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EVALUATION OF DIVERSITY AND GENETIC STRUCTURE AS STRATEGIES FOR CONSERVATION OF NATURAL POPULATIONS OF *Dalbergia nigra* (Vell.) Allemão ex Benth.

SILVA JÚNIOR, A. L. da; CABRAL, R. L. R.; SARTORI, L.; SOUZA, L. C. de; MIRANDA, F. D. de; CALDEIRA, M. V. W.; MOREIRA, S. O.; GODINHO, T. de O. Evaluation of diversity and genetic structure as strategies for conservation of natural populations of *Dalbergia nigra* (vell.) Allemão ex Benth. **CERNE**, v. 26, n. 4, p.435-443, 2020.

HIGHLIGHTS

The ISSR molecular markers were effective to estimate diversity and genetic structure.

There is moderate to high genetic diversity for *D. nigra*.

Most of the genetic diversity was influenced by individuals from Flona of Pacotuba.

The evaluated populations are structured.

Keywords:
Brazilian rosewood
Forest conservation
ISSR
Molecular marker

ABSTRACT:

The evaluation of diversity and genetic structure allows us to verify with precision the effect of evolutionary and anthropic processes on species. The objective of this research was to evaluate the divergence and the genetic structure of two natural populations of *Dalbergia nigra*, using molecular markers Inter Simple Sequence Repeats (ISSR). Leaf samples were collected from two populations, located in the National Forest of Pacotuba and the Private Natural Heritage Reserve of Cafundó. Eight ISSR primers were used, which resulted in 97 bands, with 68.04% of polymorphism. Based on the joint data, the values of 0.33 for the Nei index (H*) and 0.50 for the Shannon index (I*) indicated moderate to high genetic diversity, being influenced by the presence of genetically dissimilar individuals in the National Forest of Pacotuba. Most of the genetic divergence was intrapopulational (85.96%), with moderate differentiation between populations (Φ_{ST} = 0.1404). The estimated historical gene flow between the fragments was low ($\hat{N}_m$ = 3.21) when compared with results from other tree species, and the genetic structuring analysis separated the populations into two groups, corresponding to the two populations evaluated. The results indicate a small genetic share among populations, however, populations are becoming structured. The satisfactory levels of genetic diversity benefit the use of the trees as matrixes for programs of restoration and recovery of degraded areas, connectivity of landscapes, and sustainable use of forest resources.

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INTRODUCTION

Environmental and anthropic factors influence the diversity patterns of plant species. The loss and fragmentation of habitats associated with selective logging have been considered as the main threats to the conservation, diversity, and genetic structure of tropical forests (Macedo et al., 2012; Tapia-Armijos et al., 2015). The Atlantic Forest is a classic example of how anthropic modification has resulted in isolation and reduction of previously connected and well-established populations (Ribeiro et al., 2016).

Currently, it is estimated that only 6% of the Atlantic Forest is protected by Conservation Units (CU's) (Campanili and Schaffer, 2010), in addition, the intense fragmentation of habitats has even altered the ecological dynamics of protected areas, impairing the maintenance of species. For the existing vegetation to be conserved, maintenance and proper management of the remaining individuals are necessary (Martins et al., 2016). The determination of management and conservation actions requires knowledge of the ecological aspects and genetic composition of species, such as the diversity and genetic structure of populations (Hamrick, 2012; Gois et al., 2018).

The species *Dalbergia nigra* (Vell.) Allemão ex Benth., popularly known as Brazilian rosewood, is a tree that stands out for its ecological and economic potential. Because it has an abundance of seeds capable of colonizing the most diverse environments, it can be widely used in the restoration and recovery of the degraded environment, also helping in the soil nutritional condition once it is a nitrogen-fixing species. Also, the high-quality wood in terms of durability, handling, and commercialization, promote *D. nigra* as the best Brazilian wood (Martinelli and Moraes, 2013).

Dalbergia nigra is endemic to Brazil with a wide distribution in the Atlantic Forest (Lima, 2015). Due to the substantial biome fragmentation and the intense predatory exploration that occurred in the past, mainly from the selective cutting of the largest individuals, it is estimated that 30% of the original populations of this species were lost, causing a decrease in its genetic diversity (Martinelli and Moraes, 2013). For these reasons, *D. nigra* is classified as vulnerable on the Red List of Threatened Species of International Union for Conservation of Nature (IUCN) (IUCN, 2020).

