



Fine-scale spatial genetic structure of *Dalbergia nigra* (Fabaceae), a threatened and endemic tree of the Brazilian Atlantic Forest

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Abstract

The Atlantic Forest is one of the most diverse ecosystems in the world and considered a hotspot of biodiversity conservation. *Dalbergia nigra* (Fabaceae) is a tree endemic to the Brazilian Atlantic Forest, and has become threatened due to overexploitation of its valuable timber. In the present study, we analyzed the genetic diversity and fine-scale spatial genetic structure of *D. nigra* in an area of primary forest of a large reserve. All adult individuals ($N = 112$) were sampled in a 9.3 ha plot, and genotyped for microsatellite loci. Our results indicated high diversity with a mean of 8.6 alleles per locus, and expected heterozygosity equal to 0.74. The co-ancestry coefficients were significant for distances among trees up to 80 m. The S_p value was equal to 0.017 and indirect estimates of gene dispersal distances ranged from 89 to 144 m. No strong evidence of bottleneck or effects of human-disturbance was found. This study highlights that long-term efforts to protect a large area of Atlantic Forest have been effective towards maintaining the genetic diversity of *D. nigra*. The results of this study are important towards providing a guide for seed collection for *ex-situ* conservation and reforestation programmes of this threatened species.

Keywords: *Dalbergia nigra*, Atlantic Forest, conservation genetics, fine-scale spatial genetic structure, S_p statistic.

Received: April 2, 2012; Accepted: August 2, 2012.

Introduction

The Brazilian Atlantic Forest is one of the most diverse ecosystems in the world (Morellato and Haddad, 2000), with an estimated plant diversity of fifteen thousand species, 45% of which are endemic (Stehmann *et al.*, 2009). This ecosystem is a hotspot for biodiversity conservation, since its high species richness and endemism have been subject to intensive anthropogenic disturbance for centuries (Myers 1988; Myers *et al.*, 2000). Currently this ecosystem is highly fragmented, and only about 7.5% of 1,227,600 km² of original forest remains (Myers *et al.*, 2000). Approximately 83% of the remaining fragments of Atlantic Forest have an area of less than 50 ha, while only 0.03% exceed 10,000 ha (Ribeiro *et al.*, 2009a). According to Ribeiro *et al.* (2009a), the effort allocated to the conservation of large remnants of primary forest should be a priority,

since the preservation of sensitive species depends exclusively on the conservation of these larger fragments. Moreover, large fragments promote the preservation of core areas that are sources of seeds for both surrounding areas and reforestation programmes (Ribeiro *et al.*, 2009a).

In tropical forests, anthropogenic changes to the landscape affect pollen and seed dispersal. Although trees are less vulnerable to human-induced interference than other organisms (Hamrick, 2004), tropical trees are extremely dependent on animals for pollination and seed dispersal, which might be highly affected by the degradation of this habitat (Pacheco and Simonetti, 2000; Wang *et al.*, 2007). Hence, alterations in the wildlife associated to tropical forests have large impacts to the mating system and spatial genetic structure (SGS) of trees, especially in fragmented populations (Murawski and Hamrick, 1992; Hall *et al.*, 1994; With *et al.*, 1999; Pither *et al.*, 2003). SGS, which is the nonrandom distribution of genotypes in a given space, is often a consequence of multiple processes, such as historical demographic changes, selection and limited gene flow (Vekemans and Hardy, 2004; Randall *et al.*, 2010). In

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plants, gene flow through the pollen and seeds determines whether alleles are widely or locally dispersed (Bradshaw, 1972; Levin and Kerster, 1974). However, the dispersion distance is small compared to the area covered by plant populations, which can promote fine-scale SGS (Veekmans and Hardy, 2004; Lopez-Gallego and O'Neil, 2010). Seed dispersal more strongly influences genetic structuring of populations than pollen movement (Chung *et al.*, 2003). When long-distance pollen movement occurs, restricted seed dispersal might result in the grouping of half-sibs near to the maternal plant (Berg and Hamrick, 1994). Moreover, if both pollen movement and seed dispersal are limited, this will result in inbreeding and an increase in SGS (Sokal and Wartenberg, 1983; Hamrick and Nason, 1996). In contrast, if only short-distance pollen dispersal occurs, wide and independent seed dispersal will ensure the distribution of genetic variation, leading to weak or near random structuring (Dewey and Heywood, 1988; Loiselle *et al.*, 1995; Chung *et al.*, 2000; 2003). Furthermore, fine-scale SGS studies allow us to infer the extent of gene dispersal within species indirectly. This method is especially convenient for tropical trees, as it is quite difficult to directly monitor pollen and seed movement in these species (Born *et al.*, 2008).

