Genetics and Molecular Biology, 36, 4, 533-539 (2013)

www.sbg.org.br

Research Article

AFLP marker analysis revealing genetic structure of the tree *Parapiptadenia rigida* (Benth.) Brenan (Leguminosae-Mimosoideae) in the southern Brazilian Tropical Rainforest

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Abstract

Parapiptadenia rigida is a tropical early secondary succession tree characteristic of the Tropical Atlantic Rainforest. This species is of great ecological importance in the recovery of degraded areas. In this study we investigated the variability and population genetic structure of eight populations of *P. rigida*. Five AFLP primer combinations were used in a sample of 159 individuals representing these eight populations, rendering a total of 126 polymorphic fragments. The averages of percentage of polymorphic loci, gene diversity, and Shannon index were 60.45%, 0.217, and 0.322, respectively. A significant correlation between the population genetic variability and the population sizes was observed. The genetic variability within populations (72.20%) was higher than between these (22.80%). No perfect correlation was observed between geographic and genetic distances, which might be explained by differences in deforestation intensities that occurred in these areas. A dendrogram constructed by the UPGMA method revealed the formation of two clusters, these also confirmed by Bayesian analysis for the number of K cluster. These results show that it is necessary to develop urgent management strategies for the conservation of certain populations of *P. rigida,* while other populations still preserve reasonably high levels of genetic variability.

Keywords: Tropical tree, genetic diversity, population genetics, conservation, AFLP.

Received: April 20, 2013; Accepted: June 20, 2013.

Introduction

The Atlantic Rainforest is considered one of the five most important biodiversity hotspots in the world (Myers *et al.*, 2000) and one of the most threatened biomes of the planet. For centuries the Atlantic Rainforest has been subjected to intense human disturbance that has intensified in the last seven decades, causing the fragmentation of large forest areas and leaving behind, in the majority of its area, only small disconnected forest fragments that are almost entirely isolated and surrounded by extensive anthropogenic matrices, such as pasture, monoculture and areas of urban development (Fundação SOS Mata Atlântica, 2002). Hence, the long-term survival of endemic species in this biome will depend on their ability to persist in these environments and our ability to manage and conserve them in degraded landscape (Fahrig, 2002).

Forest fragmentation exposes populations to ecological and genetic problems caused by inbreeding and loss of variation due to the reduction of effective population size that can lead to genetic drift. The loss of genetic variability that results from such events can cause a decrease in the reproductive ability, disease resistance and genetic plasticity, making it more difficult for natural populations to adapt to environmental change and turning them more susceptible to extinction (Heywood and Stuart, 1994). After habitat fragmentation, the majority of species still remains in the fragments for some time, however, the problems caused by the imbalance of the ecosystem favors the dominance of few or a single species. Nevertheless, small forest fragments still have significant value for biodiversity, although they are influenced by the size, shape and degree of isolation between them (Turner and Corlett, 1996). In this context, studies on animal and plant populations in forest fragments gain increasing importance to address issues like the loss of biodiversity that can cause great harm to future human generations (Wilson and Frances, 1997). In order to establish strategies for the conservation of species and ecosystems, further knowledge of the genetic variability in such populations is needed (Botrel *et al.*, 2006).

Parapiptadenia rigida (Benth.) Brenan. (Leguminosae-Mimosoideae), is a deciduous, heliophyte, allogamous, monoecious, early secondary tree species that grows on various soil types and is recommended for the re-

