grouping types of individuals. The classification will not be on the basis of predetermined characters (whether these are genetic, or morphologic, or physiological, or geographic, or ecological, etc); on the other hand, the characters capable to differentiate the individuals which are the object of the study, must come from the same process of classification.

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The Genetic Structure of *Sorocea bonplandii* in Southern Brazilian Forest Fragments: AFLP Diversity

By A. R. RUSCHEL[1),2),*)], R. O. NODARI[2)] and B. M. MOERSCHBACHER[1)]

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Abstract

To analyse possible genetic erosion due to fragmentation in the Brazilian Atlantic Forest ecosystem, we investigated the genetic diversity within and among one large and six small populations of one of the key species of this ecosystem, *Sorocea bonplandii*, using AFLP analyses of 468 plants. Eight primer pairs yielded 299 polymorphic fragments for analysis. *S. bonplandii* was characterized by an unusually high genetic diversity within the species and also within individual populations, around 94% of the total genetic diversity occurred within populations. Genetic distances between populations were low in spite of extensive fragmentation. Genetic distance was significantly correlated with geographical distances between fragments, but these differences may have existed before fragmentation. Our results have direct implications for sustainable management of *S. bonplandii*, indicating that conservation strategies might be based on a random sample of trees taken throughout the Atlantic forest. However, the minimum population size required for maintaining the huge genetic diversity of this species is unknown. In order to establish a sustainable management plan for the species, further ecological studies are needed.

Key words: dioecious plant, forest fragmentation, genetic diversity, Moraceae, Subtropical Atlantic Forest.

[1)] Department of Plant Biochemistry and Biotechnology, University of Münster, Hindenburgplatz 55, 48143 Münster, Germany.

[2)] Departamento de Fitotecnia, Universidade Federal de Santa Catarina, C. P. 476, Florianópolis CEP 88040-900 SC, Brazil.

[*)] Corresponding author: ADEMIR R. RUSCHEL, Phone: +55-9132041234, Fax: +55-32772888. E-mail: arruschel@yahoo.com.br.

Introduction

The Atlantic forest, classified fifth among the hotspots of global biodiversity containing endemic species (MEYER

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et al., 2000), is one of the richest ecosystems in Brazil. It encompasses around twenty thousand plant species of which 40% are endemic. Today, only around 7.5% of the original area of 1,227,600 km^2 remains, mostly as fragmented and isolated forest areas. The pressure on these small forest habitats is extremely high, since 70% of the Brazilian people are living within the area originally covered by Atlantic forest (IBAMA, 2002). As the continuous process of deforestation and forest fragmentation increase the risk of biodiversity loss, actions to protect this ecosystem are urgently needed. Knowledge-based, sustainable forest management strategies are needed so that the inhabitants of tropical forest areas are not forced to inefficiently use and destroy the remaining forested lands.

In order to analyze genetic diversity at the population level in forest remnants of the Alto-Uruguai river region (seasonal deciduous forest), we focused on a key species, *Sorocea bonplandii* (Baill.) W.C. Burger, Lanj. and Wess. Boer., (Moraceae), a small sub-canopy tree which is widespread and occurrs in high density in Atlantic forest (Lorenzi, 1998; Ruschel *et al.,* 2006).

S. bonplandii is a dioecious species, with seed and pollen likely dispersed by animals, and with a short life cycle. In a species with these characteristics, genetic erosion e.g. due to ecosystem fragmentation and disturbance should become apparent in a rather short time frame. As a non timber species, these trees have never been the subject of any logging activities so that no additional selective anthropogenic influence is overlying the results. However, *S. bonplandii* is traditionally used for arts-and-craft, medical, and food purposes so that this species lends itself as one cornerstone for a knowledge-based management plan for sustainable forest uses (Ruschel *et al.,* 2006).

Molecular markers such as allozymes and AFLP are useful tools for analyzing genetic diversity. Both methods are especially suited to analyze genetic variation below the species level, particularly in investigations of population structure and differentiation (Ferreira and Grattapaglia, 1996; Müller and Wolfenbarger, 1999; Ouborg *et al.,* 1999). The main advantage of using allozyme markers is the ability to carry out heterozygosity analyses, while AFLP markers easily generate hundreds of highly reproducible markers allowing high-resolution genotyping of fingerprinting quality (Vos *et al.,* 1995).

