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RESEARCH ARTICLE

Genetic structure of *Bertholletia excelsa* populations from the Amazon at different spatial scales

Patricia Sanae Sujii[1] · Karina Martins[2] · Lucia Helena de Oliveira Wadt[3] · Vânia Cristina Rennó Azevedo[4] · Vera Nisaka Solferini[1]

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Abstract Population genetic structure and genetic diversity levels are important issues to understand population dynamics and to guide forest management plans. The Brazil nut tree (*Bertholletia excelsa* Bonpl.) is an endemic species, widely distributed through Amazonian upland forests and also an important species for the local extractive economy. Our aim was to analyze the genetic structure of Brazil nut trees at both fine and large scales throughout the Amazon Basin, contributing to the knowledge base on this species and to generate information to support plans for its conservation. We genotyped individuals from nine sites distributed in five regions of the Brazilian Amazon using 11 microsatellite loci. We found an excess of heterozygotes in most populations, with significant negative inbreeding coefficients (*f*) for five of them and the fine-scale structure, when present, was very small. These results, as a consequence of self-incompatibility, indicate that conservation plans for *B. excelsa* must include the maintenance of genetic diversity within populations to ensure viable amounts of seeds for both economic purposes and for the local persistence of the species.

Keywords Genetic diversity · Brazil nut tree · SSR · Plant genetics · Gene flow

Introduction

The upland forests of the Amazon region extend from the Atlantic Ocean to the eastern slopes of the Andes, comprising an estimated area of 3,000,000 km^2. These forests are characterized by the presence of large canopy trees in areas untouched by floods (Sampaio 1942; Braga 1979; Tadaiesky et al. 2008), forming a mosaic of discontinuous environmental patches. Depending on the species mating system, gene flow between populations can be greatly restricted, favoring differentiation by processes of either natural selection or genetic drift. The evaluation of population genetic structure and genetic diversity levels are important to understand the effects of environmental fragmentation and its influence on population dynamics, helping to guide forest management and plant breeding programs (Erickson et al. 2004; Azevedo et al. 2007; Piotti et al. 2013; Leite et al. 2014). However, few studies have addressed the population genetic structure of Amazon rainforest tree species. The present work aims to fill in part of this gap by studying a species that occurs in upland areas.

The Brazil nut tree (*Bertholletia excels* Bonpl.) is endemic to the upland forests of the Amazon and is very important to the Amazonian extractive economy (IBGE/SIDRA 2010), since its seeds represent one of the main

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✉ Vera Nisaka Solferini
solferin@unicamp.br; veras@unicamp.br

[1] Department of Genetics, Evolution and Bioagents, Institute of Biology, University of Campinas - Unicamp, PO Box: 6109, Campinas, SP 13083-970, Brazil

[2] Departamento de Biologia, Centro de Ciências Humanas e Biológicas, Universidade Federal de São Carlos, Campus Sorocaba, Rodovia João Leme dos Santos Km 110, Sorocaba, SP, Brazil

[3] Embrapa Acre, BR 364 km 14, Caixa Postal 321, Rio Branco, AC, Brazil

[4] Laboratório de Genética Vegetal, Embrapa Recursos Genéticos e Biotecnologia, PqEB W5, Brasilia, DF, Brazil

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forest products in the states of Acre and Amapá. Besides the main trade for direct consumption, the nuts have recently been used by the cosmetics industry. The species is classified as vulnerable (A1acd + 2 cd) according to the *Red List of Threatened Species* (IUCN 2010), with habitat loss by deforestation as the main threat.

Some morphological and phenological differences are observed in Brazil nut trees from different regions of the Brazilian Amazon Basin. The flowering period was reported to occur during the dry season in the eastern Amazon and in the beginning of wet season in the southern Amazon (Maués 2002; Vieira et al. 2008). The fruit and seed yields also differ among trees from the western and southern Amazon (Wadt et al. 2005; Tonini et al. 2009). However, it is unclear whether these differences are genetically controlled and if there exists a degree of genetic differentiation among populations.

