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Author(s)	Yeni, W. N. Ratnaningrum; Sapto, Indrioko
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# Variation on Genotypes and Flowering Characters Affecting Pollination Mechanisms of Sandalwood (*Santalum album* Linn., Santalaceae) Planted on *ex-situ* gene Conservation in Yogyakarta, Indonesia

YENI W. N. Ratnaningrum<sup>1\*</sup> and SAPTO Indrioko<sup>2</sup>

<sup>1</sup>Laboratory of Forest Seed Science and Technology, Faculty of Forestry,  
Universitas Gadjah Mada, Jl. Agro, Bulaksumur 55281,  
Yogyakarta, Indonesia

<sup>2</sup>Laboratory of Forest Genetics and Tree Improvement, Faculty of Forestry,  
Universitas Gadjah Mada, Jl. Agro, Bulaksumur 55281,  
Yogyakarta, Indonesia

## Abstract

This research is a part of the long term research on sandalwood improvement strategy based on gene conservation approaches, focused on gene diversity and flowering characteristics of seven sandalwood provenances planted on *ex-situ* gene conservation in Wanagama Forest Research Station, Yogyakarta Province, Indonesia, and its effect on pollination mechanisms. Juvenile leaves sampled from any individual of each provenance where isozyme analyses then conducted with vertical polyacrilamide gel electrophoresis procedures based on David-Ornstein method with 3 enzyme systems: SHD, EST and DIA. Standard genetic distance measured following Nei and dendrogram then made following cluster analyses based on UPGMA. Parameters of flowering measured daily on each of 10 selected individual based on Ghazoul and Owens methods. Pollination mechanisms investigated by observing the attractant availability provided by 10 marked individual of each provenance. Observation was then made on plant pollinator interactions: stigmatic contact, pollen load, interplant movement, feeding behavior and visitation rate; that were measured daily based on Ghazoul and Jones and Little methods. Provenances originated from same site performed similar genotypes as well as flowering characters. All of provenances were both protandry dichogamy and heterostyly. Variation on size and color of perigonium were found among provenances between times. Generally, provenances originated from Eastern parts of Indonesia flowered earlier compared to those of central parts of Java, with shorter period as well; while those of Bromo was the latest to flower with the longest period. There were 6 types of 3 orders of insect found to visit flowers: moths, butterflies (Lepidoptera), flies (Diptera), ants, bees and wasps (Hymenoptera). Type of insect and visitation rate depended on flowering phases in term of primary (pollen and nectar) and secondary (color of perigonium and mature anthers) attractants availability. Meanwhile, preference of visitors to visit certain provenances was affected more by secondary attractant.

*Key words:* Sandalwood, gene diversity, flowering characters, pollination mechanisms, *ex-situ* gene conservation

## 1. Introduction

An economic-important endangered species native to Eastern parts of Indonesia, *Santalum album* Linn (Santalaceae) – formerly called sandalwood – has been categorized as a threatened species because of its significant degradation and/or habitat loss due to high demand on its wood and oil. This species distributed naturally along China, India, Indonesia and Phillipinne (IUCN, 1994; McKinnel, 1993). In Indonesia, this endangered-economic species is endemic in Nusa Tenggara Timur, found in several places in Timor, Sumba, western parts of Flores, Alor and Roti islands (McKinnel, 1993; Suseno, 2001), and the eastern parts of Java and Sulawesi (Kharisma, 1994). Its heartwood contains 1.5–5% of strong-specific fragrance of oil, which is widely used for wood carving, religious and

medicinal purposes (UNEP, 2007; Noatay, 2002; Hartono, 2000). Its oil has been used as irreplaceable materials for cosmetics (Hartono, 2000), prime sources of perfumes and aroma therapy (UNEP, 2007; Noatay, 2002), and was predicted to have anti-melanoma compounds (Agusta and Jamal, 2000). It brought sandalwood to be an important-economic fancy wood (Noatay, 2002) with significantly increasing price of its heartwood to be USD 1.000–1.500 per kg. A ten times increasing price from 20.000 to 200.000 Rupee even observed in the last 10 years from 1997 to 2007 (UNEP, 2007).

Earlier it was of the opinion that The Outer Arc of Banda Islands – located in the south-eastern parts of Indonesia – was the center of origin of sandalwood that is presently exist all over the world. Some researches even believed that sandalwood recently grown in India

was a result of gene introduction from Timor hundreds year ago (Van Steenis, 1971 in Riswan, 2000; Fischer, 1928, 1938 in Angadi *et al.*, 2003). In contrary, some other researchers believed that sandalwood was come from the southern parts of India (Bentley and Trimer, 1880 in Riswan, 2000). Brand (1994) concluded that sandalwood in West Timor and India are of different varieties or races, because of large genetic distance between the two countries. Meanwhile, another opinion assumed that the existing genetic difference occurred as a result of genetic differentiation caused by the bottleneck effect in natural selection processes (Angadi *et al.*, 2003).

There were 103 species belong to familia Santalaceae, in which 8 of them were listed as threatened species because of the high risk of extinction (IUCN, 1994, 2001). Three of them were endemic in South East Asia: *Santalum album* (China, India, Indonesia and Phillippinne), *S. macgregorii* (Papua and Papua New Guinea) and *Scleropyrum wallichianum* (India, Malaysia, Phillippinne, Singapore, Srilanka, and Thailand) (IUCN, 1994; GRIN, 2006). Three species (*Acanthosyris asipapote*, *Santalum album*, and *S. haleakalae*) were categorized as Vulnerable, two (*Exocarpos gaudichaudii* and *Santalum macgregorii*) were Endangered, and even one (*S. fernandezianum*) was listed to be Extinct (IUCN, 1994). The threatening of sandalwood occurred as a result of species degradation and habitat loss because of several reasons: 1) local consumption, 2) illegal-logging and wood extraction, and 3) fires. McKinnel (1993) reported the significant reduction of sandalwood population because of 1) highly exploitation, 2) land clearing, 3) uncontrolled grazing, 4) fires, 5) less natural regeneration and 6) lack of reforestation programs. This conditions brought sandalwood in category Vulnerable with criteria number A1d (VU A1d): it proven to face high risk of extinction in their nature during medium period of time; and criteria A1d refers to the species that undergo  $\geq 20\%$  (or 50% in version 3.1) reduction of population size during the last 10 years or 3 generations, and the factors causing those reduction can be handled and ended based on actual or potential exploitation level (IUCN, 1994, 2001). It implied that both species and population will be strongly affected by the human activities and/or natural hazardous in a very short period of time in the future, and therefore the category might soon switched to be Critically Endangered or even Extinct. Based on this consideration, it was recommended to establish the combination of *in-situ* and *ex-situ* gene conservation (Young *et al.*, 2000).

