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Primer Note: Microsatellite-AFLP development for *Araucaria araucana* (Mol.) K. Koch, an endangered conifer of Chilean and Argentinean native forests

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Abstract

Araucaria araucana (Mol.) K. Koch is one of the most important native species of Chile and Argentina, and also one of the most endangered. In this study, we report the development and characterization of a set of microsatellite markers in the species by means of the microsatellite-AFLP (M-AFLP) technique. A total of 25 M-AFLP derived bands, showing a typical microsatellite pattern, were selected and sequenced. Of these, 12 that contained microsatellite sequences, were used for primer extension. Six of the resulting SSR markers provided easily interpretable patterns and were used to investigate the level of genetic diversity in two populations of *A. araucana*. A total of 43 alleles were amplified. The mean overall loci of observed and expected heterozygosities for the Conguillio and Villa Araucaria populations were 0.322 and 0.443, respectively. The primers presented in this study may provide useful information for the establishment of a conservation strategy in the species.

Key words: Microsatellite, endangered species, *Araucaria araucana*, conservation.

Araucaria araucana (Mol.) K. Koch is one of the oldest conifers in South America and a representative symbol of Chilean and Argentinean forest biodiversity due to its endemicity and longevity. Likewise, it is the major tree species in the traditional lands of the indigenous Mapuche Pehuenche community, showing that both the ecological and cultural significance of araucaria forests are key elements for its conservation. The species occurs mainly in the Andean region at the border of Chile and Argentina and also in the Coastal Cordillera of Chile. *A. Araucana* is considered an endangered species due to its restricted current distribution, slow growth and low regeneration (Veblen, 1982), and it is included in Appendix I of CITES and listed as 'vulnerable' species on the IUCN Red List (1996).

Microsatellite markers have been developed in other species of Araucaria genera such as *A. cunninghamii*, *A. rulei*, *A. subulata* and *A. angustifolia* (Scott et al.,

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2003; ROBERTSON et al., 2004; SALGUEIRO et al., 2005; SCHMIDT et al., 2007); however, to the best of our knowledge, there are no SSRs specifically developed in *A. araucana*. Likewise, successful cross-species amplification of microsatellites among *A. angustifolia* and *A. araucana* has been reported (SALGUEIRO et al., 2005; MORENO et al., 2011), although the number of transferred loci with high amplification quality was low.

We report the development and characterization of SSR markers in *A. araucana* by means of the microsatellite-AFLP (M-AFLP) technique (VAN EIJK et al., 2001), which has been successfully applied in other species (YANG et al., 2011; ALBERTINI et al., 2003; ACQUADRO et al., 2005; BRUNA et al., 2008).

Genomic DNA was extracted from fresh leaves using the Qiagen DNeasy™ Plant mini Kit. Total DNA (500 ng) was restricted-ligated with *Eco*RI and pre-amplified according to VOS et al. (1995). M-AFLP selective amplifications were performed in a 20 µl reaction mix containing 1/100 of pre-amplified DNA, 50 ng of *Eco*RI+3 (E-CCA, E-CAA, E-CCC) primer in combination with 10 pmol 5'-anchored microsatellite (5'-AM) primer ($CGGC(AC)_7$, $GTGC(TC)_7$, $CGTCC(CA)_7$), 2 µl of 10X PCR buffer (Sigma), 0.2 mM dNTPs, and 0.4 U *Taq* DNA polymerase (Sigma) (ALBERTINI et al., 2003). Amplified fragments were resolved on 5% denaturing poly-acrylamide gels (OWL-systems.www.owlsci.com) and silver stained (BLUM et al., 1986). The 9 M-AFLP primer combinations applied to 6 genomic DNA samples, produced a total of 183 amplification products of which, 41 (22%) were polymorphic. Twenty-five polymorphic bands showing a typical microsatellite pattern were excised from poly-acrylamide gels and re-amplified with the same primer combination used to generate the original M-AFLP pattern. PCR products were then cloned into a pCR4-TOPO vector using the TOPO TA Cloning kit (Invitrogen) and sequenced using Big Dye Terminator 3.1 (Applied Biosystems) on an ABI 3130XL DNA Analyzer (Applied Biosystems). Sequences were edited and aligned using Vector NTI 9 (Invitrogen).

All 25 clones contained a microsatellite. The most common motif was AC (12 clones), TC (8 clones) and CA (5 clones). Thirteen of the 25 fragments did not contain additional units of SSR motif with respect to the number of repeats in the 5'-AM primer and were not used for primer design and for the second step of the procedure. Of the remaining 12 fragments, only 3 contained a perfect microsatellite, while the remaining 9 contained a compound microsatellite. A locus-specific forward primer directed towards the microsatellite motif was designed for each of the 12 selected clones using the Primer 3 software (ROZEN and SKALETSKY, 2000).

