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Microsatellite Paternity Analysis Used for Evaluation of Outcrossing Rate Among Five *Hevea* Rubber Clones in a Systematic Seed Orchard

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ABSTRACT

A first generation of synthetic rubber (*Hevea brasiliensis*) clones derived from a polycross among multiple parents was produced to establish a seed orchard that will be useful to systematically enhance cross-pollination among several clones and breed superior rubber genotypes. The objective of this study was to evaluate the flowering patterns and outcrossing rate among five *Hevea* rubber clones within this seed orchard using microsatellite markers. Five rubber clones (AVROS 2037, BPM 1, IAN 873, PB 260 and RRII 118) were systematically grown in a random design with spacing of 7 × 7 m in a seed orchard at the Phetchabun Highland Agricultural Development and Research Center, KhaoKho, Phetchabun (16°35" N and 100°57" E), Thailand. Five pairs of polymorphic microsatellite primers were used to analyze 288 seedlings derived from the seed orchard. The five microsatellite loci chosen for this study were highly polymorphic, with a mean of 5.8 alleles per locus and a combined exclusion probability of 0.988553, both of which were sufficiently high to correctly assign parentage. Individual female parents varied in their estimated outcrossing rate from 58.62 to 98.36%, while the overall outcrossing rate in the seed orchard was 79% and selfing rate was 21%. Pollen contamination was not observed in this seed orchard. The high outcrossing level and the lack of pollen contamination may be useful for the establishment of a seed production facility and for the management of hybrid production.

Keywords: *Hevea brasiliensis*, microsatellite markers, seed orchard, outcrossing rate, selfing rate.

INTRODUCTION

Hevea brasiliensis, also known as the Para rubber tree, produces natural latex rubber, for which global production reached 10.5 million t in 2010, with most rubber plantations areas located in Southeast Asia, particularly in Thailand (producing 3.25 million t in 2010), Indonesia, Malaysia, India, Vietnam and China (Saha and Priyadarshan, 2012). *Hevea* rubber is an important economic crop in Thailand. The Office of Agricultural Economics (2011) reported that *Hevea* rubber has been planted on 3.02 million ha, with about 2.04 million ha of *Hevea* rubber being harvested to produce more than 3 million t.

Hevea rubber (*Hevea brasiliensis* (Willd. ex A. de Juss) Müll.-Arg.) is an outbreeding, monoecious species (Simmonds, 1986) with

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imperfect flowers that is pollinated by insects such as midges and thrips (Rao, 1961). *Hevea* rubber can also be vegetatively propagated by a process known as green budding. The scions should be aged about 6–8 mth and are transferred to polythene bags as budded stumps. (Rubber Research Institute Department of Agriculture, 2010). The most common rootstocks for *Hevea* rubber production in Thailand are the progeny of any early introduced clones that have high heterozygosity (Kinnarat and Rattanawong, 2002). RRIM600 is the major cultivated variety of *Hevea* rubber in Thailand, constituting about 80% of plantings) (Sangsing *et al.*, 2004).

Most the clones introduced early in the history of rubber cultivation in Thailand are believed to have been lost and there is currently a high level of inbreeding depression in cultivated rubber germplasm (Nakkanong *et al*., 2008). Rao *et al*. (2008) explained that there was a significant decrease in the performance of seedlings grown in open-pollinated seed lots as they were subject to inbreeding depression due to the self-pollination of parental plants. For example, Hardner and Potts (1995) reported that the growth of self-pollinated eucalypts in terms of tree volume at age 43 mth decreased to 48% of that documented for fully outcrossed progeny. Open pollination between elite trees planted in seed orchards allows the production of seeds of high genetic quality and diversity (Pakkanen *et al*., 2000). Inbreeding depression could be minimized by interplanting several parents in a polycross seed orchard to produce a roughly random array of outbred hybrids between good parents (Simmonds, 1986). However, the main problem of open-pollinated seed is that the male parents are unknown. Molecular markers can be used to identify plant varieties and microsatellites are particularly useful for this type of study because they are locus-specific, co-dominant, highly polymorphic, technically easy to use and highly reproducible (Hayden and Sharp, 2001). Moreover, microsatellites are powerful plant screening tools for genetic mapping, genetic diversity assessment and population genetics (James *et al*., 2003; Phumichai *et al*., 2011; Triwitayakorn *et al*., 2011). The objective of the present research was to evaluate flowering patterns and the cross-pollination rate among five *Hevea* rubber clones within a seed orchard using microsatellite markers.