Genetic processes (mating, migration, selection and mutation) and its dynamics are of consideration on any of conservation efforts (Young *et al.*, 2000). Genetic drift, migration, selection and mutation acts as a four major evolutionary/genetic forces affecting allele frequencies within population (White *et al.*, 2007; Young *et al.*, 2000). Mating systems – one of the aspects of genetic processes – do not directly affect allele frequencies; but do alter allele distribution among genotypes (White *et al.*, 2007). Mating systems strongly

influenced by reproductive biology (sexual systems, incompatibility mechanisms, flowering and pollination processes) (Griffin and Sedgley, 1989; Kittelson and Maron, 2000) and spatial structure (Young *et al.*, 2000); and the interaction between affect the levels and dynamics of genetic diversity (White *et al.*, 2007; Young *et al.*, 2000). It is important to maintain the continuity of genetic processes in order to preserve the existing diversity, protect the evolutionary potentials, and improve the future diversity (Young *et al.*, 2000).

Numbers of researches showed a high genetic variation of sandalwood in Wanagama (Yuliah, 2011; Naiem and Ratnaningrum, 2010; Ratnaningrum, 2010; Haryjanto, 2009; Indrioko *et al.*, 2009; Irmawati, 2007), that was even higher compared to those that was grown both in natural habitat in Nusa Tenggara islands (Haryjanto, 2009) and India (Suma and Balasundaran, 2003), and in *ex-situ* conservation area in Watusipat, Central Java (Haryjanto, 2009; Rimbawanto *et al.*, 2006). Indrioko *et al.* (2009) and Yuliah (2011) estimated genetic variation dynamics in sandalwood natural population in Wanagama and found that there were reduction of allele frequencies at seedling level, where missing alleles were observed as well. It implied that some alleles were no more inherited to the next generation. Both researches concluded that the missing alleles occurred as a result of random genetic drift. Further research (Ratnaningrum, 2010) found that even in genetic trials, several alleles were observed in a very low frequency level. Certain alleles were found only on certain provenances, indicated that the genotypes were provenance specific. It was concluded to set the main priority on the genetic conservation of sandalwood in Wanagama that was projected to be one of gene banks in Indonesia. Further researches recommended sandalwood improvement strategy based on genetic conservation that is focused on maximizing diversity, minimizing self-mating (Naiem and Ratnaningrum, 2010), and maintaining a natural mixed mating (Yuliah, 2011; Naiem and Ratnaningrum, 2010; Indrioko *et al.*, 2009). This research is a part of the long term research on sandalwood improvement strategy based on gene conservation approaches. Research focused on gene diversity and flowering characteristics of seven provenances of sandalwood planted on *ex-situ* gene conservation in Wanagama Forest Research Station, Yogyakarta Province, Indonesia; and its effect on pollination mechanisms.

## 2. Study site

Long term improvement program on sandalwood aimed to create progeny with a high-containing-oil heartwood and high resistance of pests and diseases in a short growing period (Suseno, 2001). A sandalwood genetic conservation program in Indonesia has been initiated with the introduction of eleven thousands seedlings originated from Timor in Wanagama, Yogyakarta in 1968. Wanagama Education Forest – belongs to the Faculty of Forestry, Universitas Gadjah Mada – is a 600 ha forest research station located in Gunungkidul, Yogyakarta (150-400 m a.s.l of altitude, 7° North latitude – 8° South latitude). Soil was latosols,

hard-sticky clay texture with a very shallow solum depth. A sediment carbonate rocks dominated by limestones. It classified to C and D of Schmidt and Fergusson climatic types with 1900 mm yearly rainfall on 2 – 6 rainy months. The temperature is  $27,7^{\circ}\text{C}$  in average with 80-90% relative humidity (Anonymous, 1988).

In 1968, eleven thousands seedlings originated from Timor have been planted in Compartment 5 of Wanagama as a part of degraded land rehabilitation. Only eleven out of those 11.000 seedlings were survived. Those remained seedlings were then able to reproduce sexually, spread its offsprings widely over generations, and recently believed to be a new local landrace. At present, the remained mother trees and its offsprings – that was dispersed widely until more than 5 km in distance – dominated a 10 ha of Compartment 5 and nearby; performed a natural population of sandalwood (Anonymous, 1988; Priyanto *et al.*, 1987).

Furthermore, a genetic plantation – combined a progeny and provenance trials – was established on Compartment 17 in the end of 1993. This plantation comprises 106 progenies which were selected from seven provenances representing different climatic zones in Indonesia. Buat and Netpala provenances which have an origin in Timor Tengah Selatan - Nusa Tenggara Timur, and Tilomar provenance with the origin in Timor Timur, represented a semi-arid to arid region. Bromo provenances that was collected from Lawu mountain, Jawa Tengah, represented a tropical mountain forest region. While, Wanagama and Karangmojo provenances originated from Gunungkidul,

Yogyakarta, and Imogiri provenance from Bantul, Yogyakarta; was several local landraces represented a tropical lowland forest region (Figure 1).

From these two main sources, the genetic processes such as migration, selection and gene flow occurred over generations in Wanagama, resulting in the existence of several new groups of sandalwood. These new groups distributed widely from the lowest place along the catchment area of Oya River in Compartment 5, to the highest zone in the upper side of Kemuning Hill in Compartment 6 and 7. Numbers of researches showed a high genetic variation of sandalwood in Wanagama (Yuliah, 2011; Naiem and Ratnaningrum, 2010; Ratnaningrum, 2010; Haryjanto, 2009; Indrioko *et al.*, 2009; Irmawati, 2007) that was even higher compared to those that was grown both in natural habitat in Nusa Tenggara islands (Haryjanto, 2009) and India (Suma and Balasundaran, 2003), and in *ex-situ* conservation area in Watusipat, Central Java (Haryjanto, 2009; Rimbawanto *et al.*, 2006).

Previous studies indicated reduction of allele frequencies at seedling level, where missing alleles were observed as well. It implied that some alleles were no more inherited to the next generation. These researches concluded that the missing alleles occurred as a result of random genetic drift. Further research (Ratnaningrum, 2010) found that even in genetic trials, several alleles were observed in a very low frequency level. Certain alleles were found only on certain provenances, indicated that the genotypes were provenance specific.

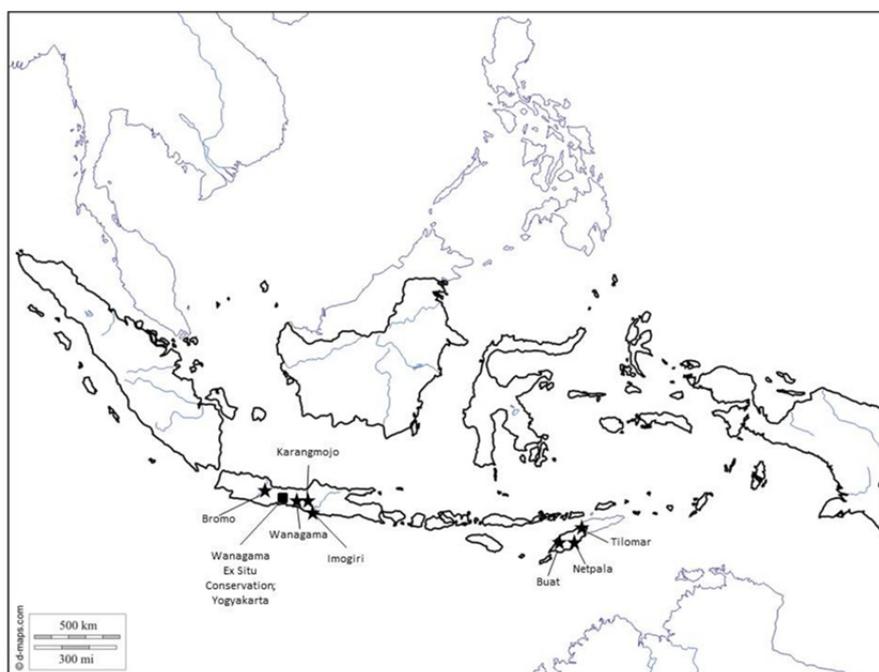


Fig. 1. Study site, Wanagama *ex-situ* gene conservation that is located in Yogyakarta Province, Indonesia, indicated by ■ symbol. Provenances where the genetic materials were collected represents by ★ symbol.