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covery of degraded forests, especially in areas of permanent preservation (Durigan and Nogueira, 1990; Vaccaro *et al.*, 1999). This species is found in the Atlantic interior forest of several Brazilian states, including Minas Gerais, São Paulo, Paraná, Santa Catarina and Rio Grande do Sul, with widest occurrence in the latter three states. It is characterized by a hard wood that is commonly used in construction, shipbuilding, carpentry and joinery, manufacture of coaches, stakes, light and telephone poles and railway sleepers (Lorenzi, 2002; Da Silva *et al.*, 2012). Despite of being a monoecious plant, *P. rigida* presents self-incompatibility (,Ribas LA, 1999, PhD Thesis, Universidade Federal de Viçosa, Viçosa) and its seeds are dispersed by wind, water or barochory, and pollen is dispersed by small and medium sized bees (Kageyama, 1992). *P. rigida* has a lifespan of around 100 years and flowering occurs around 10 years of age (Da Silva *et al.*, 2012). This species also has medicinal properties and is widely used in folk medicine for the treatment of sinusitis, cough (Franco and Fontana, 1997), vaginal infections (Korbes, 1995) and broncho-pulmonary diseases. Gum, resins and tannins can be extracted from its bark and fruit, (Cândido, 1974; Lorenzi, 2002). These factors make this species very important for restoration programs carried out in the Atlantic Rainforest.

In this study we investigated the genetic diversity within and between *P. rigida* populations in eight naturally occurring forest fragments of different sizes, using AFLP markers in an attempt to determine possible effects of fragmentation on the genetic structure and to provide subsidies for management and conservation of these populations.

Material and Methods

Sampling strategy

Leaves were collected from 159 adult individuals of *P. rigida* present in eight forest fragments distributed in eight Atlantic Rainforest remnants in the southern Brazilian States Paraná and Santa Catarina. We sampled only individuals that had reached reproductive age, and this was done during their flowering time. The minimum distance among trees was 30 meters, and individuals were sampled throughout all areas of the fragments (Table 1, Figure 1).

DNA isolation and Amplified Fragment Length Polymorphism (AFLP) reactions

Genomic DNA was isolated from approximately 0.5 g of fresh leaves using the CTAB method, as described by Doyle and Doyle (1987). The DNA concentration was estimated using a fluorometer (DyNA Quant 200, Höfer-Pharmacia), according to manufacturer instructions. An AFLP analysis was carried out as described by Vos *et al.* (1995). Briefly, 0,8 to 1.0 μg of each DNA samples were submitted to restriction digestion by *Eco*RI/*Mse*I endonucle-ases (5U each) and ligation to their respective adapters. After incubation for 16 h at 37 °C, the samples were diluted (1:10) in ultrapure water. Polymerase chain reaction (PCR) amplifications were 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 24 primer combinations. Five primer com-binations were chosen for selective PCR. The products of selective amplification were resolved by electrophoresis in polyacrylamide gels (polyacrylamide 7% acryl-

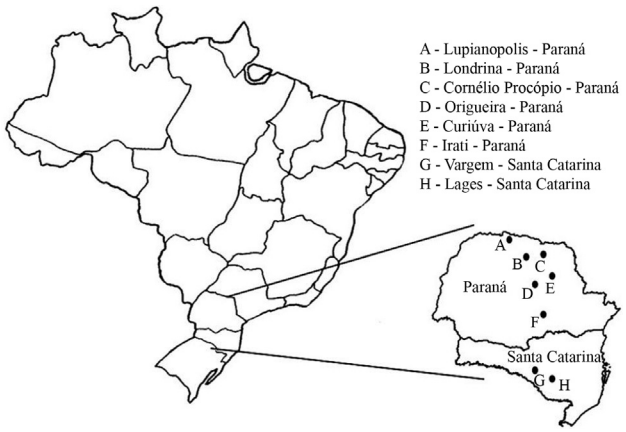

Figure 1 - Map of Brazil highlighting the States of Paraná and Santa Catarina, with the geographic representation of the eight studied populations of *Parapiptadenia rigida*.

Table 1 - Geographic information, fragment area, and vegetation type of the sampled populations.

