Indonesian Journal of Biotechnology, June, 2013 Vol. 18, No. 1, pp.26-35

Improvement of Seed Orchard Management Based on Mating System of Cajuputi Trees

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Abstract

Breeding plan of cajuputi in Indonesia is aimed to increase plantation productivity of oil yield and 1.8 cineole content. Seed orchard of cajuputi at Paliyan, Gunungkidul, established using selected and genetically improved materials, has been producing seeds for operational plantation. This seed orchard would perform optimally if the mating systems of all individuals contribute to the inheritance of all genetic potential of the offsprings. Therefore, investigation of the mating systems of cajuputi was indispensible. The study has been carried out on 10 selected mother trees and the 24 offsprings of each mother trees using 8 microsatellite markers of nuclear DNA, namely Hin-2 (100-132 bp), Hin-4 (79-114 bp), Hin-5 (128-148 bp), Hin-7 (136-224 bp), Sal-1 (93-99 bp), Sal-3 (118-219 bp), Xho-1 (96-111 bp) and Xho-4 (150-216 bp), respectively. The result showed relatively high genetic variation of the offspring (H_E=0.602, H_O=0.594) originated from parent trees in the seed orchard. Parent trees tend to outcross (t_m=0.951, t_s=0.806), although seeds originated from biparental inbred ($t_m - t_s$ = 0.145) and correlated paternity (rp=0.098) have also been observed. This genetically viable population could maintain its reproduction fitness for short term and adapt to the dynamic environmental changes for long term.

Key words: mating system, cajuputi, seed orchard, microsatellite

Introduction

Cajuputi (*Melaleuca cajuputi* subsp. *cajuputi*) is an endemic species to Indonesia and has an important role for essential oil industry. Cajuputi oil contains 15-60% of 1.8 cineole (one of monocyclic types of monoterpenes) which has medicinal properties (Turnbull, 1986; Boland *et al.*, 1991). Besides, it also contains *sesquiterpene alcohols globulol*, *viridiflorol* and *spathulenol* as main essential oils (Brophy and Doran, 1996).

Breeding program of *Melaleuca cajuputi* subsp. cajuputi has been initiated collaboration by the Centre for Forest Biotechnology and Tree Improvement Yogyakarta in collaboration with CSIRO Forestry and Forest Product Australia in 1995. This program was aimed to improve cajuput productivity, especially increasing the oil yield and 1.8 cineole content (Doran *et al.*, 1998). A progeny trial was established in Gunung Kidul and Ponorogo using genetic materials originated from natural stands in Moluccas (Buru, Seram, Ambon), Northern Territory of Australia, Western Australia, and Gundih Central Jawa. Improvement of cajuputi oil characters showed genetic gain of 1.8 cineole content (10%) and oil yield (21%) (Susanto *et al.,* 2003). The progeny trial has been converted into seed orchard to produce genetically improved seed.

An ideal seed orchard should represent genetic variation of breeding population in order to transfer all improved genotypes of selected families in a relatively balanced proportion to their offspring. Thus, the

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seeds should reflect high productivity in line with the genetic gain of the improved characters. Flowering synchrony of parent trees, including anthesis, ovule receptivity, and pollen maturity, is required in order to support panmictic pollen flow and random mating within seed orchard. On the contrary, contamination of alien pollen from outside of the seed orchard should be avoided.

Information of mating system is essential in seed orchard management, especially to identify the inheritance of genetic characters of selected families and to produce improved seeds without inbreeding potential. Inbred in numerous forest tree species leads to inbreeding depression, e.g. selfing in *Eucalyptus globulus* reduced up to 48% of productivity in comparison with progenies regenerated by outcrossing (Hardner and Potts, 1995). Factors of reproductive biology such as sexual system, incompatibility mechanism, flowering and pollination process (Griffin and Sedgley, 1989; Kittelson and Maron, 2000) and spatial structure (Boshier, 2000) will influence level and dynamic of population genetic diversity.

