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Study of the genetic diversity and structure of a natural population of *Nectandra megapotamica* (Spreng.) Mez. using RAPD markers

L.S. Costa[1], L.R.S. Reiniger[1], B.M. Heinzmann[2], L.P. Amaral[2] and C.M.L. Serrote[1]

[1]Núcleo de Biotecnologia e Melhoramento, Departamento de Fitotecnia, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil
[2]Laboratório de Extrativos Vegetais, Departamento de Ciências Florestais, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil

Corresponding author: L.R.S. Reiniger
E-mail: liarsr@ufsm.br

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ABSTRACT. *Nectandra megapotamica* (Spreng.) Mez. is a tree species that naturally occurs in the Atlantic Forest, Brazil. The aim of this study was to evaluate the genetic diversity and structure of a natural population of 12 *N. megapotamica* individuals using random amplified polymorphic DNA markers. Eleven primers were used in this study, producing 81 bands, of which 98.99% were polymorphic. Analysis using STRUCTURE defined three different clusters (K = 3), results that were consistent with those of principal coordinates analysis. Both Nei's genetic diversity (h = 0.33) and Shannon's diversity index (I = 0.49) were relatively high. Analysis of molecular variance indicated that 24.89% of the genetic variability was among clusters, while the remaining 75.11% was within clusters. The Mantel test showed a weak correlation between genetic and geographic distances (r = 0.25, P = 0.105). Overall, the results revealed high levels of genetic diversity within clusters and high genetic differentiation among

clusters without any spatial pattern of genetic variability. In addition, gene flow was independent of the geographical distribution and was compatible with the hierarchical island model.

Key words: PCR; Genetic parameters; Forestry species

INTRODUCTION

The Atlantic Forest biome is one of the world's major hotspots of biodiversity. Previous studies indicated that the flora of this biome consists of approximately 20,000 known species, including about 8,000 endemic species (Mittermeier et al., 1998). Among the native species of the Atlantic Forest, those belonging to the Lauraceae family are all woody plants with high economic value for timber and essential oils.

Nectandra megapotamica (Spreng.) Mez. (Lauraceae), commonly known as canela-preta, is found in the south, southeast, and central regions of Brazil. This species is widely used in folk medicine for its antirheumatic, anti-inflammatory, and analgesic properties. As previous phytochemical studies have shown, these properties are due to the presence of phenylpropanoids (Garcez et al., 2009), tetrahydrofuran lignans, and alkaloids (Dos Santos and Gilbert, 1975). Additionally, Apel et al. (2006) suggested that the essential oils of *N. megapotamica* have pharmacological potential due to their antitumor activity and antimicrobial activity against *Staphylococcus aureus*. Environmental factors such as seasonality, temperature, water availability, radiation, nutrient availability, altitude, and atmospheric pollution can significantly alter the biological activity of various extractives; however, the chemical composition of essential oils is mainly determined by genetic factors (Gobbo-Neto and Lopes, 2007).

Molecular markers are an important tool for evaluating genetic diversity within and among species and populations. Compared to other polymerase chain reaction (PCR) markers, Random Amplified Polymorphic DNA (RAPD) markers have several advantages; they are easy, affordable, quick to assay, and require only small amounts of DNA (Bered et al., 1997). The assessment of genetic diversity in natural populations is important for the study of speciation in the tropical forests and the conservation of genetic resources (Buckley et al., 1988). The objective of the present study was to assess the diversity and characterize the genetic structure of a natural *N. megapotamica* population from the central region of Rio Grande do Sul state, Brazil, using RAPD markers.

MATERIAL AND METHODS

Plant material and DNA extraction

A total of 12 individual samples of *N. megapotamica* were collected in Itaara, Rio Grande do Sul, Brazil (Table 1) and dried at room temperature. DNA was extracted from dry leaves as described by Dellaporta et al. (1983).