City/State	Latitude	Longitude	Fragment Area	Altitude	Locality/type
Cornélio Procópio / Paraná	23°15'S	50°44'W	9 km^2	660 m	northern Paraná
Curiúva / Paraná	24°01'S	50°26'W	8 km^2	780 m	northeastern Paraná
Irati /Paraná	25°32'S	50°39'W	13 km^2	810 m	southeasternParaná
Lages/ Santa Catarina	28°11'S	50°43'W	15 km^2	920 m	mountain region of Santa Catarina
Londrina / Paraná	26°26'S	51°14'W	10 km^2	610 m	north of Paraná State
Lupionópolis / Paraná	22°45'S	51°39'W	10 km^2	600 m	northern Paraná State
Ortigueira / Paraná	24° 09'S	50°52'W	15 km^2	750 m	central-eastern region of Paraná
Vargem / Santa Catarina	27°31'S	50°53'W	5 km^2	780 m	mountain region of Santa Catarina State

amide:bis-acrylamide 29:1) for 3 h at 200 V and stained with 20% silver nitrate. A 50-bp molecular ladder (Ludwig Biotecnologia, Ltda.) was used to deter-mine the molecular weight of the fragments.

Data analysis

All individuals were scored for the presence or absence of AFLP bands (1 or 0) to construct a binary matrix. Only bands with molecular sizes between 100-700 bp and only those that could unequivocally scored for presence or absence across all individuals were considered for further analysis. The software dBoot v. 1.1 (Coelho, 2001) was used to estimate the coef-ficient of variation (CV) for the number of AFLP markers, generating a parameter that is capable of determining the reliability of the results obtained with our data. The percentage of polymorphic loci (Pp), Nei's genetic diversity (H_S; Nei, 1978), the Shannon index (H'), genetic distance (Nei, 1972), and total heterozygosity (H_T) were calculated, using POPGENE v. 1.31 (Yeh *et al.*, 2000). To test for a correlation between genetic and geographic distances, as well as for a correlation between fragment size and genetic diversity, the Pearson's Linear Correlation and the Mantel Test were employed using the software BioEstat version 5.0 (Ayres *et al*., 2007) and TFPGA version 1.3 (Miller, 1997), respectively. Analysis of molecular variance (AMOVA) was estimated using Arlequin v. 3.11 software (Excoffier *et al.*, 2005) to evaluate the distribu-tion of genetic variation within and among samples, as well as to estimate the F_{ST} and pairwise Fst indexes. A dendrogram was constructed by means of the UPGMA method implemented in POPGENE v.1.3.1 (Yeh *et al.*, 2000) and a bootstrap analysis was done utilizing dBOOT v.1.1 software (Coelho, 2001). The software STRUCTURE version 2.3.3 (Hubisz *et al.*, 2009) was used to identify the number of similar population clusters (*K*). The analysis of the number of clusters was performed using the admixture model with a burn-in and run lengths of 10,000 and 100,000 interactions, respectively. The number of clusters was determined following the guidelines of Pritchard and Wen (2004) and Evano *et al.* (2005), in the online software Structure Harvester (Earl and vonHodt, 2012).

Results and Discussion

Five selective AFLP primers generated 126 polymorphic markers with an average of 25.2 markers per pairwise combinations in 159 individuals that belonged to the eight populations of *P. rigida*. The *Eco*RI-ACG/*Mse*I-CAG and *Eco*RI-ACG/*Mse*I-CAT combinations generated the highest (28), and the *Eco*RI-ACG/*Mse*I-CTA combination the lowest numbers of well defined markers (20). The coefficient of variation calculated for the total number of markers was 7.47%, indicating that the number of markers was sufficient to perform the analysess of genetic structure and diversity (Figure 2).

So as to verify whether fragmentation had impacted the genetic variability of the populations of *P. rigida* we calculated the percentage of polymorphic loci (Pp), Nei's gene diversity (Hs) and the Shannon- Wiener index (H'), as well as the total heterozygosity (Ht; Table 2). The percentage of polymorphic loci, Nei's gene diversity and Shannon-Wiener index for all populations were Pp = 60.4, Hs = 0.217, and H' = 0.322. When comparing these with the genetic diversities found in other tropical tree species, such as *Hagenia abyssinica* (Hs = 0.30; Feyissa *et al.*, 2007), *Cedrela odorata* (Hs = 0.17; Torre *et al.*, 2008), *Aeghilla sellowiana* (Hs = 0.10; Medri *et al.*, 2010) and four other tropical tree species studied by Nybom *et al.* (2004) (Hs = 0.22), we concluded that most populations of *P. rigida* still preserve moderate levels of genetic diversity.

