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Spatial genetic structure in natural populations of *Caryocar brasiliense* Camb. (Caryocareceae) in the North of Minas Gerais, Brazil

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A B S T R A C T

Caryocar brasiliense is a widely distributed but endangered Brazilian Cerrado tree species. We studied patterns of spatial genetic structure and populational differentiation from four natural populations in the North of the State of Minas Gerais, Brazil. Genetic diversity from 240 individuals using allozyme loci was analyzed and inferred by heterozygosity index, Nason's kinship coefficient and assessment of historical population bottlenecks. We did not find evidence of inbreeding within fragments, but from the fitting tests into the infinite allele model of mutation no population was shown to be in equilibrium, indicating recent bottlenecks. Only one population (Japonvar) showed positive coancestry value indicating the tendency of increased structure among trees that are spatially closer to each other. Although the average genetic differentiation in each population pair was generally low ($D_G = 0.036$), Mantel tests showed tendency for distance dependence of genetic structure ($r_m = 0.905$, $P = 0.084$). Fragmentation may cause genetic isolation by next generations, increasing differentiation among populations. Thus, considering the practically irreversible fragmentation of populations around agricultural areas at *Cerrado* Biome, landscape management strategies for *C. brasiliense* should be considered to protect the still existent populations.

1. Introduction

Brazilian Cerrado, a woody savanna vegetation, covers an area of almost 2 million km^2 and is the second most extensive biome in South America, only exceeded by the Amazon rain forest. Currently, it is reduced to only 20% of the original area (Myers et al., 2000), due to the expansion of agricultural frontier in the last 35 years. Recently, it was classified as a hotspot for conservation priority because of its rich biodiversity, with many endemic plants and animals, and also because they are among the most endangered eco-regions on Earth (Myers et al., 2000). The vegetation is composed of grasses with relatively shallow roots and deeply rooted evergreen and deciduous woody plants, growing in oligotrophic soils and subject to frequent fires (Bucci et al., 2005). Many of the plant species found in the region possess a strong economic importance and have been utilized unsustainably by local human populations, adding a strong exploitation component that has accelerated loss of genetic diversity by habitat destruction. Nonetheless, the interest in studying genetic structure in plant species populations of the Cerrado biome is recent, as most of the works have been published in the present decade (Collevatti et al., 2001, 2003; Lacerda et al., 2001; Zucchi et al., 2003; Telles et al., 2006; Martins et al., 2006; Ramos et al., 2007).

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The conservation of the habitat biodiversity within the fragments depends on the particular capacity of the species to survive genetic bottlenecks and stochastic events. Thus genetic conservation studies play an important role as they interpret the survival mechanisms of populations threatened by the ecosystem fragmentation (Templeton et al., 1990). For this, it is necessary to access the genetic diversity and structure in the natural populations and test for associations with various characteristics of the environment or the species. Studies at landscape-level scales provide an insight into micro-evolutionary patterns by elucidating the movement of genes at a range of spatial scale (Storfer et al., 2007). At landscape-level scale between natural populations, genetic structure has been attributed to historical factors and isolation by distance (Wright, 1943). Within-population genetic structure, in addition to the ecological and evolutionary processes that affect spatial distribution patterns (e.g. seed dispersal, intra- and interspecific competition and environmental heterogeneity), significant spatial genetic structure is primarily influenced by limited gene movement via pollen and seed dispersal, local genetic drift, inbreeding and selection favoring the same or different genotypes (Vekemans and Hardy, 2004).

The aim of this study was to verify the patterns of genetic differentiation between natural populations of *Caryocar brasiliense*, a widely distributed but endangered Brazilian Cerrado tree species. *C. brasiliense* is pollinated by small territorial bat species with low flight ranges (Gribel and Hay, 1993) and the great majority of seeds are dispersed by gravity, although some seeds may be dispersed by the greater-rhea (*Rhea americana*) (Collevatti et al., 2003). We used allozyme markers, that have been successfully used to address the questions about spatial genetic structuring at landscape-level (Vieira and Carvalho, 2008; Mora-Vicente et al., 2009) and within-population genetic structure (Trapnell et al., 2008; Tarazi et al., 2009). We focused on the following questions: (1) Is there the association between genetic and geographic distances among populations? (2) What are the levels and spatial scale of relatedness in *C. brasiliense* across landscape-level and within-population? (3) Is there evidence of recent bottlenecks in the studied population that could have resulted from ancient forest fragmentation due to anthropogenic disturbance? We hypothesized that patterns of genetic variation in populations should reflect the expectation for a typically outcrossing species, namely high levels of genetic variation within and relatively low levels of differentiation between natural populations. If seed dispersal and pollen movement is widespread relative to the distance between populations the result should be an undetected spatial genetic structure by isolation by distance model.