We used the AFLP genetic markers, with the objective to assess the degree of genetic diversity of the species within and between fragmented populations and their relationship to a large, undisturbed population. As genetic distance typically increases with forest fragmentation, the large undisturbed population was expected to retain the original genetic diversity, while potential losses due to fragmentation might become apparent in the smaller populations.

Materials and Methods

Plant material, sampling, and study areas

Leaf tissue of *Sorocea bonplandii* was collected from seven forest fragments of the Atlantic Forest in Southern Brazil (*Fig. 1, Table 1*). Only one of the remnants, SPA, located at the Atlantic coast, belongs to the Tropical Atlantic Forest. All others are situated in the Alto-Uruguai river ecosystem of the Subtropical Atlantic Forest. In each fragment, young leaves were harvested from approximately 80 plants with a height of > 25 cm and a minimum distance of 15 m on a unidirectional way. Total height and diameter at breast height (DBH) of the sampled trees were measured.

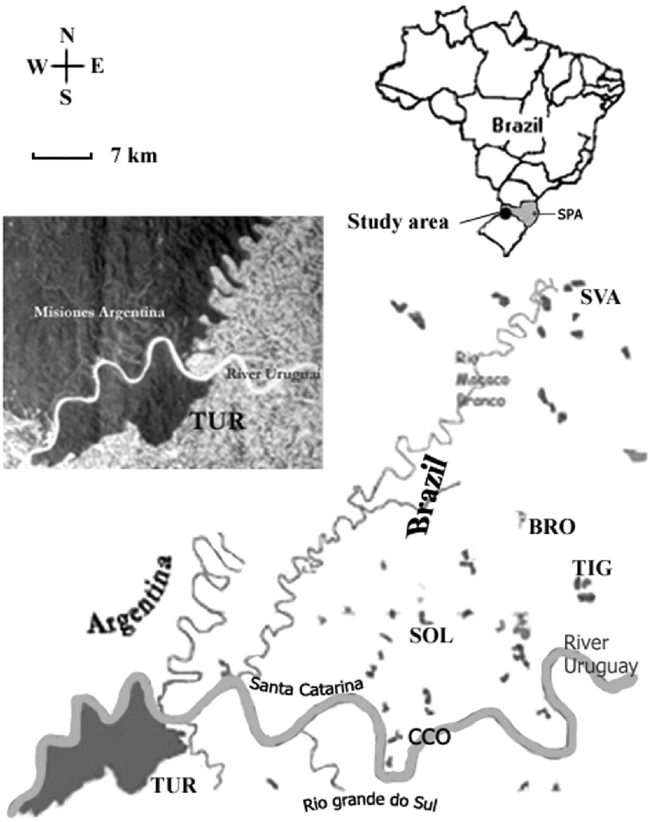

Figure 1. – Location of the remnants of the Subtropical Atlantic Forest ecosystem of the High-Uruguay river in South Brazil, including Park Turvo, the largest intact remainder of this ecosystem at the border to Argentina, and five smaller remnants (CCO, SOL, BRO, TIG, SVA) in Santa Catarina, in distances of ca. 20 to 50 km from Park Turvo, and of one remnant (SPA) of the Tropical Atlantic Forest ecosystem situated at the Atlantic coast, ca. 500 km away from Park Turvo (adapted from Fundação SOS Mata Atlântica and INPE 1998).

For each population, plants were classified in two subgroups according to diameter classes: plants with DBH > 4 cm were named 'adult plants', and plants with DBH < 2 cm and height of > 25 cm 'young plants' (*Table 2*).