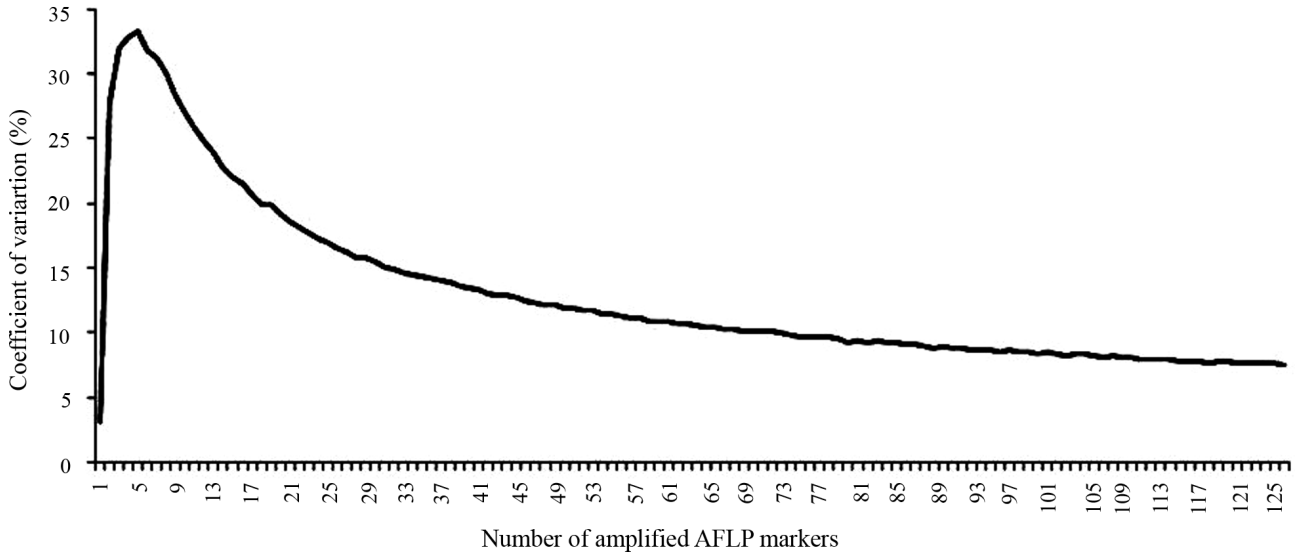

Figure 2 - Coefficients of variation for the AFLP markers.

The populations of Curiúva and Vargem (Table 2) showed the lowest percentage of polymorphic loci (Pp = 48.44 and Pp = 49.22), Nei's gene diversity (Hs = 0.176 and Hs = 0.171) and the Shannon-Wiener index (H' = 0.261 and H' = 0.257). On the other hand, the populations of Ortigueira and Irati showed higher values for these genetic parameters (Pp = 82.81 and Pp = 79.69; Hs = 0.301 and Hs = 0.287; H' = 0.444 and H' = 0.423, respectively). When comparing the genetic variability of a fragment with their respective sizes a significant correlation between these factors was observed, illustrating the impact of forest cover reduction on the genetic variability of this species (Table 1; Figure 3B). It also needs to be taken into account that in fragmented populations the genetic variability decreases slowly and may be directly correlated to the effective population size. Furthermore, according to Finkeldey and Hattemer (2007), the persistence of tree populations in tropical forest over generations is contingent on a minimal population size, as small populations are sooner or later prone to extinction. Fragmentation of natural populations can lead to evolutionary constraints due to loss of genetic variability (Frankham, 1996; Young *et al.*, 1996). These changes are reflected in the processes of genetic drift and

Table 2 - Measures of genetic variability eight populations of *P. rigida* by AFLP markers. Pp: percentage of polymorphic loci; Hs: Nei's gene diversity; H': Shannon-Wiener index; H_T: total heterozygosity.