RAPD reactions

The primers were selected according to the results obtained by Hanai et al. (2010) using *Ocotea catharinensis* (Lauraceae). A total of 11 primers were selected, based on their high polymorphic profile and good reproducibility of the generated fragments (Table 2). The amplification

reactions were performed in an AmpliTherm TX96 Plus™ thermocycler (AmpliTherm Thermal Cycler, Madison, WI, USA). Each amplification reaction (25 µL) contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 µM RAPD primer (Sigma-Aldrich, St. Louis, MO, USA), 20 ng DNA, 1.5 U/µL Taq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA), and ultra-pure water. The thermal profile consisted of an initial denaturation at 94°C for 5 min, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min, and elongation at 72°C for 2 min, with a final extension at 72°C for 6 min. Amplification products were resolved by electrophoresis on 1.5% agarose gel.

Table 1. Coordinates and elevation of the collection area (Itaara, Rio Grande do Sul, Brazil) of the 12 *Nectandra megapotamica* individuals (N1-N12) used in this study.

Sample	Latitude	Longitude	Elevation (m)
N1	29 40' 10.10" S	53 46' 19.01" W	232.28
N2	29 40' 12.05" S	53 46' 18.60" W	230.36
N3	29 40' 12.62" S	53 46' 18.87" W	239.01
N4	29 40' 12.01" S	53 46' 18.84" W	230.84
N5	29 40' 12.82" S	53 46' 19.20" W	237.09
N6	29 40' 12.70" S	53 46' 18.41" W	242.86
N7	29 40' 12.49" S	53 46' 18.19" W	244.30
N8	29 40' 12.19" S	53 46' 17.66" W	240.93
N9	29 40' 11.89" S	53 46' 17.32" W	245.98
N10	29 40' 11.83" S	53 46' 17.23" W	247.18
N11	29 40' 11.84" S	53 46' 17.70" W	242.38
N12	29 40' 12.22" S	53 46' 19.42" W	230.60

Table 2. Selected random amplified polymorphic DNA (RAPD) primers used in this study for the genetic analysis of 12 *Nectandra megapotamica* individuals along with the number of obtained bands, number of polymorphic bands, and percentage of polymorphic bands.

Primer	Sequence	Number of obtained bands	Number of polymorphic bands	Polymorphic bands (%)
OPA-02	5'-TGCCGAGCTG-3'	2	2	100
OPA-04	5'-AATCGGGCTG-3'	11	11	100
OPA-10	5'-GTGATCGCAG-3'	3	3	100
OPA-11	5'-CAATCGCCGT-3'	9	9	100
OPA-12	5'-TCGGCGATAG-3'	6	6	100
OPA-13	5'-CAGCACCCAC-3'	11	11	100
OPA-15	5'-TTCCGAACCC-3'	4	4	100
OPA-18	5'-AGGTGACCGT-3'	6	6	100
OPD-05	5'-TGAGCGGACA-3'	10	10	100
OPD-07	5'-TTGGCACGGG-3'	10	10	100
OPD-08	5'-GTGTGCCCCA-3'	9	8	88.89
Overall		81	80	98.99

Data analysis

Fragment data were scored for the presence ("1") or the absence ("0") of a band using PyElph (Pavel and Vasile, 2012). The number of genetic clusters (*K*) in the population was defined by software STRUCTURE 2.3.4 (Pritchard et al., 2000) using the admixture model default and correlated allele frequencies. Each run had a burn-in length of 500,000 followed by 500,000 Markov Chain Monte Carlo (MCMC) repetitions with 20 iterations per *K* from *K* = 1 to 12. The optimal number of clusters was determined using the ΔK method (Evanno et al., 2005). Genetic variation was calculated by Nei's genetic distance (*h*) and Shannon's information index (*I*) using POPGENE

(Yeh et al., 1997). The analysis of molecular variance (AMOVA) was calculated using Arlequin 3.5 (Excoffier and Lischer, 2010), and principal coordinates analysis (PCoA) was performed using GenAlEx 6.5 (Peakall and Smouse, 2012) to visualize the genetic relationship between clusters. The Mantel test was applied to estimate the correlation between genetic distance and geographic distance among clusters.