DNA extraction and AFLP analysis

Approximately 70 mg of dehydrated (in silica gel) leaf tissue per plant were used for extraction of total genomic DNA using the CTAB procedure described by Stefenon and Nodari (2001). An AFLP (amplified fragment length polymorphism) protocol of Vos *et al.* (1995) was applied, with minor modifications (Marschalek, 2003). DNA was restricted using the enzymes *Eco*RI (rarely cutting) and *Mse*I (frequently cutting). Thirty-two combinations of primers based on three selective

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Table 1. – Geographic location, altitude above sea level, forest area, type of ecosystem (SDF, Seasonal Deciduous Forest; ODF, Ombrophilous Dense Forest), and nature of exploitation (SE, selective exploitation; IE, intensive exploitation; WE, without exploitation) of seven Atlantic Forest fragments in Southern Brazil.

fragment*	forest ecosystem	nature of exploitation	area (ha)	altitude (m)	lat. South (S)	long. West (W)
TUR	SDF	WE	17491	290-350	27° 11' 12'- 34"	53° 51' 03" - 33"
SOL	SDF	WE	11	510	27° 05' 04"	53° 40' 07"
CCO	SDF	SE	50	320	27° 11' 25"	53° 38' 02"
SVA	SDF	SE	50	590	26° 56' 12"	53° 31' 30"
TIG	SDF	IE	12	350	27° 04' 21"	53° 27' 38"
BRO	SDF	IE	11	470	27° 04' 06"	53° 36' 54"
SPA	FOD	IE	30	300	27° - 28°	48° - 49°

* TUR – Parque Estadual Turvo, Derrubadas, RS; CCO – Sede-Capela, Itapiranga, SC; SVA – São Valentin, Descanso, SC; TIG – Tigre, Mondaí, SC; BRO – Beato Roque, São João d'Oeste, SC; SOL – Soledade, São João d'Oeste, SC; SPA - São Pedro de Alcântara, SC.

bases were tested for the second selective PCR amplification, and eight combinations were finally chosen for analysis (*Table 3*). Electrophoresis of AFLP fragments was performed on 7% (w/w) polyacrylamide gels (25 cm x 0.2 mm) on a one-dye model 4200 Licor DNA automatic sequencer.

Table 2. – Number of *Sorocea bonplandii* plants analysed using AFLP markers in seven forest remnants of Atlantic forest, Southern Brazil.

	group of plants		
population	all[1]	adult[2]	young[3]
BRO	69	24	33
CCO	68	32	31
SOL	67	32	30
SPA	42	23	15
SVA	74	28	33
TIG	70	31	35
TUR	78	29	26
total	**468**	**199**	**203**

[1] height > 25 cm.
[2] DBH > 4 cm.
[3] DBH < 2 cm and height > 25 cm.

Data analysis

Each AFLP fragment was counted as a separate putative locus and scored as present (1) or absent (0) for each plant. The number of polymorphic and monomorphic bands was counted for each primer pair, and the frequencies of their appearance was evaluated in each population. Only bands that could be read unambiguously on each gel image and that were present in the populations of all forest fragments were used for further analysis. For these loci, the diversity index according to Shannon and Wiener was calculated, then averaged for the population, and Shannon indices of different populations were compared using t-test (MAGURRAN, 1988).

Further analysis was performed using the ARLEQUIN software V.2.0 (SCHNEIDER *et al.,* 2000). Genetic diversity based on NEI's unbiased diversity statistics (NEI, 1987), and genetic distances according to NEI (1978) were calculated for each population and compared between populations. A hierarchical analysis of molecular variance (AMOVA) was performed among ecosystems, between populations within each ecosystem, and within each population, according to EXCOFFIER *et al.* (1992). The Mantel-test was used to test the signifi-

Table 3. – AFLP primer pairs and absolute and relative number of evaluated AFLP markers (bands) for monomorphic and polymorphic bands.

primer pair[1]	monomorphic (%)	polymorphic (%)	total	evaluated
E^R ATA - M CCG	27 (34)	52 (66)	79	23
E^R ATA - M CGA	34 (38)	56 (62)	90	42
E^R AGG - M CAT	55 (54)	46 (46)	101	23
E^R ATA - M CTC	39 (31)	85 (69)	124	41
E^R AAA - M CAT	40 (36)	71 (64)	111	32
E^R AAA - M CGA	20 (19)	84 (81)	104	49
E^R AAA - M CAG	25 (26)	71 (74)	96	52
E^R AAA - M CCG	20 (20)	80 (80)	100	37
total	**260 (32)**	**545 (68)**	**805**	**299**

[1] E^R, fluorescence labeled EcoRI primer (IRDye™ 800 infrared dye, LiCor); M, MseI primer.