Populations	Pp	H_S	H'
Cornélio Procópio	57.81	0.212	0.314
Curiúva	48.44	0.176	0.261
Irati	79.69	0.287	0.423
Lages	64.84	0.226	0.338
Londrina	50.78	0.180	0.268
Lupionópolis	50.00	0.182	0.270
Ortigueira	82.81	0.301	0.444
Vargem	49.22	0.171	0.257
Mean	60.45	0.217	0.322
H_T		0.278	

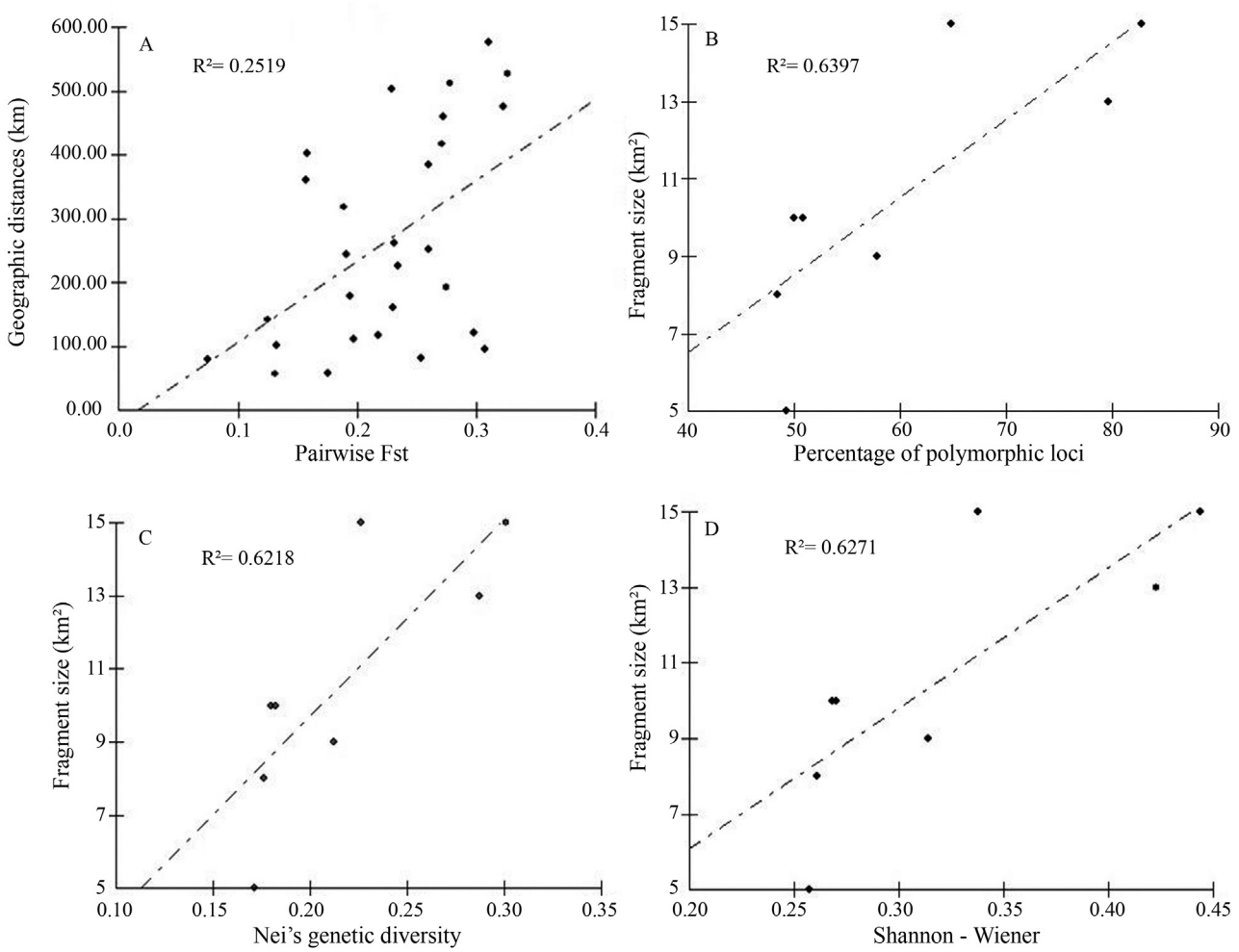

Figure 3 - Pearson's correlations for (A) geographic distances against pairwise Fst; (B) fragment size against percentage of polymorphic loci; (C) fragment size against Nei's genetic diversity; (D) fragment size against Shannon-Wiener index.

