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ESTIMATION OF POLLEN CONTAMINATION AND MATING SYSTEM IN *PINUS MERKUSII* SEEDLING SEED ORCHARD USING ALLOZYME MARKERS

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The amount of pollen contamination and mating system parameters in a *Pinus merkusii* seedling seed orchard in East Java were estimated using allozyme gene markers. By running starch gel electrophoresis of enzymes from the haploid megagametophyte side by side with the diploid embryo, the precise information on male and female gametic contribution to the seed produced in the seed orchard was identified. The estimation was based on the single-locus allelic frequency differences between seed orchard populations and an unimproved plantation of the species surrounding the orchard. The study revealed high level of pollen contamination (29.7 ± 18%) in the seed orchard. Excess homozygotes obtained from the orchard suggests that consanguineous matings have occurred. Multilocus outcrossing rates of the three blocks and four local stands ranged from 0.137 to 0.305, and single-locus outcrossing rates ranged from 0.102 to 0.244. The seed orchard design, wind direction during flowering period and flowering synchronisation could have contributed to the pollen contamination and inbreeding observed. Although pollen contamination and inbreeding may be a problem in this seed orchard, the seed may still be useful. Management considerations for such seed orchard to deal with these problems are discussed.

Keywords: Genetic gain, consanguineous mating, phenology, background pollen, mating system, outcrossing

INTRODUCTION

Pinus merkusii is one of the tropical pines that grow widely in South-East Asia (Cook et al. 2010). Its natural distribution stretches from Myanmar, Thailand, Vietnam, Laos and Cambodia, all the way down to Luzon and Mindanao Island in Philippines and Sumatra Island in Indonesia. The species is found on a diversity of soils, over large altitudinal and climatic ranges (De Laubenfels 1988). The Sumatran population is composed of three races, namely, Aceh, Tapanuli and Kerinci. The Aceh race was extensively planted in Sumatra and Java using the agroforestry taungya system (Feilberg & Søegaard 1975). Currently, there are more than 900,000 ha of *P. merkusii* plantation forests all over Java, and they have the highest priority in genetic improvement programmes for fast growth, stem straightness and resin production. To facilitate the improvement programmes of the species, a seed orchard of selected families, designed to produce frequent, abundant and easily harvested genetically improved seed, was established between 1978 and 1984 (Feilberg & Søegaard 1975).

To obtain optimum benefits from seed orchard the following conditions must be met: synchrony in floral phenology, isolation from surrounding stands, equal male and female gamete production, random mating pattern, compatibility for all crosses, and minimal or absence of self-fertilisation (Wang 2004, Chaix et al. 2010, El-Kassaby et al. 2015, Gonzaga et al. 2016). However, the most common challenges facing seed orchard management are inbreeding risks and pollen contamination (Kaya et al. 2006, Gonzaga et al. 2016). Pollen contamination influences the genetic worth of seed orchard and therefore determines the quality of seed production, which may include reduction of genetic gain (Grattapaglia et al. 2014, Funda et al. 2016, Gonzaga et al. 2016). Studies of

wind-pollinated orchards show that between 25 and 80% of seeds produced usually result from pollen contamination (Kaya et al. 2006, Torimaru et al. 2009, Bilgen & Kaya 2014). In young orchards, where pollen production is limited, the contamination rate could be even higher. However, several approaches have been used to reduce pollen contamination in seed orchards including supplemental mass pollination, bloom delay, flower or strobili stimulation, neighbourhood isolation, pollen mixing, controlled pollination and isolation by tents (Miranda et al. 2013, Grattapaglia et al. 2014, Alexander & Woeste 2016, Funda et al. 2016).

The genetic value of seed orchard also depends on the mating success of individual trees, or patterns of genetic relatedness between gametes (Gonzaga et al. 2016). This is important because mating patterns can change gene pool of the progenies. Random mating usually result in genetic equilibrium. However, this requires mating without bias on phenotypic resemblance, relatedness or physical distance, which is difficult to achieve (El-Kassaby et al. 2010). Furthermore, under non-random conditions, genetic elements may not form zygotes independently. Departures from random mating in open-pollinated progenies can result in several detrimental effects (Khasa et al. 1993). First, inbreeding depression for economically and adaptively important characteristics may result in decreased growth vigour, poor survival, susceptibility to pests, reduced seed set, and high frequency occurrence of various dwarfs and pigment abnormality (Grattapaglia et al. 2014). Second, estimates of variance components becomes biased when inbreeding occurs, resulting in erroneous estimates of heritability and genetic gain because of the greater degree of relatedness among individuals within progenies (Chaix et al. 2010). To ensure high quality seeds production, it is important to understand the mating patterns and level of pollen contamination occurring in seed orchard.