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Table 4. – Abundance of AFLP polymorphisms and frequency classes of AFLP polymorphisms (of the 299 polymorphic AFLP bands analysed in detail), in *Sorocea bonplandii* plants in different forest fragments.

fragment	frequency classes of polymorphisms (1)						
	< 5%	5 - 25%	25 - 50%	50 - 75%	75 - 95%	> 95%	Total
BRO	53 (17.9)	125 (41.7)	45 (15.1)	31 (10.4)	33 (11.0)	12 (3.9)	299 (100)
CCO	53 (17.8)	121 (40.4)	46 (15.2)	29 (9.8)	37 (12.3)	13 (4.5)	299 (100)
SOL	50 (16.7)	127 (42.6)	47 (15.7)	28 (9.2)	33 (11.1)	14 (4.7)	299 (100)
SPA	42 (14.0)	135 (45.1)	45 (14.9)	29 (9.8)	32 (10.6)	17 (5.6)	299 (100)
SVA	51 (17.0)	119 (39.7)	49 (16.4)	31 (10.4)	39 (13.0)	10 (3.5)	299 (100)
TIG	48 (16.0)	124 (41.4)	49 (16.4)	25 (8.4)	42 (13.9)	12 (3.9)	299 (100)
TUR	48 (16.2)	123 (41.2)	47 (15.9)	23 (7.7)	47 (15.8)	10 (3.3)	299 (100)
mean (%)	**49.3 (16.5)**	**124.8 (41.7)**	**46.8 (15.6)**	**28.1 (9.4)**	**37.5 (12.5)**	**12.6 (4.2)**	**299 (100)**

(1). Differences between fragments in each class of frequency are not statistically significant (ANOVA, P > 5%).

cance of the correlation between the matrices of genetic distances and geographical distances between the populations (SMOUSE *et al.,* 1986).

Cluster analysis showing the relationships among populations based on NEI's genetic distance index was performed based on the unweighted pair-group method with arithmetic averaging (UPGMA method, SNEATH and SOKAL, 1973) using NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System – version 2.1) (ROHLF, 1989).

Results

AFLP analysis of 468 *S. bonplandii* individuals was carried out using eight combinations of primer pairs, and a total of 299 polymorphic loci could be reliably scored. The size of the AFLP fragments ranged from 50 to 600 bp, most having a size of less than 300 bp.

The level of AFLP polymorphism in each population ranged from 0% (AFLP fragments 100% present) to 100% (AFLP fragments present in some but absent in others) which indicates a high degree of differentiation among populations (*Table 4*). On average, about 60% of the polymorphic loci appeared at low frequency (< 25%) within populations. The distribution of AFLP fragments across different frequency classes was similar for all populations (ANOVA, P > 0.05).

The Shannon diversity index H' for the species *S. bonplandii*, as revealed by AFLP markers, was 0.35 (*Table 5*). Generally, the sub-populations of adult and young plants present slightly higher values of diversity compared to total population diversity. The lowest diversity index was observed within SPA, although there were no statistically significant differences between populations (t-test, P >0.05). SPA in general exhibited the largest genetic distance from the total population (*Table 5*).

On average, NEI's genetic diversity value within one population was H = 0.214 ± 0.104 when all plants were included in the analysis, and very similar values were obtained when the group of adult plants or that of young plants were analyzed separately (0.214 ± 0.091 and 0.216 ± 0.107, respectively). The undisturbed forests (without exploitation – TUR, SOL) exhibited higher genetic diversity than disturbed forests (*Fig. 2a*). This difference was not seen when only adult plants were analyzed (data not shown). No clear dependency of genetic diversity from the size of the forest or from the density of *S. bonplandii* within the forest was apparent (*Fig. 2b, c*).

