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Mating system variation among populations, individuals and within and among fruits in *Bertholletia excelsa*

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Abstract

The aim of this study was to investigate variation in mating system among three Brazilian Amazon populations of the tree *Bertholletia excelsa* with different levels of anthropogenic interventions. We collected open-pollinated seeds from one natural population, remnant trees dispersed in a pasture, and trees from a plantation. Outcrossing rate not varied among the populations and indicates that all seeds were originated from outcrossing ($t_m = 1.0$). Mating among relatives was significant higher in the plantation than forest and pasture populations, probably due the fact that many trees are related in the plantation. Correlated mating was significantly higher in pasture ($r_p = 0.47$) and plantation ($r_p = 0.51$) than in the natural

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population ($r_p = 0.22$), suggesting that trees in natural population are pollinated by a higher number of pollen donors. The paternity correlation was significantly higher within ($r_{p(w)} = 0.41$) than among fruits ($r_{p(a)} = 0.18$), showing a higher probability to find full-sibs within than among fruits. The fixation index was generally lower in seed trees than in their seedlings, suggesting selection for heterozygous individuals from seedling to adult stages. Progeny arrays collected from the natural population had a lower proportion of pairwise full-sibs than in pasture and plantation and higher variance effective size (2.75) than trees in pasture (2.15) and plantations (2.22). Results highlight that seed collections for conservation, breeding and reforestation programs preferentially should be carried out in natural populations due low proportion highest variance effective size within progeny.

Key words: Brazil nut tree; Brazilian Amazon; conservation genetics; Lecythidaceae; microsatellite markers; population genetics; tropical tree species.

Introduction

The mating system is a key factor shaping the distribution of genetic variability in tree populations, since it determines how alleles are transmitted from one generation to another through reproduction. Mating systems are influenced by both genetic and environmental factors (Kalisz et al., 2004; Goodwillie et al., 2005; Good-Avila et al., 2008; Feres et al., 2012). Genetic factors are the sexual system, self-incompatibility, heterosis and inbreeding depression. Environmental factors are those affecting the behavior of pollination vectors, such as population density, distance between conspecifics, humidity, wind, and temperature (Millar et al., 2000; Fuchs et al., 2003). These both factors may affect outcrossing rates and correlated matings (Degen and Sebbenn, 2014).

Also, human disturbances such as habitat fragmentation and logging may affect mating systems by reducing the number of reproductive trees and increasing the distance between conspecifics. All these disturbances affect the amount and behavior of pollen vectors with consequences on the mating system (Fuchs et al., 2003; Lowe et al., 2005; Aguilar et al., 2008; Lacerda et al., 2008; Arruda et al., 2015). Comparing mating systems in tree populations with different degrees of human disturbance is a powerful strategy to predict how these diverse variables may affect reproduction, variance effective size and number of seed trees for seed collection. Since we expect higher relatedness and inbreeding and low effective size in seeds collected from seed trees in disturbed forests (Moraes and Sebbenn, 2011; Tambarussi et al., 2015), this approach is particularly valuable in designing sampling strategies aiming for *ex situ* conservation and breeding or even for forest restoration purposes (Degen and Sebbenn, 2014).

Generally, tree species populations display mixed mating systems (mixture of selfing and outcrossing) or are predominantly outcrossed (Ward et al., 2005; Degen and Sebbenn, 2014). Rates of outcrossing, mating among relatives, and correlated matings may vary among populations, progenies, fruits within individuals, as well as among reproductive events (Surles et al., 1990; Silva et al., 2011; Feres et al., 2012). Thus, open-pollinated progeny may represent a mixture of self-sibs, half-sibs, full-sibs and self-half-sibs (Feres et al., 2012; Degen and Sebbenn, 2014).

Bertholletia excelsa Bonpl. (Lecythidaceae) or the Brazil nut tree is one of the most important non-timber forest tree species of Amazonian forests and is considered a model species for forest conservation. Its nuts are almost exclusively harvested from mature forests and this NTFP (non-timber forest product) has enjoyed widespread and longstanding economic success in the international market (Wadt et al., 2008). This species occurs in non-flooded (*terra firme*) forests throughout the Amazon basin, preferentially on clay-sandy soils (Prance and Mori, 1979). In some regions in Eastern Amazonia, adults are concentrated in groves (Peres and Baider, 1997), but in Southwestern Amazonian individuals are randomly distributed (Wadt et al., 2005). It is a hermaphrodite species and may be self-incompatible, thus avoiding self-fertilization (O'Malley et al., 1988). Pollination is accomplished by large-bodied bees of *Bombus*, *Centris*, *Xylocopa* and *Epicharis* genera and some species of *Euglossini* (Maués, 2002). Few genetic studies have been conducted to date, and most addressed genetic diversity and structure in natural populations (Buckley et al., 1988; Sujii et al., 2015). A mating system study based on allozymes showed *B. excelsa* is predominantly outcrossing (O'Malley et al., 1988).