Genetic markers (biochemical and molecular) have been used to determine mating system parameters and estimate pollen contamination in seed orchards (Fernandes et al. 2008, Torimaru et al. 2009, Feng et al. 2010, Kaya & Isik 2010, Bilgen & Kaya 2014). Biochemical markers such as allozymes were popularly used in the 1980s and 1990s because they are easy to use, cost effective and exhibit simple Mendelian inheritance (Firas et al. 2015). These markers have been used reliably in determining origin of planting materials and clonal homogeneity in seed orchard (Ivanek et al. 2013). In addition, allozyme markers have been used successfully to assess mating patterns and pollen contamination in *Pinus brutia* seed orchards (Kaya et al. 2006). However, with the advent of modern technologies, development and application of new generation of molecular markers such as microsatellite, single nucleotide polymorphism and whole genome sequencing have provided more reliable tools for population genetic studies, complementing the use of biochemical markers (Gonzaga et al. 2016).

The aim of the present study was to determine the mating system parameters and estimate the level of pollen contamination in *P. merkusii* seedling seed orchard in Indonesia using allozyme markers. The findings and recommended management strategies for the orchard are discussed.

MATERIALS AND METHODS

Seed orchard description

The open-pollinated seedling seed orchard studied is located in the Forest District Jember, subforest district Sempolan, East Java, Indonesia, and is owned by Perum Perhutani (state forest company). The orchard was established between 1978 and 1984 as a half-sib progeny test composed of families that were derived from selected plus trees of *P. merkusii* from all over Java. The orchard is divided into six sectors (I–VI, Figure 1) based on the year of planting. Each sector is 16 ha in size, which is split into 10 blocks (1–10, Figure 1), with 1.6 ha per block. Starting in 1978, 200 families comprising 5 seedlings per family were planted in each block of sector I. They were arranged in a randomised block design with blocks as replications. In 1979, 40 families that were already tested in the previous year were planted in sector II, along with 160 new selected families. The same design was applied for sector III, IV, V and VI (Suseno 1994). The initial spacing was 4 m × 4 m but at present the distance between trees is irregular because of roguing. Roguing was done several times, and at the time the samples for this study were being collected, all blocks had varied number of families, with one or two trees per family. Even though there is no

