

Genetic differentiation in *Aspidosperma polyneuron* (Apocynaceae) over a short geographic distance as assessed by AFLP markers

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Genet. Mol. Res. 10 (2): 1180-1187 (2011)

Received October 25, 2010

Accepted December 9, 2010

Published June 21, 2011

DOI 10.4238/vol10-2gmr1126

ABSTRACT. Studies on intraspecific variation can contribute to the development of conservation strategies by identifying units of conservation for threatened species. *Aspidosperma polyneuron* is a tropical tree of seasonal semideciduous forests that is currently endangered and protected because it has been heavily logged for lumber, although it was once common in Brazil and neighboring countries. We investigated genetic structure in two samples of *A. polyneuron* collected from steep hillsides and from flat areas of a natural forest fragment in northern Paraná State, Brazil. Seven AFLP primer combinations yielded 200 markers, with a polymorphic rate of 88.5% for samples from the flat area and 99% for samples from the high declivity area. Total genetic diversity (H_T) was 0.387, while the genetic diversity

within the populations (H_s) was 0.307 and 0.372, for samples from the flat and the high declivity areas, respectively. Genetic differentiation between samples was high, with a mean F_{ST} of 0.265 and a genetic distance of 0.148, indicative of a high degree of genetic structure over a short distance. Principal coordinate analysis separated the samples into three groups of individuals; the first group included individuals from the high declivity area, the second group consisted of individuals only from the flat area, and the third group had individuals from both areas. Bayesian analysis also showed $K = 3$ clusters. The unexpected high level of intraspecific variation of *A. polyneuron* in this small forest fragment should be taken into account when evaluating the genetic impact of forest degradation on this species in other semideciduous forest fragments.

Key words: Conservation genetics; AFLPs; Genetic variability; Tropical forest fragment

INTRODUCTION

Habitat fragmentation and disturbance due to deforestation, weed invasion, fire, and the introduction of animals may directly reduce the actual size of plant populations and alter the abundance and effectiveness of pollinators and seed dispersers (Lamont et al., 1993; Aizen and Feinsinger, 1994; Kremen and Ricketts, 2000). Such factors may lead these populations to face reduction in diversity, genetic isolation and an increase in endogamy. When these processes are strengthened they may lead populations to greatly reduce their adaptability and even undergo extinction (Frankham, 1995; Newman and Pilson, 1997; Saccheri et al., 1998).

Aspidosperma polyneuron Muell. Arg (Apocynaceae) is a long-lived tropical tree species that can reach over 1200 years (Carvalho, 1994). The trees have a slow growth rate, taking 10 to 15 years to increase 5 cm in diameter at breast height and around 50 years to reach the reproductive stage (Toresan et al., 2005). This species grows to 20-30 m in height and its trunk can reach 60 to 90 cm in diameter. The species blooms from October to November with fruit maturation occurring from August to September, producing a large quantity of winged seeds with anemocoric dispersion, at intervals of 2 to 4 years. Pollen dissemination is probably carried out by moths and/or small insects (Morellato and Leitão-Filho, 1995).

With a broad range of distribution *A. polyneuron* can be found from 10° N (Venezuela) to 25° 50' S (Brazil), at altitudes of 80 to 1000 m and under mean annual rainfall of 1100 to 2500 mm (Carvalho, 1994). In Brazil, this species can be found in deep and fertile soils on ridges and slopes of seasonal semideciduous forests, in the States of Bahia, Mato Grosso do Sul, Minas Gerais, Goiás, Mato Grosso, Rondônia, and Paraná. From an ecological standpoint, *A. polyneuron* is considered to be one of the most important species for recuperation of degraded heterogeneous forests (Carvalho, 1994; Lorenzi, 2002). Nevertheless, this tree is in danger of extinction in the State of Paraná, as a consequence of the habitat fragmentation driven by agriculture and the economic exploitation of its heavy and compact wood that has been used in construction and furniture (Hatschbach and Ziller, 1995; Ribas et al., 2005). The species has also been exploited due to its chemical compounds, which include alkaloids, saponins and es-

essential oils (Schumutz, 1960; Marcondes-Ferreira Neto, 1988). The objective of the present study was to determine whether there are differences in the genetic structure between samples of *A. polyneuron* from two different microhabitats of a semideciduous forest fragment.