The level of intrapopulation SGS should be considered by conservation programmes of threatened species, since it can influence estimates of genetic parameters (Ennos and Clegg, 1982; Young and Boyle, 2000; Bittencourt and Sebbenn, 2007). Hence, it is important to establish sampling strategies for *ex situ* and *in situ* conservation procedures (Doligez and Joly, 1997; Sebbenn *et al.*, 2008; Tarazi *et al.*, 2010). Therefore, obtaining knowledge about fine-scale genetic variation provides a guideline for the management of natural populations (Hamrick and Sherman-Broyles, 1992; Williams and Hamrick, 1996).

Dalbergia nigra (Vell.) Allemão ex Benth, popularly known as Brazilian rosewood, is a legume tree that is endemic to the Brazilian Atlantic Forest. Due to more than five centuries of overexploitation of its high quality timber and biome deforestation, this species has become threatened and restricted to small fragments, parks and reserves (Costa *et al.*, 1998). Since 1998, it has been listed as "vulnerable" in the Red List of the International Union for Conservation Nature (IUCN, 2007), with international trade being prohibited (CITES, 1992). The mating system of this species is not known. Bees probably perform pollination, and seeds are dispersed by the wind (Carvalho, 1994). Although much effort is required to protect this species, few genetic studies have been performed to date (Ribeiro *et al.*, 2005, 2011; Juchum *et al.*, 2007; Resende *et al.*, 2011) and we are not aware of any study about fine-scale spatial genetic structure of *D. nigra*.

In the present study, we analyse the genetic diversity and fine-scale spatial genetic structure of *D. nigra* located in an area of continuous primary forest of a large reserve of Atlantic Forest. In a 9.3 ha plot, we sampled all adult indi-

viduals ($N = 112$) and genotyped them for microsatellite loci. Specifically, we sought to answer the following questions: (1) What is the level of genetic diversity of *D. nigra* in this primary forest area? (2) What is the extent of SGS in this population? (3) What is the distance of gene dispersal? (4) What is the required minimum distance among trees to collect seeds for conservation programmes in a way to prevent inbreeding and promote a good representation of genetic diversity? (5) Have the long-term efforts of preserving a large remnant of Atlantic Forest been effective in conserving genetic diversity of *D. nigra*?

Materials and Methods

Study area and sampling

Samples of *D. nigra* used for this study were collected in the Rio Doce State Park Biological Reserve (from 19°48'18" to 19°29'24" S and 42°38'30" to 42°28'18" W), which was implemented in 1944 (IEF, 2002) and is located in the state of Minas Gerais, southeastern Brazil (Figure 1a, b). It is the largest remnant of Atlantic Forest in this state, with a total area of 36,000 ha of native primary and secondary vegetation (Solá, 2001). The sampled area, named Campolina, comprises 130 ha of undisturbed primary forest vegetation (Ribeiro *et al.*, 2005). One plot of 9.3 ha was randomly delimited and the cambium of 112 adult trees was sampled (density = 12.0 trees/ha) for the analysis of diversity and fine-scale spatial genetic structure. The diameter at breast height (dbh) of the sampled trees ranged from 10 to 53 cm, with an average of 23.3 cm and median of 21 cm. The spatial distribution of trees (Figure 1c) was recorded using GPS Garmin 60CSx and visualised with TrackMaker 11.7 software (Ferreira Jr, 2001).

DNA isolation and genotyping

Total genomic DNA was extracted from 2.5 mg of cambium following the protocol of Novaes *et al.* (2009). Seven polymorphic nuclear microsatellite loci (*Dnig1*, *Dnig2*, *Dnig4*, *Dnig5*, *Dnig6*, *Dnig7* and *Dnig10*), which have been previously isolated and characterised by Ribeiro *et al.* (2009b), were used for genetic analyses. PCR amplifications were performed using the conditions established by Ribeiro *et al.* (2009b), which included a universal fluorescent-labelled M13 tail primer, and following the protocol described by Schuelke (2000). Microsatellite fragments were analysed in a MegaBACE 1,000 automated sequencer (Amersham Biosciences) using 0.1% Tween 20 and ROX-500 Size Standard (GE Healthcare), and scored using the MegaBACE Fragment Profiler 1.2 software (Amersham Biosciences).