Mating system could be analyzed using genetic markers. Genetic marker is any visible character or otherwise assayable phenotype, for which alleles at individual loci segregate in a Mendelian manner (White *et al*., 2007). Molecular marker application is valuable for genetic study. High variability of molecular marker is more suitable to reflect genomic variation in comparison with biochemical and morphological markers (Beebee and Rowe, 2004). One of the molecular markers suitable to estimate mating system is Simple Sequence Repeats (SSR) or microsatellite. This is a codominant marker; shows hypervariable polymorphisms; can be observed in nucleus-, chloroplast-, and mitochondria-DNA; and confirms high consistency of reproducibility (White *et al*., 2007). Observation of SSR variation is based on amplification results of tandem repeats of DNA fragment (Finkeldey and Hattemer, 2007) using developed primers adjacent to the flanking region. SSR has been applied to study genetic variation of many tropical species, such as *Gliricidia sepium* (Dawson *et al*., 1997), *Shorea curtisii* (Ujino *et al*., 1998), *Neobalanocarpus heimii* (Iwata *et al*., 2000), *Prosopis* spp. (Mottura *et al*., 2005), *Koompassia malaccensis* (Lee *et al*., 2006), *Shorea platyclados* (Ng *et al*., 2009a), *Gonystylus bancanus* (Ng *et al*., 2009b), and *Shorea leprosula* (Ng *et al*., 2009c). Some SSR primers of nuclear-DNA specified to Cajuputi has also been developed by Miwa *et al*. (2000) and showed enough polymorphisms in term of allelic richness, i.e. 2-10 alleles per locus.

A preliminary study looking at the mating system of cajuputi seed orchard at Paliyan has been carried using lower polymorphism of biochemical marker (i.e. isozyme) and arbitrary sampling trees (Kartikawati, 2008). Although this previous research resulted that cajuputi trees are mostly outcross, this information could not be used to improve the management of seed orchard due to lack information of flowering synchrony. Therefore, a comprehensive study of mating system is needed using highly polymorphism marker of SSR and selected families in peak flowering season.

The objectives of this study were to describe the genetic variation and mating system of cajuputi in half-sib seedling seed orchard at Paliyan, Gunung Kidul, Yogyakarta. Information of mating system is required to evaluate seed orchard performance and support seed orchard management to minimize inbreeding effect and increasing pollination effectiveness, so that seeds resulted from genes recombination of selected families in the seed orchard can be generated.

Materials and Methods

Samples collection

Material for the study was a seedling seed orchard of cajuputi in Paliyan, Gunung Kidul, Yogyakarta. It was established in 1998 using Randomized Complete Block Design with 19 families consisted of 10 treeplots per family and 10 blocks as replication. The initial spacing was 3m x 1.5m. After final

roguing, this seed orchard consisted of 160 individuals. Ten families evenly distributed in seed orchard were sampled. The sample trees were not isolated and produced abundant seeds. Seeds were harvested, classified and identified according to their mother trees. Then seeds were germinated and grown for two months. The family numbers sampled for mating system analysis were listed in Table 1. Leaves were collected from 10 selected mother trees and their offspring (24 progenies per mother tree).

Table 1. Family number and position of mother trees at seed orchard sampled for mating system analyses.

No	Family number	Block	Provenance
1	1	VIII	Ratgelombeng, Buru
2	5	IV	Masarete, Buru
3	8	VIII	Waipirit, Seram
4	11	IX	Pelita Jaya, Seram
5	12	III	Cotonea, Seram
6	13	V	Cotonea, Seram
7	18	VI	Suli, Ambon
8	19	VII	Wangi, Northern Territory Australia
9	23	II	N Broome, Western Australia
10	24	III	Gundih, Central Java

DNA isolation and amplification

Total DNA of each leaf sample was isolated using modified CTAB (*Cetyl Trymethyl Ammonium Bromide*) method (Zhou *et al.*, 1999). SSR markers of nuclear-DNA were applied to analyze mating system using 8 primers specifically developed for cajuputi (Miwa *et al*., 2000). Nucleotide bases of each primer were described in Table 2.

Amplification of DNA was carried out using 10 µl reaction mixtures of PCR reagents consisted of 5 ng template DNA 2 µl, 0.25 µl each primer pair, 4.65 µL H_2O, 10X buffer without $MgCl_2$ 1 µl, dNTP 0.8 µl, Amplitaq Gold 0.05 µL and 2.5 mM $MgCl_2$ 1 µl. The profile of PCR steps was initial denaturation (95°C for 10 min), 25 cycles of denaturation (94°C for 30 sec), annealing of primers (temperature of each primer was described in Table 2, for 1 min), and extension of DNA (72°C for 1 min). PCR step was finished with final extension (72°C for 5 min). Fragment length of amplified DNA (PCR product) was determined using sequencer of ABI Prism 3100 genetic analyzer (Applied Biosystem) and genotyped using Genemapper software (Applied Biosystem).