MATERIALS AND METHODS

Plant material

The *Hevea* seed orchard was established in July 2003 on a 1.372 ha field at the Phetchabun Highland Agricultural Development and Research Center, KhaoKho, Phetchabun province (16°35” N and 100°57” E, 711 m above sea level), Thailand and has been managed for seed production (Simmonds, 1986). The original buds from the five varieties—AVROS 2037, BPM 1, PB 260, IAN 873 and RRII 118—were grafted onto RRIM 600 rootstock and -planted in a random design with systematic spacing of 7 × 7 m consisting of 25 clones of each variety per plot (Figures 1a and 1b). Cross pollination occurred randomly and fruit capsules that had set were randomly covered with nets to protect them from predation. Capsules were collected from 10 clones per variety (Figure 1c), Ten samples per clone were randomly collected from within one of four directional sectors in each plant canopy. A total of 120 open-pollinated seeds were collected from each of the varieties AVROS 2037, BPM 1, PB 260, IAN 873 and RRII 118. A total of 600 seeds (120 per clone) were tested for germination and planted for later trait evaluation at the Chachoengsao Rubber Research Center, Rubber Research Institute of Thailand. Seeds were collected in late August 2010 in the morning prior to capsules shattering (Figure 1d) and seed germination tests were performed in the afternoon of the same day. Coconut dust was used for the seed germination medium in plastic baskets. Germination testing was carried out in the seed beds and after the transfer of germinating seed to the polyethylene bags. A total of 120 rubber seeds from each variety were spread over the germination

