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Genetic diversity among natural populations of *Mandevilla velutina*

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ABSTRACT

Mandevilla velutina (Mart. Ex Stadelm) Woodson (Apocynaceae) is an endemic species in the Cerrado (Brazilian Savannah), whose alcoholic extracts and root infusions are extensively used in the popular medicine to treat inflammatory diseases and against snake bites. Currently, this species has been pointed out as crucial in conservation programs. Therefore, studies on its genetic diversity, as well as the development of methodology for *in vitro* conservation in germplasm banks are imperative to avoid its extinction. The objective of this work was to investigate the intra- and inter-populational genetic variability of *M. velutina* to enhance the representativeness of germplasm banks. To this aim, we used RAPD molecular markers. The analysis of molecular variance (AMOVA) indicated that the intra-populational (81.25%) was higher than the inter-populational (18.75%) variability, which was confirmed by the Nei's Diversity Index. The PHI_{st} (0.188) and q_P (0.1586) values of genetic variation indicated high population structuring. There seems to be no direct correlation between geographic distances and genetic similarity among the three studied populations.

Keywords: *Mandevilla velutina*, medicinal plant, extinction risk, molecular markers.

RESUMO

Diversidade genética em populações naturais de batata infalível

A batata-infalível (*Mandevilla velutina* (Mart. Ex Stadelm) Woodson, Apocynaceae) é uma espécie endêmica do Cerrado, amplamente utilizada na medicina popular na forma de extrato alcoólico ou infusões do sistema subterrâneo. A planta é utilizada no tratamento de processos inflamatórios e em acidentes com serpentes. Atualmente, esta espécie é considerada prioritária em programas de conservação e, por isso, estudos de diversidade genética e métodos de conservação *in vitro* em bancos de germoplasma são relevantes para evitar sua extinção. O objetivo deste trabalho foi estudar a variabilidade genética em diferentes populações de batata infalível por meio de marcador molecular RAPD visando fornecer subsídios para a conservação da espécie em bancos de germoplasma. A análise molecular de variância (AMOVA) revelou que a variabilidade dentro das populações (81,25%) foi maior que entre populações (18,75%). Esses resultados foram confirmados pelo Índice de diversidade de Nei. As estimativas de variação de PHI_{ST} (0,188) e q_p (0,1586) indicaram alta estruturação populacional. Não foi observada uma relação direta entre as distâncias genéticas e geográficas das três populações estudadas.

Palavras-chave: *Mandevilla velutina*, planta medicinal, risco de extinção, marcador molecular.

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DNA analysis using molecular markers, such as RAPD (Random Amplified Polymorphic DNA), has been successfully used to study the genetic diversity of many plant species (Rout *et al*., 1998; Piola *et al*., 1999; Gauer & Cavalli-Molina, 2000; Bittencourt, 2000; Ciampi, 2001; Sales *et al*., 2001; Souza *et al*., 2004; Gonçalves *et al*., 2008; Gonçalves *et al*., 2009). RAPDs are valuable in studies of genetic mapping, population genetics, molecular systematics, genotype fingerprinting and marker-assisted selection in plant and animal breeding. Among its advantages, RAPDs are a low cost marker and provide an unlimited number of highly polymorphic fragments, which cover nearly the entire genomic DNA (Ferreira & Grattapaglia, 1998). Since they are not species-specific markers, RAPDs are especially suitable for the preliminary assessment of the genetic diversity in populations and/or species which little is known from in the scientific point of view, as it is the case of *Tibouchina papyrus* (Telles & Soares, 2007). RAPDs are PCR-based markers developed by Williams *et al*. (1990) that offer the opportunity to generate a large amount of polymorphism of DNA fragments spread throughout the genome, without requiring prior knowledge of the DNA-sequence target, or of species-specific primers. The technique involves the simultaneous amplification of several anonymous regions in the genome using arbitrary primer-sequences for PCR (Ferreira & Grattapaglia, 1998).

The genetic characterization of populations by means of molecular markers is based on the evaluation of differences in genetic patterns and the identification of specific bands. The different markers currently available allow us to access a considerable level of polymorphism, thus enabling the analysis of genetic variability within and between populations (Alfenas *et al*., 2006). The analysis of genetic diversity makes it possible to select genotypes of interest for further conservation in germplasm banks, aiming at preserving the genetic variability present in natural populations and decelerating the pace of genetic erosion.