RESULTS AND DISCUSSION

A total of 81 bands with molecular weights ranging from 250 to 2080 bp were amplified from the 11 RAPD primers. All primers showed a high level of polymorphism and 98.99% of the bands were polymorphic (Table 2). These results indicated that these 11 RAPD markers can be reliably used for differentiating *N. megapotamica* individuals and populations. Hanai et al. (2010) identified 94 bands when they used the same RAPDs for screening *in vitro* cultures of *Ocotea catharinensis*.

The optimal value of *K* was 3 as determined by ΔK statistic using STRUCTURE (Figure 1). The height of the ΔK values indicate the strength of the population subdivision; here, the highest value was observed for *K* = 3 and a second mode for *K* = 4. The standard deviation in LnP(K) (data not shown) also suggested the subdivision of the population into three clusters, and these results were consistent with PCoA (Figure 2).

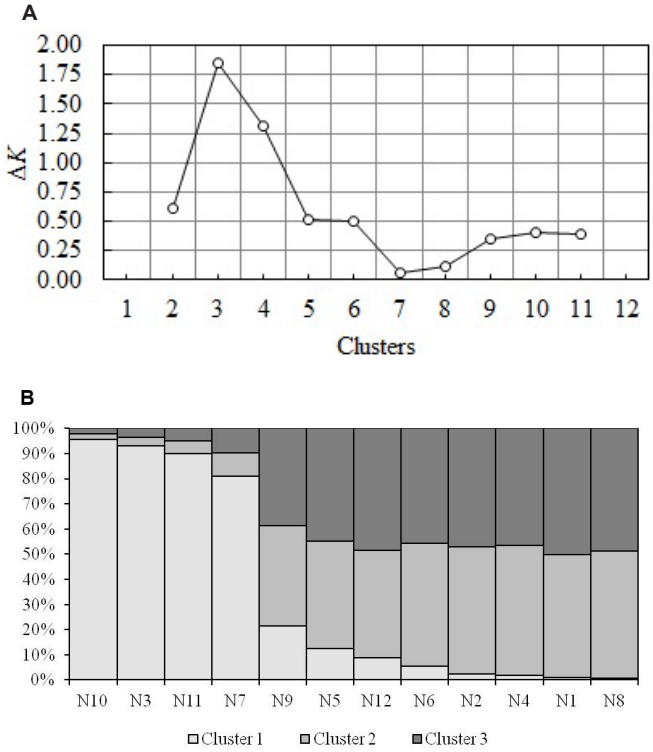

Figure 1. Δ*K* likelihood distribution **A.** Estimated population structure. **B.** Each individual is represented by a vertical column, which is partitioned into segments of different shades of grey that represent the individual estimated membership fractions in k clusters.

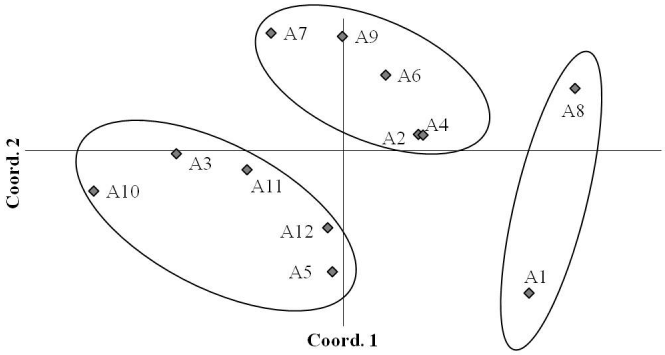

Figure 2. Genetic relationship among 12 *Nectandra megapotamica* individuals as determined by principal coordinate analysis (PCoA). Axis 1 accounts for 38.80% and axis 2 for 12.23% of the variation in the data.