Bertholletia excelsa logging is prohibited by law in Brazil, Peru and Bolivia, where this

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species is very common and many local communities rely on *B. excelsa* seed harvest for their incomes (GUARIGUATA et al., 2009). Although it is common to find *B. excelsa* in pastureland or sometimes even in plantations, the value of these scattered trees for seed collection or *in situ* conservation is debated. Studies on tropical trees has been found that isolated trees in pastures are not reproductively isolated, but such trees in general present greater selfing and correlated mating than trees occurring in continuous and forest fragments (DICK et al., 2003; FUCHS et al., 2003; LANDER et al., 2010; MORAES and SEBBENN, 2011; MANOEL et al., 2012). Selfing and correlated mating increase the relatedness and decrease the effective size within open-pollinated progenies and selfing increase the inbreeding within families and consequently the number of seed trees for seed collection is higher in isolated trees than in continuous or fragmented forest (MORAES and SEBBENN, 2011; MANOEL et al., 2012). Isolated trees in pastures are more intensively exposed to wind and lightning storms than trees in forested areas, experiencing higher mortality rates. It is anecdotally known that seed production is heavily affected, as *B. excelsa* trees within forested matrix exhibit higher levels of seed production than their pasture-plantation counterparts. Some possible explanations of the reduced seed set are the dependence on forest-dwelling bees to accomplish pollination and reduce inbreeding depression, but no empirical study to date has evaluated how *B. excelsa* reproduction can be affected in these disturbed areas. Furthermore, there is not study about the mating system of *B. excelsa* plantation. Plantation maybe an alternative for seed collection if mating are random and open-pollinated seeds present low inbreeding, due the facility to collect seeds. This study aims to fill this gap by comparing the mating system between a group of remnant trees dispersed in pasture and trees from a plantation with natural population within forested matrice. We also explore outcrossing rate variation among and within fruits in the natural populations to more deeply characterize the mating system of this important Amazon tree species. In sum, our investigation uses microsatellite markers to compare mating system in populations with different life histories: natural mature forest, a plantation, and sparse trees in pasture. We specifically tested the following hypotheses: The human activities as spatial isolation of trees in pasture and forest plantation disturb the mating system of *B. excelsa* due changes in the behavior of the pollinator vector caused by changes in reproductive population density. Thus, we tested if the selfing rate and the paternity correlation are higher in sparse trees in pasture than trees occurring in natural forest and plantations; (2) mating among relatives is lower in sparse trees in pasture than in natural population due to intrapopulation spatial genetic structure, and than in plantations due to relatedness among individuals originated from same seed trees; (3) the paternity correlation is lower among than within fruits and; (4) the levels of variance effective size are lower and inbreeding is higher in open-pollinated seeds collected in sparse trees than seed trees occurring in plantation and natural forest.

Materials and methods

Study sites

The study was carried out in *B. excelsa* populations in one mature natural forest, one-plantation, and one pastureland with remnant trees. The study sites are distributed in the southern and southwestern portion of the Amazon in the Brazilian states of Acre and Mato Grosso. In Acre, one plantation and one pastureland with sparse *B. excelsa* seed trees were sampled. To statistic analysis, we defined the plantation and isolated trees in pasture as „populations". The plantation (ACPLA) was established in 1982 in the Experimental Campus of Embrapa Acre, in Rio Branco municipality, using a spacing of 10 x 10 m among trees and occupying an area of approximately 0.2 ha (100 trees/ha). Details of the provenance and number of seed trees when seeds were collected are unknown. The plantation was not isolated as there was a natural forest in the vicinity. The sparse *B. excelsa* trees (ACPAS) remnants of a native forest of 163 ha pastureland (0.31 trees/ha) within the Santa Maria farm, BR-317, km 17, Senador Guiomard municipality. This pasture lies between two other farms and there is a forest nearby. Pasture conversion took place 15 years ago. In Mato Grosso, the study was carried out in 20 ha plot (7.5 trees/ha) installed in a natural mature forest (MTFOR) in Dal Pai farm, Itaúba municipality.

Sampling

We sampled open-pollinated seeds from 14 seed trees in MTFOR, 8 seed trees in ACPAS

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and 9 in ACPLA. In ACPAS and ACPLA we sampled ten fruits per tree, obtaining an average of 30 seeds per tree. We germinated seeds in sandboxes at Embrapa Acre nursery, which were kept moist for four months. After this period, we removed seeds from the sand, peeled and placed again in the sandboxes for germination. In MTFOR, we sampled 7 fruits from each seed tree. Seeds were peeled and put to germinate in sandboxes in a greenhouse with 50% shading. For genetic analysis, we sampled leaves from a minimum of 10 seedlings per progeny. For hierarquical analysis of mating system among and within fruits, in the MTFOR population, we kept the identity of fruits for each seedling. Leaf or trunk cambium tissues of seed trees were also collected. Leaves were dried. Tissue samples were stored at –20°C until DNA purification.

