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Cross transferability of SSRs to five species of Araucariaceae: a useful tool for population genetic studies in *Araucaria araucana*

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Abstract

Cross-species amplification of microsatellites is a common procedure to obtain suitable markers to be used in population genetic studies. Primers designed for one (source) species are used to amplify homologous loci in related (target) species. It is expected that phylogenetically close species will share a higher proportion of markers, and genetic distance could be a useful parameter to predict successful transferability between different taxonomic groups.

We analyzed twenty-two primer pairs developed for *Araucaria angustifolia* (Bertol.) Kuntze in five target species of the Araucariaceae family. The results were summarized in vectors of presence and absence of bands and compared through the Jaccard similarity index. Using the sequences of eight published genes, genetic distances between pairs of species were estimated and related to transferability rate using Pearson correlations.

Successful transfer rate ranged from 31.8 to 77.3%, being these among the highest reported for plants. The highest transfer rate was observed between the South American species. The transferability was confirmed sequencing seven fragments amplified in *A. araucana* (Molina) K. Koch, and using the best five to estimate genetic diversity parameters in a natural population of this Andean coniferous.

Key word: cross species amplification; pairwise genetic distance; conservation of microsatellite loci; Pehuén.

Resumen

Transferibilidad cruzada de microsatélites a cinco especies de Araucariaceae: una herramienta útil para estudios de genética de poblaciones en *Araucaria araucana*

La transferencia de cebadores que amplifican loci microsatélites desde otras especies es una práctica habitual para obtener marcadores adecuados para estudios de genética poblacional. Los cebadores diseñados en una especie (fuente) son utilizados para amplificar loci homólogos en especies relacionadas (blanco). Se espera que especies cercanas filogenéticamente compartan un mayor número de marcadores y la distancia genética entre especies podría ser un parámetro útil para predecir el éxito de la transferencia entre diferentes grupos taxonómicos.

Se analizó la transferencia de veintidós primers, desarrollados para *Araucaria angustifolia* (Bertol.) Kuntze, a cinco especies blanco de la familia Araucariaceae. Los resultados se resumieron en vectores de presencia y ausencia de bandas y fueron comparados a través del índice de similitud de Jaccard. Se estimaron las distancias genéticas de a pares entre las especies analizadas utilizando las secuencias de ocho genes, y se las relacionó con el índice de transferencia utilizando correlaciones de Pearson.

El éxito de la transferencia varió entre 31,8 y 77,3%, encontrándose estos valores entre los más altos reportados en plantas. El mayor índice de transferencia se verificó entre las especies sudamericanas. La transferencia fue confirmada secuenciando siete fragmentos amplificados en *Araucaria araucana* (Molina) K. Koch, y utilizando los cinco mejores para estimar parámetros de diversidad genética en una población natural de esta conífera andina.

Palabras clave: amplificación inter-especifica; distancia genética de a pares; conservación de loci microsatélites; Pehuén.

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Introduction

Microsatellites or Simple Sequence Repeats (SSRs) are among the most useful DNA markers for studying population genetic structure and dynamics (Zhang and Hewitt, 2003). Although their development has become more accessible in recent years, the cost and effort to obtain SSRs is still significant. Cross-species amplification of SSRs is therefore a common practice (*e.g.* Kayser *et al*., 1996; Kijas *et al.,* 1995; Kupper *et al.,* 2008; Lin *et al.,* 2008). It is assumed that the transferability success depends on the extent of sequence conservation in the primer sites flanking the microsatellite loci and the stability of these sequences during evolution (Zhang and Hewitt, 2003). Therefore, it is expected that the chance of successful cross-species amplification is inversely related to the genetic distance between the species (Zucchi *et al.,* 2002).