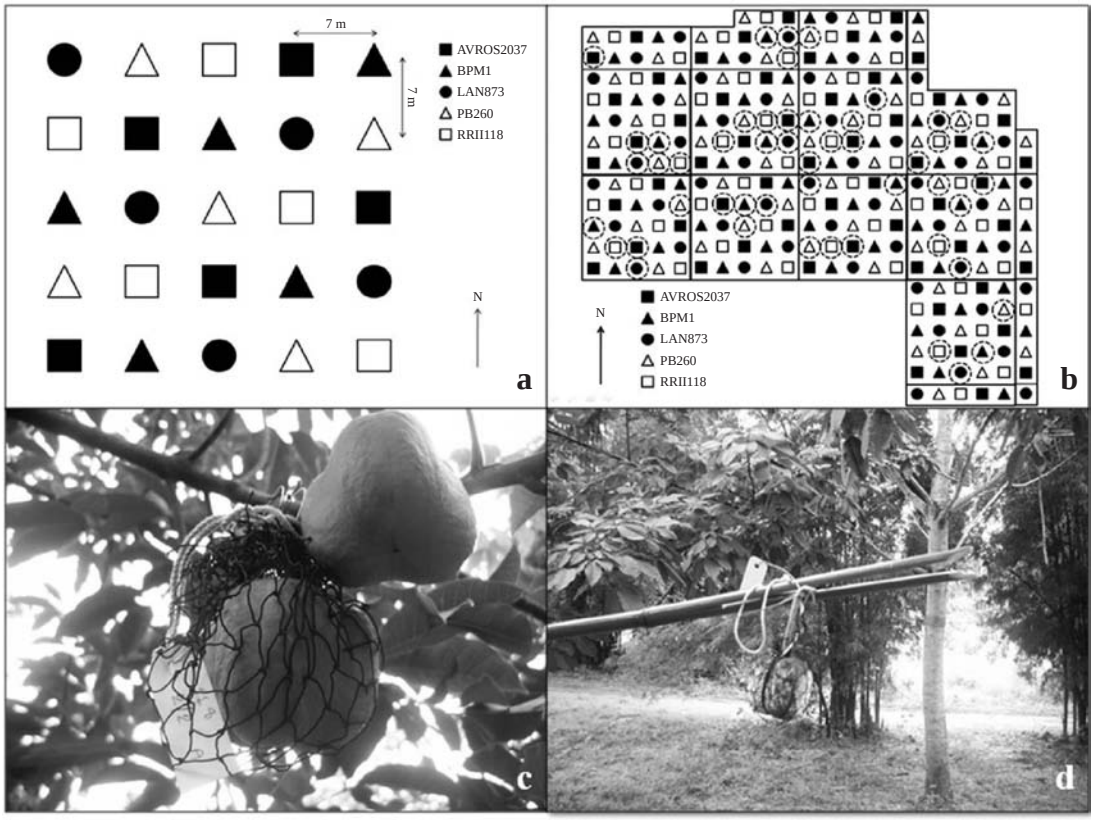

Figure 1 (a) Position of each clone in systematic plot, (b) Map of the experimental site and location of clones were collected seeds shown by dot circle (■=AVROS2037, ▲=BPM1, ●=IAN873, Δ=PB260, □=RRII118), (c) Each parent's capsule was covered by a net (d) Mature capsules were collected prior to shattering for the seed germination test.

beds in a single layer. The germination rate was assessed 2 wk after sowing. After germination, the seedlings were transferred to polyethylene bags (0.06 × 0.36 m). Seedlings were genotyped for paternity testing as explained in the next steps.

Flowering pattern

To determine whether the flowering patterns of related clones overlapped temporally, the plantation site was visited every day during the flowering period to make observations on flowering phenology using standard scores that were recorded as the first date that flowers were in bloom, the date that 50% of flowers were in bloom and the last date that flowers were in bloom (February–March 2012) as shown in Figure 2.

DNA extraction and SSR analysis

Genomic DNA was extracted from the young leaves of candidate parents and seedlings by a modified cetyltrimethyl ammonium bromide (CTAB) method, as described by Doyle and Doyle (1990), with slight modifications. The DNA quantity was assessed by comparing all samples against three standardized solutions of Lambda phage DNA (100, 250, 500 ng.μL^{-1}; Fermentas, Foster City, CA, USA), in an 0.8% agarose gel stained with ethidium bromide and visualized with UV light.

Five primer pairs—Hb34, Hb66, Hb82, Hb89 and Hb111—that could amplify polymorphic products were used for paternity analysis (Table 1). The SSR amplification reactions were

Figure 2 (a) *Hevea* rubber inflorescence, (b) Female flower, (c) Male flower, (d) First day of flowers in bloom, (e) 50% of flowers in bloom, (f) last day of flowers in bloom.

carried out in 0.2 mL tubes using a PTC-200 Thermal Cycler (MJ Research; Watertown, MA, USA). For polymerase chain reaction (PCR) amplification, samples were prepared in 10 µL reaction volumes containing approximately 10 ng of template DNA, 50 mM KCl, 20 mM Tris-HCl buffer (pH 8.0), 1.5 mM $MgCl_2$, 0.2 µM of each primer, 0.4 mM of each dNTP and 0.5 unit of *Taq* DNA polymerase (Fermentas). The reaction mixture was subjected to PCR amplification using the following PCR program: 5 min at 94 °C, then, 30 cycles of 94 °C for 1 min; 50 °C, 58 °C, 58 °C, 58 °C and 60 °C for the primer pairs Hb34, Hb66, Hb82, Hb89 and Hb111, respectively for 45 s; and 72 °C for 1 min, followed by 7 min at 72 °C. After amplification, PCR reactions were mixed with 10 µL of loading dye (95% formamide, 0.25% Bromophenol blue and 0.25% xylene cyanol) and denatured. Two µL samples of the PCR reactions were separated by electrophoresis in 6% denaturing polyacrylamide gels (Sequi-Gen1 GT Nucleic Acid Electrophoresis Cell; Bio-Rad; Hercules, CA, USA) at 40 W constant power for 2 h and were visualized by silver staining as described by Benbouza *et al.* (2006). The band sizes were estimated by comparison with a 10 bp DNA ladder (Invitrogen Corp.).