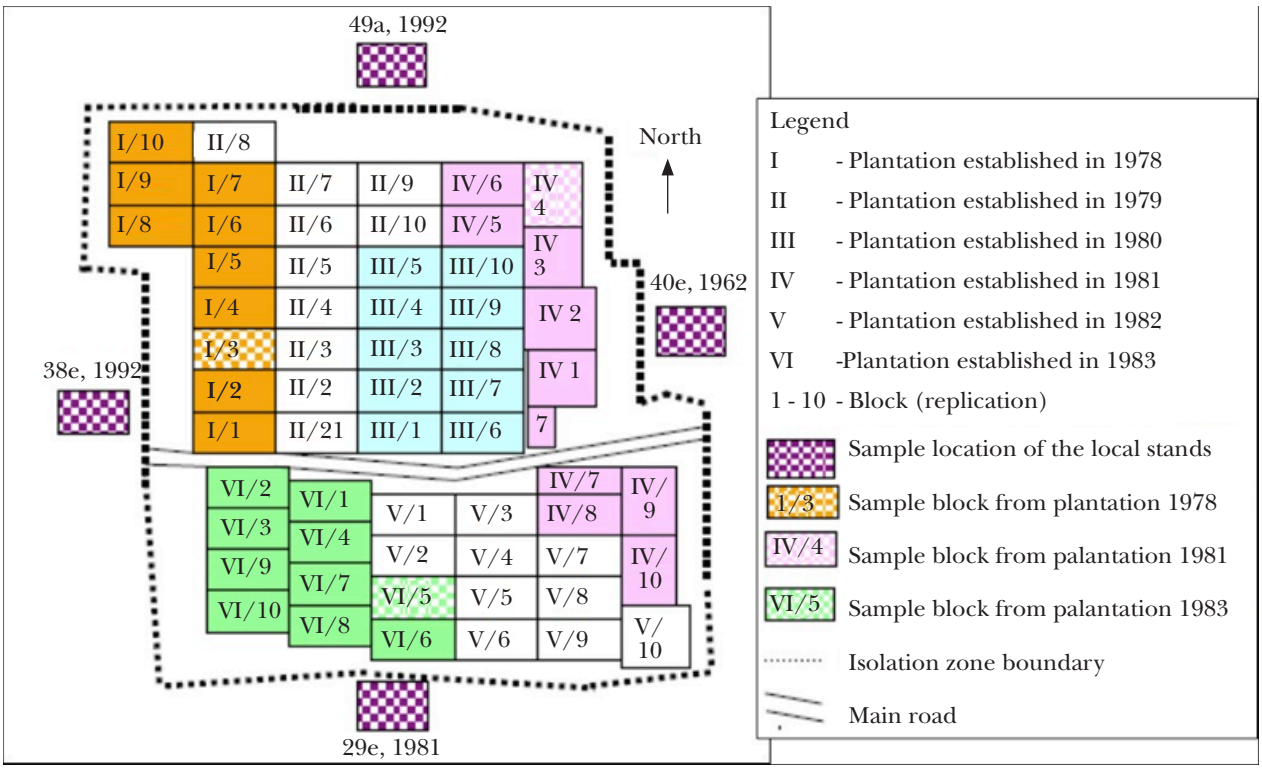

Figure 1 Sketch map of the open pollinated seedling seed orchard of *Pinus merkusii* in Jember, East Java, Indonesia

accurate record of the total number of families and trees left after roguing, the final objective of the management plan was to have 50 families with one tree per family in each block.

The orchard is surrounded by a 200 m wide strip isolation zone, which consists of a plantation of different tree species of different ages. These trees include *Racosperma mangium* (planted in 1986 and 1983), *Calliandra callothyrsus* (1984), *Eucalyptus deglupta* (between 1986 and 1987), *Eucalyptus urophylla* (between 1981 and 1985), *Leucaena leucocepala* (1985), *Santalum album* (1986), *Swietenia mahagoni* (1981), *Tectona grandis* (1988) and mixed forest (1985). Beyond the isolation zone, there are unimproved woodlots (referred here as unimproved stands) of different forest tree species of various ages, but mainly *P. merkusii* that were planted for wood and resin production.

Seed collection

Cones were collected from both *P. merkusii* trees in the seed orchard and unimproved *P. merkusii* trees in the stands surrounding the orchard, which are the putative source of pollen contamination (Figure 1). Cones were collected in August and September 1997. From inside the orchard, cones were obtained from trees in three different blocks that were randomly sampled from different years of plantation as shown in Figure 1. They were from sector I plantation of 1978, block 3 (I/3); sector IV plantation of 1981, block 4 (IV/4); and sector VI plantation of 1983, block 5 (VI/5). Forty trees that represent 40 families were sampled per block. From the area surrounding the orchard, 20 pine trees in total were sampled from stands 38e, 40, 49a and 29e, which were located west, east, north and south of the orchard respectively. The cones were air dried under sunlight, separated by family and then seeds were extracted, cleaned and stored at 4 °C until analysis at University of Alberta.

Electrophoretic procedures and data analyses

Ten seeds per tree were assayed by starch gel electrophoresis (12% starch). The probability of incorrectly identifying a heterozygote at a particular locus is $(1/2)^{k-1}$, where k is the megagametopytes assayed per tree. With 10 seeds assayed per tree, the probability of misclassifying

a heterozygote was 0.2%. Both megagametophyte and embryo tissues were run side-by-side in starch gel electrophoresis. In this way, genotype contribution from the male and the female parents to the seeds could be identified. The electrophoretic procedures, banding patterns of the allozymes and the mode of inheritance were described previously (Suwarni 1999, Suwarni et al. 1999).

