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# Genetic diversity of *Anadenanthera colubrina* Vell. (Brenan) var *cebil*, a tree species from the South American subtropical forest as revealed by cpSSR markers

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#### Abstract

Anadenanthera colubrina var cebil is a tree species native to the Upper Parana Atlantic Forest where human activities have severely impacted causing deep fragmentation. Microsatellites are not available in this species. Therefore, the first objective of this study was to generate chloroplast simple sequence repeats (cpSSR) by cross-species transfer. Understanding the evolutionary dynamics of subdivided populations is an important matter. In this way, a first approach to the characterization of the haplotypic diversity within and between populations as well as the genetic structure of native Argentinean populations were the main goals of this study.

Twenty four individuals from two populations of the Misiones province were studied and four cpSSR *loci* were tested. Two of them exhibited polymorphic patterns leading to the identification of 11 cpDNA haplotypes with high mean genetic diversity (GD = 0.73). The minimum spanning network defined three clear groups which can be assigned to at least three subpopulations. AMOVA indicated that the total variance showed the highest percentage of variation (48%) within subpopulations with a fixation index ( $\mathbf{F}_{ST}$ ) statistically significant ( $\mathbf{F}_{ST} = 0.520$ ; p < 0.05). Brown's two *loci* component analy-

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sis indicated that substructure population is present. Jost's differentiation global index  $(D_{est})$  was 0.049 while  $D_{est}$  pairwise comparison reflected a certain level of genetic structure.

The high diversity level detected in the adult trees of *A. colubrina* var *cebil* from the populations under study could be due to recent human influence. In this way, the reduction in population size caused a reduction in the number of trees leading to surviving trees showing the historical diversity of the populations analyzed.

Key words: Upper Parana Atlantic Forest, Curupay, intraspecific genetic diversity, cpSSR cross-species transfer.

#### Introduction

The study of the genetic diversity distribution, population structure and evolutionary features of a species native range is of particular relevance when evaluating their long-term variability and fitness (GóMEZ et al., 2002).

Anadenanthera colubrina Vell. Brenan var cebil, (Fabaceae, Mimosoideae) known locally as curupay, is a tree species native to the South American subtropical forest. Curupay trees can be found in the Upper Parana Atlantic Forest, which extends from Brazil into the northern part of Argentina, where human activities have had a severe impact leading to deep fragmentation. These trees can reach up to 35 m in height, with flowers arranged in hermaphrodite inflorescences and long legume fruits with narrow and flattened seeds (JUSTI-NIANO and FREDERICKSEN, 1998; CIALDELLA, 2000). Their floral traits show that pollination is performed by bees and seed dissemination occurs over short distances by anemochory – autochory after pod dehiscence (JUSTI-

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NIANO and FREDERICKSEN, 1998; ABRAHAM DE NOIR et al. 2002). Curupay has high potential for sustainable forest management, and could play a key role in the recovery of degraded forest areas (JUSTINIANO and FREDERICKSEN, 1998).

Further exploration of the evolutionary dynamics of subdivided populations is of key importance since the remaining natural areas continue to become increasingly smaller and fragmented (LAROCHE and DURAND, 2004). Disturbed habitats could threaten many plant and animal populations through possible erosion of their genetic variability by different mechanisms such as bottlenecks, genetic drift and inbreeding (LAROCHE and DURAND, 2004).

The organization of the circular chloroplast DNA (cpDNA) genome of land plants is extremely conserved, with genes usually occurring in the same order (GRIVET et al., 2001). Chloroplast DNA variation is predominantly maternally inherited in most Angiosperms (HARRIS and INGRAM, 1991) and this seems to be the case in A. colubrina var cebil. The non-recombinant, uniparental inheritance and haploid nature of this organelle genome makes it a useful tool for evolutionary studies (PROVAN et al., 2001) as well as for the examination of historical colonization processes based on cpDNA variation (CAVERS et al., 2004). cpDNA acts as a single inheritable unit (DOULATY BANEH et al., 2007), and the assignment of a cpSSR "haplotype" to any given accession is a very straightforward process due to the high copy number of cpDNA molecules per cell along with the lack of recombination in the chloroplast genome (BRYAN et al., 1999a). Non-coding intron and intergenic spacers are particularly variable and contain microsatellite and other kinds of polymorphisms even between closely related individuals and taxa in a range of plants groups (CESARE et al., 2010).