Table 2. Cajuputi primers for amplification

Microsatelite locus	Reverse (R) and forward (F) primer pair sequences (5'-3')	Repeat type	Size range (bp)	Annealing temperature (°C)
Hin-2	R:ACCGTCAACCACCACTGTTTG F:GCCAGCAGTGATTAGAGCATC	$(GCC)_5(GCT)\ (GCC)_4$	116-130	57
Hin-4	R:TTTGGCGTGCTCAGAGCTCT F:CACCCCAAATATTCCACTCTC	$(GA)_{10}AA(GA)_1$	91-110	57
Hin-5	R:GTTTGCCAAATCCATTACGGTC F:CAATGATATTCACGTAGTCGGTG	$(AAG)_3(ATT)(AAG)_6$	146-134	57
Hin-7	R:TCACTACCATGTAGGTGCTCC F:TTACAAACATACCTCTGGCCAG	$(CT)_{14}…(CA)_{23}$	168-226	57
Sal-1	R:AGTCCCAGTCGTCAACAGAG F:CCATCAAAGACAAAAGAGCGTC	$(CGG)_6$	91-97	57
Sal-3	R: GCATCATCATCGAGCTGCATG F:ACCAGTGACTAATCGGGTGTG	$(GT)_{20}(GC)_3GT(GA)_{27}$	139-192	57
Xho-1	R:AGGTGGTGATGGACGAGCTG F:GTCGCATTGACATCCGAAGCG	$(GGC)_6(GGT)(GGC)$	103-117	60
Xho-4	R:AATCCGCGACTGTGCAGAGG F:CTCAAGCCGATGTTCCTCGC	$(GT)_{41}(GA)_{23}$	155-283	60

Source: Miwa *et al*. (2000)

Data analysis

Genetic variation of progenies of the seed orchard was estimated based on genotypes of amplified DNA using SSR markers. Expected heterozogosity (H_E), observed heterozigosity (H_O), and fixation index (F_{IS}) (Finkeldey and Hattemer, 2007) were calculated for each locus.

Mating system was analyzed based on mixed mating model and genotypes were compiled for each locus as procedure of MLTR software (Ritland, 2002). Analysis was performed with 1000 bootstrap replications. The following parameters were calculated: the multi locus population outcrossing rate (t_m), the single locus population outcrossing rate (t_s =), likelihood of mating between relatives / biparental inbreeding (t_m-t_s =), the correlation of paternity / fraction of siblings that share the same father (r_p), and effective number of pollen donors (N_{ep}).

Results and Discussion

Genetic variation

The SSRs showed polymorphisms for all primers (see Figure 1) and confirmed allelic richness of cajuputi as described by Miwa *et al*. (2000). Range of fragment length of amplified DNA (PCR product) of each primer are as follows: Hin-2 (100-132 bp), Hin-4 (79-114 bp), Hin-5 (128-148 bp), Hin-7 (136-224 bp), Sal-1 (93-99 bp), Sal-3 (118-219 bp), Xho-1 (96-111 bp) and Xho-4 (150-216 bp), respectively. Therefore, mating system of cajuputi could be analysed based on its genetic variation.

Genetic variation in a seed orchard plays important role, since population with high level of genetic variation can improve its adaptability to environmental changes and natural selection, such as pest and disease resistance, tolerance to pollutant, struggling against competitor and predator (Frankham *et al*., 2002). The research resulted high genetic variation of effective pollen pool of cajuputi seed orchard at Paliyan, Gunung Kidul, namely H_E = 0.602 (Table 3) and H_O = 0.594 (Table 4), respectively. The fixation index was very low (0.013) and showed that allele and genotype frequencies tend to stay on Hardy-Weinberg Equilibrium. It indicated that this population could naturally regenerate and keep its genetic structure to the next generation (Frankham *et al*., 2002) and support conservation of genetic resources.

In term of mating system, parent trees of genetically conserved population tend to perform random mating indicated with closed to zero fixation index (F_{IS}) value.