Despite its importance for both sustainable development and conservation strategies, a demographic study of this species (Peres et al. 2003) showed aging populations, with just a few or no juvenile trees, especially in central and eastern Amazonia. This suggests a history of intensive seed gathering that has caused a disjunction of the populations of this species. However, further studies found no evidence of overexploitation (Wadt et al. 2008; Scoles and Gribel 2011; Oliveira 2011), while others found high levels of juveniles in cultivated areas (Cotta et al. 2008; Paiva et al. 2011) in western Amazonia. These results, although contradictory, highlight the importance of studying this species across the Amazon Basin.

Aiming to contribute to a better understanding of the biology of *B. excelsa* and to generate information to support management plans for species conservation, we evaluated the genetic structure of natural populations throughout the Amazon Basin at local and regional scales. At the intrapopulation scale, this study addressed the fine-scale spatial structure and levels of inbreeding. At the large scale, comparing individuals from different populations, we tested if the species conforms to a stepping-stone model.

Materials and methods

Species

The Brazil nut tree, *B. excelsa* Bonpl. (Lecythidaceae), is the only species of the genus. Trees are found only in upland forests in the Amazon region and form groups called *castanhais*, where the trees are very common and the adults are randomly distributed (Mori and Prance 1990; Peres and Baider 1997; Wadt et al. 2005). At maturity, *B. excelsa* is a very tall, emergent tree, which can live up to 996 years according to radiocarbon dating (Vieira et al. 2005). The species is outcrossing, with hermaphrodite flowers that are pollinated mainly by medium or large bees from the Apidae and Anthophoridae families (Maués 2002; Cavalcante et al. 2012). Fruits fall below the crown and the seeds are dispersed mainly by agoutis (*Dasyprocta* sp.). These scatter-hoarding rodents open the fallen fruits and bury some nuts 5–10 m away from the collection site for later feeding but occasionally they may disperse seeds as far as 200 m (Paiva and Guedes 2008; Haugaasen et al. 2010). Seed predation by monkeys, squirrels, and parrots has also been observed (Trivedi et al. 2004); according to some authors, they can also act as seed dispersers (Peres and Baider 1997; Shanley and Medina 2005), although this has yet to be fully studied.

Sampling and DNA extraction

We sampled 378 adult trees of *B. excelsa* from nine sites in five regions of the Amazon. Sites were 2–2109 km apart (Fig. 3a). Region A is situated in the southwestern Brazilian Amazon, regions B and C are in the central area, and regions D and E are located in the north and northeast, respectively. Each site was named a priori as a population. Sampling was done in areas of native forest where local residents extract Brazil nuts. Three of the sites were on private property, while six others were conservation units for sustainable use (authorization SISBIO 16317-1). As the species is able to resprout (Paiva et al. 2011), at each site the samples were taken at least 10 m apart, to avoid collecting clonal material. From each sample site, 34–46 adult trees were collected and geo-referenced (Online resource 1).

A 2-cm-diameter disk of vascular cambium was collected from the trunk at a height of around 1.3 m from the ground. The cambium material was preserved in 1 ml of transport buffer (300 µl of CTAB buffer 2 %; 700 µl of absolute ethanol) kept between 4 and 10 °C until DNA extraction. The genomic DNA was extracted from the cambium disks using the 2 % CTAB procedure (Doyle and Doyle 1987).

Microsatellite genotyping

Individuals were genotyped with eleven microsatellite markers, seven of which were developed by Sujii et al. (2013), and four developed by Reis et al. (2009). Both the conditions of amplification reactions and the characterization of loci are as described previously (Sujii et al. 2013).

The microsatellite loci were amplified using primers marked with different fluorochromes and the fragments resolved with an ABI 3700 automated DNA analyzer (Applied Biosystems, Crescent City, CA, USA). The peaks of fluorescence were identified with GeneScan software (Applied Biosystems version 3.7, 2001, Crescent City, CA,

USA) and genotyping was performed with the program Genotyper (Applied Biosystems version 2.0, 1996, Crescent City, CA, USA).

Data analysis

Genetic diversity and fine-scale spatial genetic structure

The genetic diversity of each population was characterized using estimates of the average number of alleles per locus (A), allelic richness (R_S) (Petit et al. 1998), allele frequencies, and observed (H_o) and expected (H_e) heterozygosities. The inbreeding coefficient (f) was calculated as an estimator of Wright's coefficient of inbreeding (F_{IS}) (Weir and Cockerham 1984), with its significance evaluated by permutations. The strict Bonferroni correction (Holm 1979) was applied to achieve correct estimates of type I errors in evaluating the significance of the f estimates. These analyses were carried out using FSTAT software (Goudet 1995). Confidence intervals for f estimates were obtained by employing 1000 bootstrap replications using the software GDA 1.0 (Lewis and Zaykin 2001).