Microsatellite analysis

Total genomic DNA extraction was performed using a CTAB protocol described in DOYLE and DOYLE (1990), employing an automatic tissue disruptor. DNA was quantified after electrophoresis on 1% agarose gel by comparison to known phago lambda DNA standards concentrations. DNA samples from seed trees and their progenies were genotyped with seven or eight microsatellite markers (*Table 1*). PCR (Polymerase Chain Reaction) amplification was performed separately for each locus, using 3 ng or 5 ng of DNA; 1x Taq polymerase buffer (10x, 10 mM Tris-HCl, pH 8.3, 50 mM KCl); 0.25 mM dNTPs; 0.25 mg/ml BSA; 1U of Taq DNA polymerase, 0.23 mM of each primer and ultra-pure water, in a final volume of 13 µl. Mato Grosso and Acre studies were done independently in different laboratories so we used markers and different PCR cycling protocol (*Table 1*). To Acre, the analyzes were performed at Embrapa Acre's Molecular Biology Laboratory (Labmol) and to Mato Grosso, in the Embrapa Genetic Resources and Biotechnology's (Cenargen) Genetics Laboratory. In Labmol amplifications were performed in a MJ Thermocycler 96+ Biocycler according to the conditions described in DON et al. (1991). PCR fragments were separated through capillary electrophoresis (Fragment Analyzer, FS 96, Advanced Analytical). Genotypes were determined using ProSize 2.0 software (Advanced Analytical). In Cenargen's Genetics Lab, the forward primers were labeled with fluorescent dyes and the PCR was performed on Veriti thermocycler (Applied Biosystems). A multiplexing step with two loci was assembled to perform genotyping. For each

Table 1. – Microsatellite markers (SSR) used to genotyped *Bertholletia excelsa*.

Population	SSR	PCR program	Reference
ACPLA and ACPAS	Bex02, Bex03, Bes13, Bes14, Bes18, Bes19, Bex27, and Bex37	The PCR conditions were optimized using a touchdown program (DON et al., 1991). Initial denaturation at 95° C for 5 min, 19 cycles of 95° C for 1 min, 62° C for 1 min, and 72° C for 1 min, decreasing the denaturation temperature after cycle 1 by 0.8° C every cycle followed by 9 cycles of 95° C for 1 min, 52° C for 1 min, and 72° C for 1 min. Finally 1 cycle of 95° C for 1 min, 55° C for 1 min, and 72° C for 10 min for final extension.	REIS et al., 2009
MTFOR	Bex02, Bex06, Bet09, Bet12, Bet14, Bet15, and Bet16	Initial denaturation at 94° C for 5 min; 35 cycles of: denaturation at 94° C for 1 min, annealing of primers at 56° C for 1 min, extension at 72° C for 1 min and 30 sec; and a final extension at 72° C for 7 min.	REIS et al., 2009; SUJII et al., 2013

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multiplex, 1 µl of each reaction was added to 9 µl of Hidi and 1 µl of ROX internal marker. This mixture was denatured for 5 min at 95°C and taken to an automatic analyzer ABI 3730 (Applied Biosystems). The data were interpreted using GeneMapper software. The allelic size rounding was conducted by AlleloBin software (PRASANTH et al., 2006). The amplified products were diluted and separated through electrophoresis in 5% polyacrylamide gels in an automatic DNA sequencer ABI Prism 377 XL, following the manufacturer's instructions (Applied Biosystems Inc.). Molecular weight standards ROX and TAMRA (Applied Biosystems Inc.) were used to estimate the size of alleles. Genetic data were collected and analyzed using Genescan and Genotyper programs (Applied Biosystems Inc.).

Analysis of mating system

The mating system at population and individual seed tree levels were analyzed according to the mixed mating and correlated mating models, using the MLTR 3.1 program (RITLAND, 2002). Estimates were based on the Expectation maximization numerical method (EM). The estimated indexes were: gene frequencies of pollen and ovules, fixation index of seed trees (F_m), multilocus (t_m) and single-locus outcrossing rates (t_s), mating among relatives ($t_m - t_s$), selfing correlation (r_s) and paternity correlation (r_p). In MTFOR, the multilocus paternity correlation was also estimated within ($r_{p(w)}$) and among ($r_{p(a)}$) fruits. The 95% confidence intervals (95% CI) of the estimated indexes were calculated based on 1,000 bootstraps among individuals within progeny as re-sampling unity.