### 3. Materials and Methods

To measure genetic diversity, juvenile leaves were sampled from each individual of each provenance planted at Compartment 17 in the middle of 2009. Isozyme analyses were then conducted with vertical polyacrilamide gel electrophoresis procedures based on David-Ornstein method (Sheido, 1993) with 3 enzyme systems: SHD (E.C. 1.1.1.25.), EST (E.C. 3.1.1.) and DIA (E.C. 2.6.4.3.). Standard genetic distance measured following Nei (1987) and dendrogram were then made following cluster analyses based on unweighted pair-group method with arithmetic mean (UPGMA).

Observation on flowering and pollination mechanisms was done over 12 flowering periods from 2005 to 2010. To observe flowering phenology, each of 10 individual trees of seven provenances were marked and observed. Parameters on flowering (floral initiation, flowering phases of development, duration time of each developmental phase) were then measured daily based on Ghazoul (1997) and Owens *et al* (2001) methods.

Pollination mechanisms were observed during a whole flowering season in a daily based observation to record flower visitors and plant-pollinator interactions. Attractant availability was observed on 10 marked individual of each provenance, and the observation was then made to record type, abundance, and activity time interval of flower visitors. Observation was then made on plant pollinator interactions including stigmatic contact, pollen load, interplant movement, feeding behavior and visitation rate, that were measured daily based on Ghazoul (1997) and Jones and Little (1983) methods.

### 4. Result and Discussion

The goals of any of conservation programs are to maintain genetic diversity and minimize processes that might reduce the variation (Neel *et al*, 2001). Understanding how the diversity formed and predicting the diversity changes that might happen in the future are the important aspects on designing conservation programs and strategies. Knowledge on all aspects of mating systems is of importance on determining genetic diversity and gene dynamics among generation (Neel *et al*, 2001), estimating gene flows within and among population, assessing the best ways to conserve and utilize the variation (Young *et al*, 2000), designing conservation forms and efforts (Bernardello *et al*, 1999; Bawa and Hadley, 1990; Owens *et al*, 1991), and understanding causes and indicators of extinction (Kaye, 1999). Numbers of researches have been done with emphasize on the role of mating systems on determining genetic variation structures within and among population. This information helps to determine priority in *in-situ* conservation and genetic materials collection, and design mating strategies for *ex-situ* and re-introduction plantation (Neel *et al*, 2001).

#### 4.1 Genetic diversity and genetic distance

Basically, variation existed as a result of differences in growth environment, plant genetics and interaction built in between (Zobel and Talbert, 1984). Tree species

commonly have a high level of genetic diversity at taxon, individual and population levels, with a high level of heterozygosity and proportion of polymorphic loci, compared to those of annual species (Young *et al*, 2000). Level of gene diversity varied and affected by mating systems: a higher diversity found on taxon with predominant outcrossing mating systems, and in contrary, naturally selfing taxon has a low level of genetic diversity but high in an inter-population variation. Genetic variation within population observed to be higher compared to those of among population; with exception to mass or production plantation, particularly for those which regenerated by vegetative propagation (Griffin and Sedgley, 1989). Species with limited distribution, fragmented or isolated population usually have a very high genetic differentiation among population. In contrary, species having a very wide distribution will perform high variation among region compared to those of population within region (Young *et al*, 2000).

In Wanagama, genetic variation observed to be lower in natural population with  $H_o$  0.333 (Yuliah, 2011) and  $H_e$  0.345 (Yuliah, 2011) to 0.487 (Ratnaningrum, 2010); compared to those of genetic trials with  $H_o$  0.392 to 0.558 and  $H_e$  0.287 to 0.409 observed in this research, respectively. In this genetic trials, high genetic variation ( $H_T$  0.403) was mostly found within provenance ( $H_S$  0.366), while variation between provenances was very low ( $D_{ST}$  0.037). The  $G_{ST}$  showed that only 9.2% of variation exist between provenance. These levels is observed to be higher compared to those that was grown in natural population in Nusa Tenggara islands with  $H_o$  0.323 (Haryjanto, 2009), in *ex-situ* conservation in Watusipat, Central Java with  $H_o$  0.391 (Rimbawanto *et al*, 2006), and in several provenances in India with  $H_o$  0.130 (Suma and Balasundaran, 2003), respectively.

The low genetic variation in Wanagama conservation indicated close inheritance among provenances. Nei's standard genetic distance (Nei, 1987) (Table 1) showed closest inheritance between Buat and Netpala, and it can clearly be understood as both provenances were originated from the same site, Timor Tengah Selatan. But it was surprisingly found that both provenances were not closely inherited to Tilomar provenance that was originated from Timor Timur. Tilomar provenance was related closely to Wanagama that was believed as the local landrace in Gunungkidul. Referring to the establishment history of sandalwood in Wanagama, it

Table 1. Genetic distance of seven provenances of sandalwood planted on *ex-situ* genetic conservation in Wanagama based on Nei's standard genetic distance (Nei, 1987)

Provenance	Netpala	Karangmojo	Bromo	Wanagama	Tilomar	Imogiri
Buat	0.013	0.021	0.035	0.073	0.078	0.131
Netpala		0.026	0.050	0.119	0.107	0.191
Karangmojo			0.018	0.084	0.060	0.140
Bromo				0.122	0.087	0.181
Wanagama					0.055	0.021
Tilomar						0.092

was known that the seedlings were collected from Timor (Anonymous, 1988). So it is clearly understood that provenance Tilomar and Wanagama landrace were came from the same origin. This reason also explained why Wanagama is not closely related to Karangmojo, even when both landraces were grown at the same site in Gunungkidul region.