The fine-scale spatial genetic structure (SGS) for each population was analyzed based on the estimate of the pairwise kinship coefficient between pairs of individuals (F_{ij}), using the estimator of J. Nasson, as it weighs the allele contribution and is not biased by low frequency alleles (Loiselle et al. 1995). Average pairwise F_{ij} estimates were plotted against pairwise spatial distances, considering the distance classes of 50 or 100 m, depending on the population. For each distance interval, the standard deviation (SD) of the average F_{ij} estimates was calculated using the Jackknife method with 1000 replications of loci, which was also used to calculate the 95 % confidence interval of the pairwise kinship ($CI_{95\ \%} = F_{ij} \pm 1.96$ SE) for the null hypothesis of no genetic structure ($F_{ij} = 0$). The overall extent of SGS in each population was quantified by calculating $S_p = b{-}\log/(1{-}F_1)$, in which $b{-}\log$ is the slope of the linear regression between the pairwise kinship and the logarithm of spatial distance between pairs of individuals, and F_1 is the average pairwise kinship between all individuals in the first distance class, which includes all the neighboring pairs (Vekemans and Hardy 2004). The null hypothesis of absence of structure ($b{-}\log = 0$) was tested by the Mantel test and significance obtained by 1000 bootstrap replications. All computations were carried out using the SPAGeDi 1.2 program (Hardy and Vekemans 2002).

Population genetic structure

The genetic structure was characterized using θ as an estimator of Wright's F_{ST} (Weir and Cockerham 1984) between each pair of populations. Fisher's exact test and Bonferroni correction were also performed (Holm 1979) to test the significance of θ, using the FSTAT software (Goudet 1995).

Inferences of the population structure, the number of genetically homogeneous populations, and the assignment of the individuals in each population were done with the program Structure (Pritchard et al. 2000), which uses a Bayesian approach to analyze multi-locus genotype data. The model allows for genome mixing, the number of groups (K) varied from 1 to 12, for 600,000 replications with the first 100,000 being discarded (burnin), and 10 independent repetitions performed. For detection of the most probable number of genetically homogeneous populations, the descriptive statistics of Evanno et al. (2005) was used. This statistic is based on the rate of change in the probability of the data between successive K values. The K value, which best represents the structure of populations, may be identified by the peak value of ΔK.

The unbiased Nei's genetic distance (Nei 1978), which minimizes bias from small samples, was estimated for each pair of sampled populations. Cluster analysis using genetic distances was performed using UPGMA (Sneath and Soakal 1973) and the neighbor-joining method (Saitou and Nei 1987). The consistency of the nodes was evaluated with 1000 bootstrap replicates. The degree of fit of each tree to the matrix of genetic distances was quantified by the proportion of variation in the genetic distance matrix that is explained by the tree (R^2), using the TreeFit software (Kalinowski 2009).

The Analysis of molecular variance (AMOVA) within and between groups was performed in Arlequin 3.5 (Excoffier et al. 2005). The groups were based on the assignment test results from the Structure program (Pritchard et al. 2000).

The correlation between the pairwise Rousset's genetic distances (Rousset 1997) and the geographic distances of populations was evaluated using the Mantel test with 1000 permutations, using the program IBD (Bohonak 2002). This program generates estimates of F_{ST} between all pairs of populations using Weir's (1990) estimator and converts these to Rousset's (1997) distance $F_{ST}/(1{-}F_{ST})$. The geographic distance was log transformed to test a two-dimensional stepping-stone model fit (Rousset 1997).