To estimate the level of pollen contamination, the genotype of each sample tree was inferred from its progeny array for five allozyme loci encoding four enzyme systems. The four enzymes were isocitric dehydrogenase (IDH, EC 1.1.1.42), malate dehydrogenase (MDH, EC. 1.1.1.37), phosphoglucomutase (PGM, EC. 2.7.5.1) and shikimate dehydrogenase (SKDH, EC. 1.1.1.25). To estimate mating system parameters, six allozyme loci from five enzyme systems were analysed, which included the previous four enzymes plus adenilate kinase (AK, EC.2.7.4.3).

The allele frequencies of the five allozyme loci (*Adh-2, Pgm-1, Pgm-3, Mdh-2* and *Skdh-1*) for maternal parents (ovule pool), paternal parents (pollen pool) and background stands were calculated using the Multilocus Mating System program (MLTR) version 1.0 (1997). The pollen contamination rate was estimated using migration model (El-Kassaby & Ritland 1986). A chi-square contingency test was used to determine the heterogeneity of gene pools at p = 0.05. Using the migration model, frequencies of the most common allele in the maternal tree population, together with frequencies of the orchard (block) outcrossing pollen gene pools and the outside source (unimproved stand) were used to compute the contamination rate at locus *i*:

$$\hat{m}_i = \frac{p_i - r_i}{q_i - r_i}$$

where $\hat{m}_i$ = estimated migration rate of pollen at the i^{th} locus or the proportion of unimproved stand pollen present in the orchard, r_i = frequency of the most common allele in the orchard ovule pool at the i^{th} locus, p_i = frequency of the same allele in the orchard outcrossing pollen pool at the i^{th} locus, and q_i = frequency of the same allele in the outside source at the i^{th} locus. Since *p*, *r* and *q* are independent samples of genes, the variance of $\hat{m}_i$ is computed using the differential approximation without covariance terms as follows

$$v\left(\hat{m}_i\right) = \left[\frac{P_i\left(1-P_i\right)}{N_p}\right]\left[\frac{1}{q_i - r_i}\right]^2 + \left[\frac{r_i\left(1-r_i\right)}{N_r}\right]\left[\frac{\left(q_i - p_i\right)}{\left(q_i - r_i\right)^2}\right]^2 + \left[\frac{q_i\left(1-q_i\right)}{N_q}\right]\left[\frac{p_i - r_i}{\left(q_i - r_i\right)^2}\right]^2$$

where N_p = number of pollen (number of embryos), N_r = number of ovule (number of trees × 2), and N_q = number of foreign pollen (number of trees in the unimproved stand × 2). Assuming that the migration estimates are approximately independent among loci, the minimum variance estimate of migration on an average over loci is the weighted average:

$$\hat{m} = \left[\sum_{i-1}^{n} \frac{1}{v_{\hat{m}_i}}\right]^{-1}\left[\sum_{i-1}^{m} \frac{\hat{m}_i}{v_{\hat{m}_i}}\right]$$

where n = number of loci studied. The variance of the minimum variance estimate is

$$v\left(m\right) = \left[\sum_{i-1}^{n} \frac{1}{v_{\hat{m}_i}}\right]$$

We used MLTR program to determine multilocus outcrossing rate (t_m) and single locus outcrossing rate (t_s) based on the six polymorphic loci (*Ak-1, Adh-2, Pgm-1, Pgm-3, Mdh-2* and *Skdh-1*). Standard errors of the outcrossing estimates were calculated using 200 bootstraps (re-sampling between families). Wright's fixation index, observed heterozygosity (*Ho*) and expected heterozygosity (*He*) was estimated for each block and unimproved stand using POPGENE version 1.21 (1997). Wright's fixation index (*F*), which measures the deviation of observed heterozygotes from the proportion expected under Hardy–Weinberg equilibrium was calculated as

$$F = 1 - \frac{Ho}{He}$$

The proportion of the expected heterozygotes was calculated following the formula:

$$He = \frac{2N}{2N-1}\left(1 - \sum_{i=1}^{n} pi^2\right)$$

where N = number of embryos sampled from the population and p_i = frequency of the i^{th} allele at

the locus. The chi-square (χ^2) test was employed to test the excess or deficiency of observed heterozygotes relative to Hardy–Weinberg proportions for statistical significance.