#### 4.2 Plant Reproductive Biology

Mating or breeding systems is a reproductive system of a given species (Schlegel, 2003) which describes the probabilities between two gametes of different organisms to be mated to develop zygote (Zobel and Talbert, 1984). This systems is a combination of a whole sexual characteristics that is directly influencing plant genetic composition among generation (Kittelson and Maron, 2000), and is significantly affecting number and distribution of genetic variation within population (Young *et al.*, 2000). Plant mating systems vary with the reproductive biology and spatial structure of a species, which combine to influence the levels and dynamics of genetic diversity (Griffin and Sedgley, 1989; Bawa and Hadley, 1990; Ghazoul, 1997; Young *et al.*, 2000). Consequently, designing implementation plan and strategy of genetic conservation needs the understanding on reproductive biology processes, spatial structures, and how the interaction built in among those components to determine pattern of gene flow and genetic variation (Young *et al.*, 2000). Aspects of reproductive biology include sexual systems, incompatibility mechanisms, flowering characters and pollination mechanisms. While, spatial structures determine arrangement and biological function of floral organs (Griffin and Sedgley, 1989; Young *et al.*, 2000), which in turn regulate flowering type and pollination model of species (Griffin and Sedgley, 1989; Owens *et al.*, 1991).

Characteristics of mating systems strongly influence the genetic diversity. Aspects of mating systems such as selfing rate, outcrossing rate and inbreeding depression significantly contribute to genetic diversity, and therefore have direct effect to conservation (Neel *et al.*, 2001). Proportion of selfing and crossing within population is affected by genetic mechanisms such as self-sterility (Kittelson and Maron, 2000; Sage *et al.*, 1999) and self-incompatibility (Young *et al.*, 2000; Griffin and Sedgley, 1989), flowering characters and pollination mechanisms (Griffin and Sedgley, 1989; Bawa and Hadley, 1990; Ghazoul, 1997), and sexual systems (White *et al.*, 2007). Flowering characters comprises flowering ontogeny and phenology (Owens *et al.*, 1991; Griffin and Sedgley, 1989) and floral spatial structures and arrangements (Young *et al.*, 2000; Griffin and Sedgley, 1989; Owens *et al.*, 1991). Sexual systems affect the proportion of selfing and crossing in such ways as the separation of floral sexes, that could be temporally (protandry and protogyny dichogamy) or spatially (monoecy and dioecy) separated (White *et al.*, 2007).

As a high selfing brought into the inbreeding depression, plants perform various natural mechanisms manifested in the model of flowering, pollination and

fertilization, that is aimed to maintain or enhance crossing (Jones and Little, 1983). In self-compatible species, typically flower has a natural mechanism to minimize selfing through the separation of its floral sexual organs that could reveal in temporal and spatial ways. Plant with temporal separation mechanism attempted to separate the maturity of its sexual organs by time, so that the pollen and stigma were mature at a different time. In plant with spatial separation mechanism, stigma and pollen separated by a different place, such as a monoecy (male and female organs emerged on different flower within the same tree) or dioecy (male and female organs risen on a different tree); or there were specific floral architecture where stigma separated away from its pollen, such as those found on heterostylous types (White *et al.*, 2007; Griffin and Sedgley, 1989).

Various studies focused on compatibilities and pollination mechanisms of tropical forest trees indicating strong barriers to inbreeding; leading to the conclusion that there was a preference to be a predominantly outcrossed species, even a low level of inbreeding was also observed (Young *et al.*, 2000; Bawa and Hadley, 1990). Up til now, there is still very limited information on the breeding systems on any of tropical forest trees species. A detailed information has only been made for the economic-important species such as *pinus*, *eucalypts* and *leucaena*; whereas for most other species there were no data available, as studies has never been conducted yet even in a preliminary observation (Young *et al.*, 2000).

A long term research on the reproductive biology of sandalwood in Wanagama has been conducted since 2005 on various aspects such as flowering ontogeny and phenology (Naiem and Ratnaningrum, 2011; Ratnaningrum, 2010; Prasetyaningtyas, 2005), floral structure and morphology (Ratnaningrum, 2010; Lestari, 2006; Nalvien, 2006), sexual systems and the maturity of sexual organs (Naiem and Ratnaningrum, 2011; Ratnaningrum, 2010), pollination mechanisms (Ratnaningrum, 2010; Ratnaningrum and Prehaten, 2005), incompatibility mechanisms and crossing ability (Naiem and Ratnaningrum, 2011; Ratnaningrum, 2010; Haryanto *et al.*, 2005), and the effects of environmental factors on flowering (Naiem and Ratnaningrum, 2011; Ratnaningrum, 2010; Ratnaningrum and Prehaten, 2005). This integrated works brought into the similar conclusion that the reproductive biology of sandalwood is genetically specific as it is strongly controlled by genetic factors.

##### 4.2.1 Flowering characters

Provenances with similar genotypes that were collected from same site performed similar characters on floral structure and morphology, sexual organs maturation, flowering ontogeny and phenology (anthesis and flowering period, flowering frequency, and floral initiation) as well. All of provenances was both protandry dichogamy in sexual systems and heterostyly in spatial structure. Variation on size and color of perigonium was found among provenances between times. Generally, provenances collected from

eastern parts of Indonesia flowered earlier compared to those of originated from Java, with shorter period as well; with the exception to Bromo that was the latest to flower with the longest period (Table 2, Figure 2).

In this research we also observed sexual systems, that was referring to any of parts or position where the sexual organs emerged in plant that might in turn affect the mating systems; and the spatial structures, that was determining arrangement and biological function of floral organs. Transformation of floral parts during the developmental phases was also observed to be

provenance/genotype specific (Figure 3).

Genotypes control was also observed on sexual organs maturity and longevity (Table 3) and floral structure (Table 4), where each provenance performed specific characters. Variation found on floral structure and arrangement as well as sexual organs maturity and longevity (Table 3). However, both protandrous dichogamous in sexual systems and heterostyly in spatial structure were observed in all of provenances. Variation on size and color of perigonium was found among provenances between times.

Table 2. Flowering ontogeny and developmental phases on seven provenances of sandalwood planted on *ex-situ* gene conservation in Wanagama, Yogyakarta Province, Indonesia

