



Effect of selective logging on genetic diversity and gene flow in *Cariniana legalis* sampled from a cacao agroforestry system

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ABSTRACT. The fragments of the Atlantic Forest of southern Bahia have a long history of intense logging and selective cutting. Some tree species, such as jequitibá rosa (*Cariniana legalis*), have experienced a reduction in their populations with respect to both area and density. To evaluate the possible effects of selective logging on genetic diversity, gene flow, and spatial genetic structure, 51 *C. legalis* individuals were sampled, representing the total remaining population from the cacao agroforestry system. A total of 120 alleles were observed from the 11 microsatellite loci analyzed. The average observed heterozygosity (0.486) was less than the expected heterozygosity (0.721), indicating a loss of genetic diversity in this population. A high fixation index ($F_{IS} = 0.325$) was found, which is possibly due to a reduction in population size, resulting in increased mating among relatives. The maximum (1055 m) and minimum (0.095 m) distances traveled by pollen or seeds were inferred based on paternity tests. We found 36.84% of unique parents among all sampled seedlings. The progenitors of the remaining seedlings (63.16%) were most likely out of the sampled area. Positive and significant spatial genetic structure was identified in this

population among classes 10 to 30 m away with an average coancestry coefficient between pairs of individuals of 0.12. These results suggest that the agroforestry system of cacao cultivation is contributing to maintaining levels of diversity and gene flow in the studied population, thus minimizing the effects of selective logging.

Key words: Spatial genetic structure; Microsatellites; Atlantic Forest; Jequitibá rosa; *Cariniana legalis*

INTRODUCTION

Cariniana legalis Mart. O. Kuntze (Lecythidaceae), popularly known as jequitibá rosa, is one of the largest trees of the Atlantic Forest, reaching 60 m in height. Its wood has proven commercial value (Carvalho, 1994). This species has been designated in the vulnerable category to extinction (IUCN, 2012), occurring in a limited number in natural habitats (Siqueira et al., 1986) with a population density of 0.8 trees/ha (Harritt and Jesus, 1987).

Reductions in population size can cause loss of alleles and reduced heterozygosity (Young et al., 1996). Populations with reduced size and density contribute to an overall reduction in allele diversity, which becomes intensified when these factors are associated with reduced dispersal of pollen and seeds due to fragmentation effects. The fragmented populations may become genetically and demographically isolated, causing local extinction of species (Nason and Hamrick, 1997).

The best-known commodities grown in agroforestry systems are coffee and cacao, which have multistrata shading systems combining native forest, timber, and fruit. Agroforestry systems contribute to conservation of biodiversity, providing habitat for many species due to landscape connectivity and edge reduction among forests and agricultural areas (Harvey and González Villalobos, 2007). In Bahia, the cacao is grown under the shade of large native trees in an agroforestry system known as 'cabruca'. Studies have shown that the cacao agroforestry system in southern Bahia harbors a considerable number of species, such as small mammals (Pardin, 2004), birds (Faria et al., 2006), ants (Delabie et al., 1999), bats (Faria and Baumgarten, 2007), ferns, and frogs and lizards (Faria et al., 2007). Some authors have argued that traditional agroforestry practices can contribute to *in situ* conservation of biodiversity through the conservation of tree species on farms, reducing pressure on remaining forests, and thus favoring a natural habitat for several species (Acharya, 2006; McNeely and Schroth, 2006). Agroforestry systems have helped to increase the population density of species through identifying and promoting strategies that aim at sustainable land use that are consistent with the maintenance of local biodiversity (Fifanou et al., 2011).

Gene flow among populations is essential for the microevolution of species, since it allows them to operate as independent evolutionary units (Husband and Barrett, 1996). Gene flow is one of the essential factors in the composition of genetic diversity within a population and between different generations (Dow and Ashley, 1998). A major pathway of gene flow in plant populations has been through pollen dispersal (Hamrick and Nason, 2000). The pollination mechanisms associated with the spatial structure of trees determine how individuals recombine their genes in successive reproductive events, which strongly affects the genetic characteristics of populations (Nason and Hamrick, 1997).