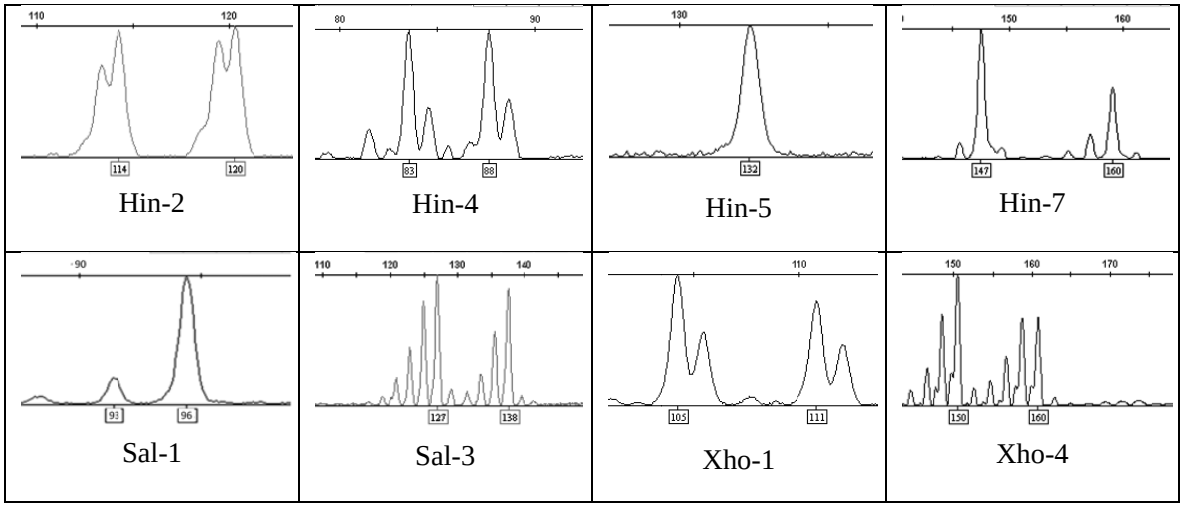

Figure 1. Examples of electropherogram of fragment length (in base pairs) resulted by amplification of each SSR primer on cajuputi.

Table 3. Expected heterozigosity (H_E) of the offspring observed in ten families

Family number	Expected heterozigosity (H_E) of each loci								
	Hin-2	Hin-4	Hin-5	Hin-7	Sal-1	Sal-3	Xho-1	Xho-4	Total
1	0.768	0.842	0.382	0.817	0.547	0.765	0.645	0.840	**0.701**$^{+}$
5	0.643	0.755	0.642	0.745	0.465	0.701	0.509	0.618	0.635
8	0.645	0.757	0.487	0.838	0.082	0.833	0.674	0.845	0.645
11	0.565	0.740	0.509	0.566	0.467	0.573	0.513	0.778	0.589
12	0.490	0.675	0.643	0.795	0.590	0.511	0.363	0.672	0.592
13	0.198	0.654	0.363	0.642	0.370	0.677	0.299	0.839	**0.505***
18	0.160	0.742	0.638	0.736	0.478	0.706	0.395	0.828	0.585
19	0.483	0.688	0.551	0.565	0.370	0.705	0.573	0.780	0.589
23	0.399	0.730	0.494	0.774	0.551	0.645	0.518	0.691	0.600
24	0.392	0.742	0.260	0.700	0.451	0.723	0.604	0.790	0.583
Total	0.474	0.733	0.497	0.718	0.437	0.684	0.509	0.768	0.602

Note: $^{+}$= highest heterozigosity; *= lowest heterozigosity

Table 4. Observed heterozigosity (H_O) of the offspring observed in ten families

Family number	Observed heterozigosity (H_O) of each loci								
	Hin-2	Hin-4	Hin-5	Hin-7	Sal-1	Sal-3	Xho-1	Xho-4	Total
1	0.833	1.000	0.375	1.000	0.458	0.792	0.708	0.792	**0.745**$^{+}$
5	0.875	0.917	0.500	0.833	0.583	0.500	0.652	0.667	0.691
8	1.000	0.875	0.292	0.261	0.083	0.917	0.609	0.875	0.614
11	0.917	0.917	0.458	0.458	0.417	0.417	0.542	0.667	0.599
12	0.667	0.958	0.167	0.917	0.375	0.417	0.417	0.708	0.578
13	0.125	0.458	0.292	0.583	0.250	0.583	0.333	0.792	**0.427***
18	0.167	0.583	0.583	0.708	0.375	0.750	0.458	0.917	0.568
19	0.583	0.458	0.292	0.708	0.208	0.750	0.750	0.667	0.552
23	0.458	0.875	0.208	0.792	0.208	0.750	0.783	0.625	0.587
24	0.458	0.875	0.125	0.875	0.292	0.708	0.625	0.667	0.578
Total	0.608	0.792	0.329	0.714	0.325	0.658	0.588	0.738	0.594