MATERIAL AND METHODS

Characterization of the study area

The study area covered 1.7 km², at an altitude of 464 m, of a seasonal semideciduous forest fragment located on the Doralice Farm in Ibiporã County, Paraná State, Brazil (23°16'S and 51°03'W). This area is surrounded by monoculture crops, orchards and grasslands. The fragment exhibits an area of high declivity, close to the banks of the Tibagi River and, further ahead, the topography gradually softens until becoming almost flat. Whereas the flat portion of the fragment displays deep clay soil, with a high level of humidity and a lower availability of light under the canopy, the high declivity area presents soil that is weaker in nutrients, shallower with rocky outcroppings (Soares-Silva and Barroso, 1992). Plant density also varied between the two portions of the fragment, with the flat area exhibiting greater canopy cover and higher density of individuals than in the high declivity area (Costa, 2009).

Plants of *A. polyneuron* were sampled from the high declivity area and the flat area, at a minimal distance of about 200 m between areas, taking into account plants in the pre-reproductive phase, with a maximum stature of 2 m. Young leaves of 30 individuals were sampled from each area at a minimum distance of 30 m between individual plants to minimize sampling of siblings.

DNA isolation and amplified fragment length polymorphism (AFLP) reactions

Genomic DNA was isolated from approximately 0.5 g fresh leaves using the CTAB method, as described by Doyle and Doyle (1987). AFLPs were carried out as described by Vos et al. (1995). Briefly, DNA samples were submitted to restriction with *EcoRI*/*MseI* endonucleases (5 U each) and binding to their respective adapters. After incubation for 16 h at 37°C, samples were diluted (1:10) in ultrapure water. Polymerase chain reaction (PCR) amplification was carried out using pre-selective primers complementary to the adapters with addition of one 3' nucleotide and diluted 1:10. For selective amplification, an initial screening was carried out with four individuals from each area using 16 primer combinations. Seven primer combinations were chosen for selective PCR: *EcoRI*-AGC/*MseI*-CAC, *EcoRI*-AGC/*MseI*-CAG, *EcoRI*-AGC/*MseI*-CAT, *EcoRI*-AGC/*MseI*-CTA, *EcoRI*-ACG/*MseI*-CAT, *EcoRI*-ACG/*MseI*-CAC, and *EcoRI*-AGC/*MseI*-CA. The products of selective amplification were separated into polyacrylamide gels 7% (29:1) acrylamide:bis-acrylamide for 3 h at 200 V and stained with 20% silver nitrate. A 50-bp molecular ladder (Ludwig Biotecnologia, Ltda.) was used to determine the molecular weights of the fragments. Amplified fragments of between 50 to 1000 bp were used to create a presence/absence matrix.

Data analysis

Estimation of allele frequencies and genetic diversity of populations using dominant

markers may prove to be problematic (Zhivotovsky, 1999; Alexander et al., 2004). The possibility of statistical deviations could be eliminated through highly polymorphic data (Krauss, 2000). It is already known that, in order to obtain precise estimates of population genetic parameters, a high number of AFLP loci should be used, with a minimum of 30 individuals per population (Tero et al., 2003). Therefore, we used the dBoot v 1.1 software (Coelho, 2001) to estimate the coefficient of variation (cv) for the number of AFLP markers, generating a parameter that is capable of determining the reliability of the results of statistical analyses. The percentage of polymorphic loci (P_p), Nei's genetic diversity (H_s ; Nei, 1978), Shannon index (I), and genetic distance (Nei, 1972) were calculated, using POPGENE v. 1.31 (Yeh et al., 2000). Analysis of molecular variance (AMOVA) was estimated using Arlequin v. 3.11 (Excoffier et al., 2005) to evaluate the distribution of genetic variation within and among samples, as well as to estimate the F_{ST} index. Principal coordinate analysis (PCoA) was used to evaluate the distribution of genetic distance in clusters with the FAMD software (Schlüter and Harris, 2006). The reliability of these clusters was tested with a Bayesian clustering analysis using the STRUCTURE software (Pritchard et al., 2000).