Characterization of new microsatellites is costly and technically demanding considering they must be isolated de novo from species being examined for the first time (PEAKALL et al., 1998; KAUNDUN and MATSUMOTO, 2002; ZANE et al., 2002). Fortunately, DNA regions flanking microsatellite loci are relatively conserved and primers designed in a source species have been used successfully to amplify SSRs in closely related taxa (KAUNDUN and MATSUMOTO, 2002). In the case of chloroplast microsatellites (cpSSRs), repetitive flanking regions are even more conserved across genera, allowing for the design of universal chloroplast primers potentially useful for a wide range of species (CATO and RICHARD-SON, 1996). Chloroplast microsatellites inherit several important and unique characteristics from the organelle genome in which they occur (EBERT and PEAKALL, 2009).

Maternally inherited markers are the most suitable for phylogeographic reconstructions since they allow the study of seed movement. However, paternally inherited plastid DNA polymorphism could provide insights into past genetic processes (MARCHELLI et al., 2010). The pattern of genetic differentiation varies whether it considers maternal or paternal plastid inheritance. In the first case, it has been reported low intraspecific genetic variation (MARCHELLI and GALLO, 2006; PASTORINO et al., 2009). On the other hand, MARCHELLI et al., (2010) have reported very low genetic differentiation in *Araucaria araucana*, as expected for a paternally inherited plastid that moves with pollen grains.

Despite the value of chloroplast markers in population genetics and the growing number of studies developing and applying cpSSRs, their use is still largely centered on economically important plants and their relatives. The potential of cpSSRs to offer unique insights into ecological and evolutionary processes in wild plant species remains to be fully realized (EBERT and PEAKALL, 2009).

Considering that microsatellites have not been described for *A. colubrina*, the first objective of this study was to generate cpDNA markers for this novel species by cross-species transfer from *Nicotiana tabacum*. These markers would be subsequently used to further explore the haplotypic diversity within and among populations as well as the genetic structure present in native Argentinean populations of *A. Colubrina*.

## **Materials and Methods**

## Plant material

Two populations of A. colubrina located in Santa Ana  $(27^{\circ}25'55.92"S55'34'16.68"W$ , altitude 153 m asl) and Candelaria  $(27^{\circ}26'58.2"S55'44'20.22"W$ , altitude 104 m asl), in the province of Misiones (Argentina), were sampled (*Figure 1*). The distance between populations was nearly 17 km and two subpopulations approximately 3 km apart were considered within each population. Trees over 0.20 m in normal diameter were considered as adult trees. Between four and seven adult trees were sampled for each subpopulation. In some cases trees were only 3 m apart due to their absence in some areas. The low number of sampled trees is a consequence of the high levels of deforestation in the region. Leaves collected were conserved with silica gel in labeled plastic bags.



Figure 1. – Location of A. colubrina var cebil populations under study.  $\bullet$ : Santa Ana,  $\blacktriangle$ : Candelaria.

Table 1. – Characteristics of four novel microsatellite loci isolated from A. colubrina var cebil.

Locus	Primer sequences (5'-3')	T <sub>a</sub> (°C)	Allele size range (bp)	N° alleles	Repeat motif	Genebank Accession number
АсСстр3	CAGACCAAAAGCTGACATAG GTITCATTCGGCTCCTTTAT	45	50	1	(T) <sub>8</sub>	FJ560722
AcCcmp5	TGTTCCAATATCTTCTTGTCATTT AGGTTCCATCGGAACAATTAT	47	130	1	$(T)_7 C(A)_3 T(A)_{10}/(A)_{10}$	FJ560723
AcNtcp8	ATATTGTTTTAGCTCGGTGG TCATTCGGCTCCTTTATG	52	352-363	6	(T) <sub>10</sub>	FJ560724
AcNtcp9	CTTCCAAGCTAACGATGC CTGTCCTATCCATTAGACAATG	50	439-459	5	(T) <sub>6</sub> /(A) <sub>7</sub> /(A) <sub>8</sub>	FJ560725

Table 2. - Allelic frequencies in the studied subpopulations of A. colubrina var cebil.