Results

Genetic diversity and fine-scale SGS

All populations had moderate levels of genetic diversity (Table 1). The populations of region D (at the north of the Amazon basin) possessed the greatest allelic richness. The

Table 1 Estimates of genetic diversity in populations of *B. excelsa*, obtained with 11 microsatellite markers

Population	*N*	Number of alleles	*A*	R_S	H_e	H_o	*f*
A1	46	69	5.91 (±0.868)	4.36 (±0.819)	0.649 (±0.062)	0.677 (±0.080)	-0.043^{ns} [−0.201; 0.119]
A2	46	72	6.18 (±0.952)	3.88 (±0.846)	0.641 (±0.067)	0.645 (±0.087)	-0.005^{ns} [−0.190; 0.172]
B1	35	40	3.36 (±0.338)	3.00 (±0.311)	0.551 (±0.017)	0.809 (±0.072)	−0.486* [−0.704; −0.175]
B2	35	50	4.18 (±0.4469)	3.77 (±0.438)	0.581 (±0.031)	0.802 (±0.068)	−0.427* [−0.643; −0.102]
C	46	52	4.36 (±0.312)	3.84 (± 0.257)	0.573 (± 0.024)	0.775 (± 0.085)	−0.328* [−0.555; −0.088]
D1	45	65	5.55 (±0.623)	5.30 (±0.544)	0.672 (±0.040)	0.712 (±0.056)	-0.062^{ns} [−0.150; 0.044]
D2	46	67	5.73 (±0.0702)	5.26 (±0.639)	0.677 (±0.039)	0.691 (±0.038)	-0.036^{ns} [−0.116; 0.064]
E 1	34	59	5.00 (±0.884)	3.80 (±0.797)	0.612 (±0.064)	0.736 (±0.073)	−0.245* [−0.427; −0.021]
E2	46	65	5.55 (±0.867)	3.84 (±0.738)	0.598 (±0.043)	0.700 (±0.057)	−0.188* [−0.359; −0.004]

Sample size, *N;* average number of alleles per locus, *A*; allelic richness based on a sample size of 23 individuals, R_S; observed heterozygosity, H_o; and expected heterozygosity, H_e (with respective standard errors); and inbreeding coefficient, *f* (with confidence intervals)

ns not significant

* Significant ($p < 0.001$)

inbreeding coefficient was significantly negative in five populations, indicating an excess of heterozygotes (Table 1).

A significant fine-scale SGS was observed in the A1 population for the first distance class (Fig. 1), although there were also significant overall SGS in populations, A2, D1, D2, and E1, in which the values of S_p varied between 0.012 and 0.033.

Population genetic structure

Private alleles with frequencies higher than 5 % were found in at least one population from each region, and after grouping the samples by region the number of private alleles was even higher (Table 2), indicating that populations from the same region share alleles not found in other regions (Table 3). The estimates of pairwise θ were high (0.10–0.25) and significant ($p < 0.001$) between populations of different regions (Table 3), and populations from the same region showed very low levels of differentiation ($\theta \leq 0.014$).

For the population assignment analysis, the best support was for four groups, since $K = 4$, where the ΔK was the highest ($\Delta K_4 = 42.6$), with a low likelihood variance in each replication and little variation between different replicates. However, as for $K = 2$ and $K = 5$, the ΔK values were higher than the others ($\Delta K_2 = 21.3$; $\Delta K_5 = 17.1$).

When samples were organized into two groups, one comprising the individuals from region A and the other, the remaining regions, the samples from region E showed some admixture with region A (Fig. 2). When $K = 4$, one group was composed of the populations from region A, the second comprised B and C, and the last two were formed by samples from regions D and E. For $K = 5$ (Fig. 2), each group comprised the individuals from each region. In all cases, in spite of signs of gene flow between groups, no individual was assigned to a group different from the sample origin.

The dendrograms obtained by the UPGMA and the neighbor-joining methods show four main groups (Fig. 3): (i) region A, in the west of the Amazon basin; (ii) regions B and C, in the center; (iii) region D in the north; and (iv) region E in the northeast. The two methods provided slightly different topologies, and the neighbor-joining algorithm produced a best-fit tree ($R^2 = 0.979$) than the UPGMA ($R^2 = 0.756$). In both dendrograms, group A is more distant from the others and groups B and C are closely related.

The AMOVA was performed considering four groups of populations, according to the population assignment test and the dendrograms. This analysis showed that 86.1 % of the total variation was within groups and 12.06 % was among groups ($p < 0.01$) (Table 4).