The mating system parameters were used to estimate the effective number of pollen donors among and within fruits ($N_{ep} = 1/r_p$), within fruits ($N_{ep(w)} = 1/r_{p(w)}$) and, among fruits ($N_{ep(a)} = 1/r_{p(a)}$), following RITLAND (1989), mean coancestry coefficient within progeny ($\Theta = 0.125 (1+F_m)[4s + (t^2_m + t_m sr_s)(1+r_p)]$, where s is the selfing rate ($s = 1 - t_m$) following SEBBENN (2006). The variance effective size within progeny was estimated from the variance of gene frequencies due to genetic drift (σ^2_p), as derived by COCKERHAM (1969): $\sigma^2_p = [(n-1)/n]\Theta + (1+F_o)/2n]\, p(1-p)$, where n is the sample size within progeny, p is frequency for a given neutral allele, and F_o is the average inbreeding coefficient. As in an idealized population (without relatedness among individuals and inbreeding) under random mating, the corresponding σ^2_p value for a group of n offspring is $\sigma^2_p = p(1-p)/(2n)$, in which we can assume for an arbitrary reference population this variance as $\sigma^2_p = p(1-p)/(2N_e)$. Thus, we can equate both σ^2_p expressions to

Table 2. – Average inbreeding and mating system estimates in *Bertholletia excelsa* populations with 95% confidence intervals in parentheses (95% CI). Values with dissimilar letters in the same line are significantly different, according to the 95% confidence interval.

Index	MTFOR (95% CI)	ACPAS (95% CI)	ACPLA (95% CI)
Total number of seed trees: n_{st}	14	8	9
Total number of seedlings: n	320	94	97
Seed tree fixation index: F_m	0.02 (0.02 to 0.02)A	0.06 (0.06 to 0.06)A	0.02 (0.02 to 0.02)A
Seedlings fixation index: F_o	0.23 (0.09 to 0.38)A	0.14 (0.09 to 0.18)A	0.04 (0.01 to 0.08)B
Multilocus outcrossing rate: t_m	1.00 (1.00 to 1.00)A	1.00 (1.00 to 1.00)A	1.00 (1.00 to 1.00)A
Mating among relatives: $t_m - t_s$	0.04 (0.02 to 0.04)A	0.02 (0.01 to 0.03)A	0.08 (0.05 to 0.10)B
Correlation of selfing: r_s	0.11 (0.11 to 0.12)A	0.11 (0.10 to 0.12)A	0.11 (0.10 to 0.12)A
Correlation of paternity: r_p	0.22 (0.11 to 0.27)A	0.47 (0.31 to 0.58)B	0.51 (0.38 to 0.61)B
Effective number of pollen donors: N_{ep}	4.5 (3.7 to 6.0)A	2.1 (1.7 to 3.2)B	2.0 (1.6 to 2.7)B
Coancestry within progeny: Θ	0.153 (0.146 to 0.159)A	0.184 (0.164 to 0.197)B	0.189 (0.172 to 0.201)B
Variance effective size: N_e	2.75 (2.60 to 2.93)A	2.15 (2.11 to 2.48)B	2.22 (2.09 to 2.32)B
Number of seed trees: m	54 (51 to 58)A	67 (61 to 71)B	68 (63 to 72)B

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derive the variance effective size within progeny as,

$$N_e = \frac{0.5}{\Theta\left(\frac{n-1}{n}\right)+\frac{1+F_o}{2n}}.$$

This expression was used for computing N_e for population and progeny arrays. To determine whether there was inbreeding in seedlings, we used the within population fixation index, $F_o = 1-(H_o/H_e)$, where H_o and H_e are observed and expected heterozygosities, respectively (NEI, 1977). However, due to the fact that plants within progeny inherited at least one of the maternal alleles, which may result in an overestimation of gene frequencies of maternal alleles, F_o for mean population and progeny array was estimated using the H_o estimated for each population and mean progeny array, and H_e estimated from gene frequencies of pollen pool of each population. The H_o was estimated using FSTAT program (GOUDET, 1995) and H_e by $H_e = 1-\Sigma p^2$, where p is the frequency of each allele of a given locus in pollen pool (NEI, 1977). In Θ and N_e calculation, negative F_m and F_a estimates were assumed to be zero, because these indexes were derived from the inbreeding coefficient (which ranges from 0 to 1) and not from the fixation index (which ranges from –1 to 1). The minimum number of seed trees required for harvesting seeds for conservation purposes was estimated by $m = N_{e(r)}/N_e$, where $N_{e(r)}$ is the required effective population size (SEBBENN, 2006). We assumed a $N_{e(r)}$ of 150 (LACERDA et al., 2008). The 95% CI of these indexes was estimated using lower (l) and upper (u) values of the parameters estimated by bootstraps:

Table 3. – Inbreeding and mating system indices at the individual level in three *Bertholletia excelsa* populations (MTFOR, ACPAS, and ACPLA).