Locus	Allele	Candelaria A	Candelaria B	Santa Ana A	Santa Ana B	Total
	352	0,000	0,000	0,143	0,000	0,042
	354	0,000	0,000	0,143	0,000	0,042
A aNtan 8	358	0,333	0,000	0,143	0,000	0,125
Астисро	361	0,500	0,571	0,000	1,000	0,500
	363	0,167	0,429	0,000	0,000	0,167
	NA	0,000	0,000	0,571	0,000	0,167
	439	0,000	0,000	0,714	0,000	0,208
	445	0,167	0,000	0,000	0,000	0,042
AcNtcp9	449	0,333	0,000	0,286	0,500	0,292
	452	0,500	0,571	0,000	0,500	0,375
	455	0,000	0,429	0,000	0,000	0,125

#### DNA extraction

Dry young leaves (about 5 g) were ground to a fine powder with liquid nitrogen using a ceramic pestle. Next, genomic DNA was isolated using the Rapid One Step Extraction (ROSE) method described by STEINER et al. (1995), modified as follows: 1% of polyvinylpolypyrollidone (PVPP) was added to the extraction buffer and organic washes were performed (GARCÍA et al., 2007). DNA was precipitated by addition of cold isopropanol and 3M NaCl.

DNA concentration was estimated electrophoretically against a known amount of  $\lambda$  DNA marker used as standard.

### Primer selection

Four chloroplast *loci* were assayed using the universal primers pairs Ccmp3 and Ccmp5 developed by WEISING and GARDNER (1999) and primers pairs Ntcp8 and Ntcp9 developed by BRYAN et al. (1999b). These primers were intended to amplify four different *loci* according to the conserved nature of intergenic or intronic regions in higher plant chloroplasts (CHENG et al., 2003). Oligonucleotide sequences described in *Table 1* were synthesized by the Operon Tech Corporation.

## Polymerase chain reaction (PCR) assay

Amplification reactions were carried out in a final volume of 30 µl containing 0.2 mM dNTPs, 2.5 mM MgCl<sub>2</sub>, 2 µM of primer, 1x reaction buffer (750 mM Tris-HCL pH 8.8, 200 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Tween 20), 0.75 units of *Taq* polymerase (#EP0402 Fermentas) and approximately 17 ng of DNA template. Amplifications were performed using a Techne PHC – 3 Dri – Block ® Cycler as follows: (i) initial denaturation at 94 °C for 4 min; (ii) 30 cycles of denaturation at 94 °C for 30 s, annealing temperature [T<sub>a</sub>] for 1 min and extension at 72 °C for 30 s; and (iii) final extension at 72 °C for 5 min. The T<sub>a</sub> values for the different primers were: 52 °C (Ntcp8), 50 °C (Ntcp9), 47 °C (Ccmp3) y 45 °C (Ccmp5).

## Visualization of PCR products

DNA amplification was evaluated by gel electrophoresis using agarose (2%) and TRIS Borate EDTA (TBE) buffer (1x). Gels were run at 5 v/cm, stained with Ethidium Bromide (0.5 µg/ml), and visualized under UV light (SAMBROOK et al., 1989).

Allele identification was carried out in 6% denaturing polyacrylamide gels (43 cm  $\times$  35 cm  $\times$  0.4 cm) electrophoresed in 0.5 X TBE buffer at 75W for about

*Table 3.* – Haplotypes defined by sizes of alleles (in bp) and haplotypic total frequency.