The genetic distance between populations of *S. bonplandii* was low, with a similarity above 93% (*Fig. 3*). Again, SPA was the population with the greatest dis-

Table 5. – Shannon diversity index (H´) based on polymorphic AFLP loci of *Sorocea bonplandii* populations and sub-populations of adult and young plants in Atlantic forest, Southern Brazil. Numbers in parentheses represent the frequencies of the diversity among population ($D' = [1 - H'_{Population} / H'_{Total}]$ x 100).

diversity within and among populations (1)			
population	all	adult	young
BRO	0.323 (7.60)	0.338 (6.84)	0.337 (7.88)
CCO	0.320 (8.62)	0.330 (9.18)	0.340 (7.14)
SOL	0.327 (6.60)	0.341 (5.91)	0.337 (7.97)
SPA	0.308 (11.82)	0.322 (11.17)	0.326 (10.92)
SVA	0.324 (7.27)	0.337 (7.11)	0.333 (9.04)
TIG	0.319 (8.89)	0.343 (5.56)	0.322 (12.08)
TUR	0.324 (7.27)	0.345 (4.87)	0.331 (9.61)
all	**0.350**	**0.363**	**0.366**

(1). Differences between Shannon indices are not statistically significant (t-test, P > 0.05) (MAGURRAN, 1988).

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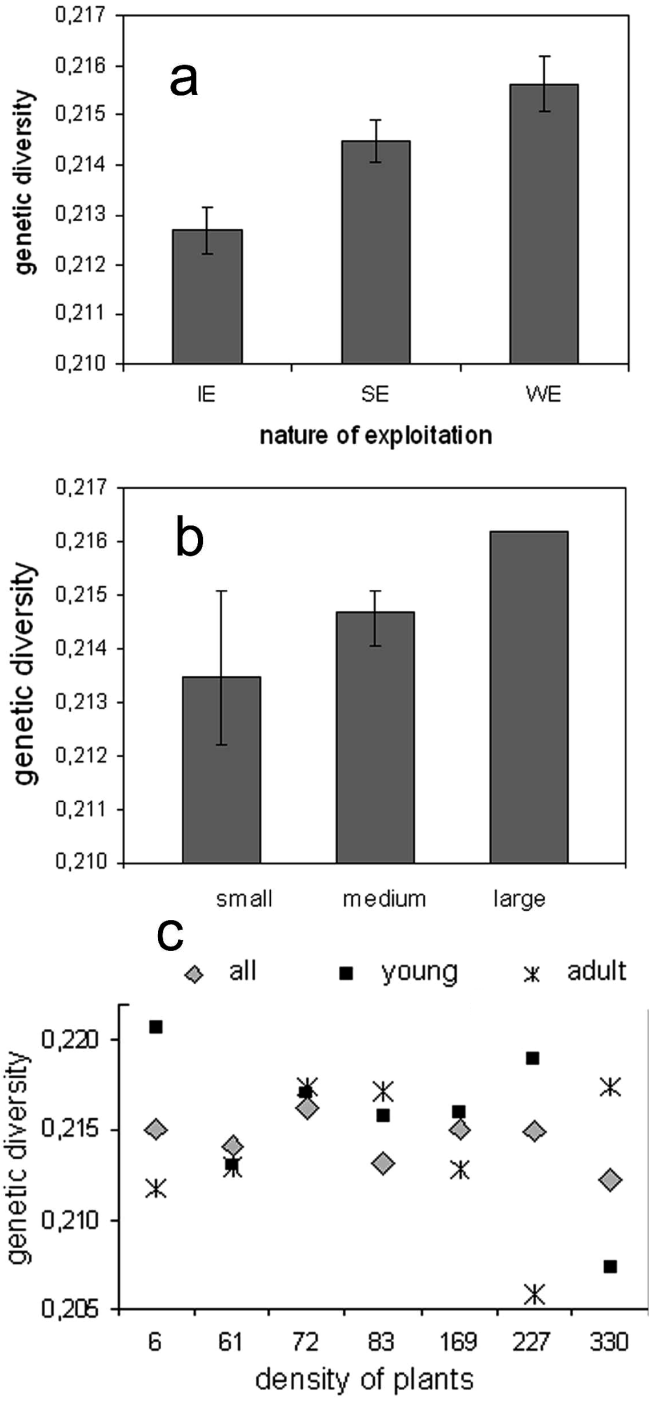

Figure 2. – Distribution of genetic diversity (NEI's index) of *Sorocea bonplandii* populations in the Atlantic forest, Southern Brazil, as influenced by: **a**, the intensity of past exploitation (IE, intensive exploitation; SE, selective exploitation; WE, without exploitation); **b**, the forest size (small, ca. 11 ha; medium, ca. 50 ha; large, 17490 ha); and **c**, the density of plants per hectare. Bars denote range of data.

