

Artigos

Analysis of genetic diversity in two plantations of *Pinus caribaea* var. *hondurensis*

Análise da diversidade genética em dois plantios de *Pinus caribaea* var. *hondurensis*

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ABSTRACT

Forest tree breeding activities in plantations with exotic species implies several instances of material selection where genetic variation can be affected. The objective of the work was to verify the genetic variability present in two plantations of *Pinus caribaea* var. *hondurensis* by microsatellite markers, initially designed for *Pinus taeda*. Thus, 299 individuals were analyzed using eight polymorphic microsatellite markers. The results indicate that both plantations have adequate levels of genetic diversity that are representative of the *Pinus* genus. Through the Bayesian method, it was possible to detect two different population genetic structure ($K = 2$) between both plantations analyzed. In conclusion, this study suggests that microsatellites markers are useful tools to monitor genetic variation in genetic breeding programs. The genetic diversity estimated in both plantations is similar and typical for *Pinus* plantations, and as expected, there was a slight decrease in genetic variability in the commercial plantation in comparison with the base plantation.

Keywords: SSRs (Short Sequence Repeats); Caribbean pine; Agroforestry; Biotechnology

RESUMO

As atividades de melhoramento genético florestal em plantios com espécies exóticas envolvem várias instâncias de seleção de material, onde a variação genética pode ser afetada. O objetivo do trabalho foi verificar a variabilidade genética presente em duas plantações de *Pinus caribaea* var. *hondurensis* através de marcadores microssatélites, inicialmente desenhados para *Pinus taeda*. Assim, 299 indivíduos foram analisados utilizando oito marcadores microssatélites polimórficos. Os resultados indicam que ambas plantações têm níveis adequados de diversidade genética que são representativos do gênero *Pinus*. Através do método Bayesiano, foi possível detectar duas populações genéticas estruturadas ($K = 2$) entre as duas plantações analisadas. Em conclusão, este estudo sugere que os marcadores microssatélites são ferramentas úteis para monitorar a variação genética nos programas de melhoramento genético. A diversidade genética estimada em ambas plantações é semelhante e típica das plantações de *Pinus*, e como se esperava verificou-se uma ligeira diminuição da variabilidade genética na plantação comercial, em comparação com a plantação base.

Palavras-chave: SSRs (Repetições de Sequência Curta); Pinheiro do Caribe; Agrofloresta; Biotecnologia

1 INTRODUCTION

Multiple species of the genus *Pinus* are widely used in homogenous reforestation programs in different parts of the world, due to their great adaptation to different climatic regimes and the application of their products (wood, fibers, cellulose) (AGUIAR *et al.*, 2011). *Pinus caribaea* Morelet is an important and widely planted tropical conifer, its natural range covers from northern Mexico to southern Nicaragua, including the islands of Cuba and the Bahamas. This species has been extensively used as an industrial planting in tropical and subtropical areas around the world (TAMBARUSSI *et al.*, 2010). Nevertheless, forest breeding activity with introduced species involves several instances of material selection, in which genetic variation can be reduced. Inbreeding is a likely result in progeny produced in plantations of a newly introduced exotic species and could be increased in advanced generations. Inbreeding reduces genetic variability and low levels of genetic variation damage the evolutionary adaptation processes in a changing environment. Populations with low genetic variability are not adequate as a basis for breeding program populations (NEALE; WHEELER, 2019).

The incorporation of molecular markers in breeding programs seeks to optimize the structure of improvement/production plantations, increase the information needed to decide on the infusion of new materials and improve the selection strategies (MARCUCCI POLTRI *et al.*, 2010). Among them, microsatellite markers have been indicated for genetic variability and population genetics studies, since they are co-dominant and constitute one of the most polymorphic molecular marker currently available. The use of selectively neutral molecular markers allows quantifying characteristics of genetic variability within and between populations (GRATTAPAGLIA; RESENDE 2011). Neutral markers have proved to be useful to study phylogeographic and gene flow patterns in conifers (BAGNOLI *et al.*, 2009; GONZÁLEZ-MARTÍNEZ *et al.*, 2010) and are increasingly being used to infer demographic history in tree species (DAÏNOU *et al.*, 2010).