Haplotype	cpSSR /	locus	Total Frequency	
	AcNtcp8	AcNtcp9	100001100quenty	
H1	358	449	0.0833	
Н2	358	445	0.0417	
Н3	361	449	0.1250	
H4	363	452	0.1250	
Н5	361	452	0.2500	
H6	361	455	0.0833	
H7	363	455	0.0417	
H8	NA	449	0.0417	
Н9	354	439	0.0417	
H10	NA	439	0.1250	
H11	352	439	0.0417	

NA = null allele.

60 min. Polyacrylamide gels were silver stained and a 100 bp Ladder (BIORAD) was used for allele discrimination. Allele sizes were determined using the GQmol software available at <u>http://www.ufv.br/dbg/gqmol/gqmol.</u> <u>htm</u> (SCHUSTER and CRUZ, 2004).

#### Sequencing of PCR fragments

Amplified products from two individuals out of thirty samples analyzed were sequenced using forward and reverse primers in a MegaBACE<sup>1000</sup> sequencer. Products were previously purified using the GFX PCR DNA and Gel Band Purification Kit (General Electric Healthcare). Sequences were edited using the Chromas lite 2.01 software available at <u>http://www.technelysium.com.au/ chromas\_lite.htlm</u> and aligned using the BioEdit sequence alignment editor software available at <u>http:// www.etoology.net/index.php/software/genetics/100-bioedit-709.html</u> (HALL, 1999).

#### Data analysis

Different haplotypes were obtained by combining alleles from different *loci*. The terms *locus* and allele will be used to refer to a cpSSR region or a variant in size at a given accession, respectively. Not all primers used yielded amplification products for all individuals analyzed. On account of this, a null allele was considered when no product was detected after the amplification reaction using DNA from different extractions. Null alleles were taken into account for haplotypic definition. Allelic frequency was obtained for each *locus*. Haplotypic frequencies were obtained for each subpopulation and for all individuals. The number of shared haplotypes was estimated by direct counting.

Gene diversity was estimated as

$$GD = (1 - \sum pi^2)/(n-1)$$

where *n* is the number of alleles and *p* is the frequency of the *i*th allele (NEI, 1987). Due to the non-recombinant nature of the chloroplast genome, cpDNA haplotypes were treated as alleles at a single *locus*. This assessment was developed using the POPGENE software package (version 1.32) available at <u>http://www.ualberta.ca/~fyeh/</u> (YEH and BOYLE, 1997).

Multilocus association variances were partitioned into single-locus and two-locus components following Brown's analysis for multilocus associations among and within subpopulations (BROWN and FELDMAN, 1981). In this analysis, single-locus and two-locus components were measured by their contributions to the variance in the number of heterozygous *loci* in two randomly chosen gametes. Variance components in the number of heterozygous *loci* determined for single *locus* effect were: Mean gene diversity (MH), Variance of diversity (VH) and Wahlund's effect (WH). Variance components in the number of heterozygous loci determined for two-locus effect were: Mean linkage disequilibrium (MD), Wahlund's effect (WC), Interaction between MD and WC (AI), Variance of disequilibrium (VD) and Covariance of interaction (CI).

A minimum spanning network among haplotypes was constructed based on the number of different alleles between haplotypes. This assessment was developed using the ARLEQUIN 3.5 software package available at <u>http://cmpg.unibe.ch/software/arlequin3/</u> (Excoffier et al., 2005). The network was drawn using the HAPSTAR-0.5 software available at <u>http://www.fo.am/hapstar</u>.

A hierarchical analysis of variance was used to partition the total variance into covariance components among individuals within subpopulation variation, variation among subpopulation within population and variation among populations by an Analysis of Molecular Variance (AMOVA) (EXCOFFIER et al., 1992). The departure of this analysis is a genetic distance matrix between haplotypes based on the number of different alleles. The significance of covariance components was tested using 999 permutations arbitrarily fixing the *P*-values at 0.05. The fixation index ( $F_{\rm ST}$ ) was calculated for pairwise subpopulations. These analyses were performed using GENALEX, a user-friendly cross-platform

Table 4. – Haplotypes shared between studied subpopulations of A. colubrina var cebil.

