ESTERASE POLYMORPHISM IN REMANANT POPULATIONS OF *Aspidosperma polyneuron* Müll.Arg. (APOCYNACEAE)[1]

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ABSTRACT – The population genetic structure of the endangered tree species *Aspidosperma polyneuron* Mull.Arg. (Apocynaceae) was reported based on analysis of esterase polymorphism in two remanant populations. Allelic variation was detected at three isoesterase loci (*Est-3*, *Est-9*, and *Est-10*). The proportion of polymorphic loci for both populations was 30% and deviation from Hardy-Weinberg equilibrium was observed for the *Est-3* locus observed in the northern population. Segregation distortion and the lower level of observed and expected heterozygosity in this population were attributed to founder genotype. The high genetic identity values for northern and northwestern populations are in accordance with the low levels of interpopulation genetic divergence demonstrated by the $F_{(ST)}$(0.03) value. The $F_{(IS)}$ value (0.23) indicated moderate levels of inbreeding. *A. polyneuron* can be indicated as an example of endangered species suggesting high genetic variation in contrast to the low genetic variation reported for endangered species. The esterase isozymes may be a good genetic marker for studies of natural *A. polyneuron* populations.

Key words: Apocynaceae, genetic diversity, isoesterases, isozymes, 'peroba-rosa'
Running title: Esterase isozymes in *Aspidosperma polyneuron*.

POLIMORFISMO DE ESTERASES EM POPULAÇÕES REMANESCENTES DE Aspidosperma polyneuron Müll.Arg. *(APOCYNACEAE)*

RESUMO** – A análise do polimorfismo de isozimas esterases foi usada para reportar a estrutura genética de duas populações remanecentes da espécie de árvore em extinção **Aspidosperma polyneuron Müll.Arg. (Apocynaceae)**. Variação alélica foi detectada em três locos de isoesterases (Est-3, Est-9, e Est-10). A proporção de locos polimórficos de ambas as populações foi de 30%, sendo observado um desvio do equilíbrio de Hardy-Weinberg no loco Est-3 na população da região norte do Estado do Paraná. Uma distorção na segregação e um mais baixo nível de heterozigosidade observada e esperada nesta população foram atribuídos ao efeito do genótipo fundador. Os valores altos de identidade genética das populações do norte e noroeste do Estado estão de acordo com o baixo nível de divergência genética interpopulacional demonstrado pelo valor de* $F_{(ST)}$ *(0,03). O valor de* $F_{(IS)}$ *(0,23) indicou moderado nível de endocruzamentos. **A. polyneuron** pode ser indicada como um exemplo de espécie em extinção apresentando variação genética alta, contrastando com a variação genética baixa reportada sobre espécies em extinção. As isozimas esterases podem ser um bom marcador genético em estudos de populações naturais de **A. polyneuron.

*Key words: **Apocynaceae**, diversidade genética, isoesterases, isoenzimas, 'peroba-rosa'*
*Running title: Esterase isozymes in **Aspidosperma polyneuron.***

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1. INTRODUCTION

Aspidosperma polyneuron Müll.Arg. (Apocynaceae) is a species known as "peroba-rosa" in the Southern region of Brazil, State of Parana (PR), and as 'perova', 'peroba amargosa', 'peroba rajada', 'peroba-açu', 'peroba comum', 'peroba-do-rio', 'peroba paulista', 'peroba mirim', and 'peroba miúda', in other states of Brazil (INOUÉ et al., 1984). This tree species has been considered in danger of extinction (VALENTINI et al., 1999). The factors involved in its decline are habitat destruction by human activity and the commercial preference for its wood. The wood of 'peroba-rosa' is durable and has been employed for house and furniture building since the 1970s (NOGUEIRA, 1977).

In the State of Parana, the remanant populations of *A. polyneuron* are limited to small isolated sites and the study of these populations has mainly focused on morphological characteristics of flowers and fruits (CONSTANTINO, 1990), and experimental culture of seedlings obtained by seed germination (SOUZA and MOSCHETA, 1992). Studies of genetic diversity in *A. polyneuron* are important for the conservation of this species and for breeding work. However, no information about biochemical markers and the genetic variability of this species is available in the specialized literature. Estimates of plant genetic diversity are necessary if we are to understand the forces that affect the genetic organization of natural plant populations (GITZENDANNER and SOLTIS, 2000). Maintaining the existing genetic diversity in populations is one of the most important measures for species conservation.