Genetic diversity and bottleneck detection

Allelic frequencies, the number of alleles (A), and observed and Hardy-Weinberg expected heterozygosities (H_O and H_E) were computed using Arlequin v.3.1 software

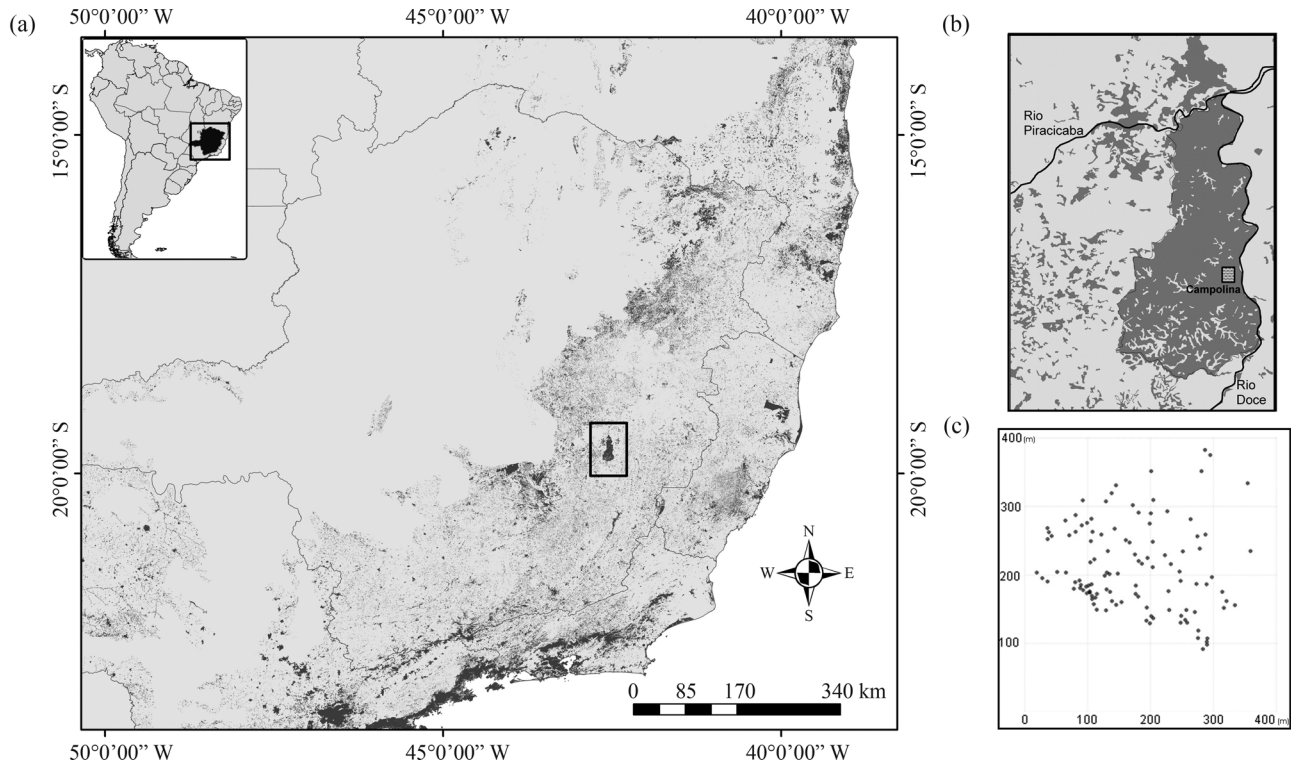


Figure 1 - Map of part of southeastern Brazil showing remaining areas of Atlantic Forest, including the Rio Doce State Park (a); the Rio Doce State Park and the location of Campolina population (a) and the distribution (in meters) of the 112 adult individuals of *Dalbergia nigra* sampled within a 9.3 ha plot of the Campolina population (c).