	Candelaria A	Candelaria B	Santa Ana A	Santa Ana B
Candelaria A	-			
Candelaria B	2	-		
Santa Ana A	1	1	-	
Santa Ana B	2	1	0	-



*Figure 2.* – Distribution of the eleven haplotypes in the sampled subpopulations of *A. colubrina* var *cebil*.

package available at <u>http://www.anu.edu.au/BoZo/</u> <u>GenAlEx/</u> (PEAKALL and SMOUSE, 2006)

An alternative differentiation measure (D), which is independent of heterozygosity, has been proposed by JOST in those cases where the  $F_{ST}$  index could be biased by high allelic diversity values. This measure quantifies diversity at a gene *locus* as the inverse of gene identity (1/J), equivalent to the effective number of alleles (RYMAN and LEIMAR, 2009). Values were calculated using SMOGD vsn. 1.2.5 available at <u>http://www.ngcrawford.com/django/jost/</u> (CRAWFORD, 2010).

## Results

## cpSSR in A. colubrina var cebil

The four microsatellites obtained belong to two different microsatellite types: two perfect mononucleotide  $[(T)_8 \text{ and } (T)_{10}]$  and two compound imperfect repeats  $[(T)_7C(A)_3T(A)_{10}/(A)_{10}]$  and  $(T)_6/(A)_7/(A)_8]$  (*Table 1*). Sequences have been renamed and deposited in the GenBank public database with the accession numbers indicated in *Table 1*.

#### cpSSRs alleles and chloroplast haplotypes

Four cpSSR *loci* were analyzed and two of them (AcNtcp8 and AcNtcp9) showed polymorphic products in the twenty four accessions examined. *Loci* AcNtcp8 and AcNtcp9 contained six and five alleles respectively with *locus* AcNtcp8 also exhibiting null alleles (*Table 2*). Allele 361 showed the highest frequency for *locus* AcNtcp8 whereas allele 452 showed the highest frequency for *locus* AcNtcp9 (*Table 2*). Both alleles are present in Candelaria A, Candelaria B and Santa Ana B while

Santa Ana A had three unique alleles. This subpopulation also showed null alleles (*Table 2*). Eleven haplotypes were found as a result of different combinations of alleles from the two polymorphic *loci* (*Table 3*) with haplotype 5 displaying the highest frequency (H5=0.2500) (*Table 3*). After subpopulation pairwise analysis Candelaria A and Candelaria B shared two haplotypes while Santa Ana A and Santa Ana B shared none (*Table 4*).

The distribution of the eleven haplotypes across the region analyzed showed a complex pattern. Haplotypes H1, H3 and H5 were scattered among three of the four populations examined (*Figure 2*). One out of eleven haplotypes was unique to Candelaria A and Candelaria B (H2 and H7 respectively), four were unique to Santa Ana A (H8, H9, H10 and H11) whereas Santa Ana B did not show any unique haplotypes. One common haplotype (H5) was shared by three of the four subpopulations analyzed (*Figure 2*).

#### *Genetic diversity*

The genetic diversity analysis yielded a high value (GD = 0.73). Brown's analysis for multilocus associations among and within subpopulations indicated that the single *locus* effect results in a high mean gene diversity (MH = 0.446) and a low variance in diversity (VH = 0.053). On the other hand, the two-*locus* component results in Wahlund's effect (WC = 0.077) were lower than the interaction of WC and MD (AI = 0.013) (*Table 5*).

The minimum spanning network of haplotypes is shown in *Figure 3*. Some haplotypes were group-specific as is the case with H1, H8, H9, H10 and H11 present in Santa Ana A and H4, H5, H6 and H7 present in Candelaria B. It is worth noting that haplotypes H4 and H5

 $Table \ 5.$  – Brown's analysis for multilocus associations among and within subpopulations.