These primers were used in combination with *Mse*I+0 primer for amplifying an aliquot of the restriction-ligation reaction. These reactions were performed in a 20 µl mixture containing 1 µl of restriction ligation as template, 50 ng *Mse*I primer, 10 pmol forward primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1U of *Taq* DNA polymerase (Sigma), and the manufacturer-supplied buffer. Cycling parameters were: 94 °C for 5 min followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 60 s at 72 °C, with a final step of 10 min at 72 °C. Only single band products were cloned and sequenced. Sequences were then used for designing locus-specific reverse primers. The 12 SSR primer pairs were used for genotyping analyses.

Amplifications were performed in a 10 µl reaction volume containing 0.2 mM dNTP, 3 mM $MgCl_2$, 0.2 µM of each primer, and 0.04 U/µL *Taq* DNA polymerase (Invitrogen). Cycling parameters were: 8 min of denaturation at 94 °C, followed by 35 cycles of denaturation at 94 °C for 50 s, annealing at 57 °C–60 °C for 45 s, elongation at 72 °C for 1 min, and finally one cycle at 72 °C for 8 min. Amplified samples were analysed using ABI PRISM 3100 DNA sequencer. Data were analyzed employing GeneScan and Genotyper software programs (Applied Biosystems).

Out of the 12 M-AFLP-derived SSRs, only 6 were revealed to be codominant and polymorphic with an easily interpretable pattern (*Table 1*). Polymorphism was

Table 1. – Characteristics of the six M-AFLP-derived SSR markers used in *A. araucana*.

Locus	Primer pair sequences (5'-3')	Repeat motif	Allele size range (bp)	Ta (°C)	PIC	GeneBank Accession nº
Aara02	F: GGTTGAGCTAACCACCCAAG R: TCCTTTGCAACATTCCCTTC	$(TC)_5(CAT)_3$	196-240	60	0.348	JN054698
Aara04	F: GAATTCCCTAGTAAGTTTGAGGAA R: GTTCCCAACGGTGCTGTAGT	$(ATC)_3(AG)_6$	84-124	57	0.487	JN054699
Aara05	F: TCAATGGAAAAATATATTACGGTATGT R: TTCCAACATCAACTGCTAATCAA	$(CA)_{10}$	82-103	60	0.188	JN054700
Aara07	F: CACCATACTTCTTCACACTCTCA R: CTCCATATGAAGAGTCACATTCTAC	$(CA)_{10}(TA)_3$	86-90	60	0.362	JN054701
Aara09	F: ATTGGTCATCAGCCAAAGAA R: TAAGGTTCTGACCGCTTGCT	$(GA)_{25}$	225-242	58	0.761	JN054702
Aara10	F: ACTCCATATGAAGAGTCACATTCTAC R: TCAGGAAACAGCCTAGACCA	$(TA)_7(TG)_{14}$	143-173	57	0.281	JN054703

Ta, specific annealing temperature in °C; PIC, polymorphism information content.

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Table 2. – Characteristics of the six microsatellite loci evaluated in two populations of *A. araucana.*

	Total (n = 57)				Conguillio (n = 30)				Villa Araucaria (n = 27)			
Locus	Na	Ho	He	F_{IS}	Na	Ho	He	F_{IS}	Na	Ho	He	F_{IS}
Aara_02	6	0.193	0.385	0.505*	6	0.267	0.479	0.447*	2	0.111	0.283	0.611*
Aara_04	10	0.193	0.503	0.617*	8	0.067	0.433	0.848*	7	0.333	0.572	0.422*
Aara_05	4	0.105	0.195	0.460*	3	0.133	0.187	0.292*	2	0.074	0.201	0.636*
Aara_07	2	0.54	0.48	- 0.132	2	0.533	0.427	- 0.254	2	0.518	0.509	- 0.019
Aara_09	11	0.754	0.791	0.019	11	0.7	0.832	0.145	4	0.814	0.7	- 0.169
Aara_10	10	0.158	0.287	0.456*	8	0.233	0.305	0.239*	5	0.074	0.273	0.732*
Average	7.17	0.324	0.440	0.320	6.33	0.322	0.443	0.286	3.67	0.320	0.423	0.369

Na, number of alleles per locus; Ho, observed heterozygosity; He, expected heterozygosity; F_{IS}, inbreeding coefficient. * $P < 0.05$.