(Excoffier *et al.*, 2005). The fixation indices (F_{IS}) were estimated using Arlequin. Scoring errors due to the presence of null alleles, stuttering or large allele dropout were tested with Micro-Checker software (van Oosterhout *et al.*, 2004). The frequencies of null alleles were also estimated using Cervus v.3.0.3 (Marshall *et al.*, 1998). The null allele frequencies were high for the loci *Dnig1* and *Dnig6* (around 32% and 33%, respectively). These loci were removed from kinship, substructure and bottleneck analyses. In the diversity analyses, estimates were performed under three conditions: 1) including loci with null alleles, 2) excluding these loci and 3) including all loci but with correction of null alleles by the Brookfield method (Brookfield, 1996) implemented by Micro-Checker (van Oosterhout *et al.*, 2004). According to Dakin and Avise (2004), about 90% of all studies include loci with null alleles in the analyses, but few of these incorporate statistical corrections to accommodate possible null alleles effects.

To check whether low heterozygosities were caused by the Wahlund effect, the population substructure was investigated using the Bayesian method with MCMC (Markov Chain Monte Carlo) estimation implemented in Structure v.2.2 (Falush *et al.*, 2007). We used an admixture model, which was run 10 times for one to four clusters, $k = 1$ to 4, using burn-in lengths of 100,000 and run lengths of 100,000 MCMC steps.

The program Bottleneck v.1.2 (Piry *et al.*, 1999) was used to detect the presence of recent bottlenecks in the studied population. The bottleneck test assumes that population reductions cause a faster decline in the number of alleles than expected heterozygosity in the Hardy-Weinberg equilibrium (H_E). Thus H_E becomes greater than H_{Eq} (heterozygosity at mutation-drift equilibrium), since H_{Eq} is calculated based on allele number (Cornuet and Luikart, 1996). Three mutation models were employed: 1) the Infinite Allele Model - IAM (Kimura and Crow, 1964); 2) the Stepwise Mutation Model - SMM (Ohta and Kimura, 1973), and 3) the Two-Phase model - TPM (Di Rienzo *et al.*, 1994). Di Rienzo *et al.* (1994) found that mutational changes in SSR primarily result in an increase or decrease of one repeat unit (SMM). As rare and important events of larger magnitude also occur (IAM), these authors proposed a mixed model (TPM). We applied the TPM with a variance of 12 (Piry *et al.*, 1999) and different percentages of single-step mutations (60-95%). We ran 1,000 iterations and tested significance with the Wilcoxon signed-rank test. According to Piry *et al.* (1999), this is the most powerful and robust test when using only few (< 20) polymorphic loci.

Fine-scale spatial genetic structure and dispersal estimate

To visualise and describe the genetic structure within populations, the co-ancestry coefficient (synonymous to

kinship coefficient), ρ_{ij} , was plotted against the natural logarithm of the distance between individuals, $\ln(r_{ij})$, over distance classes set between individuals (r) (Loiselle *et al.*, 1995; Hardy, 2003; Vekemans and Hardy, 2004). The co-ancestry coefficient is the probability of a random gene of individual i being identical by descent to a random gene of individual j . Theoretically, in a two dimensional space, ρ_{ij} tends to decrease almost linearly with $\ln(r_{ij})$ (Hardy, 2003; Vekemans and Hardy, 2004). The magnitude of the SGS can be quantified by the Sp statistic, which is a ratio between b_{\log} value and mean kinship coefficients among individuals of the lower distance class adopted for the coefficient calculation (Vekemans and Hardy, 2004). The development of the Sp statistic for SGS quantification, based on a decrease of kinship among individuals with increasing spatial distance (Vekemans and Hardy, 2004), allows the strength of SGS to be compared among different species and studies, in addition to investigating isolation by the distance process.