Seed-tree	n	F_m	F_o	t_m ±SD	$t_m - t_s$ ±SD	r_p ±SD	N_{ep}	Θ	N_e
MTFOR-1	19	-0.47	0.14	0.99–0.01	0.07–0.01	0.07+0.02	14.3	0.136	3.15
MTFOR-2	13	-0.12	0.13	0.96±0.04	0.05±0.03	0.18±0.06	5.6	0.156	2.67
MTFOR-3	14	-0.33	0.30	0.99±0.00	0.05±0.01	0.20±0.07	5.0	0.152	2.67
MTFOR-4	11	-0.20	0.38	0.94·0.05	0.06·0.04	0.18±0.03	5.6	0.160	2.40
MTFOR-5	22	-0.08	0.45	0.95±0.05	0.08±0.04	0.29±0.10	3.4	0.171	2.55
MTFOR-6	15	-0.12	0.28	0.99–0.00	0.06–0.01	0.21+0.07	4.8	0.153	2.69
MTFOR-7	19	-0.02	0.53	0.90±0.07	0.02±0.06	0.08±0.02	12.5	0.159	2.61
MTFOR-8	16	-0.68	-0.02	0.99–0.00	0.05–0.00	0.20+0.05	5.0	0.152	2.88
MTFOR-9	19	-0.31	0.24	0.96±0.04	0.07±0.03	0.13±0.06	7.7	0.150	2.86
MTFOR-10	45	0.16	0.30	0.98–0.03	0.05–0.02	0.07+0.03	14.3	0.161	2.92
MTFOR-11	51	-0.75	0.16	1.00±0.90	0.02±0.00	0.10±0.04	10.0	0.138	3.42
MTFOR-12	22	-0.73	0.07	1.00±0.00	0.06±0.01	0.08±0.02	12.5	0.135	3.26
MTFOR 13	26	0.88	0.20	0.95±0.08	0.02±0.07	0.05±0.01	20.0	0.143	3.11
MTFOR-14	28	-0.51	0.12	1.00–0.00	0.06–0.01	0.29+0.06	3.4	0.161	2.85
ACPAS 7	12	0.11	0.20	1.00±0.00	0.01±0.01	0.39±0.15	2.6	0.174	2.39
ACPAS-11	12	0.11	0.09	1.00–0.00	0.12–0.04	0.51+0.17	2.0	0.210	2.11
ACPAS 12	11	0.11	0.04	1.00±0.00	0.01±0.01	0.39±0.16	2.6	0.174	2.44
ACPAS-1	12	0.01	0.10	1.00–0.00	0.02–0.00	0.11+0.10	9.1	0.140	2.87
ACPAS 5	12	0.21	0.20	1.00±0.00	0.11±0.04	0.35±0.16	2.9	0.169	2.44
ACPAS-3	12	-0.40	0.18	0.98–0.06	0.07–0.05	0.31+0.17	3.2	0.167	2.47
ACPAS-18	11	-0.24	0.09	1.00±0.00	0.09±0.04	0.06±0.04	16.7	0.133	2.94
ACPAS-20	12	-0.00	0.21	1.00±0.00	0.03±0.01	0.70±0.18	1.4	0.213	2.04
ACPLA-30	12	-0.40	-0.15	1.00–0.00	0.02±0.01	0.08±0.37	12.0	0.135	3.02
ACPLA-45	11	0.16	0.18	1.00±0.00	0.11±0.03	0.92±0.17	1.1	0.278	1.63
ACPLA-34	8	0.31	0.08	1.00–0.00	0.06±0.02	0.81±0.26	1.2	0.297	1.52
ACPLA-76	12	-0.33	0.01	1.00±0.00	0.07±0.02	0.67±0.20	1.5	0.209	2.14
ACPLA-47	11	-0.23	-0.06	1.00–0.00	0.04±0.01	0.55±0.20	1.8	0.194	2.26
ACPLA-50	12	-0.12	0.16	1.00±0.00	0.13±0.04	0.10±0.34	10.3	0.137	2.88
ACPLA-71	8	0.19	0.05	1.00–0.00	0.15±0.05	0.23±0.35	4.3	0.184	2.21
ACPLA-51	11	-0.10	0.12	1.00±0.00	0.17±0.03	0.31±0.27	3.2	0.164	2.51
ACPLA-54	12	0.74	0.02	1.00–0.00	0.34±0.05	0.98±0.11	1.0	0.431	1.14

n is the sample size; F_m and F_o are the fixation index of seed trees and seedlings, respectively; t_m is the multilocus outcrossing rate; $t_m - t_s$ is the mating among relatives rate; r_p is the multilocus correlation of paternity; N_{ep} is the effective number of pollen donors; Θ is the coancestry coefficient within progeny; N_e is the variance effective size; SD is the standard deviation.