Components of variance in the N° of different naplotypes					
Component		Variation %			
	Mean gene diversity (MH)	0.446			
Simple <i>locus</i> effect	Variance of diversity (VH)	0.053			
	Wahlund's effect (WH)	-0.101			
	Mean disequilibrium (MD)	0.023			
	Wahlund's effect (WC)	0.077			
Two- <i>loci</i> effect	Interaction between MD and WC (AI)	0.013			
	Variance of disequilibrium (VD)	0.038			
	Covariance of interaction (CI)	-0.015			
Total variance	(MH+VH+WH+MD+WC+AI)	0.512			
Average variance	(MH+MD+Al+VD+Cl)	0.505			





*Figure 3.* – Minimum Spanning Network for the chloroplast DNA haplotypes among subpopulations of *A. colubrina* var *cebil*. Haplotypes are represented with the same symbols as in *Figure 2* and their origin is included.

Table 6. – Analysis of Molecular Variance (AMOVA) from a genetic distance matrix between haplotypes based on the number of different alleles.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	1	105331.576	105331.576 Va	17
Among subpopulations within populations	2	110816.983	55408.492 Vb	35
Within subpopulations	20	214674.024	10733.701 Vc	48
Total	23	430822.583	22383.852	100

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Table 7. – Fixation index for different hierarchical levels and statistical significance.

Fixati	on index	Significance values
F <sub>RT</sub>	0.175	0.079
$\mathbf{F}_{\mathbf{PR}}$	0.419*	0.012
F <sub>ST</sub>	0.520*	0.005

\* Statistically significant (999 permutations).

were also present in other subpopulations. Santa Ana B only showed two haplotypes (H3 and H5) which were also present in other groups. Haplotype H1 was located at the center of the network.

AMOVA indicated that the total variance was distributed mainly within subpopulations where the highest percentage of variation (48%) was found (*Table 6*). The fixation index ( $F_{\rm ST}$ ) was statistically significant with a high value ( $F_{\rm ST}$ =0.520; p<0.05) (*Table 7*). The  $F_{\rm ST}$  comparison between Candelaria A and Santa Ana B subpopulations did not show significant genetic differentiation and a similar situation was observed between Santa Ana A and Santa Ana B (*Table 8*).

The Jost differentiation global index ( $D_{est}$ ) was 0.049. In most cases,  $D_{est}$  comparison values between subpopulation pairs were zero except in those cases where Santa Ana A was involved (*Table 9*).

### Discussion

To our knowledge, this study represents the first analysis of cytoplasmic DNA diversity within Argentina's northeastern native species. The use of *universal* primers has notorious benefits in terms of development costs and time requirements as a strategy for accessing cpSSR variation in novel species (EBERT and PEAKALL, 2009). All four cpSSRs tested were able to yield PCR amplification products successfully in *A. colubrina*.

Ntcp primers were developed from mononucleotide simple-sequence repeats in the N. tabacum chloroplast genome. These primers were tested in other members of the Solanaceae where they revealed high levels of interspecific chloroplast DNA variation (BRYAN et al., 1999b). However, Ntcp9 was excluded from the genetic diversity analysis in Vitellaria paradoxa due to problems with allele detection in gel-based assays. On the other hand Ntcp8 was included in this study and showed four alleles (FONTAINE et al., 2004). Only five out of thirty six Ntcp primers (namely Ntcp8, Ntcp9, Ntcp37, Ntcp39, and Ntcp40) generated PCR amplification products and polymorphism was observed at each *locus* except for Ntcp40 in phylogenetic studies in yams (CHAÏR et al., 2005). These studies indicated that Ntcp primers could not be considered strictly universal but species-specific cpSSR primers. Null alleles within Ntcp8 were detected in A. colubrina var cebil, probably due to changes in the nucleotide sequence of microsatellite's flanking regions which prevent primer annealing during the amplification process. Such variations occur at a non-negligible rate (CHAPUIS and ESTOUP, 2007).

The four novel A. colubrina var cebil microsatellite sequences were rich in A/T, in agreement with the numerous chloroplast sequences deposited in GenBank (POWELL et al., 1995; EBERT and PEAKALL, 2009).