The understanding of the effect of environmental disturbances on the genetic divergence of *D. nigra* will allow us to establish strategies for its conservation *in situ* and *ex situ*. Information on the distribution of genetic diversity among and within populations contributes effectively to the conservation of genetic resources (Templeton, 2011; Potter et al., 2017).

One of the widely used tools for the quantification of these parameters is the molecular markers (Filippos, 2016). Among them, the dominant markers, such as the Inter Simple Sequence Repeat (ISSR), have been used successfully in the quantification of diversity and genetic structure in the genus *Dalbergia*, allow detecting the genetic variability among the evaluated individuals (Wang et al., 2010; Ginwal et al., 2011; Yulita et al., 2020).

This research aimed to evaluate the diversity and genetic structure of two natural populations of *D. nigra* in Conservation Units. This information will be important to define strategies for the management of CU's and conservation of the species, in addition to the identification of future matrixes for use in the restoration and recovery of degraded areas, connectivity of landscapes and sustainable use of forest resources.

MATERIAL AND METHODS

Sampling strategy

The *D. nigra* samples were collected in two Conservation Units (CU's) located in the Southern Espírito Santo State: The National Forest of Pacotuba, with 449.72 ha (Flona of Pacotuba - 20°45' S; 41°17' O), and the Private Natural Heritage Reserve of Cafundó, with 517 ha (PNHR Cafundó - 20°43' S; 41°13' O). The studied areas are 4 km distant from each other, and both present the Seasonal Semideciduous Forest vegetation type. These CU's were selected due to their representativeness of environmental preservation in the region and, historically, have suffered environmental disturbances such as selective logging, local fires, and agricultural activities (ICMBIO, 2011) (Figure 1).

Leaf samples were collected from 24 *D. nigra* adult trees, 12 individuals from each population. The sampling carried out taking into account the distribution of populations and the geographical distance of at least 100 m between individuals, avoiding kinship (Sebbenn, 2002).

DNA extraction and genotyping via ISSR

The genomic DNA was extracted by the CTAB method developed by Doyle and Doyle (1990), adjusting the concentrations to 1% polyvinylpyrrolidone (PVP) and 2% cetyltrimethylammonium bromide (CTAB). The determination of the concentration and purity values of the extracted DNA was estimated by spectrophotometry performed with the NanoDrop 2000C equipment (Thermo Scientific), adopting the ($1.80 \leq A_{260}/A_{280} \geq 2.00$) ratio as quality criteria (Aguilar et al., 2016).

To perform the PCR assays, aliquots of the individuals' DNA were used at a final concentration of 10 $ng{\cdot}\mu L^{-1}$ with