The use of allozyme variation has a long tradition in population genetics, and more recent application in conservation biology (GITZENDANNER and SOLTIS, 2000). Starch gel electrophoresis for isozyme analysis is of significant value as a method for determining genetic diversity in plants of economic importance because selective neutrality at isozyme loci can be assumed in most cases, and therefore the product of these loci provide a measure of relatedness among the plants that have been severely altered by natural or experimental selection processes (KARHU et al., 1996; STORFER, 1996). Nonspecific esterases are usual markers in genetic studies of animals, plants and microorganisms because they are easy to detect and appear to be highly polymorphic (DAVIS, 1964; MANGOLIN et al., 1997; RESENDE et al., 2000; MACHADO and CASTRO-PRADO, 2001). For most esterases a rather general substrate specificity is observed, indicating that they may have a broad biological function.

In the present study we reported esterase polymorphism as a biochemical marker in order to obtain preliminary data on the genetic variability present within and between two remanant populations of the *A. polyneuron* species.

2. MATERIAL AND METHODS

Samples of young leaves (8-10 mm) of *A. polyneuron* were collected from two different populations in the northern and northwestern regions of the State of Paraná (PR), Brazil (BR). The northern population consisted of 15 plants of medium height (4-5 m) established on the campus of the state University of Londrina (Londrina, PR) which were obtained from seedlings grown from germinated seeds in green house. The seeds were collected from plants previously native to this region but that were cut for different reasons (dying from diseases or urbanization processes).

The northwestern population consisted of 48 native plants naturally conserved for 54 years in the "Horto Florestal Dr. Luiz Teixeira Mendes" situated in an urban area (approximately 37 hectares) of Maringa (Maringa, PR). The "Horto Florestal" is an area protected from destruction established to conserve native and specific rare taxa, which often occur in small isolated populations or fragmented populations that have been reduced in size. The distance between Londrina and Maringa is 100 km. The plant height of the Maringa population ranged from 3 to about 10 m.

The electrophoretic evaluations were carried out on samples consisting of young leaves collected from each 'peroba-rosa' tree. The leaves were individually homogenized with a glass rod in an Eppendorf microcentrifuge tube using 60 ml of 1.0 M phosphate buffer, pH 7.0, containing 5% PVP-40, 0.01 M DTT (dithiothreitol), 10 mM sodium metabisulfite, 50 mM ascorbic acid, 1.0 mM EDTA, and 0.5% β-mercaptoethanol solution (RESENDE et al., 2000). After homogenization, the samples were centrifuged at 25,000 rpm for 30 minutes at 4 °C in a Sorval 3K-30 centrifuge.

The esterase isozymes (EST; EC 3.1.1._) were analyzed by procedures originally described by Resende et al. (2000). The supernatants were absorbed with

Whatman No. 3MM paper strips (5 x 6 mm) vertically inserted into a 14% starch gel (penetrose-30®) prepared in 0.01 M Tris and 0.0028 M citric acid buffer, pH 7.5. In the electrode chambers we used 0.1 M Tris and 0.028 M citric acid, pH 7.5. Electrophoresis was carried out at 4 °C for approximately 5-6 hours at 35 mA (8.5 V/cm of gel).

4-Methylumbelliferyl esters (acetate and propionate) and α–naphthyl acetate and ß-naphthyl acetate were used as substrates (TASHIAN, 1969, modified by RESENDE et al., 2000) for isoesterase detection and comparative analysis of the plants from each population. 4-Methylumbelliferyl acetate (4 mg) or propionate was dissolved in 500 ml acetone and the volume was completed to 10 ml using twice-distilled water. After staining with 4-methylumbelliferyl esters the gels were washed with tap water and separately incubated for 30-60 min in a solution containing 50 ml 0.05 M sodium acetate, pH 6.5, 40 mg fast blue RR salt and 4 mL 1% a-naphthyl acetate or 4 mL 1% ß-naphthyl aceatae (RESENDE et al., 2000).

Genetic variability in the two *A. polyneuron* populations was analyzed using the BIOSYS-1/A Computer Program for the Analysis of Allelic Variation in Genetics (SWOFFORD and SELANDER, 1989). Allele frequencies, mean heterozygosity and mean number of alleles per locus, percentage of polymorphic loci, and genetic similarity were calculated for the two *A. polyneuron* populations.

3. RESULTS

Esterase isozyme patterns of *A. polyneuron* populations from the Northern (NO) and Northwestern (NW) regions of Parana State indicated a total of six loci (*Est-1, Est-2, Est-5, Est-8, Est-9, and Est-10*) using 4-methylumbelliferyl acetate, eight loci (*Est-1, Est-2, Est-3, Est-4, Est-5, Est-7,Est-9, and Est-10*) using 4-methylumbelliferyl propionate, four loci (*Est-1, Est-5, Est-6, and Est-8*) using 4-methylumbelliferyl butyrate, and six loci using α-naphthyl acetate (*Est-2, Est-3, Est-5, Est-6, Est-9, and Est-10*) and ß-naphthyl acetate (*Est-2, Est-3, Est-4, Est-5, Est-9, and Est-10*) as substrates.