Note: $^{+}$= highest heterozigosity; *= lowest heterozigosity

Random mating would potentially involve all genetic materials of selected mother trees in seed orchard and transmit them to progeny generation. This is very important since seed orchard is categorized as the best seed source of genetically improved materials. Ideally, all trees should have a role as member of effective population number, so that they can contribute their gamets while producing seeds. Genes are maximally recombined by random mating of all parent trees.

Mating system

MLTR analysis on cajuputi revealed high level of multi locus and single locus outcrossing rate (t_m = 0.951 and t_s = 0.806, respectively). The significant difference between both of them ($t_m - t_s$ = 0.145, P < 0.05) indicated that 14.5% of seed resulted from outcrossing was inbred. Biparental inbreeding was significant but relatively low (r_p = 0,098, P < 0,05). There were 10.2 effective pollen donors (N_{ep}).

According to Ritland (2002) t value range from t=0 (inbreeding/selfing) to t=1 (outcrossed). When t value is <1, it indicates inbreeding either selfing or mating of related individuals (effective selfing). In the case of selfing, all locus will have similar effect, while in effective selfing, mean single locus (t_s) value will significantly lower than mean multi locus (t_m) (Brown *et al.*, 1985).

In general, tropical tree species tend to be outcrossing with high value of outcrossing rate (t>0.8) (Murawski *et al.*, 1994; Doligez and Joly, 1997). There are a number of physiological and genetic factors that triggered plant to be outcrossed. According to Patterson *et al*. (2004) factors such as flowering synchrony, flower abundance, pollinators and position of flowers in the crown are all have effect on outcrossing. Genetically, self-incompatibility and spatial structure of flowers favor outcrossing.

In the case of cajuputi trees in this study, it appears that physiological factor known as temporal separation, that is different time of maturity between female and male flowers, is causing the outcrossing pattern. According to Doran *et al*. (1998), cajuputi flowers are protandry, whereby pollen reach maturity before the pistil is ready for pollination. This condition minimizes the chance of selfing. In cajuputi trees, there is also a mechanism known as self incompatible, whereby flowering trees will be prevented from selfing, whilst de Nettancourt (1977) stated that plant which have self incompatible trait will not be able to produce zygote from selfing.

The outcrossing of cajuputi was observed by comparing the reproductive success amongst families resulting from controlled pollination of different families (outcrossing) and of the same families (selfing) (Kartikawati, 2005). It was reported that Index of Self Incompatibility/ISI was very low (ISI=0.05) indicating that cajuput is self incompatible. Possible mechanism for self incompatibility is the different receptivity between ovule and pollen of the same trees. According to Baskorowati *et al.* (2010), in *Melaleuca alternifolia* there is a phenomena called late acting self incompatibility in which fertilization between ovum and pollen of the same tree is prevented by the following mechanism, (1). Pollen tube cannot reach the ovum/*ovarian inhibition*; (2). Pollen tube can reach the ovum but prevented from fertilization/*pre-fertilization inhibition;* (3). Pollen tube can reached the ovum and fertilization takes place but the embryo is not developed/*post-zygotic rejection.* This mechanism is a reproductive strategy of the species to maintain genetic diversity from generation to generation by preventing mating between related individuals.

Another factor that affects the mating system is the presence of pollinator. Kartikawati (2008) found several insects as potential pollinators for cajuputi flower, especially Hymenoptera and Lepidoptera groups. The abundance of pollinators and their behavior influence the genetic flow in the seed orchard. Pollinator behavior that visits receptive flowers of the same individual tree is thought to be responsible for the present of inbred seeds. It was reported that in one individual tree, up to 300 spikes were found receptive simultaneously.

When synchronize reproduction is taking place in a seed orchard, all trees will produce flowers of which the pollen are ready to fertilize receptive ovum all at the same time. However, pollinators may have special behavior in which they will only visit certain individual trees because of unsynchronized flowering. This was demonstrated by the percentage of seeds that have shared male parent (correlated paternity) of up to 9.8%. Although this may not be indicative of inbreeding, this situation may increase the chance of inbreeding in the seed orchard.