This study aimed to compare the genetic variability present in a base plantation of *Pinus caribaea* var. *hondurensis* included in a genetic improvement program with a commercial plantation by microsatellites molecular markers. These results will contribute to the knowledge of the genetic diversity variability through different practical instances of a forest breeding program.

2 MATERIALS AND METHODS

2.1 Plant material

Pine needles were obtained from different plantations of *Pinus caribaea* var. *hondurensis* planted in different trials in the Argentinean Northeast (NEA). The trials were established with seeds collected from individuals growing in natural populations and came from the National School of Forestry Science (ESNACIFOR, Honduras) in the year 1980, and from the current Oxford Forestry Institute (England) former CFI Oxford, in the year 1973. Both trials were installed in Bella Vista, province of Corrientes, and belong to the Experimental Station of the National Institute of

Agricultural Technology (INTA EEA Bella Vista) (28° 30' 23.82" S and 59° 2' 32.11" O). The design was a Randomized Complete Block with five repetitions, plots of 9- 25 per plot (a distance of 3.5 m). From these two trials, 169 individuals (N = 169) were collected for further studies, called from now BASE PLANTATION (the plantations belonging to INTA). The commercial tree plantation, from now on referred as: COMMERCIAL PLANTATION, corresponds to a plantation of select phenotypic of commercial origin belonging to the Company PINDO S.A. (N = 130), located in Puerto Esperanza, Misiones, Argentina (26° 1' 35.48" S and 54° 1' 45.38" O).

2.2 DNA Extraction

Total genomic DNA was extracted from adult leaf tissue following a CTAB (Hexadecyltrimethylammonium bromide) modified protocol and kit: Nucleo Spin plant II Macherey-Nagel®, based in the SDS buffer. DNA quantification was performed with a spectrophotometer at 280 nm (Nanodrop ND-1000®). Twelve of twenty-four microsatellites *loci* of the PtTX series (designed for *Pinus taeda fluorescent-labeled*) have been selected based on the amplification products, unequivocal and congruent with the data presented in the bibliographical antecedents (VILLALBA, 2010). The polymerase chain reaction (PCR) has been conducted in a GenePro® Thermal Cycler E-96G.

2.3 PCR and Molecular markers

Reaction mixture: 10x Invitrogen buffer (1x), 10 mM each of dNTPs, 10 mM of each primer, and 5 units/ul of *Taq polymerase* enzyme (Invitrogen®). The MgCl₂ concentrations varied according to the needs of each pair of oligonucleotides. As template, 20 ng/ul of DNA dissolved in sterile bidistilled water were used. A typical touchdown PCR program consisted of the following steps: five minutes at 94°C, followed by 10 or 20 touchdown cycles, depending on the need for each pair of oligonucleotides, with the following conditions: 94° C (1 Min), drop in temperature to 1°C or 0.5°C (10 or 20 touchdown cycles, respectively) and 72°C (1 min). This step

was continued with 30 cycles of amplification at 94°C (1 min), followed by 1 min annealing, according to the annealing temperature specific for each primer, 72°C (1 min), and a final extension of 10 min at 72°C. The amplification products were analyzed using an ABI 3130xl Automatic Capillary Sequencer (Applied Biosystems®, USA), belonging to the Genomic Platform of the Institute of biotechnology (INTA Castelar, Hurlingham, Buenos Aires, Argentina). The electropherogram was analyzed using GeneMapper® 4.0 program.

