



Mating system in a *Hevea brasiliensis* population by isozyme loci

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ABSTRACT - The multilocus mixed mating model and correlated mating model were used to analyze the mating system of a natural *Hevea brasiliensis* (Willd. Ex Adr. of Juss.) population from the State of Acre. Seeds of 37 mother trees were planted in a progeny test in Selvíria, state of Mato Grosso do Sul. Six to 24 individuals per family were evaluated in allozyme electrophoretic analysis. Significant differences between pollen and ovule frequencies were detected in three loci, indicating that the pollen pool was heterogeneous among trees. The multilocus outcrossing rate was estimated at 0.962, indicating allogamy predominance in the population. The correlation of paternity was high (0.351), indicating that 34% of outcrossing progenies are full-sibs. The estimate of the mean coefficient of coancestry among plants within families was 0.177, confirming the mixture of different degrees of relatedness within families.

Key words: Tropical tree species, correlated mating, coancestry, inbreeding.

INTRODUCTION

The mating system of a species determines how genetic information is transmitted from one generation to the next (Wright 1921) and therefore partially determines the genetic structure of its populations. Species with a mating type that spreads pollen over great distances maintain the greatest part of genetic diversity within populations, opposite to predominantly selfing species that spreads pollen over short distances and maintain the greatest part of diversity among populations (Hamrick 1989). Such information is fundamental in breeding and forest conservation programs for the selection of

populations and the estimation of the coefficient of relatedness among plants within progenies in order to estimate genetic parameters, collect samples for *ex situ* conservation and collect seeds for environmental reforestation.

Hevea brasiliensis (Willd. Ex Adr. of Juss.) (Euphorbiaceae) or rubber tree is a tropical tree species of high economical value for Brazil. Latex, the raw material of rubber is extracted from it as well as other commercially important by-products. The species is endemic to the Brazilian Amazon region (lat 7° N to 15° S) where it occurs along river banks and in seasonally flooded forest areas (Lorenzi 1992). The hermaphrodite flowers are pollinated

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by insects of the family Ceratopogonidae (Heleidae) and thrips that cross-pollinate over short distances (Gonçalves et al. 2001). A previous isozyme loci-based estimation of the apparent outcrossing rate (0.645) indicated that the species has a mixed mating system (Paiva et al. 1994). However, the model used to estimate the outcrossing rate, the classical mixed mating model, was based on the assumption of inbreeding equilibrium. The model of inbreeding equilibrium assumes that the inbreeding coefficient does not change from one generation to another and that all inbreeding observed in a generation can be ascribed to selfing. These presuppositions are not likely to apply to tree species owing to the strong inbreeding depression these species are generally subjected to (Sorensen 1997, Wang et al. 2004), altering the inbreeding coefficient between generations, and due to the frequently detected spatial genetic structure within populations, creating another form of inbreeding, biparental inbreeding.

Therefore, the aim of this study was to estimate the outcrossing rate and various other mating parameters in the same *H. brasiliensis* population studied by Paiva et al. (1994), using six isozyme loci and a mixed mating model that does not require the supposition of inbreeding equilibrium (Ritland and Jain 1981), together with a correlated mating model (Ritland 1989) that allows a separation of the progeny fractions created by random matings of the biparental matings.

MATERIAL AND METHODS

Plant material

The material used in this study came from a test with *H. brasiliensis* progenies installed on 6/12/91, on the Fazenda de Ensino e Pesquisa of the Faculdade de Engenharia/UNESP, in Selvíria, state of Mato Grosso do Sul (MS), aiming at the genetic *ex situ* conservation of the species and future use in improvement programs. The test included 37 progenies distributed in three replications with approximately 10 plants per plot. The seeds of open pollination that were used in the test came from two interlinked natural areas close to Rio Branco, state of Acre (AC), along the highway BR 364, in the Reserva Florestal de Catuaba (km 22), which belongs to the Universidade Federal do Acre (UFAC) and from an area of EMBRAPA/UEPAE-RioBranco (km 14). In view of the state of forest conservation, the sample population of *H. brasiliensis* can be considered a wild population. In these areas *H. brasiliensis* occurs in low stand density (approximately