Developmental Phases	Time of developmental processes (days)						
	Buat	Netpala	Tilomar	Wanagama	Imogiri	Karangmojo	Bromo
<b>1. Floral buds initiation</b>							
a. Differentiation of flower buds	6.8 – 8.6	7.2 – 8.4	7.6 – 9	8.2 – 10.4	8 – 10.2	8.6 – 10.2	9.6 – 11.8
b. Maximum elongation of buds	16.4 – 16.8	16.4 17.2	17 – 17.8	17.6 19.2	17.8 18.8	18.2 – 19.4	19.4 – 21.6
c. Maximum swollen of buds	8.2 – 9.2	8 – 10	8.6 – 10.2	8.8 – 11.2	9–11	9.2 – 11.4	9.8 – 13
<b>2. Bud development prior anthesis</b>							
a. Anthers reaching maturity	2 – 3	2 – 3	2.2 – 3.4	3.6 – 4.4	2.4 – 4	3.4 – 4.6	4 – 6
b. Early anthesis, blooming of buds, opening of 4 outer petals	4-5 hours	4-5 hours	4-5 hours	4-5 hours	4-5 hours	4-5 hours	5-6 hours
<b>3. Pollination and fertilization</b>							
a. Fully anthesis, stigma receptive, polination occurs	5.2 – 6.6	5.2 – 6.6	5.6 – 7	7.2 – 9	6 – 8.6	7 – 8.8	7.2 – 10.2
b. Fertilization, drying of pistil and perigonium	2.2 – 3.4	2.2 – 3.6	2.8 – 4	4 – 5.4	3 – 5	4 – 5.4	4.2 – 6.8
c. Transformation of flower into fruit morphology, ovary develop into green-young fruit	3 – 4.8	3.2 – 4.2	3.6 – 4.2	4.8 – 6.2	3.6 – 5.8	4.6 – 6	5.2 – 8.2
<b>4. Fruit development to maturity</b>							
a. Development of fruit to maximum size	22 – 24	21.8 – 23.8	22.2 – 24	22.4 – 25.2	22.2 – 24.6	22 – 25	24.2 – 28.6
b. Maximum size of fruit; green in color	10.6 – 13.2	10.2 – 12	10.8 – 13.6	11.2 – 14	11 – 13.6	11.4 – 14.4	13.2 – 17
c. Fruit development until maturity; dark-purple to black in color	20 – 21	20.6 – 23	22.2 – 23.8	23.4 – 24.8	22.6 – 24.2	24.2 – 24.6	26.6 – 26.8
	96.8–110.2	96.8–111.8	102.6–117	111.2–129.8	105.6–125.8	112.6–129.8	123.4–150

Table 3. Maturity and longevity of sexual organs of sandalwood flowers during two years of measurement (2009 and 2010)

Provenance	Year of 2009			Year of 2010		
	Duration of stigma receptivity (day)	Duration of pollen longevity (day)	Duration of anthesis (day)	Duration of stigma receptivity (day)	Duration of pollen longevity (day)	Duration of anthesis (day)
Buat	6.3	2.5	9.4	5.2	2.0	10.0
Tilomar	5.6	5.5	10.6	4.8	5.0	11.0
Wanagama	9.2	4.1	14.8	8.6	2.8	14.4
Imogiri	6.0	5.9	11.4	4.8	5.2	13.6
Karangmojo	7.0	7.4	14.4	6.2	5.8	14.2
Netpala	6.3	2.5	9.4	5.4	2.0	10.2
Bromo	7.2	4.5	15.4	6.0	4.0	17.0

Table 4. Floral structure of sandalwood flowers during two years of measurement (2009 and 2010)

Provenance	Size of sexual organs			
	Year of 2009		Year of 2010	
	Length of stylus (mm)	Length of filament (mm)	Length of stylus (mm)	Length of filament (mm)
Buat	1.53	1.81	1.56	1.92
Tilomar	1.46	1.90	1.52	1.98
Wanagama	1.52	1.89	1.44	1.68
Imogiri	1.41	1.51	1.42	1.50
Karangmojo	1.50	1.65	1.44	1.66
Netpala	1.49	1.93	1.52	1.92
Bromo	1.49	1.58	1.48	1.58

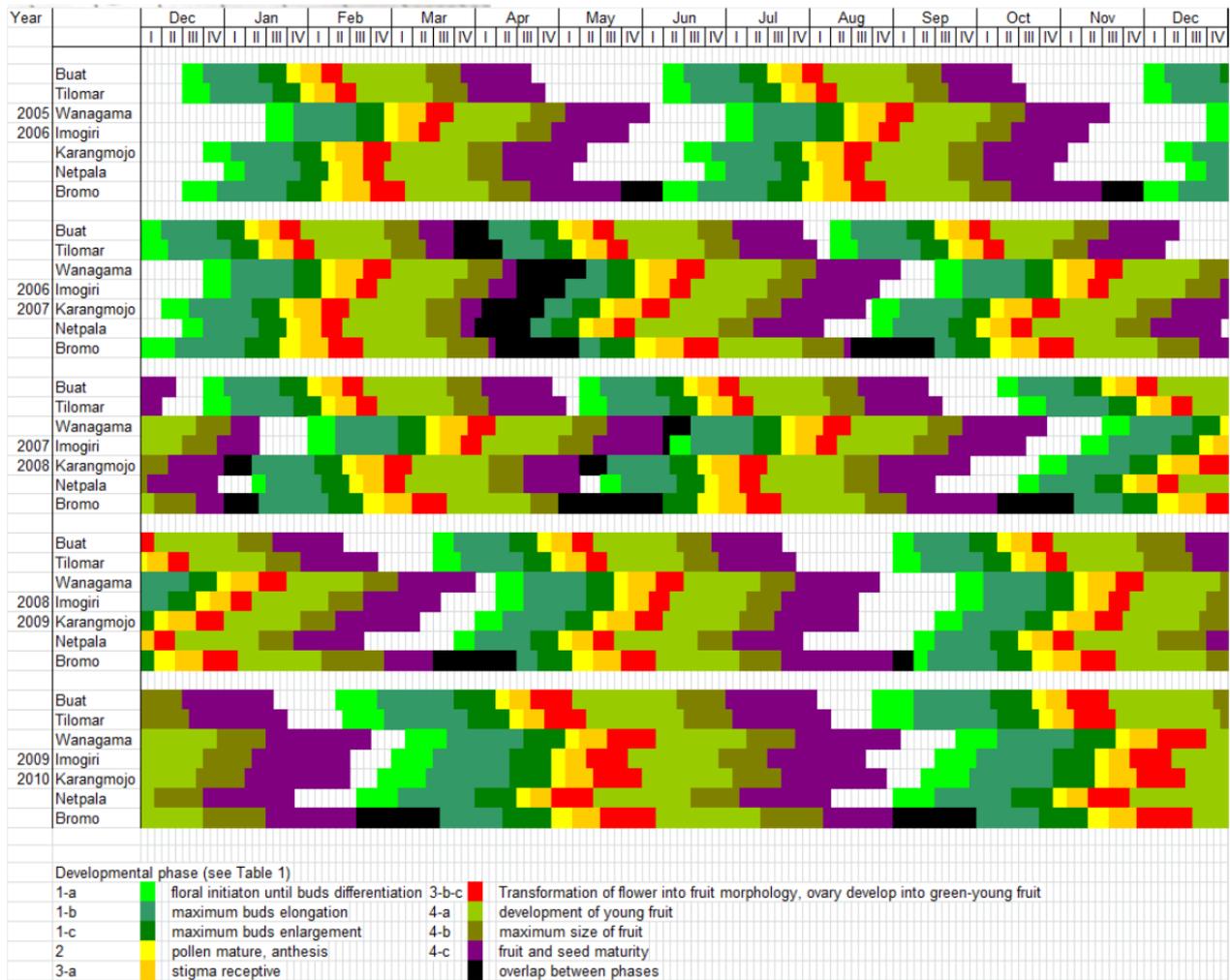


Fig. 2. Flowering phenology on seven provenances of sandalwood planted on *ex-situ* gene conservation in Wanagama, Yogyakarta Province, Indonesia during 12 flowering periods from year 2005 to 2010. Flowering of sandalwood was strongly controlled by both genetic and environment factor. Extreme temperature increment lead to (1) shorter flowering period, (2) higher flowering frequency, and (3) decrement of flower, fruit and seed abundance. In contrary, the increase of yearly rainfall affected to (1) longer flowering period, (2) later floral initiation, (3) shorter stigma receptivity and pollen longevity, (4) longer anthesis period, (5) longer reproductive organs, and (6) paler color of perigonium.