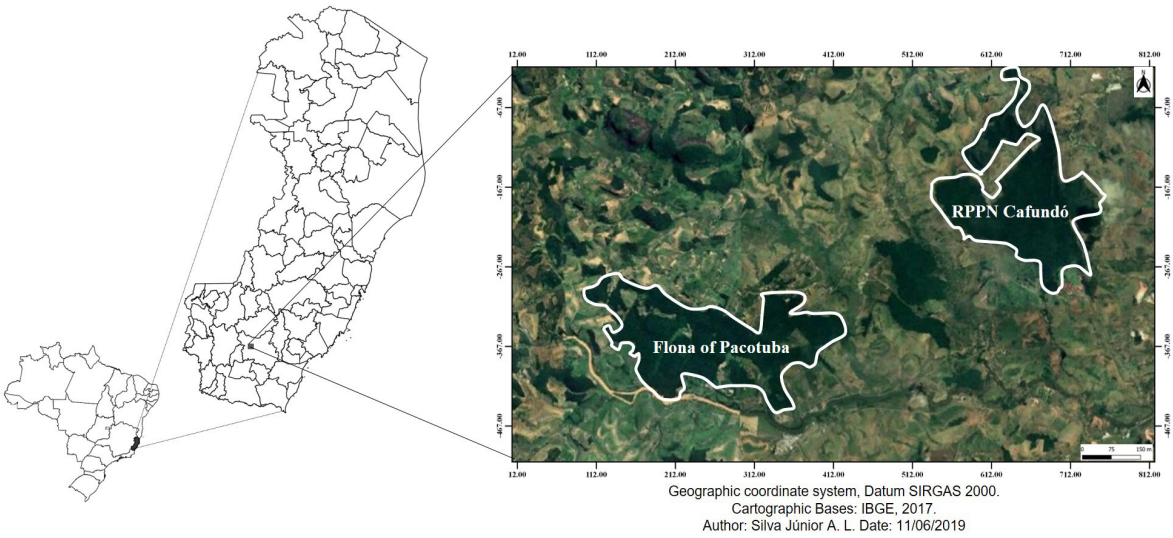

FIGURE 1 Location map of the studied areas, showing the Conservation Units: Flona of Pacotuba and PNHR Cafundó.

eight ISSR primers (UBCs 807; 809; 810; 811; 812; 818; 822 and 836), developed by the University of British Columbia. The total reaction volume was 20 μL, containing: 1X Buffer (10 mM Tris-HCL (pH 8.5) and 50 mM KCl), $MgCl_2$ (2.5 mM), dNTP (1 mM), primer (0.2 μM), 1 unit of Taq DNA polymerase and 50 ng of genomic DNA.

The fragment amplifications were performed in a thermocycler (Applied Biosystems, model Veriti), with initial denaturation step of 5 min at 94 °C, followed by 35 denaturation cycles (94 °C for 45 sec), annealing (52 °C for 45 sec) and extension (72 °C for 90 sec) and a final elongation of 72 °C for 7 min. Subsequently, the amplification products were separated by electrophoresis on a 2% agarose gel, at an electrical charge of 100 volts for 4 hours. The gels were submerged in ethidium bromide solution (0.50 μg/mL) for 30 min, photographed under UV light on a photocumenter (ChemiDoc MP Imaging System - Bio Rad) and separated according to the molecular weight by the 100 bp ladder marker (Ludwig Biotechnology).

Statistical analysis

Amplification result was converted into a binary matrix. Each primer was evaluated for the total number of bands (TNB), the number of polymorphic bands (NPB), percentage of polymorphic bands (PPB) and size variation of the fragments generated in base pairs (SVF). The polymorphic information content (PIC) was also accurate according to Weiler et al. (2010).

To estimate genetic diversity among individuals and between populations, the number of observed alleles (A_o), number of effective alleles (A_e), genetic diversity of Nei (H*) and Shannon diversity index (I*) were estimated. The average number of migrants per generation (N_m) was also calculated, which estimates the gene flow between populations. All of these statistical parameters were calculated using the Popgene software version 3.2 (Yeh and Boyle, 1997).

From the binary matrix, the genetic dissimilarity matrix was generated through the arithmetic complement of the Jaccard coefficient. The genetic dissimilarity was used to make the dendrogram, developed by the method of grouping unweighted arithmetic means (UPGMA), with a cut-off point following the proposed by Mojema (1977), with $k = 1.25$. The consistency checking between the values matrix and the formed clusters was determined by the co-phenetic correlation coefficient (CCC). For these analyzes, the Genes software (Cruz, 2016) was used the dendrogram was generated by the software R (R Development Core Team, 2016), associated with the use of vegan (Oksanen et al., 2018), cluster (Maechler et al., 2019), dendextend (Galili et al., 2020), factorextra (Kassambara and Mundt, 2020), ggpubr (Kassambara, 2020), cowplot (Wilke, 2019), gridExtra (Auguie and Antonov, 2017).