one individual per hectare). The mother trees that originated the seeds grew along the tapping road, which normally begins at one point, takes a somewhat sinuous course and returns to the point of origin. Trees growing closely to each other were avoided when the trees were chosen. The seeds of mother trees 1 to 25 were collected along a tapping road of the Reserva Catuaba and number 26 to 33 along another road. Seeds of the matrixes 34 to 37 were collected from beside a road in the EMBRAPA area. Seed collection was restricted to an area within a radius of 10 to 15 m from the mother tree, aiming to ensure the origin of the seed from the mother plant.

Electrophoresis

Young leaf tissues of 6-24 plants per family, amounting to a total of 29 families, were evaluated by isozyme electrophoresis, using 12% starch gel as culture medium. The laboratory procedures such as extraction, running and revelation conditions of the isoenzymes were based on underlying study of Paiva et al. (1994) and Alfenas (1998). Five enzymatic systems were evaluated: 6-phosphogluconate dehydrogenase (6-PGDH – EC 1.1.1.44), alcohol dehydrogenase (ADH – EC 1.1.1.1), Endopeptidase (ENDO – EC 3.4.22.16), isocitrate dehydrogenase (IDH – EC 1.1.1.42), and phosphoglucoisomerase (PGI – EC 5.3.1.9). The loci were sequentially designated by the allele of greatest anodic migration as 1, the next 2, and so forth. The genetic base of the loci was not tested by controlled crossings, but the phenotypes the selected enzymes expressed were consistent with the subunit structure and genetic interpretation of other studies into plants, as documented by Alfenas (1998).

Statistical analysis

The mating system was analyzed using the mixed mating model of Ritland and Jain (1981) and correlated mating model of Ritland (1989), performed on MLTR software (Ritland 1997). The analyses were realized in two steps: first the population estimates of the fixation index of the mother trees (F_m), multilocus outcrossing rate (t_m), unilocus outcrossing rate (t_s), outcrossing rate between related individuals ($t_m - t_s$), allelic frequencies of ovules and pollen (o and p), correlation of selfing (r_s), and correlation of paternity (r_p) were established and secondly the individual estimates of outcrossing rate per mother tree (i). The parameters were estimated by the Newton-Raphson method of maximum likelihood. Once the mother trees were

not genotyped, the mother genotype was inferred from the segregation of the progenies, using the method of the most likely maternal genotype (Brown and Allard 1970). The standard error of estimates was calculated based on 500 bootstrap resamplings. In the population analyses, the resampling units were the families and in the estimate of the individual outcrossing rate per mother tree the resampling units were plants within families. To verify where the estimates differed statistically from the unit (t_m and) or from zero (t_m , t_s , r_e and r_p), the standard error of the parameter means was estimated at 95% probability, 1.96SE (standard error).

The random matings were tested by the estimate of the genetic divergence between the allelic frequencies of ovules and pollen pool, using F_{xy} statistics. The statistical significance of F_{xy} values for each locus was obtained by the chi-square test χ^2 , proposed by Workman and Niswander (1970), where n is the number of gametes in the two groups (pollen and ovules), k is the number of alleles and s the number of groups.