Two alleles were observed in each of the *Est-3* (*Est-3*^{1} and *Est-3*^{2}), *Est-9* (*Est-9*^{1} and *Est-9*^{2}), and *Est-10* (*Est-10*^{1} and *Est-10*^{2}) loci for the NO and NW populations (Table 1). The allele 2 variants were found to have a faster electrophoretic mobility than the allele 1. The proportion of polymorphic isoesterase loci for both *A. polyneuron* populations was 30%. Allele frequencies per locus are listed in Table 1.

Chi-square analysis showed no differences between observed genotypic frequencies and expected Hardy-Weinberg frequencies for the *Est-3*, *Est-9*, and *Est-10* loci of the NW population, but departure from expected equilibrium was observed in the NO population for the *Est-3* locus (Table 2). Deviation from Hardy-Weinberg expectation represented by the fixation index (*F*) was greater at the *Est-3* locus of the NO population (*F* = 0.70).

Table 1– Allele Frequencies and Mean Heterozygosities for the Three Esterase Loci (*Est-1*, *Est-9*, *Est-10*) in the NW (Northwestern region) and NO (Northern region) Populations of *Aspidosperma polyneuron*

Tabela 1– *Freqüências dos Alelos e Heterozigosidades Médias Para os Três Loci de Isozimas Esterases (Est-1, Est-9, Est-10) nas Populações NW (Região Noroeste do Estado) e NO (Região Norte do Estado) de Aspidosperma polyneuron*

	Locus					
	NW (N=48)			NO (N=15)		
Allele	***Est-3***	***Est-9***	***Est-10***	***Est-3***	***Est-9***	***Est-10***
1	0.521	0.552	0.469	0.333	0.367	0.633
2	0.479	0.448	0.531	0.667	0.633	0.367
Mean heterozygosity						
H_o	0.500	0.354	0.396	0.133	0.333	0.467
H_e	0.504	0.500	0.503	0.460	0.480	0.480
H_o / locus =0.125					H_o / locus =0.093	
H_e / locus =0.151					H_e / locus =0.142	
Mean number of alleles/locus =1.3					Mean number of alleles/locus =1.3	
% polymorphic loci = 30				% polymorphic loci = 30		

H_o, observed mean heterozygosity; H_e, expected mean heterozygosity

Table 2 – Comparison of Genotypic Frequencies for the Three Esterase Loci (*Est-1*, *Est-9*, *Est-10*) in the NW (Northwestern region) and NO (Northern region) Populations of *Aspidosperma polyneuron*

Tabela 2 *– Comparação das Freqüências Genotípicas para os Três Loci de Isozimas Esterases (Est-1, Est-9, Est-10) nas Populações NW (Região Noroeste do Estado) e NO (Região Norte do Estado) de Aspidosperma polyneuron*

	NW					NO				
Genotype	Obs. number	Exp. number	χ^2	df	*F*	Obs. number	Exp. number	χ^2	GL	F
Est-3 $^{1/1}$	13	12.895				4	1.552			
Est-3 $^{2/1}$	24	24.211				2	6.879			
Est-3 $^{2/2}$	11	10.895				9	6.552			
			0.00 NS	1				5.828**	1	
					-0.002					0.700
Est-9 $^{1/1}$	18	14.505				3	1.897			
Est-9 $^{2/1}$	17	23.989				5	7.207			
Est-9 $^{2/2}$	13	9.505				7	5.897			
			3.317 NS	1				0.658 NS	1	
					0.284					0.282
Est-10$^{1/1}$	13	10.421				6	5.879			
Est-10$^{1/2}$	19	24.158				7	7.207			
Est-10$^{2/2}$	16	13.421				2	1.897			
			1.635NS	1				0.000 NS	1	
					0.205					-0,005

** Significant for $P< 0,01$;
Not Significant for $P< 0,01$.

Genetic diversity calculated by NEI' s (1972) genetic identity (*I*) or distance coefficient (*D*) showed high *I* and low *D* values for the two *A. polyneuron* populations (Table 3). The high genetic identity values for the NO and NW populations are in accordance with the low levels of interpopulation genetic divergence demonstrated by $F_{(ST)}$ (Table 3). *F*-statistic values calculated for the two populations indicated no differences in allele fixation between the two populations.