In Brazil, especially in the Brazilian Savannah, numerous species have been listed as priorities in conservation programs and studies of genetic diversity, because of the high degree of endemism and devastation that this biome faces. In this context, we found the species

Mandevilla velutina (Mart. Ex Stadelm) Woodson, whose natural populations are shrinking to alarming levels. That spices is used in popular medicine as alcoholic extracts or infusions of the underground part to treat inflammatory processes and accidents with poisonous snakes (Almeida *et al*., 1998).

Our objective was to study the genetic variability of *Mandevilla velutina* to provide useful information for the *ex situ* conservation of is natural populations.

MATERIAL AND METHODS

Mandevilla velutina leaves were collected in the municipalities of São Carlos, Santa Catarina State, Pedregulho, São Paulo State, and Araxá, Minas Gerais State, Brazil, comprising in total 67 individuals. Leaves of each accession were packed separately, in indentified falcon tubes containing blue silica gel, and then stored at -20ºC, until DNA extraction. Plants were collected at random and the geographical location data were mapped by the Global Positioning System (GPS). An exsiccate was deposited in the Herbarium of Medicinal Plants, University of Ribeirão Preto (HPM-UNAERP, Ribeirão Preto, São Paulo State) under the number Nº. 0013.

Young leaves were used for genomic DNA extraction, according to Doyle & Doyle (1987). DNA was purified by re-suspension in 500 µL 1 M NaCl solution and incubation in water bath at 65°C for 5 minutes. Next, DNA was kept at 4°C for 30 minutes and centrifuged at 12,000 rpm for 5 minutes. The supernatant containing the DNA was transferred to another tube and 350 µl of Isopropanol were added. After a 10-minute rest, the DNA precipitated and was centrifuged at 12,000 rpm for 5 minutes. Then, the pellet was washed twice with 70% ethanol, followed by centrifugation at 12,000 rpm for 5 min. After drying for one hour at room temperature, the pellet was re-suspended in 100 µl of Milli-Q water. For quantification, DNA was applied to an agarose gel 1% (w/v), stained with Ethidium bromide and illuminated under ultraviolet light. Bands were compared to standard DNA (lambda phage) of known concentrations.

To standardize the RAPD protocol, DNA samples were evaluated initially with 100 primers (Operon Technology and Life Biosynthesis Incorporated), from which 11 were selected (Table 1). Only reproducible bands in different analysis were considered. Weak amplifications that eventually occurred were excluded. Control samples, containing all reaction products except DNA were evaluated to check for non self-amplification or presence of contaminants. At the end, we developed a matrix of binary data containing the absence or presence of amplified fragments.

The standardization of the PCR parameters for *M. velutina* produced the following RAPD reaction: 3 µL of Tp 10X; 3 µL of dNTPs 2.5 mM; 1.8 µL of $MgCl_2$ 25 mM; 7 µL of primers 10 ng μL^{-1}; 0.2 µL of Taq polymerase 5 u μL^{-1} and 2 µL of DNA 5 ng μL^{-1} in a final volume of 30 µL. The amplification was performed in a thermal-cycler as follows: two cycles at 94°C for 2 minutes, one cycle at 37°C for 1 minute, one cycle at 72°C for 2 minutes and 33 cycles at 94°C for 10 seconds, 40°C for 20 seconds (annealing temperature) and 72°C for 2 minutes.

For the analysis of genetic variability, the binary data (presence or absence of bands) obtained from RAPD, were used to estimate the allele frequencies based on the correction proposed by Lynch & Milligan (1994). Then, a descriptive analysis of the total variability was carried out by calculating the percentage of polymorphic loci, assuming the Hardy-Weinberg equilibrium. We used the software POPGENE (Yeh *et al*., 1999). Nei´s genetic distances (1973, 1978) were used in the cluster analysis, carried out by the UPGMA (unweighted pair-group method with arithmetic means) method (Legendre & Legendre, 1998).

The variance among and within populations was studied also by AMOVA (Analysis of Molecular Variance), as proposed by Excoffier *et al*. (1992). We used TFPGA (Miller, 1997), AMOVA-PREP 1.01 (Miller, 1998) and WINAMOVA 1.04 (Excoffier, 1992). Allele frequencies were also submitted to analysis of variance (Weir 1996; Telles, 2000).