RESULTS AND DISCUSSION

Genetic variability of samples

Seven selective primer combinations, applied to 60 individual *A. polyneuron* plants, yielded 200 reproducible AFLP markers with an average number of 28.57 markers per primer combination. The cv for the total number of markers was below 1%, showing the high consistency of the AFLP data. The percentage of polymorphic loci was 88.5% for samples from the flat area and 99% for samples from the high declivity area. The values of H_s (Nei, 1978) were 0.307 and 0.372 for samples from the flat and the high declivity areas, respectively. Total genetic diversity (H_T) was 0.387 and I were 0.460 and 0.549, for samples from the flat and the high declivity regions, respectively (Table 1). Torezan et al. (2005) used random amplified polymorphic DNA (RAPD) markers to assess genetic variation in samples *A. polyneuron* from six forest fragments, including five fragments that had been severely impacted by anthropic exploitation and one fragment within a conservation unit that is free of anthropogenic factors. The authors found fairly low values of $H_T = 0.278$, even when only the conservation unit was considered ($H_s = 0.285$ and $I = 0.387$), compared to those in this study (Table 1). The genetic diversity in the flat area of the Doralice farm forest fragment had recently suffered the anthropogenic effects of selective logging of adult trees. However, no dramatic effects on genetic variability are visible in the remaining plant population of the next generation, as demonstrated by the estimated genetic parameters.

Table 1. Genetic parameters estimated on the basis of 200 AFLP markers for two samples of *Aspidosperma polyneuron* from a forest fragment on the Doralice Farm, in Iporã County, Paraná State, Brazil.

| Population | P_p | H_s | I |
|------------------|-------|-------|-------|
| Declivity | 99.0 | 0.372 | 0.549 |
| Flat | 88.5 | 0.307 | 0.460 |
| H_T | 0.387 | | |
| Genetic distance | 0.148 | | |

Percentage of polymorphic loci (P_p), genetic diversity within areas (H_s), Shannon index (I), total genetic diversity (H_T), and genetic distance (Nei, 1972).

Several studies regarding genetic variability in tropical trees have shown similar results to the ones presented here. De Carvalho et al. (2008) using RAPD markers reported high genetic variability in a population of *Luehea divaricata* Mart. found in a declivity area, while Pither et al. (2003) found *I* values varying from 0.320 to 0.380 for *Terminalia amazônia* (J. Gmell) Exell. In *Swietenia macrophylla* King, Gillies et al. (1999) found an *I* of 0.350, and Aide and Rivera (1998) found a value of 0.590 for *Poulsenia armata* (Miq.) Standl. Comparatively, it can be demonstrated that *A. polyneuron* of the fragment on the Doralice Farm maintains a good level of genetic variability.

Genetic structure

AMOVA showed that 73.52% of the genetic variability was distributed within the sampled areas and 26.48% between areas (Table 2). *Aspidosperma polyneuron* is an allogamous species, probably fertilized by moths and/or small insects (Morellato and Leitão-Filho, 1995), and with wind dispersed seeds (Carvalho, 1994). As stated by Maguire et al. (2002), it is common for cross-fertilization species to present a higher percentage of variability within groups than among groups. Considering the plants studied, which were sampled from two areas located within a short (about 200 m) distance of each other, it is important to highlight the incidence of individuals of this species between areas, evidencing the occurrence of gene flow between the sampled areas.

Table 2. Analysis of molecular variance (AMOVA) applied to AFLP markers, for two samples of *Aspidosperma polyneuron* from one forest fragment of the Doralice Farm, in Ibiporã County, Paraná State, Brazil.

| Source of variation | d.f. | Sum of squares | Variance components | % of variance |
|---------------------|----------|----------------|---------------------|---------------|
| Between populations | 1 | 362.501 | 9.00197 | 26.48** |
| Within population | 73 | 1824.085 | 24.98747 | 73.52 |
| Total | 74 | 2186.587 | 33.98944 | |
| Fixation index | F_{ST} | 0.26485 | | |

d.f. = degrees of freedom. ** $P < 0.01$ (significance test from 1023 permutations).

Low levels of genetic differentiation have been observed between populations of tropical tree species within a short distance of each other (Hamrick and Loveless, 1989; Merzeau et al., 1994; Leonardi and Menozzi, 1996; Takahashi et al., 2000; Mariette et al., 2002). Nonetheless, in *A. polyneuron*, the genetic variability (Table 1) and genetic differentiation between areas were high ($F_{ST} = 0.265$; Table 2) for samples within a short distance of each other.