RESULTS

The estimates of allele frequencies for maternal parents or ovule pool (r), outcrossing pollen parents (p) in the seed orchard, and background stands (q) are presented in Tables 1, 2 and 3 respectively. The chi-square contingency test was conducted to test the variability of gene pools between each of the three blocks inside the seed orchard and between the four stands surrounding the orchard. The outcrossing pollen and ovule pools were significantly heterogeneous among the seed orchard blocks (Table 1). The four background stands were also found to be heterogeneous (Table 2).

Table 1 The estimation of the outcrossing pollen pool allelic frequencies (p) among the three blocks in the *Pinus merkusii* seed orchard

Locus	Allele	Block			Chi-square
		I/3	IV/4	VI/5	
Idh-2	1	0.810	0.869	0.876	20.38**
	2	0.043	0.025	0.000	
	3	0.147	0.106	0.124	
Mdh-2	1	0.954	0.866	0.962	130.89**
	2	0.046	0.134	0.038	
Pgm-1	1	0.672	0.928	0.895	
	2	0.328	0.049	0.105	145.52**
	3	0.000	0.023	0.000	
Pgm-3	1	0.536	0.647	0.516	16.22**
	2	0.464	0.353	0.484	
Skdh-1	1	0.510	0.539	0.540	12.55**
	2	0.174	0.155	0.228	
	3	0.316	0.306	0.232	
Np		400	400	400	

**Significant at 0.01 level

Table 2 The estimation of the ovule pool allelic frequencies (r) among the three blocks in the *Pinus merkusii* seed orchard

Locus	Allele	Block			Chi-square
		I/3	IV/4	VI/5	
Idh-2	1	0.788	0.837	0.837	1.2*
	2	0.050	0.025	0.038	
	3	0.162	0.138	0.125	
Mdh-2	1	0.837	0.825	0.825	0.06
	2	0.162	0.175	0.175	
Pgm-1	1	0.800	0.837	0.887	5.95**
	2	0.175	0.112	0.112	
	3	0.025	0.050	0.000	
Pgm-3	1	0.650	0.538	0.512	3.51*
	2	0.350	0.463	0.488	
Skdh-1	1	0.825	0.563	0.512	27.79**
	2	0.125	0.325	0.225	
	3	0.050	0.112	0.262	
Nr		80	80	80	

*Significant at 0.05 level, **significant at 0.01 level

Table 3 The estimation of allele frequencies among the four background stands

Locus	Allele	Background stand				Chi-square
		East	South	West	North	
Idh-2	1	0.955	0.591	0.982	1.000	43.97**
	2	0.000	0.036	0.00	0.000	
	3	0.045	0.373	0.018	0.000	
Mdh-2	1	0.913	0.613	0.900	0.938	20.96**
	2	0.087	0.387	0.100	0.062	
Pgm-1	1	0.913	0.627	0.882	0.883	14.92**
	2	0.087	0.373	0.118	0.117	
	3	0.000	0.000	0.000	0.000	
Pgm-3	1	0.504	0.700	0.752	0.519	8.05**
	2	0.496	0.300	0.248	0.481	
Skdh-1	1	0.577	0.542	0.804	0.832	14.46**
	2	0.304	0.262	0.157	0.098	
	3	0.120	0.197	0.040	0.070	
Nq		40	40	40	40	

**Significant at 0.01 level

Therefore, each local stand can be considered as a source of background pollen separately that contributes to the contamination rate in each block of the seed orchard.

The estimated level of pollen contamination from adjacent unimproved stands in the seed orchard for block–local stand combinations ranged from 8 to 33.5% (Table 4). The rate of the contamination average over blocks varied from 10.3 to 30.8%, with major contribution coming from the east and north local stands, and minor contamination from the west and south side (Table 4). The contamination rates average over stands varied from 22.0% in block VI/5 to 50.5% in block IV/4. The overall estimate of pollen contamination rate in this orchard was 29.7 ± 18% and all the background pollen sources contributed to this level of contamination (Table 4), with significant contribution from the east and north local stands.

Estimates of observed and expected heterozygosity (*Ho*, *He*), Wright's fixation index (*F*), and χ^2 test of Hardy–Weinberg equilibrium for the six loci at every local stand and block in the seed orchard are presented in Table 5. The fixation index ranged from -0.040 (locus *Ak-1*, block I/3) to 1.00 (*Idh-2* and *Mdh-2* in all local stands and seed orchard blocks). The chi-square test on the fixation index rates indicated significant excess of homozygosity at all stands and blocks for all loci except for *Ak-1*.

