

## ORIGINAL ARTICLE

# Pollen and seed dispersal of Brazil nut trees in the southwestern Brazilian Amazon

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## ABSTRACT

Pollen and seed dispersal patterns greatly influence the spatial distribution of plant genetic diversity. Microsatellite-based parentage analysis provides accurate estimates of contemporary gene dispersal. Although most tropical trees have been shown to exhibit widespread pollen dispersal, few studies have estimated contemporary gene dispersal after seedling establishment. *Bertholletia excelsa* (Lecythidaceae) is pollinated by large-bodied bees, while previous seed-tracking experiments suggest their seeds are mainly dispersed across very short distances by scatter-hoarding rodents, who primarily act as seed predators. Here we used parentage analysis to provide contemporary estimates of pollen and seed dispersal in *B. excelsa* recruits. We examined six 25-ha plots located in two natural stands in the Acre River valley, in the southwestern Brazilian Amazon. We used 11 microsatellite markers to estimate genetic diversity and fixation index parameters in adults, seedlings and saplings. Genetic diversity was moderate and did not differ across size classes or sampling locations. We assigned pollen and seed parents for < 20% of the recruits, indicating that most events of realized gene flow occurred beyond our 25-ha plots. Only 10 parentage assignments were confirmed with 80% confidence. Pollen distance ranged from 33 to 372 m and seed dispersal from 58 to 655 m. Actual seed-dispersal distances were far greater than the estimates obtained in previous seed-tracking experiments. Thus, studies encompassing larger sampling areas are necessary to determine a more representative spatial scale of *B. excelsa*'s pollen and seed dispersal capacity in natural stands.

**KEYWORDS:** *Bertholletia excelsa*, gene flow, genetic diversity, pollen flow, seed flow

## Dispersão de pólen e sementes da castanheira no sudoeste da Amazônia brasileira

### RESUMO

Os padrões de dispersão de pólen e sementes influenciam a distribuição espacial da diversidade genética. Muitas espécies arbóreas tropicais apresentam ampla dispersão de pólen, mas poucos estudos avaliaram fluxo gênico a partir de plântulas. *Bertholletia excelsa* (Lecythidaceae) é polinizada por abelhas e as sementes são dispersas por roedores do tipo *scatter-hoarders* (que estocam recursos em diferentes pontos de sua área de vida), que atuam primariamente como predadores de sementes. Experimentos de remoção de sementes tem mostrado que a dispersão de sementes por esses roedores é espacialmente limitada. Nosso objetivo foi obter estimativas de dispersão de pólen e sementes em *B. excelsa* a partir da análise de parentesco de regenerantes. Nós estudamos seis parcelas de 25 ha, em duas áreas de floresta nativa no vale do Rio Acre, no sudoeste da Amazônia brasileira. Parâmetros de diversidade genética e índice de fixação foram estimados em adultos, varetas e plântulas com 11 marcadores microsatélites. A diversidade genética foi moderada e não diferiu entre classes de tamanho ou entre localidades. A paternidade foi determinada em menos de 20% dos regenerantes, indicando que a maioria dos eventos de fluxo gênico ocorreu em distâncias maiores que as encontradas nas parcelas de 25 ha. As distâncias de pólen variaram de 33 a 372 m e as de dispersão de sementes variaram de 58 a 655 m. As distâncias de dispersão obtidas neste estudo excedem em muito as estimativas obtidas em experimentos de remoção de sementes. Estudos envolvendo áreas maiores são necessários para que possamos aprofundar nosso conhecimento sobre capacidade de dispersão de pólen e sementes em populações naturais de *B. excelsa*.