The analysis of molecular variance (AMOVA) was performed using the software Arlequin version 3.5 (Excoffier and Lischer, 2010). However, the number of groups (*k*) generated for the study sample, was obtained by the Bayesian approach carried out in the program Structure 2.3.3 (Falush et al., 2007). For the structuring analysis, the following parameters were established: for each *k* value, 20 runs were carried out, with a pre-established number of groups, with a *k* value ranging from 1 to 5, according to the proposed by Evanno et al. (2005), and 10.000 Monte Carlo simulations via Markov Chains (MCMC). The data generated from this analysis were plotted in the Structure Harvester software (Earl

and Vonholdt, 2012), to define the most likely *k* value according to the Δ*k* method (Evanno et al., 2005). After selecting the best *k*, a consensus of the 20 interactions was made using the Clumpp software (Jakobsson and Rosenberg, 2007) and then the graphic result was obtained using the Distruct software (Rosenberg, 2004).

RESULTS

Descriptive analysis

Genotyping generated 97 bands in total, of which 66 were polymorphic, corresponding to 68% polymorphism. The UBC 809 primer presented the highest number of total and polymorphic bands, while the UBC 822 primer presented the lowest values. The UBC 809 primer also obtained the highest percentage of polymorphic bands (75%), meanwhile, UBC 807 presented the lowest PPB (54.54%) (Table 1).

Markers efficiency and genetic diversity

For the *loci*, we observed that the averages of the polymorphic information content (PIC), which measures the efficiency of the markers, were similar for both populations (0.36 and 0.37), as well as for the joint data (0.34) (Table 2).

The individual evaluation showed that Flona of Pacotuba had a greater number of observed and effective alleles (A_o and A_e) when compared to PNHR Cafundó. The parameters that determine the degree of genetic diversity among individuals (H* and I*) also indicated greater divergence within the population of the Flona of Pacotuba.

For the joint data, the number of observed alleles (A_o) was two alleles per *loci*, with an average of 1.57 of effective alleles (A_e). The H* and I* indices showed a small increase in genetic diversity when the data were evaluated together (H*= 0.33; I* = 0.50).

The cluster analysis performed by the UPGMA method separated the individuals into four groups. The largest group was composed of 14 individuals, with 12 accessions collected from the PNHR Cafundó and two from Flona of Pacotuba. Two other smaller groups with five and four individuals, respectively, were formed exclusively by materials from Flona of Pacotuba; and the last group presented a single individual, also from Flona of Pacotuba (Figure 2). The co-phenetic correlation coefficient (CCC) was 78%, indicating consistency between the matrix of values and the formed groups.

Genetic structuring

The evaluation of the population structure carried out by AMOVA resulted in the global

TABLE 1 ISSR primers selected for *Dalbergia nigra*, including sequence (5'-3') for each primer, the total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (PPB) and size variation of the fragments (SVF) determined by a 100 bp molecular weight marker.

Primer	Sequence (5'-3')	TNB	NPB	PPB (%)	SVF (max-min)
UBC 807	AGAGAGAGAGAGAGA GT	11	6	54.54	1300 – 300
UBC 809	AGAGAGAGAGAGAGA GG	16	12	75.00	1300 – 370
UBC 810	GAGAGAGAGAGAGAG AT	13	8	61.53	1500 – 350
UBC 811	GAGAGAGAGAGAGAG AC	13	9	69.23	1500 – 190
UBC 812	GAGAGAGAGAGAGAG AA	15	11	73.33	1400 – 400
UBC 818	CACACACACACACAC AG	11	8	72.72	1150 – 450
UBC 822	TCTCTCTCTCTCTCT CA	7	4	57.14	1300 – 700
UBC 836	AGAGAGAGAGAGAGA GYA	11	8	72.72	1300 – 390
AVERAGE	-	-	-	68.04	-
TOTAL	-	97	66	-	-

* A = Adenine; T = Thymine; C = cytosine; G = Guanine and Y = (C or T).

TABLE 2 Polymorphic information content (PIC), number of observed alleles (A_o) and effective (A_e), and genetic diversity estimated by the Nei (H*) and Shannon (I*) indices estimated for *loci*, populations, and joint data.