Estimation of genetic distance was used to define the degree of similarity between species and to determine cross-species SSR success rate in birds, cetacean and frogs (Primmer *et al.,* 2005). Transferability of primers was attempted among species within different genus (*e.g.* Guidugli *et al.,* 2010; Hendre *et al.,* 2008; Heesacker *et al.,* 2008; Zhang *et al.,* 2007), families (*e.g.* Holmen *et al.,* 2005; Brown *et al.,* 2005; FitzSimmons *et al.,* 1995), orders and classes (*e.g.* Rico *et al.,* 1996) of different organisms, resulting less efficient among plants (Rosetto, 2001). However transferability of nuclear microsatellite loci across species was successful in eucalyptus (Myrtaceae, Zucchi *et al.,* 2002), olives (Oleaceaes, Rallo *et al.,* 2003), oaks (Fagaceae, Barreneche *et al.,* 2004; Durand *et al.,* 2010), carob tree (Fabaceae, Mottura *et al.,* 2005), rubber tree (Euphorbiaceae, Feng *et al.,* 2009) and loblolly pine (Pinaceae, Liewlaksaneeyanawin *et al.,* 2004), among others.

Araucariaceae family, whose origin was estimated to be 308 ± 53 Ma (Late Carboniferous) (Liu *et al.,* 2009), includes three genera: Araucaria, Agathis and Wollemia, today restricted to the Southern hemisphere (Hill and Scriven, 1995). Most species occur in the ecozones of Indomalaya and Australasia. Two species are distributed in South America: *Araucaria angustifolia* and *Araucaria araucana* (both belonging to the section Araucaria). *A. araucana* (Pehuén) is endemic to the South American temperate forests. Its distribution range along the Andes spans between 37° 27' S and 40° 03' S latitude and it is also found in two isolated populations in the Chilean coastal mountains. It is mostly dioecious; pollen is dispersed by wind and seeds by gravity and small rodents. *A. araucana* is classified under IUCN (International Union for Conservation of Nature and Natural Resources) guidelines as vulnerable (Farjon and Page, 1999) and currently it is officially protected in CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora). In this species, several studies on genetic diversity were performed, using isozymes (Gallo *et al.,* 2004; Ruiz *et al.,* 2007), RAPD (Bekessy *et al.,* 2002) and chloroplast DNA (Marchelli *et al.,* 2010) markers. However, for gene flow and fine-scale genetic structure it is necessary to have highly polymorphic co-dominant genetic markers like SSRs.

Nuclear microsatellite loci were developed for different species within Araucariaceae and their transferability to other species within the family was evaluated (Robertson *et al.,* 2004; Scott *et al.,* 2003; Salgueiro *et al.,* 2005; Schmidt *et al.,* 2007). Salgueiro *et al.* (2005) evaluated transferability of all the microsatellite primers developed from Australasian species of the family available until that moment, to both South American species, finding only two successful transferences. Accordingly, they developed six new SSR loci that were reported as useful to *A. araucana*. However, and in agreement with the observations of Scott (2004) in other species of the family, we observed a significant failure in some of these primers when they were screened widely in the Andean species. Therefore, a proper set of highly polymorphic microsatellite markers for gene flow studies in *A. araucana* does not exist. For this reason, an effort to obtain these markers through cross-species amplification of microsatellites developed in *A. angustifolia* (Schmidt *et al.,* 2007) was made.

Additionally, this set of primers was tested in other phylogenetically more distant species within the Araucariaceae family, also to evaluate the relationship between cross-species amplification using genetic distance. For this purpose, four species of the genus Araucaria (*Araucaria angustifolia, A. bidwillli* (Molina) K. Koch*, A. cunninghamii* Aiton ex D. Don*, A. heterophylla* (Salisb. Franco) and one of the genus Agathis (*Agathis alba* Rumph. ex Jeffrey) were considered. According to the phylogenetic relationships, the expectation is that most of the microsatellite loci developed in *Araucaria angustifolia* would be successfully transferred to *Araucaria bidwilli* and *Araucaria araucana*, with a similar degree of polymorphism in the later. For the other phylogenetically more distant species, a lower transferability rate is expected. To test this hypothesis, the amplification success of each microsatellite was

evaluated in relation to the genetic distance between the target and the source species. Genetic distance between species was estimated using two mitochondrial (*coxI* and *atpI*), four chloroplast (*rbcL, matK, rps4* and *cp16S*) and two ribosomal genes (*18S* and *26S*). We demonstrate that there is not a significant correlation between cross-amplification success and genetic distance among these members of the Araucariaceae family.