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$N_{ep(l)} = 1/r_{p(u)}$, $N_{ep(u)} = 1/r_{p(l)}$, $N_{ep(a)(l)} = 1/r_{p(a)(u)}$,

$N_{ep(a)(u)} = 1/r_{p(a)(u)}$, $N_{ep(w)(l)} = 1/r_{p(w)(u)}$, $N_{ep(w)(u)} = 1/r_{p(w)(l)}$, $P_{ss(l)} = (1-t_{m(u)})^2$,

$P_{ss(u)} = (1-t_{m(l)})^2$, $P_{hs(l)} = t^2_{m(l)}(1-r_{p(u)})$, $P_{h(u)} = t^2_{m(u)}(1-r_{p(l)})$, $P_{fs(l)} = t^2_{m(u)}(1-r_{p(l)})$,

$P_{fs(u)} = t^2_{m(u)}\, r_{p(u)}$, $P_{shs(l)} = 2t_{m(u)}(1-t_{m(u)})$, $P_{shs(u)} = 2t_{m(l)}(1-t_{m(l)})$,

$\Theta_{(l)} = 0.125(1+F_{m(l)})\,[4s_{(l)} + (t^2_{m(u)} + t_{m(u)}\, s_{(l)}\, r_{s(l)})\,(1+r_{p(l)})]$,

$\Theta_{(u)} = 0.125(1+F_{m(u)})\,[4s_{(u)} + (t^2_{m(l)} + t_{m(l)}\, s_{(u)}\, r_{s(u)})\,(1+r_{p(u)})]$,

$N_{e(l)} = 0.5/\{\Theta_{(u)}[n-1)/n] + (1+F_{o(u)})/2n\}$,

$N_{ep(u)} = 0.5/\{\Theta_{(l)}[n-1)/n] + (1+F_{o(l)})/2n\}$,

$m_{(l)} = N_{e(r)}/N_{e(u)}$ and $m_{(u)} = N_{e(r)}/N_{e(l)}$.

Results

Inbreeding

In all populations the mean fixation index of seed trees (F_m) was significantly negative (*Table 2*). At the seed tree level, F_m was smaller than zero in most of the seed trees, although some presented values higher than zero, indicating in this case inbreeding and variation among trees (*Table 3*). On the contrary, the fixation index of seedlings (F_o) was significant higher than zero in all populations, ranging from 0.04 to 0.23 (*Table 2*). The fixation index within progeny (F_o) was higher than zero in all the ACPAS progenies and in 93% of MTFOR progenies. In ACPLA we observed a slightly larger variation in F_o among trees, as 78% of the progenies had greater than zero. Comparing F_m and F_o, the F_m was significantly lower than F_o in all populations, suggesting selection for heterozygous individuals from seedling to adult stages.

Mating system

The population outcrossing rate (t_m) was not significantly lower than unity (1) in the populations (*Table 2*). The mating among relatives rate ($t_m - t_s$) was significantly greater than zero in all populations (*Table 2*), but was signifi-

Table 4. – Paternity correlation and effective number of pollen donors among and within fruits in MTFOR population.

Progeny	$r_{p(w)}$ ±SD	$r_{p(a)}$ ±SD	$N_{ep(w)}$	$N_{ep(a)}$
1	0.08+0.49	0.07−0.02	13.2	13.9
2	0.81±0.23	0.08·0.00	1.2	13.0
3	0.52±0.35	0.17±0.06	1.9	5.8
4	1.00±0.01	0.10±0.01	1.0	9.6
5	0.40+0.25	0.25−0.08	2.5	4.0
6	0.94±0.22	0.15±0.05	1.1	6.6
7	0.44+0.40	0.07−0.02	2.2	14.3
8	0.35±0.24	0.18±0.04	2.9	5.4
9	0.31±0.38	0.11±0.04	3.2	8.8
10	0.35±0.14	0.04±0.02	2.8	24.4
11	0.16±0.10	0.08±0.04	6.2	12.2
12	0.08±0.44	0.08±0.02	13.3	12.3
13	0.13±0.37	0.05±0.01	7.8	22.2
14	0.23±0.25	0.29±0.06	4.4	3.5
Mean (95% CI)	0.41 (0.26-0.58)	0.18 (0.05-0.31)	2.4 (1.7-3.8)	5.6 (3.2-20.0)

$r_{p(w)}$ and $r_{p(a)}$ are the paternity correlation within and among fruits, respectively; $N_{ep(w)}$ and $N_{ep(a)}$ are the effective number of pollen donors within and among fruits, respectively; SD is the standard deviation; 95% CI is the 95% confidence interval.

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cantly higher in ACPLA (0.08) than ACPAS and ACPLA. At the level of seed trees, $t_m - t_s$ was significant higher than zero in 12 progeny from MTFOR (86%), six from ACPAS (75%) and all from ACPLA (100%), suggesting biparental inbreeding within progeny arrays (*Table 3*). The selfing correlation (r_s) was low and not significantly different among populations, suggesting low variation in individual outcrossing rate (*Table 2*). The paternity correlation within and among fruits (r_p) was significantly higher than zero in all the populations, revealing that progeny arrays have some full-sibs seedlings (*Table 2*). MTFOR natural population present significant lower r_p and higher effective number of pollen donors (N_{ep}) than ACPLA and ACPAS human altered populations. The r_p and N_{ep} were also quite variable among progenies in all the three populations (*Table 3*). The mean coancestry coefficient within progeny (Θ) was significantly lower and variance effective size significant higher in MTFOR than in ACPAS and ACPLA (*Table 2*). The coancestry within progeny (Θ) and variance effective size (N_e) were also quite variable in all the populations. The number of seed trees required for seed collection (m) to ensure that an effective population size of 150 would be retained in progeny arrays was slightly lower in MTFOR (54) than ACPAS (67) and ACPLA (68).