2. Materials and methods

2.1. Study species and sampled site

Caryocar brasiliense is an endangered Brazilian Cerrado tree species classified as one of the most important and useful plant species from the Cerrado due to its high ecological and economic importance. The seeds are surrounded by a woody endocarp coated with a yellow fleshy mesocarp rich in oil and vitamin A, and are eaten by several wild animals. *C. brasiliense* is completely outcrossed, with hermaphroditic flowers pollinated by small glossophagine bats (Collevatti et al., 2001; Gribel and Hay, 1993). Despite its ecological and economic importance, Cerrado fragmentation and the higher frequency of fire due to agricultural practices increased by the intense commerce of its fruits have been affecting recruitment, population size and dynamics of this species (Araújo, 1994; Collevatti et al., 2001).

Two hundred and forty individuals from four natural populations of *C. brasiliense* were sampled in the North of Minas Gerais (Table 1). The ‘Francisco Sá’ population possesses less evidence of anthropogenic disturbance than the other populations. Despite such ‘status’ of conservation, this population has been fragmented due to agricultural expansion, resulting in an ‘island’ surrounded by an ‘ocean’ of crops and *Eucalyptus* sp. and *Pinus* sp. plantations. All the other populations are highly fragmented and disturbed areas only with isolated remnant individuals in a pasture (*Brachiaria* spp.) for livestock rearing. Furthermore, these populations possess a strong economic importance and have been used unsustainably by local human populations. In each of these populations 60 individual adults were sampled; expanded leaves were collected and stored at −80 °C. The spatial location of the trees was determined by the use of GPS equipment.

2.2. Enzyme extraction and electrophoresis

Leaf samples were cut and crushed with a mortar and pestle in a phosphate-polyvinylpyrrolidone extraction buffer. Discontinuous system vertical allozyme electrophoresis in polyacrylamide gel was performed using 7.5% page gels and carried out at 4 °C over 210 min (at 80 mA and 300 V). Ten enzyme systems showed banding patterns that could be reliably scored.

Table 1
Levels of genetic diversity and characterization of the spatial genetic structure (SGS) of *Caryocar brasiliense* in four natural populations. n, sampled individuals; $\hat{H}_o$, observed heterozygosity; $\hat{H}_e$, Nei’s gene diversity; f, mean fixation index; F_{ij}, kinship coefficient for first distance class; Sp, extent of SGS. SD, standard deviations; CI, confidence intervals, $^*P < 0.05$ (probability level of 5%).

Populations	Longitude/latitude	n	$\hat{H}_o$ (SD)	$\hat{H}_e$ (SD)	f (CI)	F_{ij}	Sp
Japonvar	16°00′25″S/44°16′26″W	60	0.739 (0.031)	0.522 (0.009)	−0.421*[−0.5; −0.2]	0.014	0.0082*
Montes Claros	16°52′55″S/43°59′50″W	60	0.817 (0.017)	0.530 (0.005)	−0.548*[−0.6; −0.4]	0.007	0.0005
Francisco Sá	16°19′17″S/43°18′49″W	60	0.583 (0.039)	0.450 (0.011)	−0.299*[−0.3; −0.1]	0.008	0.0014
Bocaiúva	17°07′59″S/43°53′47″W	60	0.743 (0.034)	0.495 (0.008)	−0.5088*[−0.6; −0.3]	0.009	0.0029

These enzymes were alcohol dehydrogenase (E.C.1.1.1.1, *locus Adh*), β-esterase (EC 3.1.1.1, *locus* β-*Est*), β-galactose dehydrogenase (E.C.1.1.1.48, *locus Gldh*), glutamate dehydrogenase (E.C.1.4.1.3, *locus Gtdh*), isocitrate dehydrogenase (E.C.1.1.1.42, *locus Idh*), malate dehydrogenase (E.C.1.1.1.37, *locus Mdh*), malic enzyme (E.C.1.1.1.40, *locus Me*), peroxidase (E.C.1.11.1.7, *locus Per*), sorbitol dehydrogenase (E.C.1.1.1.14, *locus Sdh*) and shikimate dehydrogenase (E.C.1.1.1.25, *locus* Skdh). Staining protocols and the genetic basis of allozyme banding patterns were inferred from segregation patterns with reference to typical subunit structure and conceptual methods (Wendel and Weeden, 1989).

