Genetic diversity among natural populations of *Mandevilla velutina*

Bianca Waléria Bertoni1; Ana Valéria de Souza1; Ronaldo Biondo1; Suzelei de C França1; Mariana PC Telles2; Ana Maria S Pereira1

1UNAERP-Dept Biotecnologia de Plantas Medicinais, Av. Costátilbe Romano 2201, 14096-900 Ribeirão Preto-SP; 2Universidade Católica de Goiás-Lab. Genética e Biodiversidade, Goiânia-GO; apereira@unaerp.br

**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 (0.188) and q (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.

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 Stadem) 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 its 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 indented Falcon tubes containing blue silica gel, and then stored at -20°C, until DNA extraction. Plants were collected at random and the geographical location data were mapped by the Global Positioning System (GPS). An excisicate was deposited in the Herbarium of Medicinal Plants, University of Ribeirão Preto (HPM-UNAERP, Ribeirão Preto, São Paulo State) under the number N. 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 centrifuged at 12,000 rpm for 5 minutes. The supernatant containing the DNA was transferred to another tube and 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 analyses 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, 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, 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 clustogenic species. PHI 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, values ranging from 0.027 to 0.283, 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
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 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 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 São 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.
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.

REFERENCES

ALFENAS AC. 2006. Eletroforese e marcadores bioquímicos em plantas e microorganismos. Vigosa: Editora UFV. 627p.

ALMEIDA SP; PROENÇA CEB; SANO SM; RIBEIRO JF. 1998. Cerrado: Espécies vegetais úteis. Planaltina: EMBRAPA-CPAC. 464 p.

BATISTINI AP. 2006. Diversidade morfológica, genética e química de populações naturais de Anemopaega arvense (Vell.) Stellf. Jaboticabal: UNESP. 96p (Tese doutorado).

BITTENCOURT JVM. 2000. Variabilidade genética em populações naturais de Maytenus ilicifolia por meio de marcadores RAPD. Universidade Federal do Paraná. 58p (Tese mestrado).

CIAMPIAY. 2001. Uso de marcadores moleculares nos estudos de genética de populações de espécies florestais. In: III SIMPOSIJO DE RECURSOS GENÉTICOS PARA AMÉRICA LATINA E CARIBE. Anaí... Londrina, IAPAR. p. 19-22.

DOYLE JJ; DOYLE JL. 1987. Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

EXCOFFIER L; MOUSSE PE; QUATTRO JM. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131: 479-491.

EXCOFFIER L; WINAMOV A 1.04: Analysis of Molecular Variance. 1992. (Programa para computador livre distribuído pelo autor) Disponível em <www.marksgeneticssoftware.net/amovaprep.htm>. Acessado em fevereiro de 2005.

MILLER MP. AMOVA-REP 1.01: 1998. A program for preparation of AMOVA input from dominant-marker of raw data (programa para computador livre distribuído pelo autor) Disponível em <www.marksgeneticssoftware.net/amovaprep.htm>. Acessado em fevereiro de 2005.

NEI M. 1973. Analysis of gene diversity in subdivided populations. Proceedings of the National Academy of Sciences of the United States of America 70: 3321-3323.

NEI M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583-590.

PIOLA F; ROHR R; HEIZMANN P. 1999. Rapid detection of genetic variation within and among in vitro propagated cedar (Cedrus libani Loudon) clones. Plant Science 141: 159-163.

ROUTE GR; DAS P; GOEL S; RAINA SN. 1998. Determination of genetic stability of micropropagated plant of ginger using Random Amplified polymorphic DNA (RAPD) markers. Botanical Bulletin Academia Sinica 39: 23-27.

SALES E; NEBAUER SG; MUS M; SEGURA J. 2001. Population genetic study in the Balarie endemic plant species Digitalis minor (Scrophulariaceae) using RAPD markers. American Journal of Botany 88: 1750-1759.

SOLÉ-CAVAAM. 2001. Biodiversidade molecular e genética da conservação. In: MATTIOLI SR. (ed) Biologia Molecular e Conservação. Ribeirão Preto: Editora Holos, p.172-192.

SOLFERINI VN; SELIVON D. 2001.
Polimorfismos de isoenzimas. In: MATIOLI SR. (ed) Biologia Molecular e Conservação. Ribeirão Preto: Editora Holos, p.139-142.

SOUZA LMFI; KAGEYAMA PY; SEBBENN AM. 2004. Estrutura genética em populações fragmentadas de Chorisia speciosa St. Hil (Bombacaceae). Scientia Forestalis 65: 70-79.

TELLES MPC. 2000. Diversidade genética e estrutura populacional de cagaita (Eugenia dysenterica DC.) do sudeste de Goiás. Goiânia: Escola de Agronomia e Engenharia de Alimentos – Universidade Federal de Goiás, 129p (Tese mestrado).

TELLES MPC; SOARES TN. 2007. DNA fingerprinting no estudo de populações de plantas do cerrado. In: PEREIRA AMS (ed). Recursos Genéticos e Conservação de Plantas Medicinais do Cerrado. Ribeirão Preto: Legis Summa Ltda, p. 109-145.

WEIR BS. 1996. Genetic Data Analysis II. Sunderland: Sinauer Associates.445p.

WILLIAMS JGK; KUBELIK AR; LIVAK KJ; RAFALSKI JA; TINGEY SV. 1990. DNA polymorphism amplified by arbitrary primers is a useful genetic markers. Nucleic Acids Research 18: 6531-6535.

YEH FC; R. BOYLE T. 1999. POGENE 1.32: Population Genetic Analysis. Programa para computador livre distribuído pelos autores. Disponível em: <ftp://ftp.microsoft.com/Softlib/MSLFILES/HPGL.EXE>. Acesso em: 25/02/2002.

ZIMBACK L; MORI ES; KAGEYAMA PY; VEIGA RFA; MELLO JUNIOR JRSM. 2004. Estrutura genética de populações de Trichillia pallida Swarts (Meliaceae) por marcadores RAPD. Scientia Forestalis 65: 114-119.