The coefficient of coancestry within families of open pollination was estimated by the correlation of relatedness among plants within families (r_{xy}), based on Ritland (1989):

$$r_{xy} = 0.25(1 + F_p)[4s + (t_m^2 + t_m s r_s)(1 + r_p)]$$

where F_p is the fixation index in the parent population. As in a population without inbreeding and relatedness of a diploid species, $r_{xy} = 2\theta_{xy}$ (Lynch and Walsh 1998), we have $\theta_{xy} = r_{xy} / 2$. The fixation index in the families (F) was calculated by the expression

$$F = \frac{(H_e - H_o) + \frac{1}{2n} H_o}{H_e - \frac{1}{2n} H_o} \quad (\text{Weir 1996}),$$

where H_e is the expected heterozygosity in Hardy-Weinberg equilibrium, $H_e = [2n/(2n-1)](1 - \sum p_i^2)$, n the number of sampled individuals, p_i the frequency of allele i in a particular locus according to Nei (1978) and is the observed heterozygosity, $H_o = 1 - \sum p_{ii}^2$, where p_{ii}^2 is the frequency of homozygous genotypes ii in a particular locus. To verify if the mean value differed from zero, the confidence interval was estimated at 95% probability by bootstrap resampling, using 10.000 locus resamplings, processed on software GDA (Lewis and Zaykin 1999).

RESULTS AND DISCUSSION

The isozyme systems (malate dehydrogenase - MDH, shikimate dehydrogenase - SKDH and leucine aminopeptidase - LAP) used earlier by Paiva et al. (1994)

in plantlets of *H. brasiliensis* did not present enzyme activity in the adult phase. The systems that provided an interpretable resolution pattern were 6-PGDH, ADH, ENDO, IDH, and PGI. System 6-PGDH showed two activity zones, while the others presented only one. The six studied loci were polymorphic in the population. Locus *Endo-1* was biallelic, *6-Pgdh-1*, *6-Pgdh-2* and *Adh-1* were triallelic and *Idh-1* and *Pgi-2* were tetra-allelic.

Three (*6-Pgdh-1*, *6-Pgdh-2* and *Pgi-2*) to six loci presented significant differences at 95% probability between pollen and ovule pools (Table 1), indicating either a heterogeneous set of pollen that fecundated the individual trees or differences in the individual outcrossing rate among mother trees. In some cases one sampled mother tree was eight kilometers apart from the next, which explains the spatial heterogeneity in the allelic frequencies of pollen pools among trees. Selfings and biparental inbreeding can also generate heterogeneity in the allelic frequencies of outcrossed pollen pool among different trees.

The fixation index estimated for the mother trees ($F_m = 0.04$) was statistically not different from zero (Table 2), indicating that the mother genotypes are found in proportions of the Hardy-Weinberg equilibrium (HWE). The fixation index estimated in the progenies ($F_o = 0.162$) was positive and significantly different from zero, suggesting an excess of homozygous progenies. The excess of homozygous progenies in hermaphrodite species such as *H. brasiliensis* can be caused by selfing and crossing between related trees. The estimation of the coefficient of inbreeding that could have been caused by selfing in inbreeding equilibrium, $F = s(2 - s)$ shows that selfing could have caused a maximum inbreeding of 0.019, as opposed to 0.142 observed, that can be attributed to mating between related trees (0.142). Since biparental inbreeding in descents is equal to the coancestry coefficient between crossed parents (Lindgren et al. 1996), inbreeding seems to be a consequence of the crossing of relatives between half-sibs (0.125) and full-sibs (0.250). Still, no significant differences were detected between the multilocus and unilocus outcrossing rate ($t_m - t_s = 0.014$).

The absence of inbreeding in the mother trees and the presence in the families indicate selection against homozygous progenies. Absence of inbreeding or excess of heterozygous trees in the adult generation and presence of inbreeding in the progenies generation have also been observed in other studies realized with tree species (Millar et al. 2000, Sebbenn et al. 2000, Alves et al. 2003).

Individual outcrossing rates (Figure 1) varied from

0.61 to 1.0 among trees, with a J-shaped frequency distribution, in other words, a large proportion of high individual outcrossing rates ($t \geq 0.9$) and small to low individual outcrossing rates. Such variations could have been caused by a variation to self-incompatibility, variations in the flowering phenology and pollinator performance, visiting flowers of one and the same tree intensely before going to another.