(a)



(b)

Transformation on floral morphology of Buat Provenance during *anthesis*: inflorescences (a), phases of anthesis: early phase (b), fully anthesis and late phase (c).



(c)



(a)



(b)



(c)



(d)

Transformation on floral morphology of Netpala Provenance during *anthesis*: inflorescences (a), phases of anthesis: early phase (b), fully anthesis (c) and late phase (d).



(a)



(b)

Transformation on floral morphology of Tilomar Provenance during *anthesis*: inflorescences (a), phases of anthesis: early phase and fully anthesis (b), and late phase (c).



(c)



(a)



(b)



(c)



(d)

Transformation on floral morphology of Bromo Provenance during *anthesis*: inflorescences (a), phases of anthesis: early phase (b), fully anthesis (c) and late phase (d).



(a)



(a)



(b)



(b)



(c)



(c)



(d)



(e)

Transformation on floral morphology of Imogiri Provenance during *anthesis*: inflorescences (a), phases of anthesis: early phase (b), fully anthesis and late phase (c).

Transformation on floral morphology of Karangmojo Provenance during *anthesis*: inflorescences (a), phases of anthesis: early phase (b), fully anthesis (c) and late phase (d, e).



(a)



(b)



(c)



(d)



(e)

Transformation on floral morphology of Imogiri Provenance during *anthesis*: inflorescences (a), phases of anthesis: early phase (b), fully anthesis and late phase (c).

Fig. 3. Transformation on floral morphology during *anthesis*: from early phase, fully anthesis to the late phase.

#### 4.2.2 Pollination Mechanisms

Pollination refers to the deposition of pollen grain onto the stigma, that could be cleistogamy when stigma pollinated by pollen from the same flower, or chasmogamy when pollen came from different flower (Ghazoul, 1997; Griffin and Sedgley, 1989). Based on the type of pollination agents, the pollination processes derived into (1) biotical pollination, when the pollen transfer was mediated by biotic agents such as insects, birds and bats; and (2) abiotic pollination which refers to the pollination processes mediated by abiotic factors such as water and wind (Ghazoul, 1997; Owens *et al.*, 1991; Bawa and Hadley, 1990; Griffin and Sedgley, 1989; Jones and Little, 1983).

Pattern of genetic variation was strongly influenced by plant pollination mechanisms (Bawa and Hadley, 1990; Griffin and Sedgley, 1989). Regarding to this, it is important to understand the plant function as a part of population and more complex ecological system. In the context of biotical pollinated species, pollination is a result of the series of interaction built in between flowering plants and its pollinators that are conditioned by environment prior and during anthesis (Ghazoul, 1997; Griffin and Sedgley, 1989). Therefore, the success of pollination required the ability of agents to establish number of interactions with plant resulting in the pollen transfer. In the other side, plant should have the ability to ensure the continuity of visit and interaction, that in turn resulting in the pollen transfers.

Due to its responsibility to attract pollinators, flowers product some attractants. Attractant is a material provided by flowering plants to establish a continuous interaction with its pollinators; that could represented as a primary or secondary attractant. Primary attractants refer to the materials provided by plants to gain a continuous visit by its pollinators; that could manifested as (1) sources of energy/foods, generally are nectars and pollens, (2) a place for nests, or (3) a suitable place for breeding. The secondary attractants are any of effects displaying by flowering plants on the effort to show its existence to its pollinators; that could represented in (1) floral colors, (2) size and shape of flowers and floral structures, or (3) scent of flowers or floral parts.

Series of works on determining pollination mechanisms of sandalwood has been done since 2005 (Ratnaningrum and Prehaten, 2005; Ratnaningrum, 2010) as a means of the abundance of flower visitors and plant-pollinator interactions. Six types of 3 orders of insect: moths, butterflies (Lepidoptera), flies (Diptera), ants, bees and wasps (Hymenoptera), were found to visit flowers. Type of insect and visitation rate depended on flowering phases in term of primary (pollen and nectar) and secondary (color of perigonium and mature anthers) attractants availability. Meanwhile, preference to visit certain provenances was affected more by secondary attractant. Bees observed to be the most effective pollinator.

##### 4.2.2.1 Flower visitors and floral attractants

Compiled records within six flowering periods (Ratnaningrum, 2010; Ratnaningrum and Prehaten,

2005) reported consistencies on type of flower visitors, while the numbers varied with the fluctuation of flowers abundance. Six types of 3 orders of insect were found to visit flowers: moths, butterflies (Lepidoptera), flies (Diptera), ants, bees and wasps (Hymenoptera); in which bees were dominated in all of flowering season, followed by ants, butterflies, moths, wasps and flies, respectively. Similar result was reported by Vereendra and Padmanabha (1996) while observing pollination mechanisms of sandalwood grown in its natural population in India, and finding approximately 15 insect species visiting flowers, in which bees and ants were the main pollinators collecting nectars secreted in perianth.

Research on sandalwood grown in both *ex-situ* conservation area (Ratnaningrum, 2010) and natural stands (Ratnaningrum and Prehaten, 2005) in Wanagama reported that type of insect and visitation rate depended on flowering phases, in term of primary (pollen and nectar) and secondary (color of perigonium and mature anthers) attractants availability. It seemed that visitor abundance was strongly related to the primary attractant availability, nectars and pollens that were provided by flowers at a different flowering phase. Visitation in early anthesis phase, when the mature pollen is in a maximum availability, was dominated by pollen-eater such as moths and flies. In fully anthesis phase when the stigma is receptive and nectars availability is maximum, the nectar-eater ants and wasps dominated the pollination activities. Visitation of butterflies and bees were observed in a similar rate both in early and fully anthesis phases.

Variation of attractant with flowering phases was found not only in a term of primary, but also secondary attractant reflected in a floral visual appearance displayed by the color of perigonium and anthers. Early phase of anthesis was dominated in color by yellowish-white, off-peach to pale pink perigonium, with bright yellow to orange mature anthers. In this early anthesis phase, visitation was dominated more by diurnal insects preferring pale and gloomy color such as wasps and flies (Figure 4). During fully to late anthesis phase, flowers were dominated more by brighter and stronger color such as dark red, maroon to dark maroon. This is a range of color spectrum that is easily caught by insect facet eyes of bees, butterflies and wasps (Figure 5). Butterflies were observed to visit flowers both at early and fully anthesis (Figure 6).

Meanwhile, preference of visitors to visit certain provenances were affected more by secondary attractant displaying by floral visual appearance, that was differs among provenances. Provenances originated from Eastern parts of Indonesia, which tend to perform paler color of perigonium, were visited regularly by flies and moths. In contrary, visitation of those two types of insect was less on provenances originated from Java Island which have brighter color of perigonium. On Java-originated provenances, visitation was dominated more by butterflies, bees, and wasps.