Although cpDNA non-coding regions show a relatively low base-substitution rate, two *loci* were polymorphic in PCR fragment size and showed several alleles in *A. colu*-

 $\label{eq:table 8.-F_{ST} pairwise comparisons of studied subpopulations. F_{ST} values below diagonal. Probability values are based on 999 permutations above diagonal.$ 

	Candelaria A	Candelaria B	Santa Ana A	Santa Ana B
Candelaria A	0.000	0.007	0.025	0.418
Candelaria B	0.450*	0.000	0.001	0.038
Santa Ana A	0.488*	0.520*	0.000	0.075
Santa Ana B	0.000	0.506*	0.417*	0.000

\* Statistically significant.

Table 9.  $-D_{est}$  pairwise comparisons of subpopulations under study.

	Candelaria A	Candelaria B	Santa Ana A	Santa Ana B
Candelaria A	-	0.000	0.118	0.000
Candelaria B		-	0.225	0.000
Santa Ana A			-	0.140
Santa Ana B				-

*brina* var *cebil*. The maximum size difference among these alleles was 11 bp for *locus* AcNtcp8 and 16 for *locus* AcNtcp9. Single nucleotide polymorphisms and insertion-deletion polymorphisms (indels) are common in chloroplast non- coding regions (CESARE et al., 2010). This would explain why other Ntcp primers such as Ntcp40 and Ntcp49 showed irregular gaps of several base pairs in *Carya illinoinensis* (GRAUKE et al., 2010). Indels would then be responsible for the observed differences in size among alleles in our study.

High haplotype diversity was detected as a result of the large number of alleles observed in both *loci*, a fact that was also reflected in the high mean gene diversity value (GD=0.73). We found 11 haplotypes in 24 trees from two populations. This result contrasts with some data published in the literature for other species. In this way, CAVERS et al. (2003) found five haplotypes in 29 populations of *Cedrela odorata* and ANDRIANOELINA et al. (2006) found 13 haplotypes in 100 individuals from 10 locations of *Dalbergia monticola* (Fabaceae).

The most frequent haplotype (H5) was defined by combination of the most frequent alleles (361 and 452) and was scattered among three of the four analyzed populations (Candelaria A, Candelaria B and Santa Ana B). Santa Ana A showed the highest number of unique haplotypes, suggesting that it is the most differentiated subpopulation.

The structure of multilocus association among and within populations was partitioned into its components. The single *locus* component analysis showed high mean gene diversity (MH=0.446) and low variance in diversity (VH=0.053). A substantial and positive interaction between MD and WC values (AI) when using two-loci component analysis indicates that the correlation of alleles between populations is coincident with the pattern observed within populations (BROWN and FELDMAN, 1981). In our study, this interaction showed that the correlation of alleles between populations did not repeat the pattern within populations reflecting that population substructure is present. In addition, this population substructure might be invoked when Wahlund's effect (WC) is high but interaction between MD and WC (AI) is low (BROWN and FELDMAN, 1981). Our present results indicate that the fixation index value  $(F_{ST}=0.52)$ strengthened this result even when the  $F_{ST}$  was calculated using a haplotypic distance matrix. The analysis of molecular variance (AMOVA) was developed to quantify the contribution of different population structure levels to genetic variation. The total variance was distributed mainly within subpopulations (48%). However, the level between populations reached 17%.

While AMOVA is a powerful and robust approach, randomization is limited by sample size (FITZPATRICK, 2009). We should also keep in mind that when using hierarchical randomization tests the number of groups at each level affects the statistical power at that specific level (FITZPATRICK, 2009). It has been suggested that evaluating all pairwise comparisons might be more informative and forthright when analyzing small numbers of populations. Due to this, some authors consider that more information might be obtained by performing separate analysis of unlinked *loci* and combining inferences across *loci* (FITZPATRICK, 2009). We were unable to perform this analysis of unlinked *loci* due to the lack of cpDNA recombination. All  $F_{\rm ST}$  pairwise comparisons (*Table 8*) showed analogous results and emphasized subpopulation Santa Ana A as the most distant subset.