The estimates of the multilocus and unilocus outcrossing rates were both high, but statistically different from the unit ($t_m = 0.962$, $t_s = 0.948$; $P < 0.05$), based on the confidence interval of the error which shows that the species is predominantly allogamous (Table 2), but reproduces with a certain selfing rate. These estimates differ significantly from the previous estimate of 0.645 (Paiva et al. 1994) for the same mating event and same population. Differences of polymorphism in the loci, of ontogenic stages (plantlets and 19-year-old trees) and differences in the estimation models can explain these differences between estimates.

Positive and significant differences in the estimate of the multilocus and unilocus outcrossing rates ($t_m - t_s$) indicate the occurrence of crossing between relatives (Ritland and Jain 1981). The difference $t_m - t_s$, 0.014 (Table 2) was positive, low and statistically not different from zero, considering a confidence interval of an error of 95% probability, suggesting the absence of crossings among relatives and the probable absence of spatial genetic structure in the population; otherwise, crossings among relatives would have been detected.

The correlation of selfing within progenies (r_s) is a measure of variation in the selfing rate among plants (Sun and Ritland 1998, Millar et al. 2000). The correlation estimate r_s was low (0.096) for *H. brasiliensis*, although statistically different from zero (Table 2). This shows that in the mean, there are small differences in the selfing rate among plants. Figure 1 demonstrates this variation clearly, as discussed above.

The correlation of paternity (r_p) measures the proportion of progenies derived from biparental matings, so, it measures the proportion of progenies of crossing that are full-sibs (Ritland 1989). The estimated correlation of paternity 0.351 was high and significantly different from zero (Table 2), indicating that 35.1% of the progenies of crossing were generated by biparental matings. This result, together with the estimate of the multilocus outcrossing rate, allow the conclusion that the *H. brasiliensis* progenies under study consisted of a mixture of selfed sibs, full-sibs

and half-sibs. The estimated proportion of selfed progenies is approximately 4% (s), 34% ($t_m r_p$) for full-sibs and 62% [$t_m (1 - r_p)$] for half-sibs. Open-pollinated progenies, composed by mixtures of different types of relatedness have been detected in other tropical tree species such as *Cariniana legalis* (Mart.) O. Ktze. (Sebbenn et al. 2000); *Esenbeckia leiocarpa* L. (Seoane et al. 2001); *Teobroma grandiflorum* (Willd ex Spreng) Shumm (Alves et al. 2003) and others.

Biparental matings imply that the number of trees that contributed effectively to pollination could be small. Accordingly, the estimate of the probable mean number of pollinators was only three ($N_{ep} = 1/r_p$). The reasons for biparental matings in *H. brasiliensis* are probably associated to the performance of the pollinators, visiting nearby trees systematically, which could in turn have to do with the low density of trees per area in the species populations (usually less than one tree per hectare); this would hamper the random pollen distribution across the population. Another possible cause could be flowering asynchronism, that is, only trees in the same phase of reproductive maturity can take part in reproduction, causing genetic drift in the reproduction process, so that at each step of the phenological flowering stages only a small part of the population would participate in the crossings. Alternatively, the low annual flower density could restrict the movement of pollinating insects, leading to high biparental outcrossing rates (Millar et al. 2000). Biparental outcrossings result in an increase in coancestry coefficient among plants within progenies, which consequently reduces the variance effective size and affects the estimates of additive genetic variance from open-pollinated families.