evaluated in 57 individuals from two *A. araucana* populations (Conguillio population from Andes Cordillera and Villa Araucaria population from Costa Cordillera); all measures of genetic statistics were calculated using Arlequin 3.1 software (Schneider et al., 2000). A total of 43 alleles were detected, with a mean of 7.17 alleles per locus (*Table 2*) and 26 private alleles (21 in the Conguillio population and 5 in the Villa Araucaria population). Polymorphic information content (PIC) ranged from 0.188 to 0.761, with a mean of 0.405 (*Table 1*). The observed (Ho) and expected heterozygosities (H_e) ranged from 0.105–0.754 and 0.195–0.791, with averages of 0.322 and 0.443, respectively (*Table 2*). The inbreeding coefficient F_{IS} showed positive and significant deviation from zero in four out the six loci and the same pattern was detected at the population level (*Table 2*). This lack of equilibrium could be interpreted as the result of the long-time fragmentation and overexploitation to which these populations have been subjected. Furthermore, deficits in heterozygotes could be attributed to the presence of null alleles. Therefore, each locus was tested with Micro-checker 2.2.3 (Van Oosterhout et al., 2004), detecting significant evidence of null alleles in loci Aara04 (0.262) and Aara10 (0.172). The linkage disequilibrium tested among 15 possible pairwise loci and considering 1000 permutations, revealed linkage disequilibrium at locus Aara04. However, the critical significance level of multiple test adjusted to Bonferroni correction indicated that all loci were genetically independent.

Results confirm that the two-step primer extension procedure based on the M-AFLP technique is a powerful strategy when the costs and the resources required to isolate SSRs markers by traditional methods cannot be justified. Furthermore, these primers may provide useful information to establish conservation strategies in Araucaria.

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Transcript abundances of LIM transcription factor, 4CL, CAld5H and CesAs affect wood properties in *Eucalyptus globulus*

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Abstract

Eucalyptus globulus is the main hardwood species grown in pulpwood plantations in temperate regions of the world. We have cloned six genes influencing wood quality including the LIM domain transcription factor (LIM), 4-coumarate-CoA ligase (4CL), coniferaldehyde 5-hydroxylase (CAld5H) and the three catalytic units of cellulose synthase (CesA), from *E. globulus*. The transcript abundances of LIM in basal stems of ten independent *E. globulus* lines showed similar patterns to those of 4CL. We investigated the correlation between gene transcript abundances and wood qualities such as Klason lignin (KL) content, syringaldehyde/vanillin (S/V) ratio and holocellulose (HC) content. Expression of the LIM and 4CL were positively correlated with KL content. A highly significant positive correlation was observed between CAld5H expression and S/V ratio. Furthermore, a ratio of the sum of the transcript abundances of three CesA1, CesA2 and CesA3 to 4CL showed a positive correlation with a ratio of HC/KL content that positively correlated with the chemically extracted fiber content in this woody plant.

Key words: LIM domain transcription factor, CAld5H, CesA, 4CL, *Eucalyptus globulus*.

Introduction

Eucalyptus species constitute the most widely planted hardwood trees in temperate and subtropical regions. Their wood is used as a raw material for the production of cellulose. Eucalyptus species have fast growth rates and the ability to adapt to a broad range of geographic locations. Eucalyptus has been listed as one of the candidate biomass energy crops (Li et al., 2008; Hinchee et al., 2009). *Eucalyptus globulus* is one of the most important commercial temperate hardwood species for the pulp and paper industries because of its wood properties and pulp production characteristics.

Plant cell walls are composed mostly of cellulose, hemicellulose and lignin. Cellulose provides strength and flexibility to plant tissue and is of great importance to the chemical, textile, pulp and paper industries. In woody plants, high levels of cellulose are synthesized and cellulose accounts for about 50% of the dry weight of wood. However, the precise biosynthetic pathway of cellulose is not well understood, although genome-level studies are underway in some model plants such as *Arabidopsis thaliana* and *Populus trichocarpa* (Endler and Persson, 2011; Kumar et al., 2009). Most structural genes involved in lignin biosynthesis have been identified in many species including woody plants (Boerjan et al., 2003). The aromatic lignin polymers commonly found in woody plants are primarily composed of two monolignols, namely coniferyl and sinapyl alcohols, which typically form guaiacyl-syringyl (G-S) lignin when polymerized. These monolignols are synthesized via the phenylpropanoid pathway, which begins with deamination of phenylalanine to form cinnamate, followed by a series of ring hydroxylations, *O*-methylations, and side-chain modifications. Lignin found in gymnosperms and ferns lack S units (Boerjan et al., 2003) suggesting that the branch leading to sinapyl alcohol biosynthesis may

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