In this study, samples were collected during peak flowering that took place in the first week of February 2011. The number of flowering trees was 128 out of 160 trees in total. This shows that even at peak flowering, there was still some unsynchronized flowering. It was assumed that pollinator behavior is not a major contributing factor for the present of seeds with shares male and female parents, because the pollinator are known to have large area of flying range.

Planting density is another factor that can influence the rate of outcrossing in a seed orchard. Murawski *et al*. (1994) reported that

in *Shorea megistophylla*, stands that have high density trees has higher outcrossing rate compared to stands which have been thinned or selectively cut. Densely populated stand can hinders pollen distribution, hence reduce the chance of outcrossing. Similar results were reported in *Kandelia candel* (Chen, 2000), *Dryobalanops aromatica* (Lee, 2000), and *Shorea curtisii* (Obayashi *et al*., 2002).

In the cajuputi seed orchard, although selective thinning has been carried out, there is still sufficient density allowing trees to outcross. Other factors, namely pollinator, stand density and flowering synchronization were all working in tandem allowing outcrossing to take place as reflected by the high outcrossing rate.

Similar results we found in *M. alternifolia (*tea tree*).* Butcher *et al*. (1992) reported an outcrossing rate of 93%. However, taxonomic relatedness is not necessarily reflected in similar mating system. For instance, *Leucaena leucocephala* is known to be self compatible whilst its relative *L. salvadorensis* is self incompatible (Boshier, 2011).

Implications to seed orchard management

Seed orchard is an established stand originated from genetically improved families, and is the source of genetically superior seeds. It imperative, that such an orchard has to be managed in such a way to ensure genetic quality of the seeds. Genetic quality of seeds from seed orchard is affected by a number of factors, including genetic diversity, fertility, mating system and combining ability of the parent trees in the orchard as well as contribution of pollinator (White *et al*., 2007).

Information of the mating system in a cajuputi seed orchard is required in order to optimize the performance of the orchard as a source of improved seeds. High outcrossing value amongst parent trees (t_m=0.951 and t_s=0.806, respectively) is a good indication for maintaining genetic diversity of the offspring. Such population can maintain reproductive fitness in the short term, and will be able to adapt to the changing environment in the long run (Frankham *et al*., 2002).

Outcrossing among trees will produce maximum diversity if random mating can take place. When all parent trees in the orchard is acting as effective population number with maximum fertility, then all allelic variation of the parent trees will be passed onto the next generation. Therefore, it is important that during final thinning (roguing) the remaining trees are unrelated and have good reproductive potential. Although the outcrossing rate is high, there are still 14.5% of the offspring that are closely related/biparental inbred ($t_m - t_s = 0.145$). This shows that there is a chance for inbreeding in the cajuputi seed orchard. Griffin and Sedgley (1989) stated that inbreeding can be minimized by manipulation of planting density to maximize the chance of outcrossing. When first established, the progeny trial consisted of 19 families with 10 treeplots and replicated in 10 blocks; the planting distance was 3m x 1.5m. After final thinning, the average distance between trees is now around 7.5m x 7.5m. This spacing is still within the range of the pollinator to transfer pollen from one tree to another.

The role of pollinator is critical to ensure successful pollination as well as gene flow within the orchard. Therefore, a good management strategy to ensure enough pollinator during peak flowering is critical (Griffin and Sedgley, 1989).

Although the level of inbreeding in the orchard is low, there is still possibility to reduce it further. One way to minimize inbreeding is to introduce new genetic materials (Wallace, 2003). Since the cajuputi seed orchard at Paliyan consisted of only 19 families, introducing new families can have beneficial effect.

In conclusion, the cajuputi seed orchard at Paliyan is mostly outcrossed and has the potential to produce good quality seeds with high genetic variation. Inbreeding was detected although at very small proportion. In order to ensure that the seed orchard will

continue to produce genetically improved seeds of high genetic variation, management strategy to allow optimum crossed pollination should be maintained.

To further improve the quality of the seed orchard, further study is recommended to examine the full potential of the parent trees to outcross, and to optimize crossing amongst unrelated families. Study on ways to influence reproductive synchronization will also be useful to improve reproductive success rate.

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