The Jost's D index was estimated as a consequence of the high diversity level detected. These global statistics did not show any genetic structure ( $D_{est} = 0.049$ ). On the other hand,  $D_{est}$  comparison between subpopulations showed some degree of differentiation. All comparisons against Santa Ana A suggested the existence of genetic structure (*Table 9*), which could be derived from the presence of unique haplotypes.

Statistical differences ( $\rm F_{ST}$  vs.  $\rm D_{est}$ ) may result from variations in the theoretical development of these mathematical tools. Currently,  $\rm D_{est}$  is considered a real differentiation measure since its index is quantified in terms of the effective number of alleles and thereby is independent of heterozygosity (JOST, 2008; RYMAN and LEIMAR, 2009). Thus,  $\rm D_{est}$  quantifies diversity at a gene *locus* as the inverse of gene identity (1/J), equivalent to the effective number of alleles (RYMAN and LEIMAR, 2009) whereas, in our study,  $\rm F_{ST}$  was estimated from the distance between haplotypes. In this way,  $\rm F_{ST}$  has been more informative than Dest when performing a cpDNA diversity analysis in *A. colubrina* var cebil.

The minimum spanning network reflected the differentiation among subpopulations. Related haplotypes identified clear groups which can be assigned to at least three subpopulations. The position of haplotype H1 in the network indicated that it is the ancestral haplotype (*Figure 3*).

It may seem surprising to find that fragmented populations display large levels of cpDNA diversity when drift and reduced gene flow should indeed reduce diversity as isolation increases. The Upper Parana Atlantic Forest was originally covered by a continuous semideciduous subtropical forest with a high diversity of plant species. The greatest threat to diversity in this region is the extreme degree of fragmentation and forest degradation. This fragmentation comes as a result of the expansion of cultivated areas (DI BITETTI et al., 2003). Starting in the '60s and '70s, the Misiones forest has been deeply impacted as a consequence of the replacement of native species by exotic ones introduced for the purpose of forest exploitation (MAC DONAGH and RIVERO, 2005). The high level of diversity detected in the adult trees of A. colubrina var cebil from the populations studied could be due to recent human impact reducing the population size. In this scenario, genetic drift needs long periods of time to fix or lose haplotypes. Therefore, the reduction in population size causes a subsequent reduction in the number of trees, *i.e.* surviving trees may be showing the historical diversity of the A. colubrina var *cebil* populations analyzed.

Considering that only 7.4% of the Upper Parana Atlantic Forest original cover still remains (JARAMILLO et al., 2005) urgent conservation strategies are required and the first step to design them is to know their genetic diversity.

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# Heritability of Yield and Secondary Traits in two populations of Para Rubber Tree (*Hevea brasiliensis*)

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## Abstract

Heritability and interactions of yield and growth traits were assessed in *Hevea brasiliensis* using full-sib progenies and clonal populations. Using parent-off-spring regression, annual mean rubber yield (ARY) and summer yield (SY) showed moderate to high heritability (ARY,  $h^2 = 34-56\%$ ; SY,  $h^2 = 36-52\%$ ). Among the yield components, girth exhibited low to moderate heritability ( $h^2 = 17-36\%$ ) while branching height showed low heri-

tability  $(h^2 = 18\%)$ . Using forty clonal genotypes, annual mean rubber yield  $(H^2 = 48\%)$ , rubber yield during peak period  $(H^2 = 47\%)$  and rubber yield during stress (or summer yield)  $(H^2 = 44\%)$  showed high estimates of heritability. Among the other yield components, except volume of latex during stress period  $(H^2 = 40\%)$ , remaining yield components showed moderate estimates for heritability  $(H^2 = 29 - 37\%)$ . Dry rubber content (DRC) based on annual mean showed very high heritability  $(H^2 = 68\%)$ , followed by DRC during stress  $(H^2 = 51\%)$ and peak  $(H^2 = 50\%)$  periods. Latex flow rate based on annual mean and peak period data showed high heritability  $(H^2 = 51\%)$  followed by latex flow rate during stress period  $(H^2 = 42\%)$ . Plugging indices of annual and

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