Paternity analysis

A total of 288 germinated seedlings, including 57, 55, 57, 56 and 55 of the varieties AVROS 2037, BPM 1, PB 260, IAN 873 and RRII 118, respectively, were used for paternity analysis. Seedlings were classified as derived from

Table 1 Microsatellite markers used for the paternity analysis and exclusion parameters.

Locus	Primer sequences	Exclusion probability						
		Number of alleles	Ho	He	PIC	First parent	Second parent	Null freq
Hb34	5'-CAAAGTTGAAAATGTTAAAGGGAA-3'	6	0.878	0.831	0.806	0.480	0.654	-0.0282
	5'-CATATCAAGAAACACAGCAAAAAA-3'							
Hb66	5'-CCAGTTAGCTTTCTCTTGTGCTT-3'	4	0.674	0.734	0.682	0.307	0.479	0.04
	5'-AAGGCATAACACTGCAGGATTT-3'							
Hb82	5'-ATAATTCCAGGCCAGTTCAAAG-3'	6	0.739	0.771	0.739	0.386	0.568	0.0281
	5'-GCTCAAAGCAACGAAAACAAG-3'							
Hb89	5'-AAACATGCACACACACAAACCT-3'	6	0.781	0.796	0.763	0.415	0.593	0.0101
	5'-CTTGCTCTTACCCCTTCCTG-3'							
Hb111	5'-ATGTATGTGTGCGCAGGAAG-3'	7	0.815	0.816	0.791	0.463	0.638	0.0007
	5'-CTGTAGTCATGGCAGCAGGA-3'							
Average		5.8	0.777	0.790	0.756	0.930449*	0.988553**	

Ho = Observed heterozygosity, He = Expected heterozygosity, PIC= Polymorphic information content, Null freq = Null allele frequency, * = Combined exclusion probability (first parent), ** = Combined exclusion probability (second parent).

self-pollination if all their alleles matched those of the known female parent. If a non-maternal allele was present at any locus, the seedling was classified as arising from cross-pollination. The CERVUS 3.0 program (Marshall *et al*., 1998; Kalinowski *et al*., 2007) was then used to identify which of five possible candidates was the male parent. For simulations of likelihood ratios and their confidence levels, 10,000 iterations were run. The proportion of loci at a likelihood ratio of 0.01 with confidence levels of 95% (strict) and 80% (relaxed) were used for paternity assignment. Allele frequency, paternity, exclusion of the second parent, number of alleles observed (Ho), expected heterozygosity (He), polymorphic information content (PIC), average exclusion probability and null allele frequency were estimated using the same program above.

RESULTS AND DISCUSSION

From each clone, 120 seeds were collected, for a total of 600 seeds from each of five varieties. Because the seeds were freshly collected, many of them germinated successfully, giving an overall germination rate of 75.5%. Yeang and Chevallier (1999) had previously demonstrated an average seed germination rate of 98.7% for the clones PR 107 and RRIM 623. More recently, the germination rate of *Hevea* rubber seeds was estimated at greater than 50% from six different *Hevea* varieties consisting of RRIM 901, RRIM 2001, RRIM 2005, RRIM 2006, RRIM 2026 and PB 260 (Daud *et al*., 2012). *Hevea* rubber seeds have relatively high water content and do not undergo dormancy, although the varieties BR. 2 and Av. 163 are exceptions. Aside from varietal differences in dormancy, the viability and performance of seedlings are otherwise strongly influenced by the traits inherited from individual parental plants. Non-dormant *Hevea* varieties generally exhibit sensitivity to very low or high temperatures (Thompson, 1979; Keleny and Van Haaren, 1976; Malaysian Rubber Board, 2009). The viability of *Hevea* rubber seeds is also strongly influenced by humid tropical climates with strong sunlight and rainfall throughout the year. Exposure of seeds to direct sunlight reduces seed moisture and consequently reduces seed viability (Sakhibun, 1981).