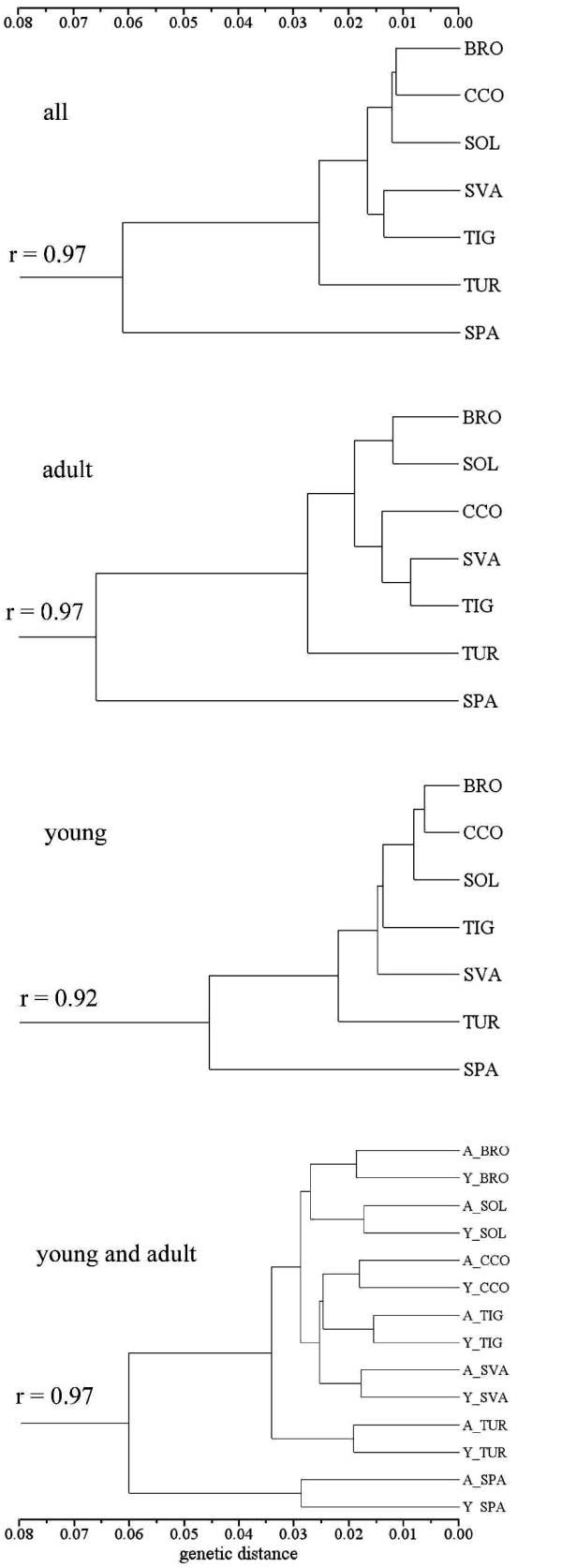

Figure 3. – Dendrogram of the relationships among *Sorocea bonplandii* populations based on NEI's unbiased genetic distances (NEI, 1978) and cophenetic correlation coefficients (r) (299 AFLP markers). Dendograms shown are based on the analysis of all plants (468 plants) in seven populations, the sub-populations of adult plants (DBH ≥ 4 cm, 199 plants), young plants (DBH ≤ 2 cm, 203 plants), and adult and young plants.

tance from the others, independently of whether all plants, or the group of adult or young plants only were included in the analysis. Similarly, TUR is always distanced from the remaining populations, while their relative grouping slightly differs depending on which group of plants is analyzed. The groups of young and adult plants of a given population always grouped together, with an average distance of about 2%.