Fine-scale SGS for the set of five loci and 112 adult individuals was assessed using linear regression of pairwise Nason's co-ancestry coefficients [or kinship coefficient, ρ_{ij} , described in Loiselle *et al.* (1995)] on pairwise spatial distances using the software SpaGeDi (Hardy and Vekemans, 2002). Although two of the seven loci were removed from the kinship estimates, five polymorphic loci, and a sample of 100 individuals, were considered sufficient to obtain close to real results (based on a model population) for SGS analysis (Cavers *et al.*, 2005). Mean multilocus kinship coefficients (ρ_{ij}) were computed for 15 distance classes ($k = 15$) spanning from 0 to 300 m, with each class 20 m wide. The numbers of pairs per distance class used in this analysis were higher or equal to 103, which are higher than the minimum number (30) recommended by Degen *et al.* (2001). The statistical significance of the autocorrelation was tested by 10,000 random permutations, with a 95% confidence interval. SGS was quantified using the Sp statistic defined by the ratio $Sp = -b_p / (1 - \rho_1)$, where ρ_1 is the mean ρ_{ij} between neighbouring individuals for the first distance class (0-20 m) and b_p is the linear regression slope of the mean kinship coefficients on the logarithm of spatial distance (0-300 m) (Vekemans and Hardy, 2004).

Wright's neighbourhood size was estimated as $N_b = -(1 - \rho_1) / b_p$, where ρ_1 is the co-ancestry coefficient between adjacent individuals for the first distance class, and b_p is the restricted regression slope within a distance class between σ and 20σ (Vekemans and Hardy, 2004). Historical gene dispersal (σ_g) was obtained through the equation $\sigma_g^2 = Nb / 4\pi D_e$. These estimates are dependent on the effective density, D_e (Vekemans and Hardy, 2004; Hardy *et al.*, 2006), which is not known for this population. However, according to Frankham (1995), previous studies have demonstrated that in natural plant populations, D_e varies among one-half to one-tenth of the density of adults (D). Thus, the

values $D/10$, $D/4$ and $D/2$ were assumed to be D_e for the Campolina population. Both Nb and σ_g^2 were estimated using SpaGeDi (Hardy and Vekemans, 2002). The 95% confidence intervals (CI) of b_p , ρ_1 , Nb , and σ_g estimates were calculated as estimate ± 1.96 s.e.. The standard errors (s.e.) were obtained by jackknifing over loci with SpaGeDi software (Hardy and Vekemans, 2002).

Results

Genetic diversity and bottleneck detection

In the analysed *D. nigra* population, the number of alleles per locus (A) ranged from two (*Dnig7*) to 15 (*Dnig6*), with a mean of 8.6 alleles per locus. The observed heterozygosities ($H_O = 0.390$ to 0.734) were lower than the expected ones ($H_E = 0.498$ to 0.835) for each locus, except for locus *Dnig7* ($H_O = 0.528$ and $H_E = 0.498$). The overall loci H_E values estimated for *D. nigra* from the three methodologies (*i.e.* considering all loci, with or without correction of null alleles with the Brookfield method and excluding loci with null alleles) were very similar, ranging from 0.707 to 0.747, showing that five loci were as informative as seven loci in the current study. The observed heterozygosities varied among these three methods, exhibiting lower values in the estimate when loci with null alleles were included ($H_O = 0.577$) compared to when they were excluded ($H_O = 0.645$). The inclusion of the loci with null alleles previously corrected with the Brookfield method produced the highest value of heterozygosity ($H_O = 0.687$; Table 1).

Intrapopulation fixation indices (F_{IS}) were positive and significant for four of the seven loci (with F_{IS} values for the four loci ranging from 0.159 to 0.512). Part of this homozygosity excess was probably due to the presence of null alleles, since the exclusion of the *Dnig1* and *Dnig6*

Table 1 - Genetic diversity and fixation indices based on seven microsatellite loci for *Dalbergia nigra* population in the Rio Doce State Park, southeastern Brazil.

Locus	A	H_O	H_E	F_{IS}	p value (F_{IS})
<i>Dnig1</i>	13	0.390	0.799	0.512	0.000
<i>Dnig2</i>	5	0.734	0.739	0.006	0.504
<i>Dnig4</i>	11	0.703	0.835	0.159	0.000
<i>Dnig5</i>	8	0.538	0.676	0.205	0.000
<i>Dnig6</i>	15	0.422	0.822	0.489	0.000
<i>Dnig7</i>	2	0.528	0.498	-0.061	0.805
<i>Dnig10</i>	6	0.723	0.786	0.081	0.070
Mean	8.6	0.577	0.736	0.217	0.000
Mean ^a	6.4	0.645	0.707	0.087	0.000
Mean ^b	8.9	0.687	0.747	0.080	0.000

A Number of alleles, H_O observed heterozygosity, H_E expected heterozygosity, F_{IS} fixation index for each locus.