Results of the mating system analysis of the *H. brasiliensis* population under study showed deviation of random matings. In terms of forest breeding, this means that the genetic covariance, i.e., the relatedness, among plants within families estimates over 1/4 of additive genetic variance. Specific biometric models must therefore be adopted to estimate the components of variances. Considering that the correlation of relatedness (r_{xy}) among plants within families of populations of allogamous diploid species without inbreeding and relatedness in the parental generation, is the double of the coefficient of coancestry (θ_{xy}) among plants within families ($r_{xy} = 2\theta_{xy}$; Lynch and Walsh 1998), r_{xy} is 0.354 (2×0.177) in the present population. This indicates that the correlation of

relatedness is 29.4% higher than expected in large random-outcrossing populations ($r_{xy} = 0.250$). Assuming that the open-pollination progenies are half-sibs would in this case however lead to a considerable overestimation of the additive genetic variance as well as of the subsequent estimates of heritabilities and gains with selection. Costa et al. (2000) estimated genetic parameters in a *H. brasiliensis* population through the inbreeding coefficient estimated by Paiva et al. (1994) to quantify the correlation of relatedness between plants within families (r_{xy}) and observed an overestimation of genetic gain, varying from 12.4 to 30.5%, when estimated through the random mating model (assuming open pollination progenies as half-sibs), compared to the mixed mating model. However, the coefficient of correlation estimated (0.18) by the authors considered that the families consisted only of mixtures of selfing and half-sibs, different from the ones presented here, which accommodate selfed sibs, half-sibs, full-sibs as much as siblings of selfing and outcrossings, though both were similar in magnitude. Thus, the Ritland model is more realistic to estimate the correlation of relatedness, adjusting all different degrees of relatedness among plants within families.

In relation to genetic conservation the results indicate that the variance effective size (N_e) retained in the progeny test is smaller than it would have been expected if the population were reproduced by random matings. For a family of infinite size, derived from random outcrossings in a population without inbreeding and relatedness in the parent generation (idealized population), $N_e = 4$. If the progeny size is finite and/or there are

deviations of random matings $N_e < 4$. For instance, a family of half-sibs originated from a non-inbred population with five plants has an effective size of 2.5 ($N_e = 0.5 / \{0.125[(5-1)/5] + 1/(2 \times 5)\}$); the expression is derived from the sampled variance in the allelic frequencies of a population with coancestry and inbreeding, according to Cockerham 1969). If the family is infinite, but there are deviations of random matings as detected here, the variance effective size is 2.8 ($N_e = 0.5 / 0.177$). Alternatively, if the family size is finite, e.g., five plants, and there are deviation of random mating, the effective size would be 2.1 ($N_e = 0.5 / \{0.177[(5-1)/5] + 1/(2 \times 5)\}$). Considering that the evaluated material consisted of a progeny test with 37 families, approximately 10 plants per plot and three replications, the mean effective size of each family was estimated at 2.6 and at 96 (37×2.6) for the total set of families retained in the germoplasm bank. Frankel and Soulé (1981) claimed that the conservation of an effective size of 50 would be sufficient to retain 90% of the actual genetic variability (expressed in terms of heterozygosity) in loci with two alleles of species of random matings without overlapping generations. In the case of tree species, the suggestion has been made of multiplying the effective size (50) twice or more times, to accommodate the superposition of generations and the deviation of random matings (Nunney and Campbell 1993). Based on the variance effective size estimated in the trials of *H. brasiliensis* families ($N_e = 96$) one can therefore assume its potential of retaining part of the genetic variability in the short term (up to approximately 10 generations).

Table 1. Estimates of allelic frequencies, genetic divergence in pollen and ovules (F_{ST}) and chi-square test (χ^2) of a *H. brasiliensis* population