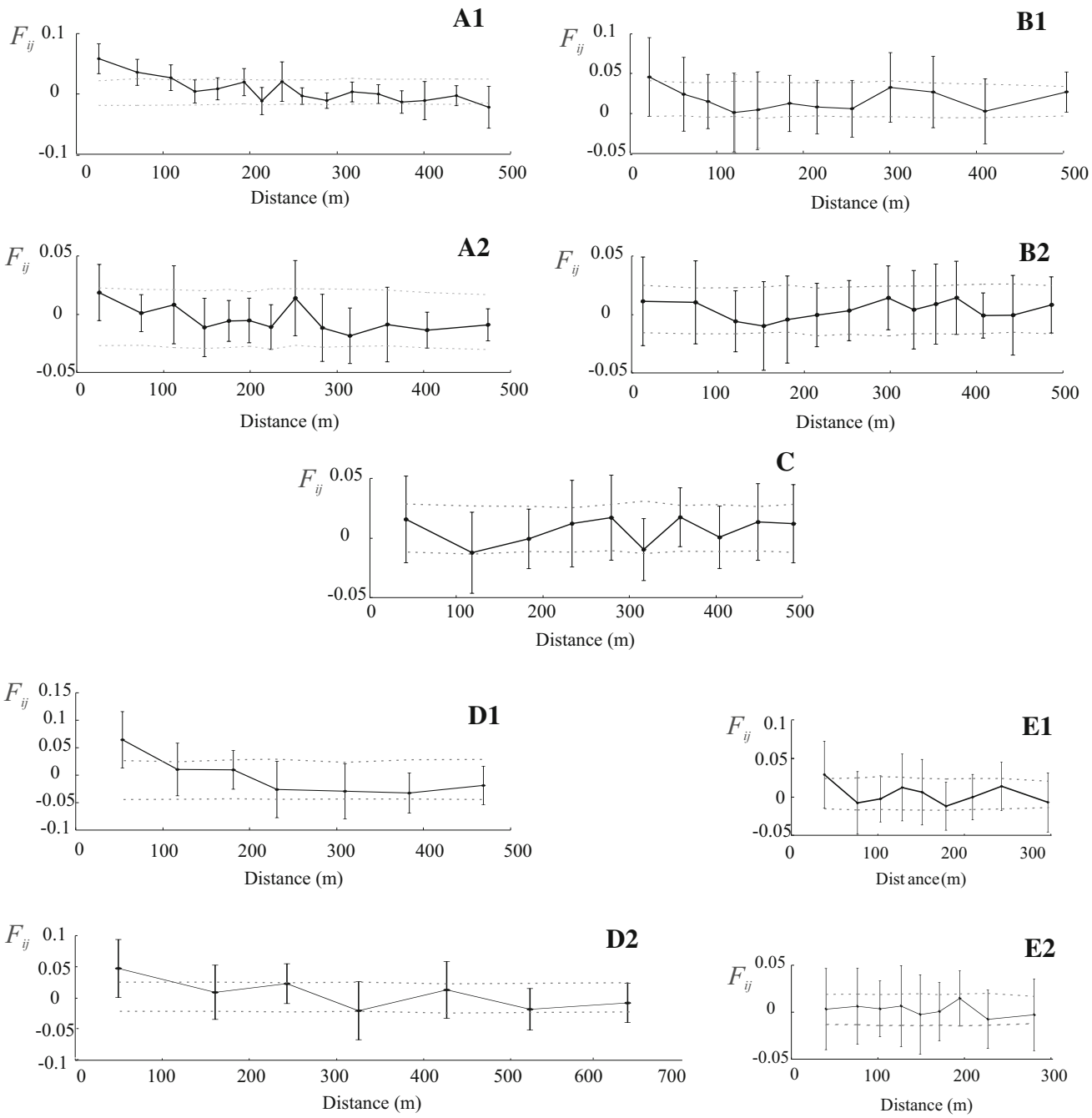

Fig. 1 Average pairwise relationship (F_{ij}) over the closest distance intervals (<700 m) for each *B. excelsa* population; bars indicate the 95 % confidence interval of (F_{ij}) and trajectory lines indicate critical values of rejection ($CV_{95\ \%}$) of the null hypothesis of absence of spatial genetic structure

As all the previous results grouped samples from B1 and B2 as well as samples from D1 and D2 and E1 and E2, they were treated as populations B, D, and E for the Mantel test, which did not show a significant correlation between pairwise genetic and geographic distances ($r = 0.38$; $p = 0.11$) and between pairwise genetic and log-transformed geographic distances ($r = 0.53$; $p = 0.09$) (Fig. 4).