2.3. Data analysis

Potential linkage disequilibrium between allozyme loci was tested in populations using FSTAT version 2.9.3.2 (Goudet, 2002) with the significance level corrected for multiple comparisons (Rice, 1989). The following genetic diversity parameters were estimated using the program FSTAT: proportion of polymorphic loci (P_L; 0.95 criterion), mean number of alleles per locus (A), observed heterozygosity ($\hat{H}_o$) and Nei's gene diversity ($\hat{H}_e$). An estimate of inbreeding levels was obtained using Wright's fixation index ($f = 1 - \hat{H}_o/\hat{H}_e$). Significance of f was assessed through estimation of the 95% confidence intervals (CI) after 1000 bootstraps. We used the BOTTLENECK 1.2.02 program (Cornuet and Luikart, 1996) to test for significant recent decreases in $\hat{N}_e$, based on the principle that populations that have gone through a severe and recent genetic bottleneck show a faster reduction in the number of alleles than in the $\hat{H}_e$ (Luikart et al., 1998). All enzyme loci are assumed to fit an infinite allele model of mutation (IAM) (Kimura and Crow, 1964). The significance was assessed using the Wilcoxon signed rank test, based on 1000 replications.

We used the genetic distance of Gregorius (D_G) instead of F_{ST} statistics to quantify the difference between the populations because F_{ST} has been proven to be a weak measure of genetic differences (Hedrick, 2005). To test for isolation by distance, pairwise ($D_G/1 - D_G$) matrices were related to geographical distances between populations. Mantel tests were used to test for significance (1000 permutations) with the software TFPGA version 1.3 (Miller, 1997). Spatial genetic structure within populations was further analyzed using Nason's kinship coefficient (Loiselle et al., 1995). This coefficient can estimate between pairs of mapped individuals x and y a ratio of probability differences of identity-in-state between homologous genes (Rousset, 2002). To test for significant deviations from random SGS, observed values for each distance class were compared to the 95% confidence interval derived from 1000 permutations. The extent of SGS was estimated using the Sp statistic following Vekemans and Hardy (2004): $Sp = -b_{log}/(1 - F_{(ij,1)})$, where b_{log} is the regression slope and $F_{(ij,1)}$ is the mean kinship coefficient between individuals belonging to the first distance interval F_{ij}. The b_{log} standard errors were obtained by jack-knifing over loci. These calculations were performed using the program SPAGeDi 1.2 g (Hardy and Vekemans, 2002).

3. Results and discussion

3.1. Genetic diversity

Ten polymorphic loci and thirty alleles were observed and analyzed. The percentage of polymorphic loci ($P_L = 100\%$) was similar for all populations. The average number of alleles per locus was $\hat{A} = 3.0$ for populations Japonvar and Montes Claros and $\hat{A} = 2.6$ for populations Francisco Sá and Bocaiúva. The high proportion of polymorphic loci and the number of alleles per locus detected here were similar to reports for other tree species in investigation about impacts of habitat degradation on genetic resources, using allozymic markers (Bacles et al., 2004; Fuchs et al., 2003; Vieira and Carvalho, 2008).

Only one potential linkage disequilibrium test out of 180 was significant which is slightly higher than what would be expected by chance at the 0.05 level, after Bonferroni correction (Japonvar, locus $Me \times Skdh$). The relationship between the observed $\hat{H}_o$ and expected $\hat{H}_e$ mean heterozygosities resulted in a negative fixation index (f) in all the fragments analyzed, indicating a greater proportion of heterozygotes (Table 1). No major allele frequency difference was detected among populations. The high gene diversity ($\hat{H}_e$) detected for the species in the fragments can be explained by the absence of rare alleles and allele frequencies in equity. Besides, the outcrossing rates in tropical tree species can have an impact on the genetic structure and can result from ecological factors, population size and density (Murawski and Hamrick, 1991).

The average genetic differentiation in each population pair was generally low ($D_G = 0.036$), suggesting that 96.4% of the variability occurs within populations (Table 2). The genetic differentiation detected in the study is in line with that reported for other tropical tree species, that is, a greater proportion of the genetic variability was found within populations (Vieira and Carvalho, 2008; White et al., 1999). Mantel tests provided tendency for distance dependence of genetic structure ($r_m = 0.905$, $P = 0.084$). Indeed, bats that pollinate *C. brasiliense* are small, territorial species with a small flight range (Gribel and Hay,

Table 2
Geographical distances (km, above diagonal) and genetic differentiation (D_G values, below diagonal) among four populations.