Loci	Flona of Pacotuba					PNHR Cafundó					Joint Data				
	PIC	A_o	A_e	H*	I*	PIC	A_o	A_e	H*	I*	PIC	A_o	A_e	H*	I*
UBC 807	0.46	1.83	1.73	0.38	0.54	0.40	1.83	1.58	0.33	0.49	0.40	2.00	1.70	0.40	0.58
UBC 809	0.39	1.83	1.58	0.32	0.47	0.34	1.58	1.33	0.20	0.30	0.33	2.00	1.50	0.30	0.46
UBC 810	0.34	1.75	1.48	0.25	0.38	0.34	1.87	1.49	0.30	0.45	0.33	2.00	1.55	0.33	0.50
UBC 811	0.34	2.00	1.46	0.26	0.40	0.39	1.77	1.54	0.31	0.45	0.31	2.00	1.53	0.31	0.48
UBC 812	0.33	2.00	1.56	0.33	0.50	0.36	1.81	1.49	0.29	0.44	0.34	2.00	1.56	0.34	0.51
UBC 818	0.36	2.00	1.63	0.36	0.54	0.37	1.25	1.15	0.09	0.14	0.33	2.00	1.57	0.33	0.51
UBC 822	0.33	1.75	1.39	0.25	0.38	0.43	1.75	1.58	0.32	0.46	0.33	2.00	1.57	0.33	0.50
UBC 836	0.40	1.87	1.63	0.35	0.52	0.39	1.75	1.49	0.29	0.43	0.36	2.00	1.61	0.36	0.53
AVERAGE	0.36	1.87	1.55	0.31	0.46	0.37	1.70	1.45	0.26	0.39	0.34	2.00	1.57	0.33	0.50

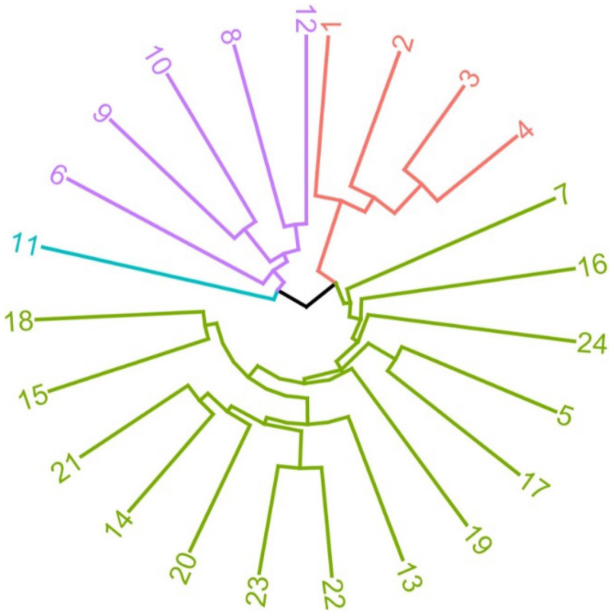

FIGURE 2 Dendrogram obtained by the UPGMA method, representing the genetic dissimilarity among 24 individuals of *Dalbergia nigra* species. The numbers 1 to 12 correspond to Flona of Pacotuba population and 13 to 24 are those from PNHR Cafundó. Cutoff point (Pc): 0.473.

estimate of Φ_{ST} = 0.1404, which means that only 14.04% of the total genetic variation was between populations, while the largest variation (85.96%), was intrapopulational (Table 3). The estimated N_m for the populations was 3.21.

TABLE 3 Analysis of molecular variance (AMOVA) between and within *Dalbergia nigra* populations.

Variation source	Degrees of freedom	Sum of squares	Components of Variance	Variation (%)
Between populations	1	23.25	1.64	14.04
Within populations	16	161.25	10.07	85.96
Total	47	184.50	11.72	
Φ_{ST} = 0.1404				

The structuring carried out in the scope of species obtained by the Bayesian approach, defined the most likely number of *k* clusters in two (*k* = 2) (Figure 3).

DISCUSSION

Performance of ISSR markers

The use of ISSR markers was efficient in identifying polymorphisms among the individuals evaluated. The observed PPB value was close to the obtained by Ribeiro et al. (2005) who found 73% polymorphism, based on allozyme markers for three *D. nigra* populations located in of the Rio Doce State Park, Minas Gerais state. Juchum et al. (2007) reported 39% polymorphism using RAPD markers for two populations, one located at PNHR Veracel and the other at Brazilwood Ecological Station, Porto Seguro, Bahia state. The history of disturbance in the areas mentioned in previous studies is similar to that evidenced in Flona de Pacotuba and PNHR Cafundó. Therefore, the differences found can be justified by the number of populations evaluated and also by the type of marker.