Since the abundance of visitors is strongly related to the number of flowers at a certain flowering period,

there is a correlation between visitor abundance and climatic factors as a mean of weather and seasonal condition. There were consistencies shown on six flowering periods that the abundance of visitors always found to be higher at the early dry season compared to

those of rainy season, with the exception on the year 2007 dry season. Extreme temperature increment occurred on January 2007 resulted in the mass flower abortion that was directly reduce visitation rate during April to August 2007 period (Figure 7; Table 5).



Fig. 4. Flies (left) and moths (right), with preference to visit flowers in paler and gloomy color, dominated visitation in the early anthesis phase.



Fig. 5. Wasps (left) and ants (right), preferring to visit flowers in brighter and stronger color, dominating visitation in the fully to late anthesis phase.



Fig. 6. Butterflies (top) and bees (below) are visiting flowers both the paler one at the early phase of anthesis (left) and the brighter one at the fully anthesis phase (right).

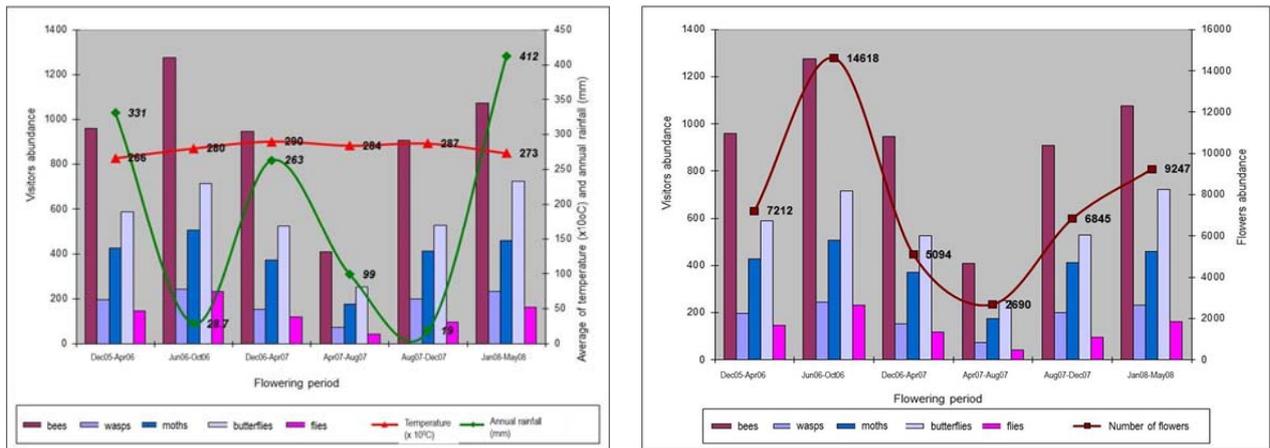


Fig. 7. Type and abundance of agents visiting sandalwood flowers in Wanagama *ex-situ* gene conservation during six flowering periods (2005 – 2008) that is correlated to environment parameters (top) and flowers abundance (below).

Table 5. Type and abundance of agents visiting sandalwood flowers in Wanagam (2005-2008)

Provenance	Type and abundance of flower visitors on two flowering phases										Flowers abundance
	Early anthesis phase					Fully anthesis phase					
	bees	wasps	moths	butterflies	flies	bees	wasps	moths	butterflies	flies	
December 2005 – April 2006											
Buat	11	0	62	18	21	43	16	23	25	10	696
Tilomar	58	6	29	25	11	101	22	15	69	7	1021
Wanagama	52	2	26	21	9	87	26	13	58	4	807
Imogiri	68	5	34	37	11	114	31	17	76	7	1278
Karangmojo	61	4	32	35	12	127	34	15	71	6	1145
Netpala	32	6	82	18	23	51	12	29	35	14	1026
Bromo	49	3	37	33	7	105	29	13	67	5	1239
June 2006 – October 2006											
Buat	18	4	92	18	34	86	16	30	21	28	1540
Tilomar	74	11	39	56	14	146	33	12	77	6	2270
Wanagama	60	12	26	48	10	126	22	11	68	4	1910
Imogiri	69	14	46	55	17	154	31	18	84	8	2410
Karangmojo	71	12	44	50	18	167	29	16	86	6	2670
Netpala	23	6	88	15	36	84	18	34	11	24	1620
Bromo	67	9	37	51	17	132	27	13	75	9	2200
December 2006 – April 2007											
Buat	24	7	49	29	19	97	16	31	50	9	910
Tilomar	57	8	31	37	12	120	27	16	67	4	978
Wanagama	21	3	11	9	4	36	6	10	26	0	316
Imogiri	17	2	16	12	2	29	8	9	22	2	398
Karangmojo	62	8	44	46	16	216	34	22	78	10	1150
Netpala	24	5	53	23	16	109	12	29	42	7	732
Bromo	39	4	31	26	13	96	12	20	58	4	607
April 2007 – August 2007											
Buat	8	0	17	10	5	22	4	13	6	3	239
Tilomar	22	2	12	30	4	42	8	12	10	0	412
Wanagama	11	0	6	18	2	35	6	3	7	0	293
Imogiri	27	5	12	39	5	54	11	10	13	0	436
Karangmojo	28	4	21	43	6	58	14	12	11	1	481
Netpala	16	2	25	22	7	30	4	17	8	6	486
Bromo	16	1	10	24	3	40	11	6	10	0	343
August 2007 - December 2007											
Buat	21	4	58	17	10	54	9	14	27	12	762
Tilomar	46	14	42	40	9	121	29	15	60	8	1290
Wanagama	29	8	35	31	3	87	17	9	46	3	819
Imogiri	49	13	43	41	4	118	25	12	49	7	1060
Karangmojo	52	13	42	45	6	129	24	14	59	5	1180
Netpala	31	5	63	19	13	66	12	22	36	11	985
Bromo	25	8	36	28	3	80	20	8	31	2	746
January 2008 – May 2008											
Buat	34	4	50	17	22	63	12	23	33	9	983
Tilomar	61	11	38	45	11	107	26	18	69	7	1350
Wanagama	52	12	32	43	8	94	19	13	61	5	1030
Imogiri	55	10	37	48	13	109	22	21	67	5	1370
Karangmojo	87	19	56	58	20	137	41	30	102	10	1870
Netpala	47	6	57	27	24	75	16	31	48	11	1320
Bromo	54	12	42	37	12	100	21	12	68	5	1320

#### 4.2.2.2 Plant pollinator interactions

##### a. Feeding behavior affecting stigmatic contact, pollen load and interplant movement

Flower visitor might be predicted as pollinator depended to its ability to ensure the pollen transfer onto the stigma (Ghazoul, 1997). Regarding to this, Griffin and Sedgley (1989) confirmed several criteria of the effective pollinator: (1) continuously visiting flowers right at the time when the pollen is mature and the stigma is receptive; (2) conducting activities at the same range of season with the flowering season; (3) visiting many flowers in many trees within population; (4) bearing sufficient pollen loads; (5) establishing continuous contact with stigma in a certain ways that ensuring the occurrence of pollination process; and (6) be present in a sufficient number.