^aExcluding loci with null alleles; ^bnull alleles corrected by Brookfield method.

loci, which have high null allele frequencies, led to a decrease in mean F_{IS} from 0.217 to 0.087 (Table 1). The correction of null alleles according to the Brookfield method (Brookfield, 1996) was effective, producing similar F_{IS} (0.080). Our results reinforce the importance of using this correction for loci with null alleles or the exclusion of these loci for fixation indices estimates.

The results obtained with the software Bottleneck under the IAM mutation model showed that H_E was significantly higher ($p < 0.05$) than H_{Eq} for three of the loci (*Dnig2*, *Dnig7* and *Dnig10*) and for all loci combined (Table 2). Using the SMM model, only the *Dnig7* locus showed significantly higher H_E than H_{Eq} , but *Dnig5* had the opposite results, i.e., lower H_E than H_{Eq} . However, the Wilcoxon test did not find any significant difference between H_E and H_{Eq} when considering all the loci combined (Table 2). Under the TPM mutation model, there were significant departures from the expected drift-mutation equilibrium in the *Dnig7* locus for all tested percentages of SMM and in the *Dnig10* locus for four percentages of SMM. However, considering all loci combined the Wilcoxon test found significant support for bottleneck in the population under TPM with 60, 70 and 80% of the single-step mutation. Under SMM and TPM with 90 and 95% of the SMM, the test was non-significant (Table 2).

Fine-scale spatial genetic structure and dispersal estimate

The Bayesian analysis performed with Structure software showed that the trees sampled were grouped in one cluster ($k = 1$), suggesting that the population harbours a single gene pool, and that the Wahlund effect is absent.

The average co-ancestry coefficient (ρ) decreased as the spatial distance between trees (r) increased, evidencing SGS in the study population (Figure 2). Co-ancestry coefficients were positive and significant ($p < 0.05$) for the distance classes 0–20 m, 20–40 m, 40–60 m and 60–80 m, with ρ values of 0.036, 0.022, 0.017 and 0.011, respectively. For the distance classes ranging from 80–100 m until

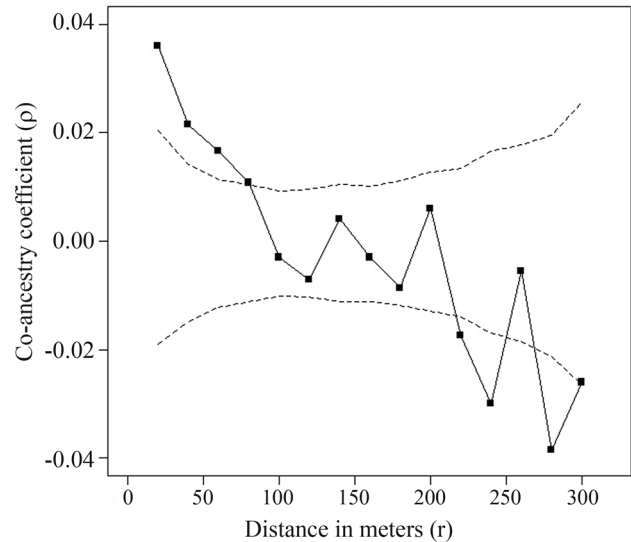


Figure 2 - Co-ancestry coefficient (ρ) for all distance classes (r) calculated according to Loiselle *et al.* (1995) for all pairs of *Dalbergia nigra* adult trees from the Campolina population at the Rio Doce State Park in southeastern Brazil. Broken lines indicate the upper and lower 95% confidence intervals (10,000 permutations).

180–200 m, the co-ancestry coefficients decreased and the p -value became non-significant, being negative and significant for some distance classes higher than 200 m (Figure 2). The linear regression slope (b_ρ) of the mean kinship coefficients on the logarithm of spatial distance (0–300 m) was negative (-0.017) and significant ($p < 0.001$). Consistent with these data, fine-scale SGS evaluated by the S_p statistic value was 0.017 (Table 3).