gene flow, which determine the degree of genetic diversity of the species (Couvet, 2002). Such effects can be observed in the populations of *P. rigida*, where the populations located in smaller fragments showed an accentuated loss of genetic variability (Tables 1 and 2; Figure 3B, C and D) when compared to the populations present in the larger fragments.

The analysis of molecular variance (AMOVA, Table 3) showed that 72.2% of the genetic variability is distributed within and 22.8% between populations. The Fst value (0.228) indicates a moderate to high genetic variation among populations, which is characteristic of allogamous tree species (Wright, 1969; Hamrick *et al.*, 1992) such as *P. rigida* (monoecious with selfincompatibility). Such distribution of the genetic variability was also observed by Mariot (Mariot A, 2000, MSc Dissertation, Universidade Federal de Santa Catarina, Florianópolis, Brazil) when analyzing four populations of *Piper cernuum* (Fst = 0.29), a species that has a similar reproductive biology as *P. rigida*. In a further comparison of our results with other studies with natural tropical populations (Paiva, 1998) we observed that most of these species preserve high within population genetic variability as observed in *P. rigida*.

In the analysis of pairwise Fst (Table 4), the populations of Lages and Vargem (80 km apart) showed the lowest genetic distance (Fst = 7.43%) while the populations of Vargem and Lupionópolis (526 km apart) exhibited the greatest genetic distance (Fst = 32.62%). The populations of Cornélio Procópio and Londrina that are distant from each other by only 58 km and the populations of Lupionópolis and Londrina, distant by 81 km, showed a Fst = 17.55% and 25.36%, respectively. Interenstingly however, the populations of Ortigueira and Lages, separated by a geographic distance of 403 km, showed a pairwise Fst of only 15.78%. These results show (Table 4) that there is no perfect correlation between the geographic and genetic distances (r = 0.459, Figure 3A), an explanation being the different fragmentation intensities that occurred in these areas. Historically, the drastic reduction of the Atlantic Rainforest occurred at different periods in the areas covered by this biome. In the plateau of Santa Catarina, this event began in the second half of the twentieth century (Vibrans *et al.*, 2008), whereas in Paraná, forest fragmentation started in the decade of 1910 and became intensified in the 1950s (Medeiros *et al.*, 2005). *P. rigida* lives around 100 years and flowering occurs around ten years of age. In our samples we collected genetic material from individuals that were already in the reproductive age, with at least one flowering time before sampling. This procedure made it possible to collect only individual that contributed to the real effective population size, even though samples were comprised of individual from many different generations.

We constructed a dendrogram using Nei's genetic distances (1978) and th UPGMA method (Figure 4A), showing the separation of two groups that were further confirmed by a Bayesian analysis for the K number of clusters (Figure 4B). One group was formed by the populations that exhibited the lowest genetic diversity (Curiúva, Londrina,

Table 3 - Analysis of molecular variance (AMOVA) using AFLP markers for eight populations of *P.rigida* distribuited in southern Brazil.

Source of variation	Degrees of freedom	Sum of squares	Variation components	Percentage of variation
Between populations	7	562.104	3.46054	22.80**
Within populations	151	1769.393	11.71783	77.20
Total	158	2331.497	15.17837	
Fixation index	F_{ST}	0.22799		

$p < 0.01$ (significance test from 1023 permutations).

Table 4 - Correlation matrix of geographical distances (km) between the populations studied, above the diagonal, and F_{ST} values between pairs of populations of *P.rigida* below the diagonal. All F_{ST} values were significant ($p < 0.05$) with 1023 permutations.