4. DISCUSSION

Despite the history of fragmentation of the *A. polyneuron* species, the isozyme esterase pattern showed a large amount of genetic variation in this species. Genetic diversity values (% polymorphic loci, observed and expected heterozygosity) were higher than mean values for dicotyledonous species reported by Hamrick and Godt (1989). Based on the high diversity and low level of population differentiation ($F_{ST} = 0.033$) we

Table 3 – Genetic Divergence Between the NW (Northwestern region) and NO (Northern region) Populations of *Aspidosperma polyneuron*

Tabela 3 *– Divergência Genética Entre as Populações NW (Região Noroeste do Estado) e NO (Região Norte do Estado) de Aspidosperma polyneuron*

locus	***I***	***D***	D_{min}	$F_{(IS)}$	$F_{(IT)}$	$F_{(ST)}$
Est-3	0.935	0.068	0.035	0.329	0.353	0.036
Est-9	0.934	0.068	0.034	0.283	0.308	0.035
Est-10	0.948	0,053	0.027	0.104	0.128	0.027
Mean				0.238	0.263	0.033

I, genetic identity (NEI, 1972); *D*, distance coefficient (NEI, 1972); *F*, fixation values (WRIGHT, 1965).

conclude that the *A. polyneuron* plants found in the remaining fragment at "Horto Florestal" (NW population) is likely to reproduce primarily sexually by outcrossed matings. The lack of major differentiation between the NO and NW populations suggests either substantial gene flow among populations or that fragmented populations have not yet been isolated long enough for genetic drift (or selection) to have caused population differentiation.

On the other hand, because most populations of *A. polyneuron* have been interconnected at least until the recent past, severely reducing population sizes could alter the mating structure within populations by increasing mating among relatives and thus increase total inbreeding ($F_{IT} = 0.263$). Fragmentation isolating previously connected populations could have deleterious effects by disrupting gene flow among populations and could exacerbate loss of diversity through drift and further increase inbreeding in remaining fragments (TEMPLETON et al., 1990; YOUNG et al., 1996; HUSBAND and SCHEMSKE, 1996).

The restoration of degraded sites to increase population sizes and to connect fragmented populations could be useful to prevent the deleterious effect of the fragmentation process. Reintroduction of plants is recommended in the restoration plan of a taxon, but the analysis of isoesterases in the NO population of *A. polyneuron* indicated that it is important to select the founder genotype to prevent a loss of genetic diversity. Isoesterase study in *A. polyneuron* showed a lower level of observed and expected heterozygosity in the NO than in the NW population. Heterozygosity and inbreeding are specific measures of diversity that should be targeted because of the direct influence of levels of diversity on fitness (HUENNEKE, 1991; LINHART and GRANT, 1996; LATTA and MITTON, 1997).

The lower level of observed and expected mean heterozygosity in the NO population than the level of observed and expected mean heterozygosity in NW population may be attributed to the effect of founder genotype or may be due to a severe reduction in size of this population since the NO population has recently recolonized its current distribution area. Foundation events usually reduce within-population diversity and could increase differentiation among populations (AUSTERLITZ et al., 2000).

Segregation distortion observed for the *Est-3* locus in the NO population of *A. polyneuron* may be also attributed to the method used to establish this population: the seedlings grown from germinated seeds in house gardens were used to establish the actual NO population. Thus, the founder genotype can produce nonrandom phenotypic distribution and an absence of Hardy-Weinberg equilibrium.

The lower number of plants sampled in the NO population seems not to be a factor leading to the absence of Hardy-Weinberg equilibrium since no evidence of random phenotypic distribution was detected by isoesterase analysis of 13 plants reaching 4-5 m in height found in the "Horto Florestal" fragment (data not shown). We examined the diversity among plants of the three age classes (11 plants higher than 8 m, 13 plants reaching 4-5 m in height, and 20 plants reaching 6-8 m in height) within the NW population and the homogeneity of genetic structure and genetic diversity among the three age classes seems to reflect the occurrence of similar reproductive events year after year (unpublished results).

Esterase polymorphism in remanant population of *A. polyneuron* suggest this species as an example of an endangered species showing high genetic variation in contrast to the low genetic variation commonly reported for others endangered species (FRANKHAN, 1995). However, we suggest that caution should be taken in performing a balanced recolonization of NO population of *A. polyneuron* species since genetic consequences of founding events may strongly affect the genetic structure of this population. Under conditions causing loss of genetic diversity and decreased gene flow, decreased heterozygosity and increased differentiation among populations are expected. Such patterns are thought to be detrimental and to increase extinction risks. Esterase polymorphism in *A. polyneuron* species can be useful as a genetic biochemical marker for choosing the founder genotypes or in order to supply the restoration program.

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