The selective logging of several species was a common practice in the Atlantic Forest of southern Bahia, which started soon after the discovery of Brazil (Dias, 2010). Studies suggest that logging caused a strong reduction in the number of individuals of existing species (Slik et al., 2002), loss of rare alleles, and reductions in the average number of effective alleles and expected heterozygosity (Silva et al., 2008). In a simulation study, Sebbenn et al. (2008) demonstrated that different tree species respond differently to logging, both with respect to demographic and genetic aspects. Therefore, forest management practices should be species-specific, taking into account growth parameters, ecological, and reproductive variables, with a long-term view for the sustainability of forests. Several species of hardwoods have an important gene bank in 'cabruca', since they are practically extinct in remaining Atlantic Forest fragments due to selective logging (Sambuichi, 2002). Currently, the occurrence of *C. legalis* in Bahia is restricted to small forest fragments and 'cabruças'. The present study aimed to evaluate genetic parameters in a natural population that was subject to selective logging in the past. Our objectives were to investigate the extent of contemporary gene flow and the spatial genetic structure in a small population of *C. legalis* sampled in a typical 'cabruca' from southern Bahia using microsatellite loci and paternity analysis.

MATERIAL AND METHODS

Vegetation sampling and DNA extraction

The census of the Barra of Cedro Farm (14°35'05"S, 39°43'20"W) population was sampled. We sampled adults and seedlings in an area of 230 ha, consisting of cocoa plantations in association with tree species in an agroforestry system known as 'cabruca' located in Pau Brasil in southern Bahia, Brazil. The landscape in this region is a mosaic of remnants of small fragments of the Atlantic Forest, and farms growing cocoa and other crops. To obtain DNA, a total of 51 *C. legalis* individuals were sampled, including 13 adults and 38 seedlings. Genomic DNA from seedlings and adults were extracted from leaf samples and cambial scrapings according to the protocol described by Doyle and Doyle (1990).

Amplification of microsatellite markers

The amplification was performed using 11 microsatellite loci, nine of which (Ces03, Ces05, Ces07, Ces10, Ces12, Ces13, Ces14, Ces16, and Ces18) were transferred from *Cariniana estrellensis* (Guidugli et al., 2009) to *C. legalis*, and two (CI05 and CI11) were developed specifically for *C. legalis*. Tails containing a portion of the plasmid M13 were added to forward primers (CACGACGTTGTTAAACGACC) for detection of amplicons by fluorescence (Oetting et al., 1995). The final volume of each polymerase chain reaction (PCR) was 13 μ L, containing 7.5 ng DNA template, 1X (NH₄)₂SO₄ buffer (Fermentas), 3 mM MgCl₂ (Fermentas), 0.25 mg/mL bovine serum albumin (Sigma), 0.25 mM dNTP (Fermentas), 0.49 μ M forward and reverse primers, 0.49 μ M tail M13 primer labeled with HEX, 6-FAM, and NED fluorescence, and 1 U Taq DNA polymerase. Amplifications were performed in the Thermal Cycler GeneAmp 9700 thermocycler (Applied Biosystems). The amplification conditions were 96°C for 2 min followed by 35 cycles of 94°C for 60 s, temperatures specific for primer annealing (Guidugli et al., 2009) for 60 s, and an extension at 72°C for 60 s. After

35 cycles, a final extension step was run at 72°C for 7 min. The PCR products were separated by electrophoresis using an ABI377 genetic analyzer (Applied Biosystems), and filter D genotyping was performed with the aid of GeneScan and Genotyper (Applied Biosystems version 3.1.2). An internal ladder standard labeled with ROX fluorescence was built using a pSPORT vector in lengths of 57, 99, 124, 157, 181, 197, 204, 283, 378, 425, and 651 bp (Brondani and Grattapaglia, 2001).