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Table 6. – Analysis of molecular variance (AMOVA) based on 299 polymorphic AFLP bands in seven populations (pop.) of *Sorocea bonplandii* plants in Atlantic forest, including subtropical and tropical Atlantic forest ecosystems (ecos.), Southern Brazil.

source of variation	d.f.	sum of squares	variance components	percentage of variation
all plants				
among pop.	6	552.84	0.90	2.74*
within pop.	461	14773.17	32.05	97.26
total		15326.01	32.95	
all plants				
between ecos.	1	173.69	1.49	4.37*
among pop. within ecos.	5	379.15	0.62	1.81*
within pop.	461	14773.17	32.05	93.82
total	467	15326.01	34.16	
adult plants				
between ecos.	1	112.24	1.59	4.65*
among pop. within ecos.	5	255.88	0.66	1.92*
within pop.	192	6140.46	31.98	93.44
total	198	6508.57	34.23	
young plants				
between ecos.	1	69.46	1.05	3.13*
among pop. within ecos.	5	238.16	0.50	1.47*
within pop.	196	6296.91	32.13	95.40
total	202	6604.53	33.68	

* $P < 0.001$.

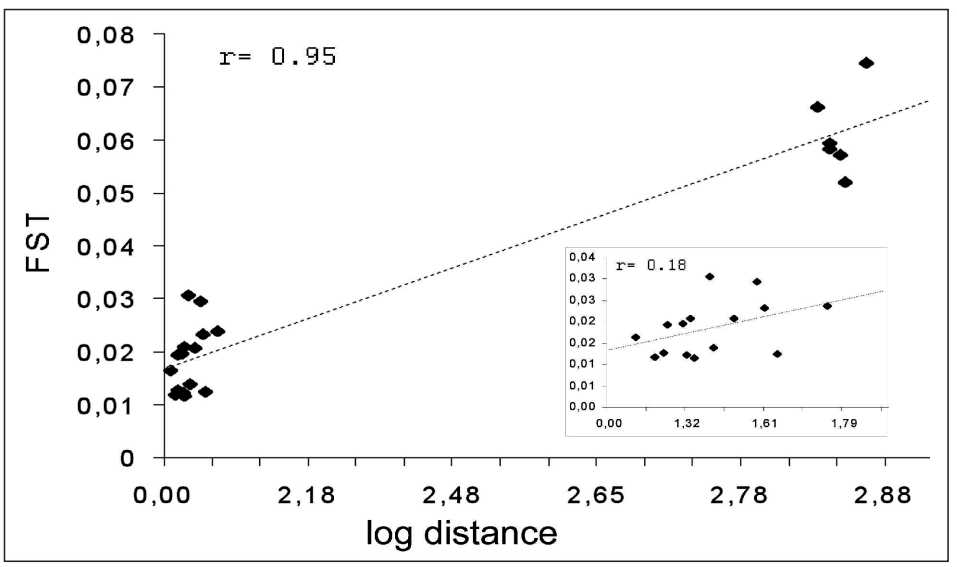

Figure 4. – Correlation between population differentiation (FST) and geographic distances for populations of *Sorocea bonplandii*, in two ecosystems of the Atlantic forest, Southern Brazil. The inset shows details for populations from the Subtropical Atlantic ecosystem with small geographic distances.

Molecular variance was analyzed by AMOVA to subdivide the observed genetic variation of *S. bonplandii* into three hierarchical levels: i) among ecosystems, ii) between populations within a given ecosystem and, iii) within a given population (*Table 6*). This analysis revealed that 93.8% of all variation was partitioned within populations, while only 4.4% was due to variation among ecosystems, and only 1.8% among populations within ecosystem. Variation among all populations of both ecosystems was 2.7% (F_{ST} = 0.0274). Both the variation between ecosystems and between populations within an ecosystem were slightly higher in the group of adult plants and slightly lower in the group of young plants, when compared to the values for all plants.

A significant correlation (P = 0.016, Mantel-test) was found between genetic distance (F_{ST}) and geographical distance (km) of populations when the remote population SPA was included, but not when the more closely spaced populations of the Subtropical Atlantic forest ecosystem alone were analysed (*Fig. 4*).

Discussion

The objective of the current study was to detect possible genetic erosion between *S. bonplandii* populations in small forest remnants of the Atlantic forest in Southern Brazil. The analysis of 299 polymorphic AFLP markers in 468 plants from seven forest remnants resulted in

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two major findings: high level of genetic diversity in all populations, and little influence of fragmentation on genetic diversity of *S. bonplandii* populations.