The use of neutral molecular markers to make inferences regarding adaptive processes is risky, unless natural selection is still in place or there is a strong connection between the selected loci and the neutral markers (Merzeau et al., 1994; Leonardi and Menozzi, 1996; Takahashi et al., 2000; Mariette et al., 2002). The AFLPs revealed a genetic distance (Nei, 1978) of 0.148 between samples of the two areas (Table 1). Therefore, it could be argued that the genetic differentiation observed between samples of *A. polyneuron* is related to differences in adaptation of the plants to ecological conditions in the two areas. For instance, the pattern of the flat area is represented by deeper soil with more humidity and higher pH, higher density of individuals, greater canopy cover, and less under canopy light availability, compared to the

high declivity area that is characterized by rocky, shallow and poor soil with lower population density. Analysis carried out on the population structure of *A. polyneuron*, in the same fragment studied here, showed that the distribution of plants was associated with the topographic gradient (Bianchini E, personal communication). Correlation between molecular differentiation and adaptive characteristics was observed in populations of *Cedrela odorata* L. (Gillies et al., 1997).

The correlation between distribution of tree species and soil and topographic variables has been presented in a variety of studies of tropical forests. Rodrigues et al. (2007) demonstrated in a community study of a seasonal semideciduous forest that the majority of species present correlation with soil fertility and texture over one topographic gradient. A similar report was presented by Carvalho et al. (2005), where they observed a reduction in the size of the forest as it grew away from the river, possibly caused by the reduction in water availability. In a study carried out in a seasonal semi-deciduous forest of the riparian vegetation of a lake, Campos and Landgraf (2001) revealed a higher diversity and abundance of plant species in areas closer to the lake.

The PCoA separated the samples into three groups of individuals (Figure 1). The first group includes individuals from the flat area; the second encompasses individuals from the high declivity area, and the last group is represented by individuals from both areas. This might be related to two random processes: first is that the seeds of the investigated species are wind dispersed; second, the pollinating agents, probably moths and/or small insects, are presumably able to fly distances greater than 200 m. The group formed by individuals from flat and high declivity areas is possibly a result of hybridization of individuals from both areas. The Bayesian analysis for the K number of clusters (K = 3) further supports the distribution of the genetic variation observed in the PCoA.

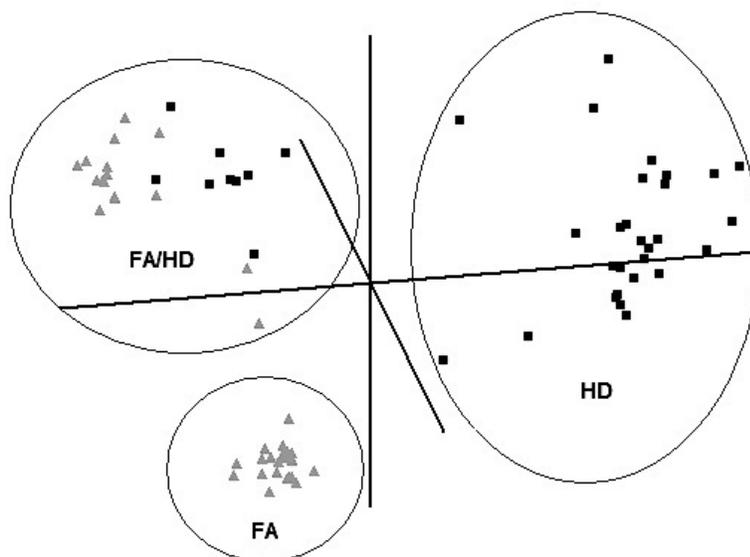


Figure 1. Principal coordinate analysis of two samples (FA = flat area; HD = high declivity area) of *Aspidosperma polyneuron* Muell. Arg. from Doralice Farm, Ibiporã County, Paraná State, Brazil. The first, second and third principal coordinates explain 30.2, 11.63, and 6.7% of the total variation, respectively.

It is interesting to note that the genetic diversity was higher ($H_s = 0.372$) for samples from the high declivity area than for samples from the flat area ($H_s = 0.307$), suggesting that the occurrence of gene flow is higher in one direction, i.e., from the flat area to the declivity area. Since samples from the flat area are located at higher altitude in relation to samples from the high declivity, the wind takes more seeds towards the declivity area than towards the flat area.

Our results showed that the forest fragment on the Doralice Farm can be considered a natural reservoir of genetic diversity for *A. polyneuron*. This knowledge is important not only in studies of genetic structure, but also for future comparative population studies of this species to evaluate the level of genetic erosion in disturbed areas. Finally, the genetic variability found within this region can be used as source of genetic diversity for future projects regarding the recovery of degraded areas.

ACKNOWLEDGMENTS

We thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), for the scholarship granted to J.O. Damasceno, and the Conselho Nacional de Desenvolvimento Científico (Process #478179/06-5) and Fundação Araucária for financial support.

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