The observed number of alleles (N_A) was 1.99, the effective number of alleles (N_E) was 1.56, *h* was 0.33, and *I* was 0.49 (Table 3). Using RAPD markers analysis, when compared to other tree species from the same family, the diversity in *N. megapotamica* could be considered high. Oliveira et al. (2008) found very similar results in *Dimorphandra mollis* (N_A = 2; N_E = 1.51; *h* = 0.30; and *I* = 0.46).

Among clusters, Nei's genetic distance ranged from 0.13 to 0.22 and Shannon's diversity index from 0.19 to 0.33 (Table 3).

Table 3. Number of individuals (N) and genetic parameters of the three clusters (*K* = 3), as determined by STRUCTURE (Pritchard et al., 2000) and principle coordinates analysis, of a natural population of 12 *Nectandra megapotamica* individuals.

Parameters	Cluster 1	Cluster 2	Cluster 3	Overall
N	5	5	2	12
Na	1.68 (0.47)	1.58 (0.49)	1.32 (0.47)	1.99 (0.11)
Ne	1.33 (0.32)	1.39 (0.40)	1.22 (0.33)	1.56 (0.32)
h	0.21 (0.17)	0.22 (0.21)	0.13 (0.19)	0.33 (0.15)
I	0.32 (0.17)	0.33 (0.30)	0.19 (0.28)	0.49 (0.18)
P%	67.9	58.02	32.1	98.77

Na = Observed number of alleles; Ne = Effective number of alleles; *h* = Nei's genetic distance; *I* = Shannon's information index; P% = Percentage of polymorphic loci.

AMOVA indicated that the highest genetic diversity was within (75.11%) rather than among the three clusters (24.89%), indicating that *N. megapotamica* is allogamous (Hamrick and Godt, 1996). In several tropical tree species, the variation within populations is greater than among populations (Zucchi et al., 2005). The pattern of distribution of the genetic diversity within the population was also observed in three species of the Lauraceae family: *Ocotea catharinensis* (80%), *Ocotea odorifera* (88%), and *Ocotea porosa* (84%) (Martins et al., 2015). Souza and Moscheta (2000) studied the breeding system of *N. megapotamica*, focusing on the flowering patterns and the synchronization of flowering, and identified the presence of an anti-selfing mechanism. Dichogamy has also been reported in other species of the Lauraceae family (Kubitzki and Kurz, 1984).

Estimates of genetic diversity among clusters F_{ST} using AMOVA indicated a high degree of differentiation between clusters 1 and 2 (0.16, $P < 0.05$) and very high differentiation between clusters 2 and 3 (0.25, $P < 0.05$). Between clusters 1 and 3, no significant differentiation was

observed (P > 0.05). These results showed that gene flow was independent from the geographical distribution and compatible with the hierarchical island model. The high and very high genetic differentiation observed between clusters 1 and 2, and 2 and 3, respectively, might be due to the relatively lower gene flow between individuals in these groups, as a result of a less synchronous activity of floral organs and pollinators. In *O. catharinensis*, *O. odorifera*, and *O. porosa*, the calculated F_{ST} values were relatively lower (0.148, 0.086, and 0.116, respectively), suggesting an effective gene flow among populations prior to habitat fragmentation (Martins et al., 2015). Zucchi et al. (2005) suggested that the variation among populations has direct conservation implications, indicating that a greater number of populations have to be sampled when the F_{ST} is high. On the other hand, if the F_{ST} value is low, a greater number of individuals by population must be sampled.

The Mantel test showed a low correlation between genetic and geographic distance among individuals (r = 0.25) that was not significant (P = 0.105), suggesting that the observed genetic differentiation was not caused by the distance. However, Zucchi et al. (2005) observed a strong spatial structure in the *Eugenia dysenterica* populations.

Overall, this study revealed the high levels of genetic diversity and high genetic differentiation among the three clusters. No spatial pattern of genetic diversity was observed between clusters, whereas the gene flow followed the hierarchical island model and was independent from the geographical distribution.

Conflicts of interest

The authors declare no conflict of interest.

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