Material and methods

Sample collection and DNA extraction

Six Araucariaceae species were studied: *Agathis alba, Araucaria angustifolia, A. araucana, A. bidwillli, A. cunninghamii* and *A. heterophylla*. The specimens were collected from different gardens in Argentina, and from a natural population of *Araucaria araucana* (Tromen 39° 37' S, 71° 20' W, 984 m.a.s.l.). Leaves from three individuals per species were sampled to test transferability of primers, and 79 individuals from one population of *A. araucana* were collected in order to evaluate polymorphism in the species. All the samples were store at –80°C until DNA extraction.

Total genomic DNA was extracted following the protocol by Stefenon *et al.* (2004), after grinding the leaves to a fine powder with a mixer mill (Retsch, Germany). DNA concentration was estimated either on 0.8% agarose gels or using a photometer.

Microsatellites analysis

Twenty-two primer pairs isolated from *A. angustifolia* (Schmidt *et al.,* 2007; Salgueiro *et al.,* 2005) were tested in the five target species. Three individuals per species were analyzed, except for *A. araucaria* where the number of individuals varied between 3 and 10, depending on the primer. Polymerase chain reaction (PCR) was carried out in a final volume of 13 µl, with 1X GoTaq reaction Buffer (Promega), 1,53 mM of polyvinylpyrrolidone (PVP, Sigma), 300 µM of each dNTPs (Invitrogen), 0,85 U of Taq Polymerase (GoTaq, Promega), 1 mM of $MgCl_2$, 0.5 µM of each primer and 5 ng of DNA. PCR thermal profile for all species was as follows: denaturation at 94°C for 4 min followed by 35 cycles at 94°C for 30s, 52-60°C for 30s, 72°C for 30s and final extension at 72°C for 10 minutes. PCRs were performed using a My Cycler thermal cycler (BIORAD). Lack of amplification under these experimental conditions was recorded as transferability failure to the target species, and no additional PCR optimization experiments were performed.

On the other hand, optimization experiments of the 22 primer pairs were carried out in *A. araucana*, testing factorial combinations of three different annealing temperatures, T_a (T_a used in source species, T_a –2°C, T_a + 2°C), and different $MgCl_2$ (1.5, 2.5 and 3.5 mM) and DNA concentrations (5, 10, 15, 20, 25 and 30 ng). The optimal combination for each primer is reported in Table 1.

PCR products were ran on 2% agarose gels with 0.5 X TBE buffer at 60 V for 10 min and at 120 V for 90 min, and visualized under blue light after staining with Syber Safe (Invitrogen). Although the amplification was ranked according to the quality of the fragments and the presence of non-specific amplification products, transference was considered successful when amplicons of the expected size range were visualized on agarose gels.

The amplified fragments obtained with the working primers were then ran on a 6% standard denaturing polyacrylamide gel. PCR products were mixed with 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol and denatured at 94°C for 5 min. Gels were ran at 80 Watt for 3 h and silver stained following the protocol by Bassam *et al.* (1991). Slippage patterns in the polyacrylamide gel are indicative of fragments of small repetitive units and therefore hint to microsatellite regions (Dowling *et al.,* 1996).

In order to confirm the presence of microsatellites, PCR products obtained with seven successful pairs of primers in *A. araucana* were sequenced (three individuals per primer). The sequence was obtained by direct sequencing (forward and reverse) using the BigDye chemistry (Applied Biosystems, Foster City, CA) and analyzed on an ABI3130XL automatic sequencer (Genomic Unit, CNIA INTA Castelar, facilities).