Populations**	CP	C	I	LA	LO	LU	O	V
CP	-	96	252	512	58	121	118	476
C	0.30743	-	160	417	111	193	57	385
I	0.25978	0.22969	-	261	244	318	142	226
LA	0.27783	0.27165	0.23089	-	504	576	403	80
LO	0.17554	0.19758	0.19122	0.22862	-	81	101	460
LU	0.29822	0.27479	0.18876	0.31035	0.25363	-	178	526
O	0.21740	0.13149	0.12501	0.15784	0.13244	0.19388	-	361
V	0.32275	0.32275	0.23457	0.07435	0.27221	0.32621	0.15740	-

** Populations: CP - Cornélio Procópio; C - Curiúva; I - Irati; LA - Lages; LO - Londrina; LU - Lupionópolis; O - Ortigueira; V - Vargem.

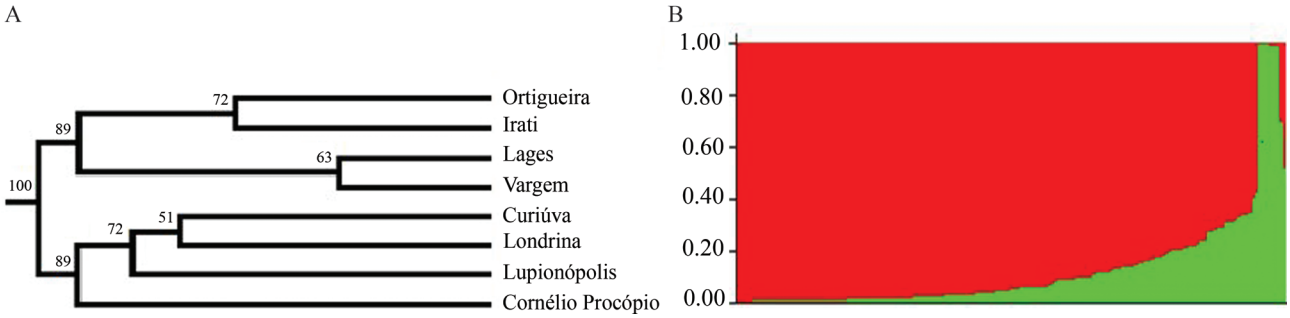

Figure 4 - Cluster analysis of the studied *Parapiptadenia rigida* pupultions. (A) Nei's genetic distance dendrogram constructed using the UPGMA method; (B) Bar plot showing the formation of K = 2 number of population clusters grouped by population identity.

Lupionópolis, and Cornélio Procópio), and a second group that was formed by the populations with the highest genetic diversity (Ortigueira, Irati and Lages), with the exception of the population of Vargem that was also present in this second group. The fact that a population such as Vargem, that has the lowest values for genetic diversity, clustered very closely with a population such as Lages, that presents higher levels of genetic diversity, may be explained by the fact that these populations are historically related and formed a continuous forest until very recently (around the 1950s), and even though the fragment with population of Vargen was highly degraded, it still shares much of its genetic diversity with the population of Lages.

It was also possible to note that the populations with the highest genetic diversity are more closely related to each other (Figure 4A), and that the populations with the lowest genetic diversity, that formed a group (with the exception of Vargem), are very distant from each other even within their group. Such a within group distance might be related to the process of genetic erosion that occurred in these populations, and which is well known to increase genetic distances among natural populations.

From this it is possible to conclude that the size of the forest remnants is directly related to the capacity of maintaining higher level of genetic diversity, and that according to Holsinger (2000), a reduced genetic variation in small populations is likely a symptom of endangerment, and that such populations require immediate management to avoid the extinction of local populations. Though reforestation efforts do occur for this species, these are still few and nowhere close to these areas that are considered natural reservoir of genetic variation for this and many other tree species.

Acknowledgments

We thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for a scholarship granted to LBS and the Conselho de Desenvolvimento Científico and Tecnológico (CNPq, grant 478179/06-5) and Fundação Araucária for financial support.

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Associate Editor: Everaldo Gonçalves de Barros