**PALAVRAS-CHAVE:** *Bertholletia excelsa*, fluxo gênico, diversidade genética, fluxo de pólen, fluxo de sementes

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## INTRODUCTION

In trees, patterns in pollen/seed dispersal and seedling establishment predominantly determine population genetic structure (Bacles *et al.* 2006). Pollen dispersal has fundamental evolutionary implications, because it significantly influences genetic processes such as gene exchange among spatially isolated populations (Wright 1946; Crawford 1984; Ennos 1994). Seed dispersal largely determines the spatial distribution of seeds and probability of seedling establishment, through the influence of density-dependent processes and other stochastic effects (Howe and Miriti 2004; Russo *et al.* 2006). Through its effects on recruitment patterns, seed dispersal is also shaping patterns of genetic structure and diversity within and between plant populations (García and Grivet 2011). For most tree species, pollen dispersal is more important than seed-mediated gene flow in terms of contribution to total gene movement (Hamrick and Nason 2000; Petit *et al.* 2005). Additionally, pollen-dispersal distances and population density tend to be negatively correlated, especially in animal-mediated pollination (Stacy *et al.* 1996; Degen *et al.* 2004; Dick *et al.* 2008).

Pollen flow patterns can be reliably characterized through paternity analysis, or progeny genotyping with microsatellites and other polymorphic molecular markers. Several paternity-analysis studies have demonstrated that most tropical trees exhibit widespread pollen dispersal (Ward *et al.* 2005), yet few studies have estimated contemporary gene dispersal after seedling establishment (Aldrich *et al.* 1998; Schnabel *et al.* 1998; Bacles *et al.* 2006; Burczyk *et al.* 2006; Martins *et al.* 2012; Baldoni *et al.* 2017). In fact, most estimates of seed-dispersal distances in tropical trees are based on seed- or fruit-removal experiments (Haugaasen *et al.* 2010; Haugaasen *et al.* 2012). Such studies, may underestimate actual dispersal distances, by failing to detect long-distance events (Haugaasen *et al.* 2012). Parentage analysis of established seedlings is a powerful strategy to provide more realistic estimates of contemporary seed dispersal (Burczyk *et al.* 2006; Martins *et al.* 2012). This technique can also estimate the relative contribution of pollen and seed movement to total gene flow.

The Brazil nut, *Bertholletia excelsa* Bonpl. (Lecythidaceae) is a large tree that occurs throughout *terra firme* forests in the Amazon basin and the Guianas (Prance 1990). Large-bodied bees capable of long-distance flight (e.g. *Eulaema mocsaryi* and *Xylocopa frontalis*) are the main flower pollinators (Cavalcante *et al.* 2012). *Bertholletia excelsa* seeds are produced predominantly by cross-pollination (O'Malley *et al.* 1988; Wadt *et al.* 2015). Fruit maturation takes 14 months, resulting in a hard, large round fruit (10–16 cm in diameter), containing 10–25 large seeds (Mori and Prance 1990). Fruits fall during the rainy season (November through January in the study region) (Evangelista *et al.* 2014), and seeds remain inside the fruit until extraction by predators capable of breaking

through the hard pericarp (Haugaasen *et al.* 2010). These seed predators include *B. excelsa*'s primary seed dispersers, the scatter-hoarding caviomorph rodents (Peres and Baidier 1997; Haugaasen *et al.* 2010) that consume and cache seeds. Seed-tracking experiments have demonstrated that dispersal distances average 5 m, and seeds are rarely buried beyond 20 m (Peres and Baidier 1997). Another fruit-tracking experiment found that rodents can move fruits 15–30 m from their original position (Haugaasen *et al.* 2012). Limited seed dispersal is a major determiner of spatial genetic structure (Hardy *et al.* 2006), resulting in seeds germinating close to the mother trees. However, adult *B. excelsa* populations exhibit a very weak spatial genetic structure across the species' natural range, suggesting that effective seed-dispersal distances might be greater than existing estimates (Sujii *et al.* 2015).

We, thus, hypothesized that seed dispersal by scatter-hoarding rodents reaches greater distances than those reported in seed- or fruit-tracking studies. Furthermore, we also hypothesized that pollen-dispersal distances are shorter in high-density stands. To test these hypotheses, we investigated two sites in the southwestern Brazilian Amazon that differ in adult density. We used 11 microsatellite loci to genotype recruits, juveniles and adults. We then performed parentage analysis to estimate pollen- and seed-dispersal distances in naturally regenerated *B. excelsa*. Finally, we compared genetic diversity and inbreeding among the recruits, juveniles and adults.