In *A. araucana,* the screening for polymorphism was done only for those primers showing a high amplification quality or displaying a high number of alleles in the source species. Between 52 and 75 individuals were amplified with five fluorescently end-labeled primers and PCR was carried out under the conditions described above. The samples were run on a MegaBace 1000 (GE Healthcare) automatic sequencer and electropherograms analyzed using the MegaBACE Fragment Profiler version 1.2 software (GE Healthcare).

Table 1. Details of cross-species amplification of 22 nuclear microsatellites developed for *Araucaria angustifolia* (source species) and tested in five Araucariaceae species (target species)

Locus	Micro-satellite motif and number of repeats	Source species				Target species																						Author
		A. angustifolia				*Agathis alba*				*A. bidwilli*				*A. heterophylla*				*A. cunninghamii*				*A. araucana*						
		Size range (bp)	Q	N	T_a (°C)	Size range (bp)	Q	N	T_a (°C)	Size range (bp)	Q	N	T_a (°C)	Size range (bp)	Q	N	T_a (°C)	Size range (bp)	Q	N	T_a (°C)	Size range (bp)	Q	N	T_a (°C)	DNA (ng)	$MgCl_2$ (mM)	
Aang 01	$(CT)_{22}$	200-260	1	5	58	100-190	2	3	54		4	3			4	3			4	3		200-250	1	6	58	10	2	Schmidt *et al.*, 2007
Aang 03	$(GA)_{13}$	240-260	1	5	58		4	3		240-260	1	3	56	240-250	1	3	56	180-240	2	3	54	240-260	1	6	58	25	2	Schmidt *et al.*, 2007
Aang 07	$(GA)_{24}$	200-280	1	3	56	190-230	1	3	56	190-220	1	3	56	190-220	1	3	56	190-220	1	3	56	190-280	3	10	54	15	3.5	Schmidt *et al.*, 2007
Aang 13	$(GA)_{20}$	200-230	4	4			4	3			4	3			4	3			4	3			4	6				Schmidt *et al.*, 2007
Aang 14	$(GA)_{27}$	150-190	1	3	56		4	3			4	3			4	3			4	3		190-210	3	5	56	15	2.5	Schmidt *et al.*, 2007
Aang 15	$(GA)_{19}$	210-290	1	3	56	160-180	2-3	3	56	210-250	3	3	56	>110	3	3	58	>110	3	3	58	190-250	3	10	59	30	2.5	Schmidt *et al.*, 2007
Aang 18	$(TC)_9$	190-320	1	3	54		4	3			4	3			4	3			4	3		200-320	1	10	54	30	1.5	Schmidt *et al.*, 2007
Aang 21	$(CT)_{12}$	190-210	1	4	56		4	3		170-190	2	3	56	160-180	2	3	56		4	3		190-210	3	6	58	10	2	Schmidt *et al.*, 2007
Aang 23	$(GA)_{19}$	180-200	1	5	54		4	3			4	3			4	3			4	3		190-210	3	6	58	25	2	Schmidt *et al.*, 2007
Aang 24	$(CT)_{19}$	160-200	1	3	58		4	3		200-250	2	3	58	380-410	2	3	58		4	3		200-230	3	5	58	25	2	Schmidt *et al.*, 2007
Aang 27	$(CT)_{12}$	160-210	1	3	58	>400	3	3	54	>260	3	3	54	>450	3	3	56	>650	3	3	56	160-210	3	8	52	30	2.5	Schmidt *et al.*, 2007
Aang 28	$(CT)_{11}$	130-170	1	3	58	170-190	2	3	56	170-190	2	3	56	170-190	2	3	58		4	3		180-200	3	10	54	15	2.5	Schmidt *et al.*, 2007
Aang 30	$(CT)_{21}$	210-230	1	4	58	360-390	2	3	54		4	3			4	3			4	3		210-230	3	6	58	10	2	Schmidt *et al.*, 2007
Aang 35	$(GA)_{10}$	200-270	4	3			4	3			4	3			4	3			4	3			4	4				Schmidt *et al.*, 2007
Aang 41	$(GA)_{12}$	170-300	1	3	58	170-200	1	3	58	170-200	1	3	58	170-200	1	3	58	170-200	1	3	54	170-200	3	8	54	30	1.5	Schmidt *et al.*, 2007
Aang 45	$(CT)_{15}$	190-270	1	3	56	310-360	2	3	54	180-200	3	3	52		4	3			4	3			4	4				Schmidt *et al.*, 2007
Ag 20	$(GA)_{12}$	240-258	1	4	57	290-310	2	3	53	400-510	2	3	55		4	3			4	3		600-650	2	3	57	10	2	Salgueiro *et al.*, 2005
Ag 23	$(TA)_5/(GT)_4$	245-259	1	4	53		4	3		250-300	1	3	53	240-260	1	3	53		4	3		240-250	1	10	53			Salgueiro *et al.*, 2005
Ag 45	$(GT)_4/AT(GT)_7$	154-168	1	4	57	>180	3	3	55	>180	3	3	55	>180	3	3	55	>180	3	3	53	400-500	3	3	53	20	3.5	Salgueiro *et al.*, 2005
Ag 56	$(TC)_{11}$	146-156	1	4	55	150-170	1	3	55	150-170	1	3	55	150-170	1	3	55	150-170	1	3	55	150-170	1	10	55			Salgueiro *et al.*, 2005
Ag 62	$(TC)_{13}$	110-130	1	4	57	>130	3	3	53	>200	3	3	53	>130	3	3	55	>130	3	3	53		4	5				Salgueiro *et al.*, 2005
Ag 94	$(CT)_{12}$	142-170	1	3	57	150-220	1	3	52		4	3		100-200	1	3	52		4	3		140-160	3	10	47	25	3.5	Salgueiro *et al.*, 2005
Number of loci with Excellent quality (Q = 1)				20			4				5				6				3				5					
Excellent and faint quality (Q = 1 + 3)				20			7				10				10				7				17					