2.4 Genetic Diversity analysis

The genetic diversity parameters of polymorphic loci were calculated by GenAEx 6.4 (PEAKALL; SMOUSE, 2006). The polymorphic information content (PIC) for each locus was estimated. The genetic structure of *Pinus caribaea* var. *hondurensis* was determined using the Bayesian clustering approach implemented in STRUCTURE v 2.3 (PRITCHARD *et al.*, 2000). This software uses multilocus genotype data to assign individuals to genetically divergent clusters. For each simulation, was used ten independent runs for each K value (K= 1-10 populations) to obtain L(K) with LOCPRIOR assigned (HUBISZ *et al.*, 2009) under the admixture model, allele frequencies correlated, with a burn-in of 100,000 steps, and Markov Chain Monte Carlo for 200,000 steps. The optimal number of K clusters was determined using the ΔK criteria. Additionally, we conducted the hierarchical analysis of molecular variance (AMOVA) to assess the level of differentiation among both plantations with GenAEx 6.4.

3 RESULTS AND DISCUSSION

3.1 Microsatellite amplification

From the PtTX series of microsatellites markers, twelve have been transferred to *Pinus caribaea* var. *hondurensis* originally designed for *Pinus taeda* (Table 1). All the amplified loci correspond to triplet repeat and low copy number motifs; this type of microsatellites markers are the ideal candidates to be transferred between conifer

species (KALIA *et al.*, 2011). They are highly conserved due to their possible functional role in eukaryotic transcription, replication, gene expression, and regulation.

Three out of four microsatellites amplified not reported previously for *Pinus caribaea* var. *hondurensis* were polymorphic (PtTX4018, PttX4092, and PtTX4098), and eight microsatellites were successfully used to compare both populations of 299 individuals.

Table 1 – Characteristics of eleven polymorphic microsatellites loci and one monomorphic used in this study in *Pinus caribaea* var. *hondurensis* plantations

<i>Pinus caribaea</i> var. <i>hondurensis</i>				
Locus	Library Type	Repeat motif	Allele size (bp)	Reference
PtTX2123	Genomic	(AGC)8	190 - 199	Elsik <i>et al.</i> 2000
PtTX2128	Genomic	(GAC)8	232 - 245	
PtTX3013	Low Copy	(GTT)10	132 - 145	
PtTX3025	Low Copy	(CAA)10	250 - 311	
PtTX3030	Low Copy	(TA)4 (GGT)10	300 - 359	
PtTX3098	Low Copy	(GTT)8	136 - 185	Kutil <i>et al.</i> 2001
PtTX3107	Low Copy	(CAT)14	152 - 172	Elsik <i>et al.</i> 2001
PtTX3116	Low Copy	(TTG)7 (TTG)5	113 - 168	
PtTX4018	UnderMethylated	(GAC)15	161 - 216	Zhou <i>et al.</i> 2002
PtTX4090	UnderMethylated	(CTT)6	188	
PtTX4092	UnderMethylated	(GAA)21	106 - 153	
PtTX4098	UnderMethylated	(GAA)7	161 - 172	

Source: Authors (2019)

3.2 Genetic diversity

The genetic diversity was characterized by different parameters (Table 2). The total number of alleles (N_a) were 61 for the BASE PLANTATION, and 58 for the COMMERCIAL PLANTATION, with a minimum of three alleles (PtTX2123-PtTX2128) and a maximum of 16 for the most polymorphic marker (PtTX3116) in both plantations.

Table 2 – Genetic diversity of two studied plantations of *Pinus caribaea* var. *hondurensis*.

BASE PLANTATION						COMMERCIAL PLANTATION				
N	Na	Ho	UHe	PIC	Locus	N	Na	Ho	UHe	PIC
160	3	0,55	0,51	0,44	PtTX2123	116	3	0,45	0,53	0,46
161	3	0,32	0,27	0,24	PtTX2128	117	4	0,46	0,5	0,43
159	7	0,55	0,62	0,56	PtTX3013	125	10	0,55	0,68	0,63
163	9	0,61	0,51	0,46	PtTX3098	120	7	0,65	0,76	0,72
169	9	0,62	0,61	0,57	PtTX3107	117	10	0,44	0,55	0,51
165	16	0,81	0,86	0,84	PtTX3116	118	16	0,72	0,86	0,84
165	9	0,77	0,64	0,61	PtTX4018	124	4	0,29	0,39	0,37
164	5	0,37	0,35	0,31	PtTX4098	113	4	0,33	0,52	0,41
163,25 (1,14)	7,62 (1,49)	0,58 (0,05)	0,55 (0,06)	0,50 (0,18)	MEAN (SD)	118,75 (1,43)	7,25 (1,58)	0,49 (0,05)	0,60 (0,05)	0,54 (0,16)