Correlated mating among and within fruits

The hierarchical paternity correlation in MTFOR (*Table 4*) was significant higher within ($r_{p(w)} = 0.41$) than among fruits ($r_{p(a)} = 0.18$). Consequently, the effective number of pollen donors was lower within fruits ($N_{ep(w)} = 2.4$) than among fruits ($N_{ep(a)} = 5.6$). At the level of seed trees, $r_{p(w)}$ and $r_{p(a)}$ were very variable among progenies ($r_{p(w)}$ ranged from 0.07 to 1.00; $r_{p(a)}$ ranged from 0.04 to 0.29), $r_{p(w)}$ and was significantly higher than zero in nine progenies as was $r_{p(a)}$ in all the 14 progenies. $N_{ep(w)}$ ranged among progeny from 1.0 to 13.3 among progenies and $N_{ep(a)}$ ranged from 3.5 to 24.4.

Discussion

Our results clearly showed human activity as spatial isolations of trees in pasture and plantation affect mating patterns in *B. excelsa* populations, as highest mating among relatives rate in plantation due greatest relatedness between reproductive trees and higher correlated matings and lower effective number of pollen donors fertilizing isolated trees and trees in plantation due the changes in population density of reproductive trees affecting the pollinators' foraging behaviour and altering pollen dispersal patterns. Consequently, the coancestry coefficient and effective size within family were also affected. We observed also individual variation in mating among relatives and correlated mating, as well as correlated mating variation within and among fruits. These results have strong implications for selection of seeds for conservation, breeding, and reforestation purposes.

Outcrossing rate and correlated mating

Our studied *B. excelsa* populations produced seeds predominantly by outcrossing, corroborating previous findings (O'Malley et al., 1988). We observed small variation in t_m among trees within populations (ranging from 0.94 to 1.0), suggesting that some degree of selfing may occur. However, the significant lower than 1.0 outcrossing rate probably occurred due the low number of seeds analyzed in same progenies. The lowest individual outcrossing rate (0.94) was observed in a progeny with only 11 seedlings. Furthermore, in a hand pollination experiment, Cavalcante et al. (2012) showed that self-pollinated flowers set no fruits. Thus, the species probably only produce seeds from outcrossing due self-incompatibility or inbreeding depression.

We hypothesized that selfing and paternity correlations would be higher in seed trees from pasture due to the absence of a forested matrix. The distance between remnant trees in a pasture may hamper the flying of pollinating bees from tree to tree, increasing geitonogamy and selfing and decreasing the number of pollen donors. Such increased selfing in pasture trees has been detected in other tropical trees (Fuchs et al., 2003; Moraes and Sebbenn, 2011; Breed et al., 2012). However, we observed no selfing in pasture trees, which indicates that a mechanism of self-incompatibility prevents inbreeding in *B. excelsa*, as was detected in a hand pollination experiment (Cavalcante et al., 2012). Additionally, because fruit set is heavily compromised in pasture trees, we believe that both the small assemblage of pollinating bees in this disturbed environment and their compro-

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mised forage behavior (visiting more flowers on the same tree) contribute to few fertilized flowers and high fruit abortion. In *B. excelsa*, fruit abortion may be a consequence of both post fertilization self-incompatibility and inbreeding depression. In fact, seeds from two of the sampled ACPAS seed trees did not germinate, perhaps due to inbreeding depression. As we genotyped seedlings, seeds that failed to germinate were not accounted for in our inbreeding estimates. The genetic load may be variable among *B. excelsa* populations Variation in germination, survival and growth traits has been observed in field trials of tree species (KÄRKKÄINEN et al., 1996; HARDNER and Potts, 1997; KOELEWIJN et al., 1999), showing that levels of genetic load vary among seed trees and populations. Furthermore, HUFFORD and HAMRICK (2003) clearly showed an increase in outcrossing rate between fertilization ($t_m = 0.79$) and seedling stages ($t_m = 0.91$) in the tropical tree *Platipodiun elegans*. Thus, our results suggest that population and individual variation in genetic load may be the cause of variation in outcrossing rates.