Diversity and spatial genetic structure in natural populations of *C. legalis*

Genetic diversity was inferred based on the number of alleles per locus (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E), and allelic richness for each locus using the FSTAT v.2.9.3.2 software (Goudet, 2002). The occurrence of rare alleles in the population (with allele frequency less than 0.05) was also detected. Diversity indices were calculated based on allelic identity within individuals (1-Qintra) and within the population (1-Qinter), using the GENEPOP software version 1.2 (Raymond and Rousset, 1995). The levels of inbreeding within samples per locus were verified by the fixation index (F_{IS}) using the FSTAT software v.2.9.3.2 (Goudet, 2002). Paternity analysis was performed from the genotypes of the 38 seedlings and 13 adults sampled using the CERVUS 3.0 software (Marshall et al., 1998). As all individuals genotyped were geo-referenced, we also calculated the distances between parents and offspring. The paternity analysis was done without prior knowledge of the alleged parents. For each seedling the most likely candidate father was identified, whose probability of paternity was indicated by the logarithm of odds (LOD) score and the Delta derivative (based on the difference between the LOD score of the most likely parent and the second most likely candidate parent). Only adults and seedlings that genotyped at least 6 of the 11 loci were considered for these analyses. Paternity analysis was estimated in accordance with strict (99%) and relaxed (95%) confidence levels.

The spatial genetic structure (SGS) was evaluated using the average coefficient of coancestry (θ_{xy}) estimated between pairs of individuals from the population. The coefficients θ_{xy} were calculated using the SPAGEDI program (Hardy and Vekemans, 2002), and the θ_{xy} values were calculated based on a set of distance classes to determine the SGS. We use distance intervals of 10 m, with a maximum distance of 100 m. Kinship coefficients were estimated following the method of Loiselle et al. (1995). To test whether the SGS deviated significantly from a random structure, the confidence interval of 95% was calculated for each observed value and each distance class using 10,000 permutations of individuals in the population.

RESULTS

The population of *C. legalis* showed a high level of allelic diversity for all analyzed loci. A total of 120 alleles were found from the 11 loci analyzed (Table 1). The Ces10 locus had the highest N_A (26) and Ces07 had the lowest (4) in the sampled population. From the total N_A observed, 60 (~50%) showed low frequency in the population (less than 0.05). The average H_O was less than the H_E , indicating that loss of genetic diversity might be occurring in the population. Based on estimates of allelic identity, diversity within individuals was lower than diversity observed among individuals within the population (Table 2). The F_{IS} was elevated in 9 of the 11 loci, indicating deviations from panmixia. In two loci (Ces05 and Cl05), the F_{IS} were negative (Table 2), suggesting a greater number of H_O than H_E in this population.

Table 1. Estimates of genetic diversity and inbreeding within populations of *Cariniana legalis* using microsatellite loci.

Locus	Number of alleles	H_E	H_O	Allelic richness	F_{IS}
Ces03	11	0.825	0.737	10.77	0.106
Ces05	5	0.541	0.868	5.00	-0.606
Ces07	4	0.225	0.066	3.82	0.704
Ces10	26	0.964	0.349	25.59	0.637
Ces12	18	0.918	0.275	17.79	0.7
Ces13	9	0.82	0.707	8.91	0.137
Ces14	12	0.797	0.179	11.94	0.775
Ces16	11	0.842	0.552	11.00	0.344
Ces18	11	0.807	0.634	10.91	0.214
Cl05	8	0.625	0.78	7.78	-0.248
Cl11	5	0.568	0.205	4.97	0.639
Mean	10.91	0.721	0.486	10.77	0.325

H_E = expected heterozygosity; H_O = observed heterozygosity; F_{IS} = fixation index.

Table 2. Estimates of genetic diversity by individual and within populations based on allelic identity over all loci, where 1-Qintra and 1-Qinter are allelic diversity within individuals and among individuals within the population and F_{IS} is the fixation index.