RESULTS AND DISCUSSION

Among the primers evaluated, we selected those that produced bands of high intensity and that revealed a high degree of polymorphism (Table 1). The 11 primers selected produced 111 bands, mostly polymorphic in the three populations, as exemplified by primer OP11 for twenty-six accessions (Figure 1). The lowest and highest numbers of bands per primer were respectively seven and 15 (Table 1).

The results of the genetic variability obtained with different statistical estimates indicated that RAPDs provided a consistent genetic analysis of the three *Mandevilla velutina* natural populations studied. Considering the 67 individuals assessed, the total percentage of polymorphic loci was 81.08%. The population from Araxá comprised the highest percentage of polymorphic loci (77.48%), followed by the populations of Sacramento (64.86%) and Pedregulho (49.55%) (Table 2). The AMOVA based on RAPD markers showed that most of the genetic variability was in the intrapopulational (81.25%) when compared to interpopulational level (18.75%), which is an interesting result, since *M. velutina* is a cleistogamic species. PHI_{st} value was 0.188 ($p<0.001$), indicating a significant structuring of the genetic variability in these populations (Table 3). The analysis of variance for allele frequencies, which was used to evaluate the structure of the genetic variability in the populations of *M. velutina*, showed q_P values ranging from 0.027 to 0.2833, with a global value of 0.1586 (IC at 95% 0.2251 and 0.1037).

Wind is the main agent of seed dispersal in *M. velutina*, which favors a long distance spread, especially because seeds are light and crowned with a dense apical tuft of 15-20 mm-long trichomes (Almeida *et al*., 1998). According to Loveless & Hamrick (1984), seed dispersal by the wind increases the variation within populations, but this depends also on the wind speed and on characteristics of the seeds. Migration

Table 1. Primers and RAPD bands obtained in the analysis of 67 accessions of *Mandevilla velutina* (primers e bandas de RAPD obtidas na análise de 67 indivíduos de batata infalível). Ribeirão Preto, UNAERP, 2007.

Primers	Sequences (5'→ 3')	Number of bands		
		Total	Polymorphic	Monomorphic
1	CAGGCCCTTC	9	5	4
2	TGCCGAGCTG	12	12	0
3	AGGTGACCGT	8	7	1
4	GTCCACACGG	9	8	1
5	CAGCACCCAC	10	10	0
6	TCGCCCAGTG	7	6	1
7	TGCCGAGCTG	8	7	1
8	GAATATGGGTGCGCTCTG	8	8	0
9	AGGGGTCTTG	13	10	3
10	AAGGGATTGTTCTGTTCGCTG	15	11	4
11	TGATTACACCAATTACCACG	12	8	4
	Total	111	92	19

Table 2. Basic descriptive statistics of three populations of *Mandevilla velutina*, assuming the Hardy-Weimberg equilibrium (parâmetros descritivos básicos de três populações de batata infalível, assumindo o equilíbrio de Hardy-Weinberg). Ribeirão Preto, UNAERP, 2007.

Population	Sample average size ($\bar{n}$)	Polimorphic loci (%)
Pedregulho	10	49,55
São Carlos	16	64,86
Araxá	41	77,48
Total	67	81,08

Table 3. Inter- and intra-populational AMOVA for 111 individuals of *Mandevilla velutina* (AMOVA entre e dentro de populações para 111 indivíduos de batata infalível). Ribeirão Preto, UNAERP, 2007.

Sources of variation	DF	SQ	MSD	Components of Variance	Total variation (%)	P	Pairwise PHI_{ST}
Among populations	2	142.22	71.11	3.37	18.75	<0.001	0.188
Within populations	61	890.84	14.60	14.60	81.25	-	-
Total	63	1,033.06					

DF: degrees of freedom (graus de liberdade); SQ: sum of squares (soma de quadrados); MSD: mean square deviations (soma dos quadrados médio); P: levels of significance of the estimates of genetic variation after 1000 permutations (nível de significância da estimativa de variação genética após 1000 permutações).

of few seeds over long distances can prevent populations from diverge. This information corroborates the results currently obtained for *M. velutina*, as most of the genetic variability was identified within populations (81.25%). In this case, it is possible to infer that populations arise from different plants. However, to confirm this statement, it would be interesting to carry out a more refined analysis of gene flow via pollen and seed, which also could define the number of maternal lineages that gave rise to these populations.