Indirect estimates of historical gene dispersal distances (σ_g) obtained by SGS analysis were 89, 109 and 144 m, while neighbourhood size (N_b) values were 60, 46 and 32, assuming effective densities (D_e) equal to $D/2$, $D/4$ and $D/10$, respectively. Lower and upper bounds for the 95% CI of the first density considered ($D/2$) for N_b were 5 and 115, while for σ_g the 95% confidence limits were 55 and 123. When considering D_e equal to $D/4$, the 95% confidence in-

Table 2 - Hardy-Weinberg heterozygosity (H_E), heterozygosity under mutation-drift equilibrium (H_{Eq}) and Wilcoxon test obtained using Bottleneck, under three models of SSR mutation: infinite allele (IAM), two-phase (TPM) and stepwise (SMM) for *Dalbergia nigra* population in the Rio Doce State Park, southeastern Brazil.

Locus	H_E	H_{Eq} under IAM	H_{Eq} under TPM (%SMM)					H_{Eq} under SMM
			60%	70%	80%	90%	95%	
<i>Dnig2</i>	0.738	0.454*	0.557	0.565	0.589	0.610	0.630	0.651
<i>Dnig4</i>	0.832	0.702*	0.702	0.803	0.810	0.826	0.833	0.848
<i>Dnig5</i>	0.675	0.614	0.614	0.714	0.737	0.756	0.769	0.789*
<i>Dnig7</i>	0.499	0.165*	0.165*	0.185*	0.194*	0.202*	0.208*	0.198*
<i>Dnig10</i>	0.786	0.516*	0.512*	0.614*	0.651*	0.676*	0.691	0.714
Wilcoxon test								
(average loci)		0.015*	0.031*	0.046*	0.047*	0.109	0.312	0.406

* $p < 0.05$.

Table 3 - Estimates of fine-scale spatial genetic structure for *Dalbergia nigra* population in the Rio Doce State Park, southeastern Brazil.

	Computed value	95% CI
ρ_1 (0-20 m)	0.036	0.0115-0.0605
p value of ρ_1	0.0007	-
bp (0-300 m)	-0.017	-0.027-0.007
N (All loci)	6216	-
Sp	0.017	-
Nb for $De = D/2$	60	5-115
Nb for $De = D/4$	46	22-70
Nb for $De = D/10$	32	NC
σ_g (m) for $De = D/2$	89	55-123
σ_g (m) for $De = D/4$	109	82-136
σ_g (m) for $De = D/10$	144	NC

ρ_1 co-ancestry coefficient in the first distance class, bp slope of the regression of co-ancestry in the logarithm of spatial distance between individuals, N neighbors pairs number, Sp statistic defined by ratio $-b_p/(1 - \rho_1)$, Nb neighbourhood size and σ_g gene dispersal distance.

tervals were 22 and 70 for Nb and 82 and 136 for σ_g . When considering D_e equal to $D/10$, the convergence of standard error was not achieved for both Nb and σ_g (Table 3).

Discussion

High genetic diversity was estimated for the population of *D. nigra* with expected heterozygosities ranging from $H_E = 0.71$ - 0.75 , depending on whether loci with null alleles were included or not in the analysis. Earlier studies also found high diversity in the Campolina population of *D. nigra* in comparison to small fragments when using allozymes ($H_E = 0.296$; Ribeiro *et al.*, 2005), sequences of chloroplast DNA (haplotypic diversity = 0.733 ; Ribeiro *et al.*, 2011) and microsatellites markers ($H_E = 0.798$; Resende *et al.*, 2011). All of these studies had a sampling strategy that was only directed towards evaluating the genetic diversity of the population through the analysis of a smaller number of trees (≤ 23) and in a larger area compared to the present study. In contrast, the current study estimated genetic diversity by sampling all the adult trees (112) in a small plot (9.3 ha). The high diversity found in all these works, irrespective of the sampling strategies and molecular markers used, highlights the high diversity of *D. nigra* in this patch of primary forest in one of the few remaining large areas of inland Brazilian Atlantic Forest.

The fixation index of the population, when excluding loci with null alleles, was small ($F_{IS} = 0.087$), but significantly different from zero ($p < 0.05$). The positive and significant F_{IS} value might be explained by the Wahlund effect or the mating system. The Wahlund effect is unlikely, as the Bayesian structure analyses indicated the presence of only one genetic cluster in the analysed area. Therefore, the most probable hypothesis is that this fixation index is caused by

mating among relatives and/or selfing. The fixation index found here was slightly different from that found by Resende *et al.* (2011), who recorded a smaller ($F_{IS} = 0.047$) and non-significant fixation index in the Campolina population, when also using microsatellite markers. The difference in fixation index estimates between the two studies is probably due to different sampling strategies. These results evidence the influence of sampling on estimates of fixation indices of a population. However, taken together, these studies suggest that *D. nigra* has mostly an outcrossing breeding system, although detailed studies about the mating system of this species have yet to be performed.