Discussion

Genetic diversity and fine-scale SGS

The average number of alleles per locus was lower than usually reported in microsatellite studies of tree species, with similar sample sizes (Collevatti et al. 2001; Bittencourt and Sebbenn 2009; Bizoux et al. 2009).

Table 2 Total numbers of private alleles in each population (AP_S) and in each region (AP_r)

Population	AP_s	Region	AP_r
A1	1	A	6
A2	–		
B1	–	B	3
B2	3		
C	1	C	1
D1	1	D	5
D2	–		
E1	–	E	4
E2	2		

Compared to other Amazonian tree species, the H_o values of the present study were similarly high and the H_e values were a little lower (Azevedo et al. 2007; Lacerda et al. 2008; Le Guen et al. 2009). The hypothesis of no inbreeding cannot be discarded as values of f were negative in all the populations. Our results provide good support for the reports of self-incompatibility mechanisms in *B. excelsa* (Müller et al. 1980; O'Malley et al. 1988; Schifino-Wittmann and Dall'Agnol 2002; Cavalcante 2008). As self-incompatibility contributes to the maintenance of genetic diversity within populations, it has to be considered in *B. excelsa* management and conservation plans.

Self-incompatibility and selection in favor of heterozygotes are described for many tree species (Hansson and Westerberg 2002; Balloux 2004; Hufford and Hamrick 2003). Since no self-fertilization occurs (Silva 2014), and the rate of geitonogamy is very low (Cavalcante et al. 2012), it is important to maintain intrapopulation genetic variability to avoid deficits in fertility and a consequent reduction in seed set. Brazilian law prohibits timber extraction from Brazil nut trees, but the intense harvesting of seeds can cause low recruitment (Peres et al. 2003). Therefore, it is advisable to leave some nuts in the forest and manage seedling placement to enhance genetic diversity and ensure suitable amounts of seeds for both economic purposes and for the local persistence of the species.

Table 3 Estimates of θ (above the diagonal) and non-biased Nei's genetic distance (Nei 1978) (below the diagonal) for each pair of populations

θ / D	A1	A2	B1	B2	C	D1	D2	E1	E2
A1		**0.014**	**0.235**	**0.210**	**0.244**	**0.150**	**0.154**	**0.154**	**0.160**
A2	0.026		**0.209**	**0.179**	**0.228**	**0.125**	**0.137**	**0.135**	**0.145**
B1	0.616	0.488		0.005	**0.058**	**0.135**	**0.139**	**0.151**	**0.172**
B2	0.551	0.415	0		**0.071**	**0.112**	**0.121**	**0.129**	**0.149**
C	0.698	0.600	0.076	0.102		**0.146**	**0.133**	**0.179**	**0.201**
D1	0.418	0.315	0.270	0.230	0.319		0.007	**0.089**	**0.107**
D2	0.442	0.366	0.286	0.260	0.283	0.013		**0.102**	**0.120**
E1	0.372	0.303	0.227	0.241	0.371	0.189	0.228		0.002
E2	0.376	0.322	0.325	0.285	0.434	0.228	0.268	0	

For θ, bold Significant ($p < 0.001$)

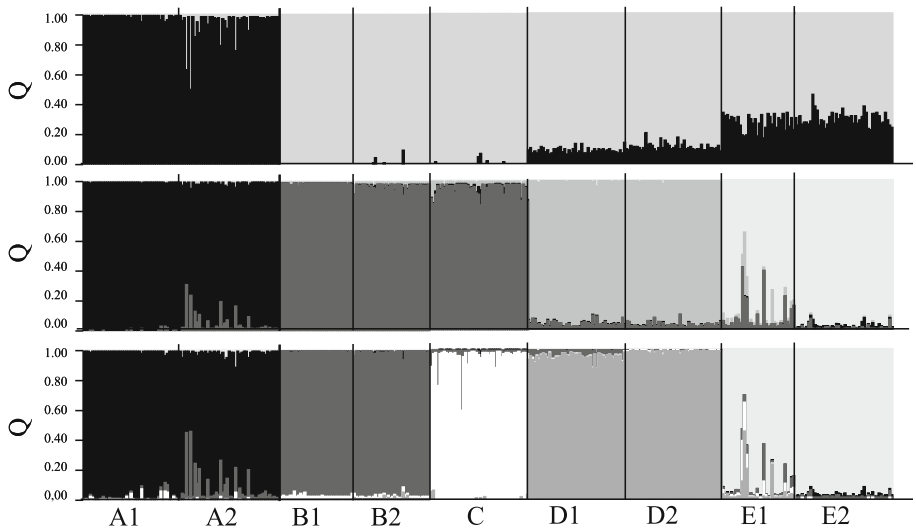

Fig. 2 Results from the population assignment test in structure, with samples ordered by population: **a** $K = 2$ (*top*); **b** $K = 4$ (*middle*), and **c** $K = 5$ (*bottom*)