Sample	1-Qintra	1-Qinter	F_{IS}
BC	0.4775	0.7133	0.3306

The paternity tests revealed 14 seedlings (36.84%) whose parents were detected with a strict confidence level of 99% (Table 3). Our results also showed that 8 of the 13 adult trees (61.53%) were contributing offspring to the population. For instance, the adult tree BC22 was shown to be the probable progenitor of five (35.71%) of the seedlings sampled in the population, indicating its reproductive potential and allelic contribution to the next generation. Gene flow via pollen and/or seed dispersal observed in the population ranged from 0.095 to 1055 m away, with an average of 333.9 m. In order to visualize the distribution of individuals in the area collected, individuals were projected on the plane (Figure 1) of geographic coordinates.

Table 3. Paternity analysis of *Cariniana legalis*.

Offspring ID	First parent non-exclusion probability	Second parent non-exclusion probability	Candidate father ID	Loci typed	Pair loci compared	Pair LOD score	Pair delta
BC17	2.68E-08	2.68E-08	BC22	11	10	1.01E+01	1.01E+01
BC19	4.42E-08	4.42E-08	BC20	10	10	5.69E+00	5.69E+00
BC21	4.41E-07	4.41E-07	BC22	11	11	8.38E-01	8.38E-01
BC23	4.44E-09	4.44E-09	BC24	10	9	3.59E+00	3.59E+00
BC25	1.02E-08	1.02E-08	BC24	10	10	4.27E+00	4.27E+00
BC30	3.11E-07	3.11E-07	BC32	11	11	1.73E+00	1.67E+00
BC33	1.47E-06	1.47E-06	BC40	10	9	4.47E+00	3.01E+00
BC34	9.70E-08	9.70E-08	BC35	10	9	3.11E-01	3.11E-01
BC36	7.38E-09	7.38E-09	BC35	10	10	8.48E+00	8.06E+00
BC43	1.04E-07	1.04E-07	BC22	11	11	6.93E+00	9.99E-02
BC45	1.24E-08	1.24E-08	BC44	11	11	1.05E+01	2.67E+00
BC47	2.02E-08	2.02E-08	BC55	9	8	5.71E+00	5.71E+00
BC48	9.15E-09	9.15E-09	BC22	11	11	1.82E+00	1.82E+00
BC52	8.48E-07	8.48E-07	BC22	11	9	4.25E+00	2.16E+00

LOD = logarithm of odds. Delta and LOD scores at strict confidence level of 99%.

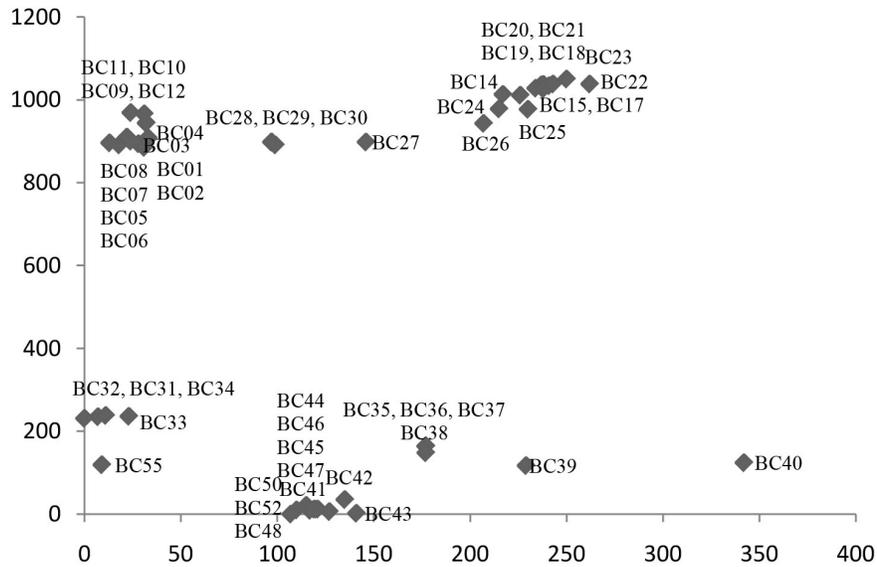


Figure 1. Dispersal in the plane of the individuals sampled in the population.

