0.993	0.960	0.998	0.972	0.963	0.962
	b	0.007	0.040	0.002	0.028	0.037	0.038
Est-3	a	0.659	0.928	0.661	0.852	0.855	0.627
	b	0.341	0.072	0.339	0.148	0.145	0.373
Dia-1	a	0.014	0.015	0.008	0.010	-	0.000
	b	0.754	0.770	0.893	0.869	-	0.766
	c	0.232	0.215	0.098	0.121	-	0.234
Dia-2	a	0.074	0.109	0.144	0.142	0.083	0.158
	b	0.919	0.728	0.837	0.775	0.825	0.804
	c	0.007	0.163	0.019	0.083	0.092	0.038
Shd-1	a	0.540	0.634	0.464	0.523	0.389	0.272
	b	0.047	0.081	0.133	0.172	0.142	0.179
	c	0.413	0.285	0.404	0.305	0.468	0.549