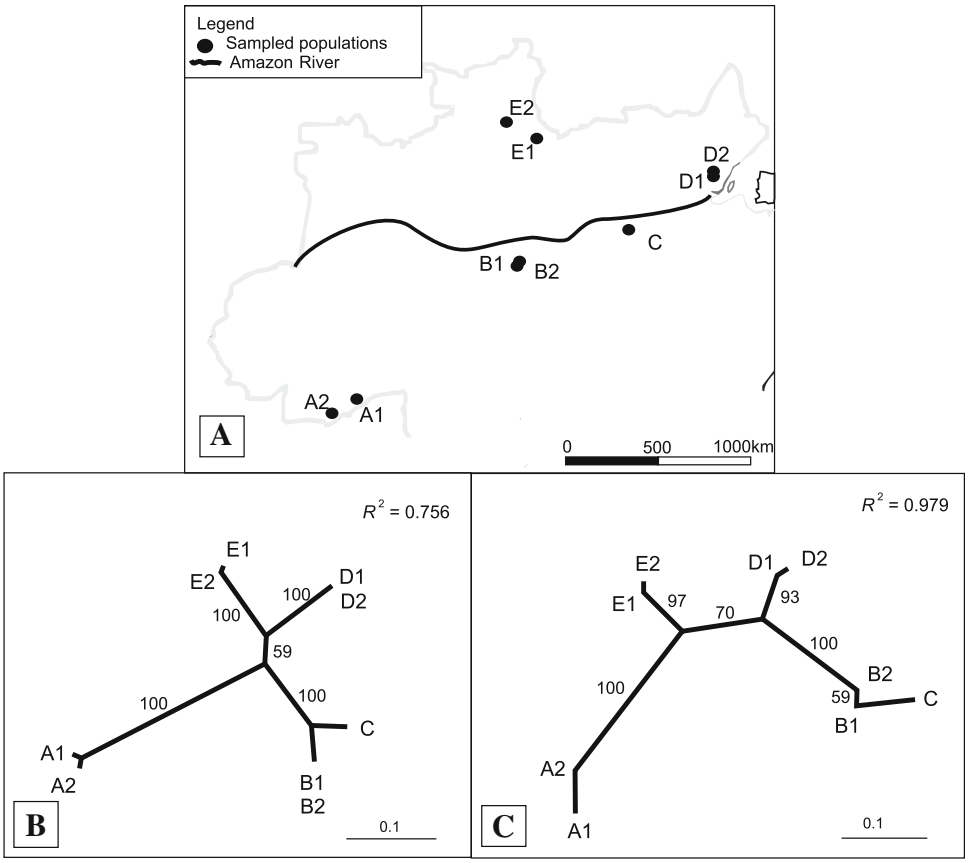

Fig. 3 **a** Sampled populations, **b** unrooted UPGMA dendrogram, and **c** unrooted neighbor-joining dendrogram generated from the genetic distances, with bootstrap values. R^2 degree of fit of the tree to the matrix of genetic distances

Table 4 Analysis of molecular variance (AMOVA) for populations organized in four groups (eastern, northern, central, western Amazon)

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation
Among groups	3	215.322	0.34421	12.06
Among populations within groups	5	34.007	0.05242	1.84
Within groups	749	1839.856	2.45642	86.10
Total	754	2089.186	2.85305	