The Wilcoxon test detected a signal of reduced effective size in the Campolina population for the IAM and TPM (60, 70, and 80% of single steps) mutation models only. According to Piry *et al.* (1999), the Wilcoxon test is the most powerful on for detecting bottlenecks when using fewer than 20 microsatellites, and the most appropriate mutation model is the TPM with 95% SMM and 5% IAM. Therefore, we consider unlikely the occurrence of a recent bottleneck in the Campolina population.

Kinship coefficients were estimated by excluding loci with high frequencies of null alleles. According to Wagner *et al.* (2006), null alleles can have a large impact on relatedness and relationship analyses. We encountered structuring in the Campolina population, which was evidenced by a decrease in the co-ancestry coefficients with increasing distance between individuals. The main cause of inbreeding in plants is the proximity of related reproductive individuals (Epperson, 2003). Co-ancestry analyses revealed structuring until a distance of 80 m between plants, indicating possible family clustering as a result of localised seed dispersal. This structuring distance is similar to that found in a population from a reserve of another Atlantic forest tree species, *Ocotea catharinensi* (Tarazi *et al.*, 2010), which has insect pollination and seed dispersed by animals. Considering the SGS obtained for *D. nigra*, seed collection for reforestation programmes of this species should use trees separated by distances of over 80 m, to avoid relatedness among seeds from different trees. The co-ancestry coefficients (ρ_{ij}) are 0.065 between first-degree cousins and 0.032 between second-degree ones (Crow and Kimura, 1970). Our results indicate that the co-ancestry value (0.036) in smaller distance classes is very close to the expected value for second-degree cousins.

The Sp value ($Sp = 0.017$) of *D. nigra* was similar that of two other tropical tree species, which exhibit insect pollination and wind seed dispersal, these being *Jacaranda copaia* ($Sp = 0.014$, Jones and Hubbell, 2006) and *Aucoumea klaineana* (mean $Sp = 0.012$, Born *et al.*, 2008). In an isolated fragment of Atlantic Forest, Sebbenn *et al.* (2011), found a higher Sp value (0.026) for the tree *Copaifera langsdorffii*, which is also insect pollinated, but with seed dispersion by birds and mammals. In a review of tree SGS, Dick *et al.* (2008) found that seed dispersal had a

major impact on tropical species. The authors found higher Sp values in plants with seeds dispersed by gravity, wind and scatter-hoarding rodents than by birds, bats or monkeys. Short-distance gene dispersal was found for *D. nigra*, with distances ranging from 89 to 144 m, depending on the effective density being considered ($De = D/2$, $D/4$ and $D/10$). These estimated gene dispersal distances are consistent with the mechanism of seed dispersion of the species (wind), the considerable Sp values and small estimates of neighbourhood sizes ($Nb = 60$, 46 and 32 for $De = D/2$, $D/4$ and $D/10$, respectively).

In summary, despite *D. nigra* being a threatened species, the studied population located in the large reserve has high genetic diversity and shows no strong evidence of a recent bottleneck. In addition, evidence of disturbance in the spatial genetic structure of this species was not found. Considering the high genetic diversity, this area could be an important source of seeds for *ex-situ* conservation programmes and/or reforestation projects. Furthermore, this area could be important for maintaining gene flow with surrounding fragments through a metapopulation structure (Resende *et al.*, 2011). In conclusion, the results of this study highlight that long-term efforts to protect a large forested area has been effective towards maintaining the genetic diversity of *D. nigra*.

Acknowledgments

We thank the Instituto Estadual de Florestas (IEF) and Rio Doce State Park for providing the facilities and licenses for sample collection. We also thank LC Resende for field assistance and LHY Kamino, M Machado and WCS Magalhães for assistance in preparing the figures. This work was supported by Brazil's Long Term Ecological Research Programme/Conselho Nacional de Desenvolvimento Tecnológico (PELD/CNPq), Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG) and Programa Nacional de Pós Doutorado/Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (PNPD/CAPES).

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Associate Editor: Dario Grattapaglia

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