## MATERIAL AND METHODS

### Study sites and sampling

The two study sites were located in the Acre River valley of the Acre state (southwestern Brazilian Amazon): Seringal Filipinas (68°39'50"W, 10°47'06"S; within Extractive Reserve Chico Mendes) and Seringal Cachoeira (68°23'20"W, 10°50'07"S; an agroextractive settlement project - PAE Chico Mendes). The sites are separated by 30 km. Cachoeira possesses more reproductive trees per ha and fewer recruits than Filipinas, but both are considered demographically healthy (Wadt *et al.* 2008), despite historical *B. excelsa* nut harvesting.

Each site contained three permanent 9-ha (300 × 300 m) plots, separated by an average of 2.2 km in Cachoeira and 0.8 km in Filipinas. Effective sampling area changed depending on tree size. Seedlings and saplings (diameter at breast height, DBH < 10 cm) were sampled in 36 subplots (25 × 25 m each) per plot. Juveniles (10 ≤ DBH < 30 cm) were sampled throughout the entire 9-ha plot. To sample adults (DBH ≥ 30 cm with signs of previous or current reproduction), plot borders were extended to encompass a sampling area of 25 ha (500 × 500 m). All individuals were georeferenced (GPS 76CXS, Garmin). The DBH of juveniles and adults was measured. Leaves or vascular cambium were collected from all sampled individuals. Sampling was performed with permission

from the Ministry of Environment (SISBIO licence # 15413/1, 03/12/2008). Immediately after collection, the leaves were dried on silica gel and the vascular cambium disks were placed in microtubes containing transport buffer (0.6 mg of ascorbic acid, 300  $\mu$ L of CTAB buffer 2% (Doyle and Doyle 1987), and 700  $\mu$ L of absolute ethanol), on ice. Upon transport to the laboratory, the vascular cambium samples were stored at -20 °C and the dried leaves were stored in silica gel at room temperature (23 °C) until DNA extraction.

### Isolation and PCR of sample DNA

A tissue disrupter (Tissue Lyser, Qiagen, Hilden, Germany) was used for DNA isolation following CTAB 2% protocol (Doyle and Doyle 1987). Samples were quantified through visual comparison with  $\lambda$  DNA standards (Invitrogen, Thermo Fisher Scientific, Vilnius, Lithuania) after 1% agarose gel electrophoresis with EtBr staining. DNA samples were diluted to a final concentration of 2.5 ng $\cdot\mu$ l<sup>-1</sup>. We used 11 microsatellite loci: Bex 02, Bex 03, Bex 09, Bex 22, Bex 27, Bex 37 (Sujii *et al.* 2013); and Bes 14, Bes 13, Bes 16, Bes 18, and Bes 19 (Reis *et al.* 2009). The PCR reaction mixture (13  $\mu$ L) included 5.0 ng of genomic DNA, PCR buffer (10  $\mu$ M Tris-HCl, 50  $\mu$ M KCl), BSA (0.25 mg $\cdot$ mL<sup>-1</sup>), dNTPs (0.25 mM), forward/reverse primers (0.27  $\mu$ M), 1 U Taq DNA polymerase (Invitrogen, Thermo Fisher Scientific), and ultrapure water. Amplification was performed in a MJ 96+ thermocycler (Biocycler, Hangzhou, China) following published procedures Don *et al.* (1991). Amplified fragments were separated through 5% polyacrylamide gel and visualized with silver nitrate staining, in accordance with previously described protocol Creste *et al.* (2001). A10-bp ladder (Invitrogen, Thermo Fisher Scientific) was used as the size standard.