Estimates of single-locus and multilocus outcrossing rates with the standard errors based on 200 bootstraps for each local stand and seed orchard block are presented in Table 6. Single-locus estimates of outcrossing rates varied from 0.102 to 0.244. The multilocus estimates of outcrossing rates ranged from 0.137 to 0.305.

DISCUSSION

Our result revealed high pollen contamination from the background unimproved populations. This would present negative genetic impact to the next generation plating materials derived from the seed orchard. These findings are similar to pollen contaminations reported for other pine seed orchards such as *P. brutia* in Antalya, Turkey (85%, Kaya et al. 2006), *P. pinaster* (52%, Fernandes et al. 2008) and Scots pine (*P. silvestris*) in Sweden (52%, Torimaru et al. 2009). Factors such as flowering synchronisation, amount of pollen production and distribution, the prevailing wind direction during flowering season and the characteristics of seed orchard isolation zone could possibly contribute to the high level of pollen contamination reported in the present study. Interestingly, *P. merkusii* native

Table 4 Estimation of the pollen contamination rates and their 95% confidence intervals

Seed orchard block	Local stand				Average
	East	South	West	North	
BI/3	0.291 ± 0.19	0.263 ± 0.14	0.090 ± 0.20	0.335 ± 0.16	0.392 ± 0.29
BIV/4	0.245 ± 0.16	-0.080 ± 0.12	0.272 ± 0.12	0.138 ± 0.13	0.505 ± 0.21
BVI/5	0.244 ± 0.15	-0.103 ± 0.10	0.142 ± 0.11	0.249 ± 0.12	0.220 ± 0.17
Average	0.308 ± 0.12	-0.217 ± 0.08	0.103 ± 0.10	0.206 ± 0.09	0.297 ± 0.18

Table 5 Wright's fixation index (*F*), observed (*Ho*) and expected (*He*) heterozygosity at single-loci, chi-square test (χ^2) and degrees of freedom (df)

Population		Gene locus	*Ho*	*He*	*F*	χ^2 (df)
Local stand	West	*Ak-1*	0.030	0.029	-0.015	0.039 (1)
		Idh-2	0.000	0.232	1.000	426.1 (3)
		Mdh-2	0.000	0.263	1.000	202.9 (1)
		Pgm-1	0.125	0.224	0.440	41.80 (3)
		Pgm-3	0.200	0.44	0.544	59.90 (1)
		Skdh-1	0.020	0.415	0.517	102.0 (3)
	North	*Ak-1*	0.015	0.015	0.0076	0.008 (1)
		Idh-2	0.000	0.000	-	-
		Mdh-2	0.000	0.296	1.000	202.4 (1)
		Pgm-1	0.035	0.054	0.346	26.50 (1)
		Pgm-3	0.190	0.443	0.570	65.70 (1)
		Skdh-1	0.185	0.423	0.562	94.10 (3)
	South	*Ak-1*	0.000	0.000	-	-
		Idh-2	0.000	0.432	1.000	429.5 (3)
		Mdh-2	0.000	0.425	1.000	201.4 (1)
		Pgm-1	0.125	0.274	0.543	455.9 (3)
		Pgm-3	0.180	0.376	0.520	54.80 (1)
		Skdh-1	0.150	0.519	0.710	143.7 (3)
	East	*Ak-1*	0.075	0.072	-0.039	0.028 (1)
		Idh-2	0.000	0.249	1.000	203.0 (1)
		Mdh-2	0.000	0.256	1.000	202.1 (1)
		Pgm-1	0.160	0.232	0.310	22.00 (3)
		Pgm-3	0.285	0.427	0.331	22.26 (1)
		Skdh-1	0.260	0.540	0.517	104.8 (3)
Seed orchard	Block (I/3)	*Ak-1*	0.113	0.108	-0.040	1.388 (6)
		Idh-2	0.000	0.34	1.000	814.3 (3)
		Mdh-2	0.000	0.276	1.000	402.6 (1)
		Pgm-1	0.258	0.278	0.074	57.70 (3)
		Pgm-3	0.270	0.439	0.385	59.60 (1)
		Skdh-1	0.125	0.259	0.516	195.6 (3)
	Block (III/4)	*Ak-1*	0.083	0.08	-0.038	0.717 (6)
		Idh-2	0.000	0.266	1.000	0.820 (3)
		Mdh-2	0.000	0.238	1.000	403.2 (1)
		Pgm-1	0.083	0.122	0.320	132.1 (3)
		Pgm-3	0.175	0.454	0.614	151.5 (1)
		Skdh-1	0.250	0.537	0.534	243.5 (3)
	Block (VI/5)	*Ak-1*	0.025	0.025	-0.013	0.058 (1)
		Idh-2	0.000	0.266	1.000	820.6 (3)
		Mdh-2	0.000	0.255	1.000	402.9 (1)
		Pgm-1	0.095	0.117	0.189	14.80 (1)
		Pgm-3	0.310	0.498	0.377	57.20 (1)
		Skdh-1	0.258	0.604	0.573	277.8 (3)