Natural populations often have high levels of genetic variation. Several events can lead to this variation, as colonization of new habitats, territories or regions; population bottleneck; genetic changes by stochastic events, such as genetic drift, mutation, natural selection; and other aspects related to the species reproduction and biology (Solé-Cava, 2001; Solferini & Selivon, 2001; Fernandes-Matioli, 2001). For estimating PHI_{st} significantly different from zero, values greater than 0.05 were considered as indicators of high population structure and vice-versa (Solé-Cava, 2001). Thus, as the estimate of variation PHI_{st} was 0.188, higher than the limit established, we concluded that there was a high population structure. This result was confirmed by analyzing the pattern of spatial variation based on the Pearson correlation coefficient (r) between the matrices of Nei's genetic distances and the geographical distances between populations. The r value for the matrices was 0.80227, which is a high magnitude.

According to Solferini & Selivon (2001), genetic distances are measures of the divergence between populations based on genotypic frequencies. When the genetic distances among the sampled populations were analyzed, we observed variations between 0.308 (the lowest value, recorded between the populations from Pedregulho and São Carlos) and 0.744 (the highest value, estimated between the populations from São Carlos and Araxá). The populations from Pedregulho and Sao Carlos, although genetically closer, are more geographically distant from each other. Thus, it appears that there is no direct relationship between genetic and geographical distances among the three *M. velutina* populations

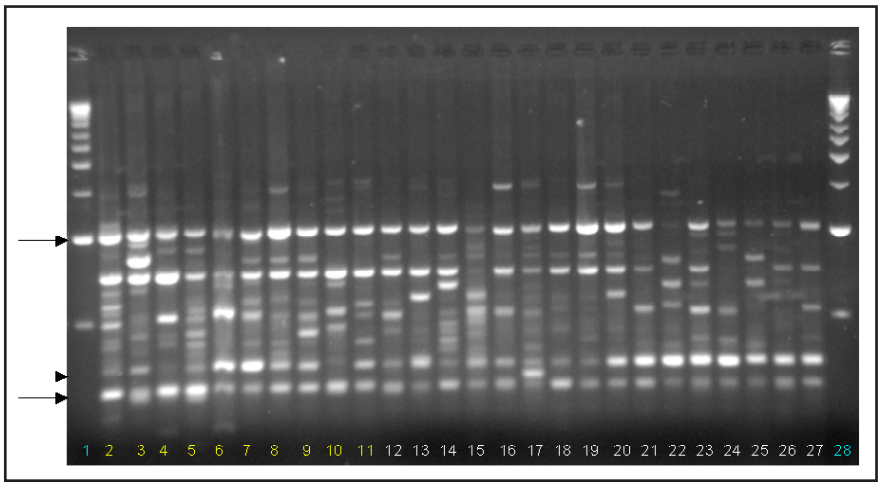

Figure 1. RAPD profile of natural populations of *Mandevilla velutina* produced by primer 11 (perfil molecular de bandas RAPD de populações naturais de batata infalível obtido com o primer 11). Ribeirão Preto, UNAERP, 2007.
* Lanes 2 to 27 correspond to ten and 16 individuals from the municipalities of Pedregulho and São Carlos, respectively (raias 2 a 27 correspondendo a dez e 16 indivíduos dos municípios de Pedregulho e São Carlos, respectivamente); Arrows indicate monomorphic bands (as setas indicam bandas monomórficas); Molecular ladders of 100 Kb in lanes 1 and 28 (marcadores de peso molecular de 100 Kb nas raias 1 e 28).

studied, as observed before for catuaba (*Anemopaegma arvense*) (Batistini, 2006).

Populations of endangered species are often structured. This is due to the environmental degradation, which promotes the formation of refuges (fragments), where small populations of these species persist without exchanging genes with individuals from not disturbed areas. Therefore, the analysis of the pattern of spatial variation is very important for conservation studies, because, if an endangered species in a given area appears structured, the conservation strategy should be to preserve the diversity of the species, preventing losses of local adaptations in the event of mixtures (Solé-Cava, 2001). Zimback *et al*. (2004) stress that the study of the genetic structure of natural populations from native species is important because it allows inferences about their current status and proposes measures to either maintain the conservation condition or to recover the genetic potential of the species. The figures of the statistical estimates presently obtained for *M. velutina* show the relevance of this work and point for the need to initiate conservation program for the species.

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