Locus	Allele	Pollen	Ovules	F_{ST}	n	df	χ^2
<i>6-Pgdh-1</i>	1	0.102	0.017	0.024	2	338	31.8**
	2	0.815	0.931				
	3	0.083	0.052				
<i>6-Pgdh-2</i>	1	0.212	0.085	0.028	2	350	39.2**
	2	0.776	0.898				
	3	0.012	0.017				
<i>Adh</i>	1	0.085	0.069	0.000	2	342	0.5
	2	0.851	0.862				
	3	0.064	0.069				
<i>Endo</i>	1	0.006	0.017	0.003	1	346	1.8
	2	0.994	0.983				
<i>Idh</i>	1	0.064	0.017	0.004	3	326	7.1
	2	0.787	0.831				
	3	0.117	0.136				
	4	0.032	0.016				
<i>Pgi</i>	1	0.086	0.051	0.006	3	348	11.6**
	2	0.799	0.763				
	3	0.094	0.169				
	4	0.021	0.017				

n = number of trees per family; ** $P < 0.01$

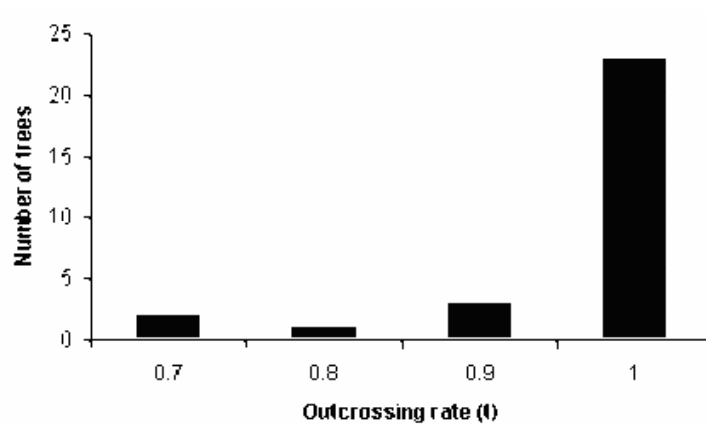
**Figure 1.** Distribution of frequency of the individual outcrossing rate in a *H. brasiliensis* population.

Table 2. Estimate of parameters of inbreeding and mating system in *H. brasiliensis*

Parameter	Mean ($IC_{95\%}=1.96SE$)
Number of families: n	29
Fixation index in mother trees: F_m	0.004 (0.000)
Fixation index in progenies: F_o	0.162 (0.068 to 0.260)
Multilocus outcrossing rate: t_m	0.962 (0.951 to 0.975)
Unilocus outcrossing rate: t_s	0.948 (0.945 to 0.951)
Outcrossing rate among relatives: $t_m - t_s$	0.014 (0.004 to 0.023)
Correlation of selfing: r_s	0.096 (0.093 to 0.099)
Correlation of paternity: r_p	0.351 (0.325 to 0.377)
Effective number of pollinators: $N_{ep} = 1/r_p$	3
Coancestry mean within progenies: θ_{xy}	0.177 (29.2%)

$IC_{95\%}$ = confidence interval at 95% probability; SE = standard error

Sistema de reprodução por locos de isoenzimas em uma população de *Hevea brasiliensis*

RESUMO - Os modelos multiloco de reprodução mista e cruzamentos correlacionados foram utilizados para a análise do sistema de reprodução de uma população natural de *Hevea brasiliensis* (Willd. Ex Adr. de Juss.) procedente do estado do Acre. Foram coletadas sementes em 37 árvores e plantadas na forma de teste de progênies, em Selvíria, MS. Desse plantio foram avaliados por isoenzimas de seis a 24 plantas por progênie. A comparação das frequências alélicas do pólen e dos óvulos revelou diferenças significativas, em três locos, indicando o pólen que contribuiu para os cruzamentos individuais não era homogêneo. A estimativa da taxa de cruzamento multiloco foi de 0,962, indicando predomínio de cruzamentos na população. A correlação paternidade foi alta (0,351), demonstrando que 34% das progênies de cruzamento eram irmãos-completos e o coeficiente médio de coancestria estimado entre plantas dentro de progênies foi de 0,177, confirmando a mistura de diferentes graus de parentescos dentro das progênies.

Palavras-chave: Espécies arbóreas tropicais, cruzamentos correlacionados, coancestria, endogamia.

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