The high level of genetic diversity of *S. bonplandii* is indicated by a high value of NEI's index (H = 0.214 ± 0.104) and a low value of Jaccard similarity index between all plants within populations (< 60%, data not shown). Reported NEI's indices of genetic diversity of gymnosperm and angiosperm species ranged from 0.037 to 0.179 (TRAVIS *et al.,* 1996; CARDOSO *et al.,* 2000; MARIETTE *et al.,* 2001; STEFENON *et al.,* 2003), Jaccard similarity typically ranged from 70 to 90% (TRAVIS *et al.,* 1996; CLEMENT *et al.,* 2002). Since *S. bonplandii* is a predominant and typical understory species in the Seasonal Deciduous Forest, it can be hypothesized that the sampled region is a center of diversity for the species which is in agreement with the high degree of diversity and with the low values of genetic similarity among individuals within population.

While the genetic diversity within the species is high, the genetic variability between populations is low in spite of extensive fragmentation. Around 94% of the genetic variation occurs within populations. Similar results (94 to 98%) were reported for fragmented populations of some other plant species (SCHIERENBECK *et al.,* 1997; REIS *et al.,* 2000; PITHER *et al.,* 2003, MEDRI *et al.,* 2003; WHEELER *et al.,* 2003) while much lower levels (49 to 58%) have also been reported (TRAVIS *et al.,* 1996; CARDOSO *et al.,* 2000). Several factors are likely to influence the genetic effects of fragmentation (LOVELESS and HAMRICK, 1984; YOUNG *et al.,* 1996), such as the extent of fragmentation and the geographical distances between the remnants, interspecific differences in longevity and pre-fragmentation abundance, the wide variety of sexual and asexual reproductive systems, the presence of seed banks, interactions with vectors of animal pollination and seed dispersal, etc. The genetic distance between populations of *S. bonplandii* clearly correlated with their geographical distance but these differences may have existed before fragmentation.

Similar results were obtained regardless of whether all plants, young plants only, or adult plants only were analysed, again indicating genetic stability of *S. bonplandii* in spite of forest fragmentation. However, the genetic diversity seems to decrease slightly with decreasing size of the fragment, and with increasing past exploitation. The past ca. 70 years of fragmentation may still be too short a time for a loss of genetic diversity caused by fragmentation to become apparent in this species. Furthermore, the history of the dynamics of fragmentation with large or small distances between remnants, and the ecological characteristics of *S. bonplandii* (RUSCHEL *et al.,* 2006) are important aspects to be considered.

Our results have direct implications for sustainable management of *S. bonplandii*, indicating that conservation strategies might be based on a random sample of trees taken throughout the Atlantic forest. However, the minimum population size required for maintaining the huge genetic diversity of this species is unknown at present. In order to establish a sustainable management plan for the species, further ecological studies are needed, such as studies on seed and pollen dispersal, ratio of female to male plants, frequencies of flowering and seed production, natural regeneration, and regeneration potential of the species following harvest. These studies might result in proposing suitable harvesting strategies.

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Allelic Diversity Revealed Through SSR Polymorphisms at the Locus Encoding HMG-CoA Reductase in Rubber (*Hevea brasiliensis*)

By T. SAHA*), C. B. ROY[1]), M. RAVINDRAN, K. BINI and M. A. NAZEER

Genome Analysis Laboratory, Rubber Research Institute of India, Kottayam 686 009, Kerala, India

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Abstract

This study was carried out to define the extent of allelic variation of 3-hydroxy-3-methylglutaryl-CoA reductase gene (*HMGR*) in wild *Hevea* accessions, based on SSR polymorphisms existing at their 3'-untranslated regions (UTRs). Existence of two microsatellite alleles and their repeat compositions was demonstrated earlier in cultivated rubber clones. Both alleles contained perfect poly $(AG)_n$ repeats interrupted by a short sequence of 12 nucleotides and allelic variation at this microsatellite locus was the result of repeat length polymorphisms. In wild populations of rubber, nine microsatellite alleles ('A' to 'I') were identified at the *HMGR* locus revealing a wide allelic diversity compared to cultivated clones. Out

[1]) Present affiliation: Plant Pathology Division, RRII.

*) Author for correspondence: Tel.: +91-481-2353311, Fax: +91-481-2353327. E.mail: saha@rubberboard.org.in

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