Source: Authors (2019)

In where: N: number of analyzed individuals; Na: numbers of alleles; Ho: Observed Heterozygosity; UHe: Unbiased Heterozygosity; PIC: polymorphism index context.

The mean number of alleles was 7.6 (SD 1.5) and 7.2 (SD 1.5), respectively. These results are similar to those reported for other pine species: in *Pinus radiata* were six alleles (N = 96); in *Pinus resinosa* and *Pinus oocarpa* (N = 502) nine alleles (N = 518) (SOTO *et al.*, 2010). In *Pinus patula* (N = 60) and *Pinus tecunumanii* (N = 108), a total of five alleles were detected, although the sample size was smaller (DVORAK *et al.*, 2009).

For populations from Mexico, three (N = 14) and four alleles (N = 60) were detected (DELGADO *et al.*, 2011). The results obtained in this work could indicate a comparable genetic diversity in both plantations of *Pinus caribaea* var. *hondurensis*. The conservation of genetic diversity is essential for many reasons; these include adaptation to environmental changes, the risks of inbreeding depression in the viability of the population, and the need to maintain genetic resources for possible future use (NEALE and WHEELER, 2019).

The genetic diversity was analyzed with the observed (Ho) and the Unbiased Expected Heterocigosity (Ho and UHe, respectively) (Table 2). The mean levels of genetic diversity observed in the analyzed plantations of *Pinus caribaea* var. *hondurensis*

(BASE PLANTATION H_o : 0.58; COMMERCIAL PLANTATION H_o : 0.49) are typical of predominantly cross-pollinated forest species (NEALE; WHEELER, 2019). They agree with the values obtained in other plantations of the same species, analyzed with microsatellites molecular marker (8 SSR).

According to Andrade Furlan *et al.*, (2007), the genetic variability of trees from the natural range revealed similar H_o values in the base plantation (0.24) and commercial plantations ($N = 25$, $H_o = 0.30$). Shepherd *et al.* (2002) reported a H_o of 0.49 (29 SSR) in plantations of the same species in Australia. The data obtained by Delgado *et al.* (2011) in two natural populations ($N = 14$ and $N = 60$) remaining and marginal of *Pinus caribaea* var. *hondurensis* in Mexico (6 SSR), showed values of H_o : 0.42. Some particularities could explain the levels of genetic diversity found in conifers: long generational intervals allow less exposure to potential bottleneck processes during the reproductive period (SANCHEZ *et al.*, 2014). Furthermore, high levels of crossbreeding are a particularly important factor in maintaining genetic diversity in the species. Another factor is that most of the forest species are monoecious, and this prevents self-fertilization because the sexes are spatially separated (in different structures and sections of the tree) and temporarily (pollen release occurs at different times of female maturation) (SEBASTIANI *et al.*, 2012). The PICs obtained were similar for each plantation. Four microsatellite markers had values greater than 0.5, and one of them: PtTX3116, showed the highest value (0.81), being the most informative marker in both plantations.