The experimental area was located 711 m above sea level and because there was no *Hevea* rubber planted nearby this helped to prevent pollen contamination from adjacent fields and

consequently, there was no source of extraneous *Hevea* rubber pollen that could have confounded data interpretation. The first date of flowering varied from 12 February to 3 March among these *Hevea* rubber clones (Figure 3). Substantial genetic variation occurred among these rubber clones for different flowering phenophases (Figure 3). In the present study, AVROS 2037 was the earliest flowering of all clones followed by IAN 873, PB 260, BPM 1 and RRII 118. Moreover, the period of flowering for BPM 1 was the same as that for RRII 118 (Figure 3). Rattanawong *et al*. (2010) also found that AVROS 2037 flowered early compared to RRII 118, which flowered early compared to PB 260. Unfortunately, it was not possible to collect data regarding the flowering period for some samples due to powdery mildew infection that occurred during the experiment.

Analysis of allele frequencies

Five microsatellite loci were evaluated for clear, unambiguous, polymorphic banding patterns in a preliminary experiment. Five microsatellite loci with a clear polymorphic profile in these five *Hevea* rubber clones were used for paternity analysis of 288 open-pollinated offspring from the five known female clones. The number of alleles per locus ranged from 4 to 7, with an average of 5.8 (Table 1). The observed heterozygosity ranged from 0.674 to 0.878, with an average of 0.777. The expected heterozygosity ranged from 0.734 to 0.831, with an average 0.790. The polymorphic information content (PIC) ranged from 0.682 to 0.806, with an average of 0.756. These average values for PIC, number of alleles and observed heterozygosity (0.756, 5.8 and 0.777, respectively) were comparable to those reported previously (Gouvêa *et al*., 2010; García *et al*., 2011; Feng *et al*., 2012), but were lower than the highest report of 17 alleles per locus over 69 SSR loci in 36 accessions of *H. brasiliensis* from the Agronomic Institute of Campinas (Mantello *et al*., 2011). The null allele frequency ranged from -0.0282 to 0.04. The combined exclusion probability was 0.930449 for the first parent in cases in which both parents were unknown and 0.988553 for