Significant SGS was found within 10 to 30 m among trees evaluated (Figure 2), showing an average coefficient of coancestry between pairs of individuals of $\theta_{xy} = 0.12$, with a similar relationship found to half-sibs ($\theta_{xy} = 0.125$). Among the distance class from 30 to 100 m, no relationship between pairs of individuals was detected within the range of 95% error estimates, indicating that the coancestry coefficients verified were significantly different from zero.

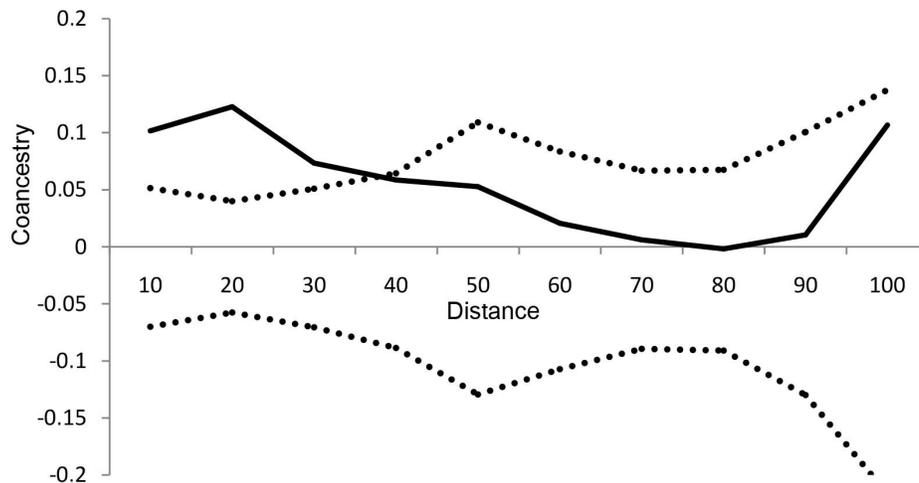


Figure 2. Correlograms of the average kinship coefficients of *Cariniana legalis* individuals for 10 distance classes with intervals of 10 m. The solid line represents the mean value. The dashed lines represent the 95%CI (two rows) of the distribution obtained from the average of 10,000 permutations of the spatial distances between all individuals.

DISCUSSION

Agricultural crop expansion and other economic human activities, such as selective cutting of trees with commercial value, have reduced tree populations throughout the world. Currently, 80% of the Atlantic Forest consists of fragments smaller than 50 ha separated by other small fragments (Ribeiro et al., 2009). Detailed studies are important for understanding the genetic effects resulting from this reduction in population density. These effects can vary among species since they depend on which ecological aspects are affected, such as the number of pollinators and the mating system. This study reflects the current situation facing many tree species along the Atlantic Forest.

Our results showed that there has been a loss in genetic diversity in the population, based on comparison of the average indices of H_E and H_O . This result corroborates with the lower observed index of genetic diversity within individuals (1-Qintra) than among individuals within the population (1-Qinter). Considering that the diversity index 1-Qintra reflects the actual diversity within a population, it is likely that this reduction is associated with a reduction in the number of adult tree individuals due to forest fragmentation and intense logging that occurred throughout the region of southern Bahia during the expansion of cacao crops in the 1960s and 1970s. The logging was compounded with a cacao crisis in the 1990s due to witches' broom disease. According to Lowe et al. (2005), a reduction in heterozygosity levels is expected in populations of trees that suffered recent reductions in effective population density. The high incidence of rare alleles (50%) observed in the population is worrying since these alleles may disappear or reduce in frequency in the population in subsequent generations. This can happen due to the effects of genetic drift, due to both the history of selective logging and due to the cutting of seedlings' understory, which is common practice in agroforestry systems of cacao. Studies have suggested that although the loss of rare alleles of some trees might result from cutting practices, with proper maintenance of seedlings, rare alleles may still remain in the general population (Silva et al., 2008).