### Statistical analysis

Individuals were grouped in three size classes based on DBH: recruits (seedlings and saplings), juveniles and adults. For each class, the following parameters of genetic diversity and inbreeding were estimated in FSTAT (Goudet 1995): average number of alleles per locus ( $A$ ), allelic richness ( $R_S$ ) according to El Mousadik and Petit (1996), expected heterozygosity ( $H_E$ ), and inbreeding coefficient ( $F_{IS}$ ). Between-class variation in genetic diversity were analyzed with the Kruskal-Wallis H test in R (R Core Team 2015). Between-class divergence ( $\theta_p$ ) per site was also estimated. The 99% confidence intervals for both and were obtained through bootstrapping over loci.

Contemporary gene flow through pollen and seed dispersal was estimated with parentage analyses and maximum likelihood in CERVUS v. 3.0.7 (Kalinowski *et al.* 2007). Possible pollen donors and seed trees of all recruits (DBH  $\leq$  10 cm) per site were determined by genotyping with at least eight microsatellites. Because all experimental plots are situated within a continuous forest containing potential

parents outside plot borders and between plots, parentage was analyzed per plot. All sampled trees with DBH  $\geq$  50 cm in extended plots were considered candidate parents of same-plot recruits. Despite being reproductive and classified as adults, trees with DBH between 30 to 50 cm were excluded to yield more conservative parentage assignments.

Parentage confidence was obtained via simulation, specifically by calculating critical delta statistics with a confidence threshold of 80%. In our simulations, 90% of candidate parents and microsatellites were correctly assigned and genotyped while 1% of loci were mistyped. A given parentage assignment was confirmed via a comparison of actual delta statistics with the simulated critical delta. In Cachoeira, combined exclusionary power for parent pairs was  $8 \times 10^{-5}$ . The parentage analysis included only samples genotyped with eight or more loci, resulting in 95 candidate parents and 26 recruits. In Filipinas, combined exclusionary power was  $3.5 \times 10^{-4}$ ; 47 candidate parents and 64 recruits were tested.

Under the assumption of limited seed-dispersal distance, the nearest adult candidate matching a recruit was classified as the seed parent. This conservative approach has been successfully used for monoecious and hermaphroditic species with known short-distance seed dispersal (Bacles *et al.* 2006; Oddou-Muratorio and Klein 2008; Nakanishi *et al.* 2009; Sebbenn *et al.* 2011; Martins *et al.* 2012). Pollen dispersal was calculated as distance between parents of a given recruit, while seed dispersal was the distance between recruits and their seed trees.

## RESULTS

Adult density was almost threefold greater in Cachoeira (1.7 adults $\cdot$ ha<sup>-1</sup>) than in Filipinas (0.62 adults $\cdot$ ha<sup>-1</sup>), but the latter contained twice as many recruits as the former. Despite sample-size differences, allelic diversity ( $A$ ) was similar across size classes and sites (Kruskal-Wallis test,  $> 0.05$  in all comparisons) (Table 1). Overall  $R_S$  and  $H_E$  did not vary significantly between sites (Kruskal-Wallis test,  $p > 0.05$ ). Additionally, we found no evidence of inbreeding (Table 1). Size classes were more similar at Cachoeira ( $\theta_p = 0.023$ ; CI<sub>99%</sub> = -0.003, 0.081) than at Filipinas, where significant between-class differentiation was observed ( $\theta_p = 0.049$ ; CI<sub>99%</sub> = 0.008, 0.131).

Each site contained five parentage lines (both parents successfully assigned), representing 7.8% and 19.2% of the recruits in Filipinas and Cachoeira, respectively (Table 2). Parentage assignments involved a minimum of 10 loci, except one assignment in Cachoeira with eight. Assignment confidence was 95% in only one test (Table 3). Few parents were present inside the 25-ha plots, indicating most actual gene flow occurred at  $> 500$  m. Estimates of pollen- and seed-dispersal distances were very similar between the two sites (Table 3; pollen:  $H = 0.27$ ,  $df = 1$ ,  $p = 0.601$ ; seed:  $H = 2.45$ ,  $df = 1$ ,  $p = 0.117$ ). Mean pollen-dispersal distance was  $198.7 \pm 110.0$  m and mean seed-dispersal distance was  $263 \pm 188.2$  m.