In contrast, our results did support the hypothesis of increased correlated matings in pasture and planting trees than in natural forest, as the number of pollen donors in pasture progenies was smaller than MTFOR. Differences in average distance among flowering trees may explain this variation amongst forest populations. While in MTFOR the average distance between trees was 16 m, in ACPAS it was 60 m. The long distance flight capacity of pollinating bees did not compensate for the advantageous higher density of flowering trees and thus larger neighboring pollinator area and number of pollen donors observed in the forest matrix (SANTOS and AISY, 2012). On the contrary, the number of pollen donors in the plantation was similar to ACPAS, even though the distance among trees in ACPLA was only 10 m. This apparently incoherent result is explained by the relatedness of trees in the plantation; seeds were probably collected from a small number of trees, which reduces the effective number of pollen donors and possibly the percentage of trees that flower every year. Higher estimates of correlated matings in isolated seed trees in pastures than in forest fragments or continuous forest has also been observed in *Paquira quinata* (FUCHS et al., 2003) and *Swietenia macrophylla* (BREED et al., 2012).

Correlated mating among and within fruits

Our results of the hierarchical paternity correlation showed that correlated matings were higher within fruits ($r_{p(w)} = 0.41$) than among fruits ($r_{p(a)} = 0.18$), indicating that on average, three pollen donors effectively fathered each fruit, but each seed tree progeny had six fathers. Thus, the probability of finding full-sibs is higher within fruits than among fruits of a given seed tree. This result is important to guide strategic seed collection, recommending the gathering of a larger number of fruits per tree as will be present in section *Recommendation for conservation and reforestation*. Our results are in line with other studies of mating system in insect pollinated tree species, where higher correlated mating within than among fruits have also been reported (SAMPSON, 1998; SILVA et al., 2011; FERES et al., 2012; MANOEL et al., 2015; TAMBARUSSI et al., 2015). However, the effective number of pollen donors varied from tree to tree and ranged from 1.0 to 13.3 within fruits and from 3.5 to 24.4 among fruits, indicating that some seed trees received pollen from fewer pollen donors than others, and that the probability of finding full-sibs within and among fruits was higher in some seed trees. *Bertholletia excelsa* is pollinated by large bees and pollen carryover from recently visited tree can be leading to many seeds within fruits having the same father and seeds from different fruits originating from different fathers (SURLES et al., 1990).

Mating among relatives and inbreeding

Mating among related trees ($t_m - t_s$) varied among populations, reaching the highest estimate in ACPLA. In general, natural populations of tree species present a particular spatial genetic structure (SGS) due to limited seed dispersal (DEGEN and SEBBENN, 2014). This leads to greater relatedness between closest trees, and may result in mating among relatives if related individuals flowering synchronous and pollinator vectors flying between near-neighbors cospecific individuals.

We also believe that in *B. excelsa* mating among relatives is the main cause of inbreeding in seedlings, as selfing is not common. Inbreeding was in higher in seedlings than in seed trees, suggesting selection for heterozygous individuals between seedlings to adult stages. This same phenomenon has been observed in

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other tree species (HUFFORD and HAMRICK, 2003; BITTENCOURT and SEBBENN, 2007) and seems to be a pattern in sessile long-lived plants such as *B. excelsa*. Inbreeding depression tends to eliminate homozygous individuals carrying deleterious alleles.

Recommendation for conservation and reforestation

The variance effective size (N_e) was higher in seeds collected from the natural population (2.75) than from pasture (2.15) and plantation (2.22). Consequently, more seed trees should be targeted for seed collection in pasture ($m = 67$) and plantation ($m = 68$) than in natural forest ($m = 54$), although these more disturbed sites will have a limited number of trees bearing fruits. The number of fruits sampled per tree is also important, as revealed by the higher correlated mating within than among fruits and highest biparental inbreeding within fruits. The number of fruits should be maximized and the number of seeds sampled per fruit should be minimized to decrease the relatedness among seeds and increase the effective size of the collected progeny array.

Despites the longstanding debate on the conservation value of remnant *B. excelsa* trees in pastures, this is the first study to date that addressed the mating system in this human altered environment. We also compared this situation with trees in a plantation and natural population. Our results support the hypothesis of partial self-incompatibility, which prevents self-fertilization but not mating among relatives. We believe that inbreeding depression is the major reason for similar rates of selfing and inbreeding observed in this human altered and the natural populations. We also hypothesized that distance between flowering trees and a forested matrix play important roles in promoting pollen flow in *B. excelsa*. To test this hypothesis, sampling pastures with varying densities of remnant trees would be necessary.

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PCR-based detection of single sequence variants from a natural collection of the non-model tree species European Aspen *Populus tremula* (L.)

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Abstract

In the present study we present and discuss the identification of species-specific SNPs to rule out any experimental influence of species-specific primer design (*Populus tremula* vs. the closely related model-species *Populus trichocarpa*) on the detectability of SNPs. Applying a species-optimized method, partial sequences of 14 genes involved in xylem cell development, xylogenesis, pectin formation, and drought stress reaction were analyzed at the genomic level. About 3 Mb of sequence information were generated by Sanger sequencing technology and 258 sequence variants were

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