Detailed observation was made on agent's behavior affecting stigmatic contact, pollen load and interplant movement that in turn ensured pollen transfers in flowering sandalwood. According to the pollen load, it was observed that butterflies and moths were able to bear pollen, even in a very small number, using a smooth feather emerged on its feet and proboscis. Both insects have sucking-type mouth that allow them to enter the flower as close as possible to collect nectars, and hence promote the intensive stigmatic contact and pollen transfer. However, their presence in a very small number make it was not possible to ensure the pollen transfer in a sufficient quantity to pollinate the entire receptive stigma. Both insects observed to move from one flower to another among different trees, hence enhance the probability of cross-pollination.

A small body with slimy surface of flies and wasps resulted in a low pollen load. Their small body imposed them to come into the flower as deep as possible to collect nectars, causing a high stigmatic contact. Unfortunately, a low pollen load resulted in a low pollen transfer onto the stigma. Their presence was also observed in a very small number. Wasps observed to fly moving among flowers and trees, hence promote the probability of cross-pollination. In contrary, flies preferred to stay at the same tree during a long period interval, although it was also observed to move among flowers.

Ants, with a so-tiny body that is small enough to reach the deepest space of entire flower parts, was found in abundant numbers almost in a whole day. It was observed to explore nectars deep in the basal portion of receptacle, walk along the stylus to reach the stigma, or quickly moving among flowers; resulting in a very high stigmatic contact. But, their small size and slimy body surface inhibit the pollen load. It was also observed to stay in the same inflorescence within the same tree that minimized the probability of cross-pollination.

A sufficient stigmatic contact and pollen load was observed only on the feeding behavior of bees. This insect belongs to family Apidae which has hairy thorax and abdomen. Their brush-structured front-feet collect pollen effectively, and their basket-like back-feet bears abundant load of pollen. They collect both nectar and pollen as the adults make a honey from nectars and

provide pollen breads to feed its larvae. It presented in a fit size to promote an intensive contacts both to stigma and anthers while entering flower to reach for nectars. This diurnal pollinator was also observed to work in a group and move among flowers and trees within population, ensuring the effective cross-pollination in a sufficient number.

##### b. Primary and secondary attractant availability

Research on sandalwood grown in both ex-situ conservation area (Ratnaningrum, 2010) and natural stands (Ratnaningrum and Prehaten, 2005) in Wanagama reported that type of insect and visitation rate depended on flowering phases in term of primary (pollen and nectar) and secondary (color of perigonium and mature anthers) attractants availability.

Floral architecture comprises any aspects related to flower size, male and female sexual organs composition, accessibilities of rewards for pollinators, and floral structures that in combination controlled plant-pollinator interactions (Ghazoul, 1997; Griffin and Sedgley, 1989). As each of visitors having specific characters on size, sensor ability, feeding behavior and rewards requirements, it is in certainty that there were a given correlation between floral architecture and its pollinator type (Faegri and van der Pijl, 1979 *in* Griffin and Sedgley, 1989). It means that any of specific floral architecture has its own specific pollinator as well. For example, small bees with sap-sucking mouth type would prefer to visit zygomorphic (semi-closed) type of flower with concealed sexual organs. Bigger bees or wasps, with bigger body parts, could only come into the bigger flowers which have dish or bowl type with exposed sexual organs. Tiny flies with sucking mouth type would also prefer flowers with dish or bowl type and exposed sexual organs, but only those in smaller size. Birds, because of its heavy body and mouth type, will only compatible to flowers with funnel type. While, a light-skinny body with proboscis mouth type makes it is easier for butterflies to collect rewards from any of flower type.

A long term research on plant pollinator interactions showed that preference to visit flower not only determined by flower structures, sexual organs position or floral color, but also affected by odor or aroma sent by certain parts of floral organ. If the floral display affected more to attract the diurnal agents, this floral odor seemed more important to nocturnal agents which conduct their activity during a night time. Those of nocturnals such as bats and moths get guidance from the floral scent to find their rewards. This is a reason why plants which flowered at night and relied on nocturnal agents to pollinate its flowers usually offer a strong aroma.

Pollinator has a sensitivity not only to distinguish a floral color but also floral structure and morphology as well (Darwin, 1876 *in* Jones and Little, 1983). The best ones were found on bees, flies and butterflies. It seemed that such plant-pollinator interaction mechanisms based on its floral color and structure is a strategy to improve its pollination efficiency by (1) enhancing the display of receptive flowers, (2) sending signals of the

attractants availability, and (3) activating the isolation mechanisms to reduce inter-specific pollen transfers.

Sandalwood flowers with its very small size, tiny and concealed stigma, less nectar and pollen, and weak odor, are facing kind of difficulties to support pollen transfers in adequate numbers. There were only several types of agents attracted to its floral display; particularly those which have skinny body with sap-sucking or sucking mouth types. Anthesis – occurred in the morning to the daytime – and an odorless flower, resulted in a less visit of nocturnal pollinators. Same cases of pollen transfer difficulties were also reported on plant having similar floral structures. *Warea carteri* grown in Lake Wales Ridge, Florida (Evans et al, 2000) and *Lactoris fernandeziana* in Robinson Crusoe islands (Bernardello dkk, 1999) – both having similar flower type and structure to sandalwood – was undergo serious problems related to pollen transfer and limited pollinators that was resulting in a high selfing-rate.

### c. Time interval of effective pollination activity

There were differences observed on the time range of activity among visitors, even all of agents were observed to be diurnal visitors. Moths found in the earliest morning at 06:00 to 08:00. Bees activity observed 1 hour later at 07:00 and seemed to have a longer interval of activity as its presence has been recorded until 10:00 am. Wasps came later at 08:00 and finished their activity at 10:00. The latest was butterflies which has just found after 09:00 and disappeared at 12:00. While, ants and flies were observed to be present at the same tree the whole day through.

Vereendra and Padmanabha (1996) found the same phenomenon in India where the pollination activities were found only before daytime in the warmer site, but it could prolonged until afternoon in the cooler area. Raju and Rao (2002) that observed the pollinators activity on *Acacia sinuata* found 4 insects species having same activity range with those observed in this research.

### 5. Conclusions

Provenances originated from same site performed similar genotypes as well as flowering characters (floral display, sexual organs maturation, and flowering phenology). All of provenances were both protandry dichogamy and heterostyly. Variation on size and color of perigonium were found among provenances between times. Generally, provenances originated from Eastern parts of Indonesia flowered earlier compared to those of central parts of Java, with shorter period as well; while those of Bromo was the latest to flower with the longest period. There were 6 types of 3 orders of insect found to visit flowers: moths, butterflies (Lepidoptera), flies (Diptera), ants, bees and wasps (Hymenoptera). Type of insect and visitation rate depended on flowering phases in term of primary (pollen and nectar) and secondary (color of perigonium and mature anthers) attractants availability. Meanwhile, preference of visitors to visit certain provenances was affected more by secondary attractant.

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