**Table 1.** Estimates of genetic diversity and inbreeding in three *Bertholletia excelsa* DBH classes in two study sites in Acre (southwestern Brazilian Amazon), obtained with 11 SSR loci.  $N$  = sample size,  $k$  = total number of alleles,  $A$  = average number of alleles per locus,  $R_s$  = allele richness based on a minimum sample size of 6,  $H_E$  = expected heterozygosity,  $F_{IS}$  = inbreeding coefficient, SE = standard error,  $CI_{99\%}$  = 99% confidence interval of  $F_{IS}$  obtained by bootstraps over loci.

	Filipinas			Cachoeira		
	recruits	juveniles	adults	recruits	juveniles	adults
$N$	64	11	47	26	21	105
$k$	46	33	54	45	50	56
$A$ (SE)	4.18 (0.63)	3.00 (0.27)	4.91 (0.78)	4.09 (0.56)	4.55 (0.58)	5.09 (0.71)
$R_s$ (SE)	2.95 (0.36)	2.88 (0.26)	3.43 (0.40)	3.36 (0.39)	3.62 (0.36)	3.50 (0.34)
$H_E$ (SE)	0.490 (0.076)	0.541 (0.054)	0.571 (0.065)	0.563 (0.062)	0.618 (0.045)	0.581 (0.058)H
$F_{IS}$	-0.049	-0.093	-0.043	0.071	0.059	-0.053
$CI_{99\%}$	-0.020 – 0.069	-0.270 – 0.070	-0.197 – 0.112	-0.006 – 0.142	-0.072 – 0.198	-0.117 – 0.010

**Table 2.** Sample size, and number and proportion of confirmed parentages of *Bertholletia excelsa* in the Filipinas and Cachoeira study sites in Acre (southwestern Brazilian Amazon).  $N$  = number of individuals; candidates = trees genotyped with at least 6 loci.

Plot	Filipinas			Plot	Cachoeira		
	N adults/N candidates	N recruits	N confirmed parentages (%)		N adults/N candidates	N recruits	N confirmed parentages (%)
1	15/11	16	2 (12.5)	5	45/42	16	3 (18.8)
3	10/8	35	1 (0.03)	6	40/40	10	2 (20.0)
4	22/17	13	2 (15.5)	7*	-	-	-
Total	-	64	5 (7.8)	Total	-	26	5 (19.2)

\* all saplings were genotyped with less than 6 loci in plot 7

**Table 3.** Pollen and seed dispersal distances estimated for *Bertholletia excelsa* recruits (seedlings and saplings) in the Filipinas and Cachoeira study sites in Acre (southwestern Brazilian Amazon).

locality	plot	recruit ID	seed tree ID	pollen tree ID	confidence	seed distance (m)	pollen distance (m)
Filipinas	1	1P07/08	95	83	80%	271	245
Filipinas	1	1-2008-6	95	93	80%	439	82
Filipinas	3	3P19	447	534	80%	167	158
Filipinas	4	4-2010-4	393	391	80%	178	310
Filipinas	4	4X3-	356	372	80%	437	101
<b>Mean (SD)</b>						<b>298 (133.5)</b>	<b>179 (97.0)</b>
<b>Median</b>						<b>271</b>	<b>158</b>
<b>Range</b>						<b>167 - 439</b>	<b>82 - 310</b>
Cachoeira	5	5LV003	D13	244	95%	58	372
Cachoeira	5	5-2010-1	D9	D36	80%	145	261
Cachoeira	5	5-2010-2	D9	D5	80%	655	33
Cachoeira	6	6P3	N48	184	80%	115	275
Cachoeira	6	6-2008-5a	N20	N19	80%	165	150
<b>Mean (SD)</b>						<b>228 (242)</b>	<b>218 (130)</b>
<b>Median</b>						<b>145</b>	<b>261</b>
<b>Range</b>						<b>58 - 655</b>	<b>33 - 372</b>