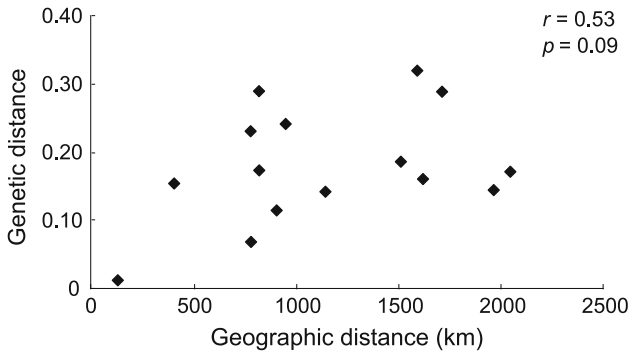

Fig. 4 Rousset's genetic distance as a function of the geographic distance between population pairs

The area corresponding to region B seems to have been greatly affected by past environmental changes (online resource 2) and its samples showed the lowest number of alleles, allelic richness, and private alleles, which may be an indication of population expansion or contraction. The distribution of several groups of organisms, such as birds and butterflies, indicates that Pleistocene climate changes influenced the present diversity of the Amazon forest (Haffer 1969; Vanzolini and Williams 1970). Our sample design was not suited to test the refugia hypothesis, even though our results for *B. excelsa* indicate that this is a topic that deserves attention.

Analysis of overall fine-scale SGS showed a small structuring for five populations (A1, A2, D1, D2 and E1),

with the highest pairwise relationship observed between individuals up to 100 m apart. The fine-scale SGS was similar to that found for other self-incompatible tree species, with pollen and seeds dispersed by animals (Vekemans and Hardy 2004). The fine-scale SGS observed in these five populations can be explained by short-distance dispersal of Brazil nut seeds. As the fruits fall from the apical branches of a crown that can reach up to 35 m in diameter (Haugaasen et al. 2010), seed dispersal is generally restricted to a few dozen meters, resulting in spatial aggregation of relatives. On the other hand, animals can carry and bury seeds over hundreds of meters (Peres and Baider 1997; Paiva and Guedes 2008) and pollinator bees can fly for hundreds or thousands of meters (Janzen 1971; Silva 2014), which explains the small F_{ij} estimates and the absence of spatial structure for the other populations. No comparisons of differential rates of gene flow by pollen and seed dispersal have been published so far.

Population genetic structure

Some authors have considered the role of humans in the distribution of Brazil nut trees throughout the Amazon, interpreting the current populations of Brazil nut trees (*castanhais*) as orchards created by pre-Colombian Indians (Clement et al. 2010; Shepard and Ramirez 2011). Our results suggest that the small-scale genetic structure of *B. excelsa* can be explained by seed and pollen dispersal; however, the structure throughout the Amazon River basin may have deeper and more complex causes. Nearby populations ($d < 200$ km) show no differentiation, with the exception of the low structuring between A1 and A2 ($d = 130$ km). This pattern of similarity between closest populations may be influenced by the behavior of pollinating bees. Although these medium- and large-sized bees can visit many trees every day and fly long distances, they tend to forage in more restricted areas when there are plenty of flowering trees, favoring short-distance pollen dispersal (Janzen 1971).

For populations separated by 700–820 km, on the other hand, we found varying degrees of structuring, ranging from very high differentiation ($\theta = 0.235$) to moderate ($\theta = 0.107$) and also low values ($\theta = 0.058$). Populations from region A have greatly diverged from the other regions (Figs. 2, 3; Table 3), probably due to their longest distance from the others and the distinct climate characteristics observed in the southern part of the Amazon forest (Sombroek 2001). The moderate to small values of population structuring (θ) observed for populations from the other regions (B, C, D, E) are similar to those obtained in studies with other long-lived, outcrossing, and widely distributed tree species (Hamrick and Godt 1996; Avise and Hamrick 1996; Lemes et al. 2003; Le Guen et al. 2009). However, we cannot exclude the possibility of an insufficient divergence time for the differentiation of close populations recently subdivided by human activities, as the Amazon has been a continuous forest for 1000 of years. Also, we cannot exclude the role of pre-Colombian Indians as "seed dispersers", as previously mentioned. Considering that the individuals were clustered in four groups corresponding to their sample region (Fig. 3), there is no evidence of a stepping-stone model fit (Fig. 4) and with respect to the reports of morphological and phenological differences among *B. excelsa* populations, our data suggest that local differentiation has taken place, which can include demographic and adaptation processes. The relative contribution of landscape, adaptation, gene flow, and genetic drift for this pattern merits further investigation. Finally, we also recommend that conservation strategies should consider the small- and large-scale population genetic structure across the Brazilian Amazon Basin.

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