Table 6 Estimate of single-locus (t_s) and multilocus (t_m) outcrossing rates and their standard errors

Population		t_s	t_m
Local stand	North	0.244 ± 0.095	0.305 ± 0.100
	East	0.215 ± 0.094	0.274 ± 0.107
	South	0.153 ± 0.087	0.281 ± 0.05
	West	0.214 ± 0.086	0.290 ± 0.084
Seed orchard	Block (I/3)	0.102 ± 0.039	0.137 ± 0.052
	Block (IV/4)	0.200 ± 0.070	0.238 ± 0.064
	Block (VI/5)	0.208 ± 0.057	0.252 ± 0.640

to Sumatra usually flower throughout the year (Siregar & Hattemer 2001). However, the trees of the present study orchard were collected from different altitudes and ecological zones (which might be representing different provenances) all over Java and perhaps this might have led to the variability in flowering phenology among the families. This is one of the assumptions that was violated while establishing the orchard, which may limit the achievement of optimum genetic gain. Generally, well-matched flowering periods among orchard genotypes are very important in reducing pollen contamination and enhancing optimum random mating within the orchard. In this regard, lack of synchrony of flowering events among individual trees within the orchard, though not evaluated in the present study, and the coincidence of some female flowers in the orchard being receptive when pollen shedding occurs from trees outside the orchard may have resulted in the high overall contamination rate. Similar conclusion of lack of sufficient pollen within the orchard was also suggested by Kaya et al. (2006) on the young *P. brutia* seed orchard in Turkey. Variation in reproductive phenology among trees within seed orchard is a significant factor promoting pollen contamination (Kess & El-Kassaby 2015).

Even though trees in the seed orchard were old enough to produce sufficient pollen, the proportion of background pollen was probably still very high, especially from the old stands. Besides, the distribution of the pollen release all over the orchard is also very important in minimising contamination. These factors, probably, together with wind direction during the flowering period might have resulted in the high level of contamination in this seed orchard. Influence of wind direction on pollen contamination has also been reported in *Pseudotsuga menziesii* seed orchard by Kess and El-Kassaby (2015). Our results showed that pollen contamination rates on average over blocks were majorly coming from the east and north sources. This could be due to the prevailing wind direction (north-east) that occurs during the flowering period, and probably the fecundity of the eastern stand (an old pine plantation of 1962). The trees from this stand might have produced large amounts of pollen that dispersed into the orchard when there was, probably, no sufficient quantities of pollen within the orchard. However, to prove the foregoing hypothesis, flowering phenology of the nearby old stand and the orchard materials together with prevailing wind direction during the flowering period need to be investigated. The trees in the isolation zone were younger than trees in the seed orchard, and most of them had not grown as tall as the *P. merkusii* trees inside the seed orchard. This might have led to lack of effective pollen barrier in the isolation zones. Ineffective barrier coupled with the typical habit of female flowers being borne on the upper crown of the pine trees might have facilitated pollen contamination in the orchard.