3.3 Genetic structure

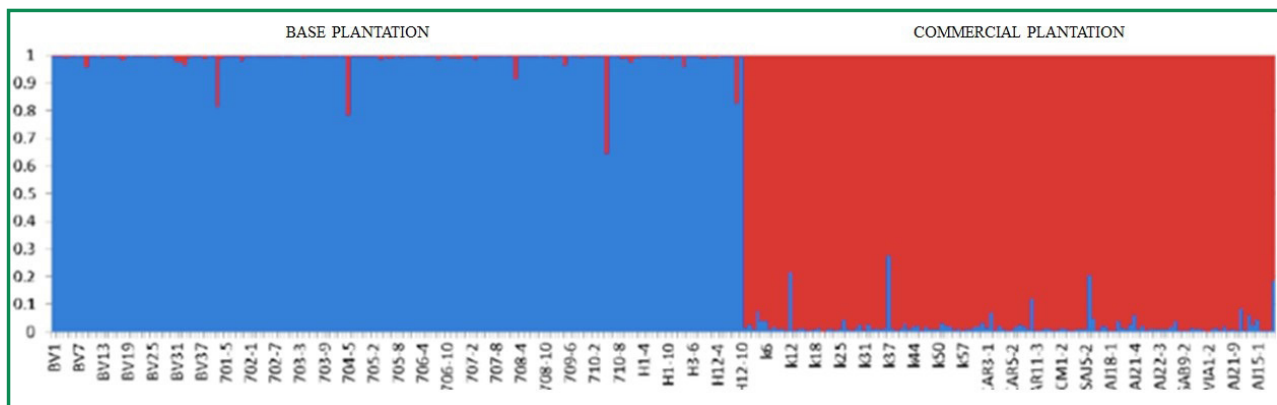
Based on the results of the Bayesian clustering, the *Pinus caribaea* var. *hondurensis* plantations were separated into two clusters ($K=2$) using STRUCTURE. The BASE PLANTATION was assigned to the first genetic cluster, whereas the COMMERCIAL PLANTATION formed the second cluster (Figure 1). This result is consistent with the AMOVA (Phipt 0.28) (Table 3). Most of the genetic variation (72%) observed in this study was due to the genetic variation of individuals within populations.

Table 3 – Analysis of Molecular Variance (AMOVA)

Source of variation	Degree of freedom	Sum of squares	Variance components	Variation (%)	p value
Among populations	1	343,9	2,2	28%	0,0001
Within populations	297	1.717,9	5,7	72%	
Total	298	2.061,1	8,08	100%	

Source: Authors (2019)

Figure 1 – Bar plot showing STRUCTURE results for K = 2



Source: Authors (2019)

In where: Vertical bars represent *Pinus caribaea* var. *hondurensis* individuals. The bars are partitioned into two color segments corresponding to the probability of belonging to one of the two genetic clusters.

The genetic differentiation between the *Pinus caribaea* var. *hondurensis* plantations was 28% (between the BASE PLANTATION and COMMERCIAL PLANTATION). This result could be due to the different origins of the seed material. The COMMERCIAL PLANTATION, as a consequence of the phenotypic selection, could present new allelic combinations. The amount of variation may well differ between quantitative traits and neutral marker loci. The level of differentiation between populations at neutral loci depends on a balance between migration and genetic drift (NEALE; WHEELER, 2019). Even low levels of migration will equalize gene frequencies between populations at

such loci. When there is diversifying selection, the balance between selection and migration can result in considerable genetic differences between populations. While migration rates are equal for all genes, selection acts differently on different parts of the genome. The artificial selection changes the genetic structures of populations in manifold ways. Successful selection for a trait changes the allelic structures at the controlling gene loci and has some correlated effects on other traits. Measuring levels of genetic variation within and among populations is a critical first step in evaluating the evolutionary biology and tree improvement potential of a species (ISIK, 2014).

4 CONCLUSION

Microsatellite markers that come from low copy number or low methylation genomic areas are highly transferable in species within the genus *Pinus*.

The genetic diversity estimated in both plantations is typical of *Pinus* plantations. The commercial plantation has a slight decrease in genetic diversity compared with the base plantation.

The Base plantation and commercial plantation have different genetic structures.

The microsatellite markers are useful tools for the monitoring of genetic variation in successive stages of forest genetic improvement.

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