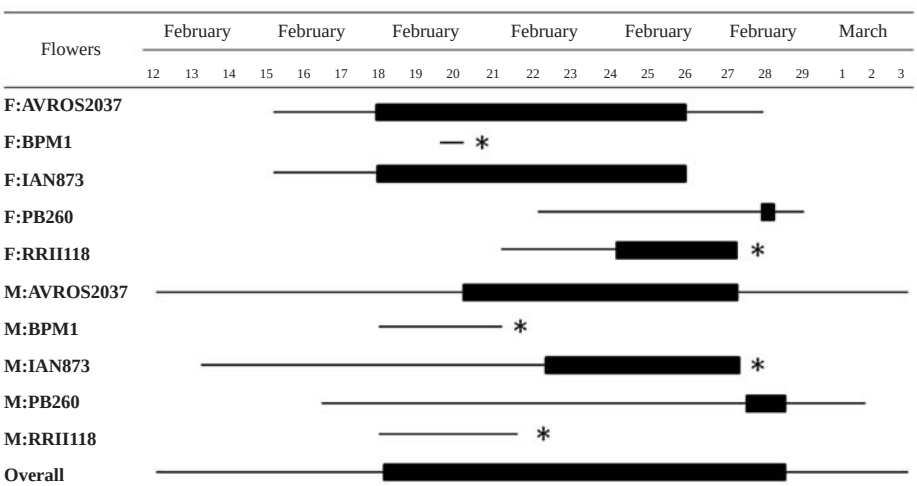

Figure 3 Phenogram showing clonal variation for duration of flowering (thin line) and 50% flowers in bloom (fat line) in a clonal seed orchard of rubber in Phetchabun Highland Agricultural Development and Research Center, Khao Kho, Phetchabun, Thailand (F = female clone, M = male clone). Data could not be collected the day after an asterisk (*) because of flower drop due to the spread of powdery mildew disease infection.

Table 2 Percentage of cross-pollination and self-pollination of the open-pollinated offspring in the seed orchard.

Female parent	Number of seedlings assayed	Outcrossing rate (%)	Selfing rate (%)	Most likely seed orchard parent contributing to cross-pollination (number offspring sired)
AVROS2037	57	84.21	15.79	BPM1(2), IAN873(30), PB260(9), RRII118(7)
BPM1	53	88.68	11.32	AVROS2037(5), IAN873(23), PB260(16), RRII118(3)
IAN873	59	64.41	35.59	AVROS2037(8), BPM1(3), PB260(20), RRII118(7)
PB260	58	58.62	41.38	AVROS2037(1), BPM1(6), IAN873(27)
RRII118	61	98.36	1.64	AVROS2037(6), BPM1(3), IAN873(37), PB260(14)
Overall	288	79	21	

the second parent when the maternal parent was known. These values were sufficiently high to correctly assign parentage. The markers therefore provided sufficient resolution for determining the pollen donors in the seed orchard.

Paternity analysis and rate of self and cross-pollination

Among the 288 seedling samples tested, the 227 seedlings were assigned as outcrossing (79%). The outcrossing rate for each female parent ranged from 58.62 to 98.36% whereas the other 61 seedling were selfing (21%) and the selfing rate for each female parent ranged from 1.64 to 41.38%. According to Simmonds (1986), the selfing rate ranges from 16 to 28% and the outcrossing rate ranges from 72 to 84% in a typical polycross seed orchard. However, Paiva *et al*. (1994) found an outcrossing rate for *Hevea* rubber in natural populations of 64.46%.

The offspring collected from the maternal AVROS 2037 clones exhibited an 84% outcrossing rate, including male parents BPM 1, IAN 873, BP 260 and RPII 118 (3, 53, 16 and 12%, respectively); However, the selfing rate of AVROS 2037 was only 16% (Table 2). The outcrossing rates of BPM 1, IAN 873, PB 260 and RRII 118 were 89, 64, 59 and 98%, respectively (Table 2). The observations on flowering pattern during January to March 2012 indicated that the RRII118 clone had the lowest selfing rate because of non-overlapping periods of blooming of male and female flowers. Furthermore, the PB260 clone had the highest selfing rate due to the 50% flower in bloom of male and female organs being in flowering synchrony.

The selfing and outcrossing rates of parental pollinations can vary due to differing self- and cross-compatibility and flowering synchrony (Yeang and Chevallier, 1999). Furlani *et al*. (2005) evaluated the mating system of a *Hevea brasiliensis* (Willd. ex A. de Juss) population in nature and found that 34% of purportedly outcrossed progenies were full-sibs. Results from the present study showed that the proportion of crossing and selfing depended on periods of flowering synchronization.

CONCLUSION

One of the most important aspects of a seed orchard is synchrony among the parental clones to assist with reproductive phenology. This determines the extent of random mating among the constituent clones and hence the genetic gain possible in the resultant progeny. Parentage analysis based on microsatellite markers permitted the identification of new interspecific hybrids and, at the same time, provided a rough idea of which *Hevea* rubber genotypes might be useful for establishing new seed orchards for inter-specific

F1 hybrid production. Further yield trials will be helpful to confirm the interspecific hybrids derived from this seed orchard.

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