The χ^2 test showed significant deficiency of heterozygotes in the seed orchard and unimproved background stands. Lower frequency of observed heterozygotes than the expected heterozygotes under Hardy–Weinberg equilibrium usually indicates the presence of inbreeding. The excess of homozygotes we found in this orchard complex suggested strong inbreeding, which necessitated further analysis of the single- and multilocus selfing rates. Similar results of limited heterozygotes in seed orchard materials have been reported by other workers (Charlesworth & Willis 2009, Kaya & Isik 2010, Hedrick 2012). We found negative value of inbreeding coefficient (*F*) only at locus *Ak-1* in

all stands and seed orchard blocks. However, the χ^2 test revealed an excess of heterozygotes in the progenies. These variations could be explained by occurrences of heterosis, over-dominance, hitchhiking effect, and embryonic and zygotic selection. Some causes of increased heterozygosity are selectively neutral such as disassortative mating (if individuals of dissimilar genotype tend to mate more often than expected under random mating), differences in allele frequency between male and female gamete pools and immigration (Hedrick et al. 2016). Conversely, positive deviation from zero observed at the other loci (positive *F*-values or excess of homozygotes) may result from Wahlund effect (differences in allelic frequencies in the effective pollen pool of each tree within one population, resulting from selfing or consanguineous matings), positive assortative mating (preferential mating among similar genotypes), selection for homozygotes, and family structure within restricted neighbourhoods (Porcher & Lande 2016). Some of these factors could be important to the present study orchard simply because the artificial stands where the trees were sampled represent a sample of the natural gene pool. At the level of this study, we cannot isolate any of the confounding factors discussed above. This would require further experiments involving controlled crosses, as well as observations on flowering phenology and effective transmission distances of pollen gametes (Hedrick et al. 2016).

The outcrossing rates showed high level of selfing in all local stands and seed orchard blocks. The mode of mating in this seed orchard complex could be classified as mixed mating pattern where both selfing and outcrossing occurred (Brown 1990). The multilocus outcrossing rate ranged from 0.137 to 0.305, and single-locus outcrossing rates ranged from 0.102 to 0.244. All the multilocus outcrossing rates estimates were higher than single-locus rates, indicating that most or all apparent inbreeding was a result of consanguineous mating, not necessarily the actual self-fertilisation (Shaw & Allard 1982). The outcrossing rate found in this study were, however, significantly lower than those reported for other conifers (Pakkanen et al. 2000, Kaya et al. 2006, Fernandes et al. 2008). Factors such as enzyme systems used and errors in genotyping of sample could also influence the accuracy of the estimation of mating system parameters and the pollen contamination levels.

The high inbreeding coefficient, and thus the significant excess of homozygotes and low outcrossing rates in this orchard complex, may be due to the layout of the seed orchard and the origin of the trees (El-Kassaby 2003). After several roguing, there were only one or two trees per family left on the site that was laid out in a family row (when the samples were collected). The planting positions of some progenies of the same family were adjacent to each other, and the families/progenies planted were replicated in each block and were also replanted in the adjacent sector. This design could have enabled crosses between progeny of the same families. Therefore, every successful fertilisation between these trees would be a consanguineous mating product. Inbreeding depression caused by selfing could result in non-viable embryos due to homozygosity of lethal recessive genes, abnormal seedlings, weak plant growth, or low seed viability (Khasa et al. 1993). The inbreeding depression caused by consanguineous mating is not as severe as self-fertilisation, but its consequences may be more critical for reforestation programmes, which rely on non-competitive plantings. The mildly inbred seedlings produced by consanguineous mating may survive past the seedling stage, but would still suffer from a loss of heterozygosity, which could lead to inferior growth and productivity as mature trees (Khasa et al. 1993).

Production of genetically improved seed is the major goal of any tree improvement programme. When pollen contamination and inbreeding take place in a seed orchard, the seed produced is obviously of uncertain genetic merit. Some management techniques that reduce pollen contamination and minimise inbreeding should be considered for the study orchard. These may include widening the isolation zone (e.g. 1.6 km or more) by converting the surrounding *P. merkusii* plantations into plantations of other species especially on the windward side, amelioration by planting the area surrounding the seed orchard with families of the same species that perform well, supplemental mass pollination, flower stimulation, girdling, bloom delay by cooling with sprinklers or control pollination and tent isolation (Funda et al. 2016). Among these management options, the most affordable strategy for Indonesia would be to establish very large seed orchards with large isolation zone. Due to variability of the environmental factors throughout the year, the level of pollen

contamination, mating system parameters and the genetic composition of seed from seed orchard may also fluctuate. Continuous testing of pollen contamination and mating patterns are probably necessary to determine the best time to harvest the seed from the orchard.

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