The inbreeding coefficients were negative in 2 of the 11 loci analyzed, indicating an excess of heterozygotes. However, the average F_{IS} of all loci was positive and high, possibly due to the reduction in the population size, mainly based on the reduced number of adults that would be contributing to offspring in the population, as demonstrated in the paternity analysis. The high inbreeding level detected in this study contrasts with other studies of populations of tree species affected by selective cutting (Cloutier et al., 2007; Silva et al., 2008). Another explanation for the high rate of the fixation average based on all loci could be the presence of null alleles.

Gene flow of pollen and/or seeds observed from the distance between adults (parents) and seedlings (descendants) ranged from 0.095 to 1055 m, with an average of 333.9 m. These analyses showed that seven of the sampled seedlings were located less than 100 m away from their possible parents, whereas the parents of the other seedlings were more than 100 m away. The remaining seedlings that were not assigned paternity might represent a low probability of paternity or that they originated from pollen flow and seeds from trees not sampled. The pollen immigration from outside the sampled farm may represent a contribution of pollen and seeds for 63.16% of seedlings sampled, suggesting long distance gene flow of pollen and/or seeds. The observed long distance pollen dispersal found in this study agrees with the observations in other species of tropical trees of low population density, thereby suggesting that the average distance of pollen dispersal is generally greater than 200 m (Nason and Hamrick, 1997;

White et al., 2002; Gaiotto et al., 2003; Dick et al., 2003; Carneiro et al., 2007; Cloutier et al., 2007; Eduardo et al., 2008). The agroforestry system to which the population of *C. legalis* is found most likely favors the action of pollen and seed dispersers. This could explain the high percentage of seedlings (63.13%) that probably originated from adult trees located in small forest fragments remaining around the sampled population.

The BC22 individual was the likely parent to five plantlets, demonstrating the greatest reproductive success in comparison with other adults in the population. Seven heterozygous loci in BC22 were observed, which shows that it can help to maintain different alleles for the next generation. This individual has a diameter of 2.2 m. Its diameter and height indicate that it is probably one of the founding individuals of this population. The distance between this individual and its descendants ranged from 19 to 1054 m. Reproductive success depends on age and high individual adaptability to local environmental conditions. The adaptability to the environment is associated with several factors, for example, greater pollinator visitation, the distribution of individuals within the population, and pollinator behavior, and thereby pollination rates (Handel, 1983). Several other factors can affect the reproductive success of plants, and further studies are needed to gain a better understanding of these mechanisms.

We verified SGS from individuals up to a 30 m distance. Individuals between 10 to 30 m distances showed coefficients of kinship resembling half-sibs. Among the 40- to 100-m distance class, no kinship was detected between individuals. This suggests that the agroforestry system probably favors the dispersal of pollen and seeds over long distances in the sampled region. These results corroborate with the average distance of 333.9 m between parents and seedlings within the population that was detected in the paternity analysis. Furthermore, selective logging in the past would have minimized the effects of structure due to the withdrawal of some related adults, and a reduction in endogamic crossing in the population. Similar results were found by Silva et al. (2008).

Based on analyses of the different indices of genetic diversity evaluated in this study, as well as contemporary gene flow and kinship analyses, despite the apparent loss of diversity found in this study, levels of diversity in the *C. legalis* population are maintained that are compatible with populations of other species of trees with good conservation status. This may be due to the small number of generations that have elapsed since selective logging. The agroforestry system of cacao 'cabruca' seems to have contributed to the maintenance of genetic diversity through gene flow within the population and between small forest fragments around the area sampled. However, for the levels of genetic diversity found in this study to be maintained in future generations, proper management is needed that takes into account the average distance of kinship in the population.

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