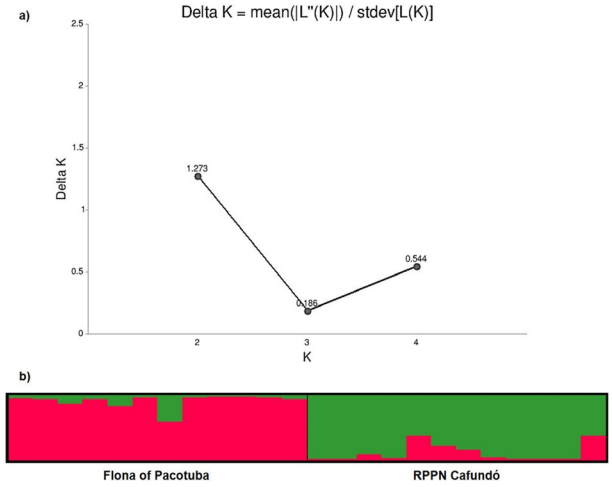

FIGURE 3 Population structure analysis (Bayesian approach) for two populations of *Dalbergia nigra*: Flona of Pacotuba (*N*=12) and PNHR Cafundó (*N*=12). a) Relationship of Δ*k* values for each *k* value. b) Genetic structure of *D. nigra* populations (indicated by different colors).

The PIC values corroborate with the PPB results. According to Chesnokov and Artemyeva (2015), for dominant markers, the PIC value between 0 and 0.25 is classified as not very informative; from 0.25 to 0.45, is moderately informative; and from 0.45 to 0.50 is highly informative. This classification determines that the markers used in this study, for individual populations, and the joint data, were moderately informative. However, the data obtained were sufficient to estimate the genetic diversity of *D. nigra* in natural populations.

Genetic diversity

The number of alleles (A_o and A_e) and the genetic diversity (H* and I*) estimated for the individual populations, were higher for the Flona of Pacotuba. Such results indicate that this population has a better distribution of alleles and, consequently, a greater genetic diversity. This fact can be directly related to

strategies carried out by the Conservation Unit (CU), such as fire prevention measures or selective cutting, the georeferencing of trees with some degree of threat, seed collection, and production of seedlings implanted in the CU itself. It should be noted that the PNHR Cafundó, despite having less genetic diversity, also represents an important source of genetic variability, having exclusive alleles, evidenced by the increase in the values of the parameters A_o, A_e, H^* and I^* of the joint data (Table 2).

For the joint data, the number of observed alleles (A_o) is equivalent to what is expected for diploid species, two alleles per *loci*. The average A_e (1.57) indicated a good distribution of alleles between populations. The results of H* and I* for the joint data revealed moderate to high genetic diversity.

The values of H* and I* found in this study were higher than those found for species of the genus *Dalbergia*, using ISSR markers. In studies with the species *D. sissoo*, mean values of 0.27 were found for H* and 0.41 for I* (Wang et al., 2010), while for *D. latifolia* species, those values were 0.16 and 0.25, respectively (Yulita et al., 2020).

The satisfactory levels found for the genetic diversity of the species indicate that it has managed to keep up with the disturbances generated by its predatory exploitation and the fragmentation of natural populations, having as a positive factor the fact that they are located in CU's, which restrict selective cutting. Studies with vulnerable and threatened species, such as *D. nigra*, demonstrated that these species can restore their population, if there is genetic diversity, knowledge and, control over the genetic structure in the occurrence areas (Martinelli and Moraes, 2013; Souza et al., 2017; Mangaravite et al., 2019). This is even more relevant because *D. nigra* is an allogamous species, that is, it preferably performs cross-fertilization benefited by entomophilous pollination, consequently increasing the genetic diversity.