## DISCUSSION

Our study revealed that *B. excelsa* seeds were dispersed beyond 500 m, a distance far greater than those estimated by previous estimates based on seed- and fruit-tracking experiments (Peres and Baidier 1997; Haugaasen *et al.* 2012). This outcome supports our hypothesis that seed dispersal agents (e.g., scatter-hoarders such as agoutis) are carrying seeds farther away than previously supposed. Similarly, parentage-based seed-dispersal distances (Martins *et al.* 2012) were far greater than estimates from seed-tracking experiments (Silva 2009) in *Carapa guianensis* (another large-seeded tree dependent on scatter-hoarder dispersers), even when the two experiments were conducted at the same site. Thus, at least for these two species, scatter-hoarder rodents are more effective at long-distance seed dispersal than previously thought.

We note that because the sampling area for candidate parents was 25 ha, and > 80% of recruits were not in the same plot as their parents, the estimate of pollen- and seed-dispersal distances was extremely conservative. In addition, plot size limited the maximum observable dispersal distance in this study, as did the small number of recruits, especially in Cachoeira. Given that sampling design and number of parentage assignments are highly influential in propagule-dispersal estimates, we recommend increasing sampling areas in future studies to better access the upper limit of *B. excelsa*'s seed-dispersal capacity in natural stands.

As Cachoeira contained three times more adults than Filipinas, we expected to assign more parents and observe smaller pollen-dispersal distances. Indeed, several studies have verified a negative correlation between mean distance among flowering trees and average pollen dispersal distance (Stacy *et al.* 1996; Degen *et al.* 2004; Carneiro *et al.* 2007; Lacerda *et al.* 2008). A recent parentage analysis in *B. excelsa* found that most pollination events occur between nearest neighbors (Baldoni *et al.* 2017), a pattern that reflects the expected foraging behavior of pollinators. In our populations, the mean distances between reproductive trees (~ 200 m in Filipinas and ~248 m in Cachoeira) matched average pollen-dispersal distance, but the low number of parentage assignments precluded statistical analysis. Given the lack of parentage for most recruits in our study, we believe pollinators are bypassing the nearest-neighbor trees and dispersing pollen at distances > 500m (maximum pairwise distance between adults in our plots). This phenomenon may occur owing to pollen carryover or asynchronous flowering (Dick *et al.* 2008) and has been observed in other low-density tree populations (Stacy *et al.* 1996; Martins *et al.* 2012).

Effective gene flow is crucial to maintaining genetic diversity in natural populations. Adults and recruits in our study had similar genetic diversity to what was observed in other *B. excelsa* populations studied in Acre (Santos *et al.* 2017) and across the Brazilian Amazon (Sujii *et al.* 2015).

Specifically, *B. excelsa* populations are less genetically diverse than other tropical trees (Azevedo *et al.* 2007; Cloutier *et al.* 2007; Raposo *et al.* 2007; André *et al.* 2008; Martins *et al.* 2012). This low genetic diversity has been confirmed in studies relying on other types of molecular markers (O'Malley *et al.* 1988; Kanashiro *et al.* 1997), suggesting that it is characteristic of this species. Moreover, the absence of inbreeding in our populations, along with the relative lack of fine-scale spatial genetic structure in natural stands (Sujii *et al.* 2015; Baldoni *et al.* 2017) are congruent with the high pollen and seed dispersal in *B. excelsa*.

## CONCLUSIONS

In this study, we used parentage analysis to provide accurate estimates of contemporary pollen- and seed-dispersal distances in natural *Bertholletia excelsa* populations. We demonstrated a high capacity of gene flow through both pollen and seed movement. Parentage analyses estimated longer seed-dispersal distances than did previous seed-tracking experiments. Indeed, most sampled recruits were not in the same plot as their parents. Based on these data, we recommend that future studies addressing contemporary gene dispersal in *B. excelsa* consider a larger sampling area.

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