Populations	Japonvar	Montes Claros	Francisco Sá	Bocaiúva
Japonvar	–	101.6	106.9	130.9
Montes Claros	0.034	–	95.8	30.2
Francisco Sá	0.051	0.029	–	107.3
Bocaiúva	0.050	0.012	0.040	–

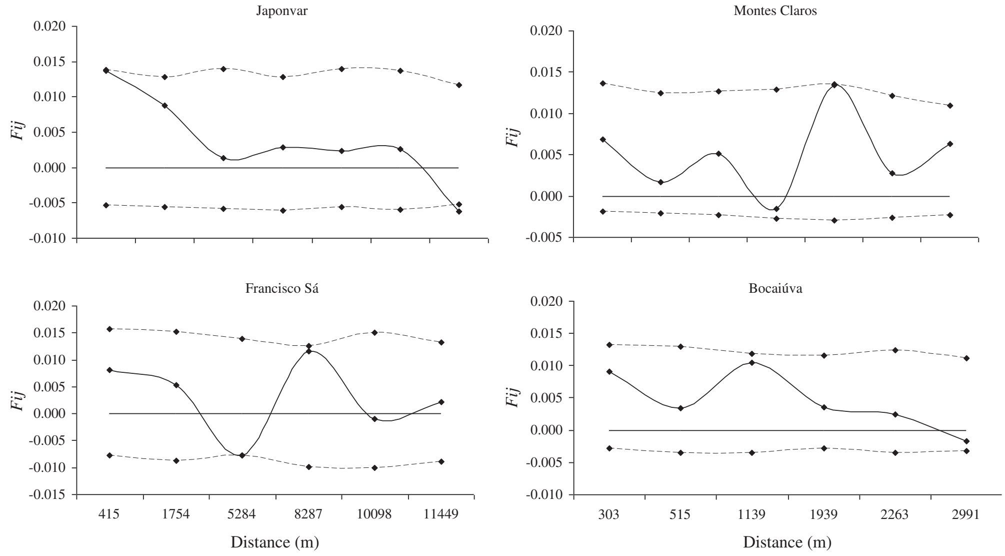

Fig. 1. Correlograms of kinship coefficients (F_{ij}) per distance classes in four natural populations of *Caryocar brasiliense*. Confidence intervals (—) around each F_{ij}-value were obtained through a jackknife procedure over loci.

1993). *C. brasiliense* individuals are spatially distributed in clumps and bat pollinators tend to forage inside the clumps, restricting gene flow. Hence, fragmentation may cause genetic isolation, increasing the differentiation among populations (Collevatti et al., 2003).

3.2. SGS

In the populations of Montes Claros, Francisco Sá and Bocaiúva coancestry values were within the range of 95% confidence limits in all of the distance classes (Fig. 1). Random genotype distribution was observed also when estimating the coancestry for individuals within Japonvar population. However, a continuous decrease in the autocorrelation values was detected with increasing distances in this population, and from 1337 m onward, it showed significant negative values, suggesting that nearby trees are genetically related and distant trees are not (Fig. 1). Positive coancestry value ($F_{ij} = 0.014$, $P = 0.050$) at 221 m indicates the tendency of increased structure among trees that are spatially closer to each other (Fig. 1). Although some seeds could be dispersed by the greater-rhea (*R. americana*), the great majority are dispersed by gravity, and tend to remain under the mother tree canopy (Collevatti et al., 2003). Indeed, the regression of pairwise kinship values on the logarithm of geographic distance had a significantly negative slope blog ($P < 0.025$) indicative of SGS in the Japonvar population ($Sp = 0.0082$).

3.3. Assessment of historical population bottlenecks and conservation genetics

From the tests to fit into the infinite allele model of mutation (Table 3), no population was shown to be in equilibrium, indicating recent bottlenecks ($P < 0.05$, Wilcoxon sign-rank test). All populations showed a significant number of loci with excessive heterozygosity, that is, the heterozygosity from the H–W proportions ($\hat{H}_e$) in the polymorphic loci was greater than the expected heterozygosity under equilibrium between mutation and drift ($\hat{H}_{eq}$). In a population recently reduced in size, the genetic diversity observed will be greater than the genetic diversity equilibrium. Therefore, the detection of recent bottlenecks corroborates historical evidence that the populations were once part of a much larger population, and can be interpreted as a consequence of the expansion of agricultural frontier resulting from human disturbance at Cerrado Biome. In

Table 3
Number of loci showing deficiency/excess of heterozygosity under IAM for bottleneck detection in *Caryocar brasiliense*.

	Populations			
	Bocaiúva	Francisco Sá	Japonvar	Montes Claros
Expected number of loci showing excess of heterozygosity	4.80	4.81	5.22	5.25
Deficiency/excess of heterozygosity	0/10	0/10	0/10	0/10
Sign test	0.00062	0.00062	0.00151	0.00158
Wilcoxon test	0.00049	0.00049	0.00049	0.00049

addition, considering the practically irreversible fragmentation of populations and the larger genetic diversity found in fragments, landscape management strategies should consider the protection of extant ones.

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