The smallest genetic distance was observed between individuals 3 and 4, from Flona of Pacotuba, which are the individuals with the shortest geographical distance (~ 100 m). The highest genetic dissimilarity value, between individual 9 from Flona of Pacotuba and individual 16 from PNHR Cafundó, was also related to the individuals' geographical distance. Although the fruits of *D. nigra* are flat, dry and long, favoring anemochoric dispersion and allowing the seeds to reach greater distances in relation to the mother plant, tree species are sometimes structured in subpopulations or demes, making them genetically more homogeneous, with a higher probability of kinship (Sebbenn, 2002; Gonçalves et al., 2019), corroborating what was observed in this study.

The partition of genetic dissimilarity and the formation of the dendrogram (Figure 2), demonstrated a close genetic relationship of some individuals from the Flona of Pacotuba (individuals 5 and 7) with the population from PNHR Cafundó. This result can be explained by the proximity of the CU's and the entomophilous pollination system of *D. nigra*, carried out mainly by bees (Silva and Costa, 2014), which may be favoring the exchange of pollen between the individuals from the studied populations.

Notwithstanding, most individuals are genetically distinct, mainly within the Flona of Pacotuba and between the two populations. These individuals can be used as seed-trees for conservation and forest breeding programs, making possible the restoration and recovery of degraded areas, the connectivity of landscapes, and sustainable use of forest resources.

Regarding the lower genetic divergence among individuals from the PNHR Cafundó, which resulted in lower population diversity, it is necessary to adopt actions that increase the genetic variability in this forest fragment. As part of a strategy, we recommend the maintenance of the adult trees, who will serve as a source of seeds associated to the planting of seedlings with genetic material from other forest fragments with similar edaphoclimatic characteristics, or from individuals genetically distinct, such as those present in Flona of Pacotuba.

Genetic structuring

According to Wright's (1978) classification, the Φ_{ST} obtained (0,14) indicated that the populations evaluated are moderately differentiated ($0.05 < \Phi_{ST} < 0.15$). Furthermore, AMOVA revealed that the greatest genetic variation is within populations.

The finding of the greatest genetic variation within populations reinforces the hypothesis of the alleles exchange between them. However, the moderate genetic differentiation between populations indicates that they tend to become genetically more distant over the generations if the gene flow is interrupted. This is an important result since we have known that small and isolated populations are strongly influenced by genetic drift (Sebbenn et al., 2011; Pence, 2016).

The number of migrants (N_m) greater than the unit ($N_m = 3.21$), as we found in this study, indicates the occurrence of gene flow between populations (Wright, 1951). However, this estimate corresponds to the historical gene flow, referring to the interaction of populations in the past, when the actual adult trees settled (Kageyama et al., 2003). Nevertheless, the N_m value found in this study can be considered low when

compared to the values described for other tree species, such as 12.70 for *Plathymenia reticulata* (Souza et al., 2017) and 13.54 for *Senefeldera verticillata* (Vieira et al., 2018), which may be favoring the moderate genetic differentiation between populations.

Additionally, the structuring analysis carried out for the species indicated the formation of two groups (Figure 3). This result corroborates with the observed in the dendrogram (Figure 2). The color contrast between populations also confirms AMOVA's results, which indicate moderate genetic differentiation. Furthermore, it is possible to visualize the sharing of green color, where red is predominant and vice versa, showing a small exchange of alleles between populations.

In general terms, the structuring in two genetic groups well divided among the populations, the moderate genetic differentiation and the small occurrence of gene flow, are conflicting contexts. However, this can be explained by the exchange of alleles favored by only a part of individuals, such as those located at the edges of forest fragments. These results also indicate that populations are becoming structured, which can make them more susceptible to genetic erosion processes.

Therefore, we suggest to increase the connectivity of forest fragments using ecological corridors and, as previously mentioned, the planting of seedlings with contrasting genetic material. Such actions can prevent genetic degradation and may gradually restore the population effective size, especially for PNHR Cafundó.

CONCLUSIONS

The ISSR molecular markers are efficient in determining the levels of polymorphisms among individuals and in detecting the genetic diversity of *D. nigra* populations. There is a moderate to high genetic diversity for the species. However, most of the genetic diversity is influenced by the presence of genetically dissimilar individuals from the Flona of Pacotuba. Additionally, the occurrence of low gene flow, moderate genetic differentiation and structuring in two groups indicate that populations are becoming structured, which can affect the maintenance of populations in the long term.

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