Size (bp) denotes PCR product size in target species. Q: quality of the amplified product (1: excellent amplification product, 2: no specific amplification, 3: multiple bands and smear or faint amplification product, 4: no amplification). N: number of individuals tested. T_a: annealing temperature. Amount of DNA and concentration of $MgCl_2$ in a final volume of 13 µl refer to *A. araucana.*

Data analysis

The results for the 22 loci were summarized for each species in a vector of 0 (no amplification) and 1 (successful amplification). Vectors were compared through the Jaccard similarity coefficient using the software NTSYS (Rohlf, 2001). This coefficient assigns more weight to the double presence of a band than to its double absence.

Uncorrected genetic distances (p-distance, Kumar *et al.,* 1993) between target and source species were estimated at *18S, 26S, rbcL, matK, rps4, cp16S, coxI* and *atpI* genes. Sequences were retrieved from GenBank, edited and aligned with MEGA (Tamura *et al.,* 2007). Additional information on microsatellite cross-species amplification was gathered from studies in other Araucariaceae species. The data were included in the comparison when (1) the source species of the microsatellite loci and the amplification success were clearly specified in the target species, and (2) *18S, 26S, rbcL, matK, rps4, cp16S, coxI* and *atpI* sequences were available for both source and target species. Overall, information regarding cross-species microsatellite transferability success for loci isolated from *A. angustifolia, A. bidwilli* and *A. cunninghamii,* available before the publication by Schmidt *et al.* (2007), was compiled and presented in Table 2 (upper part), together with our results for the 22 primers developed by Schmidt *et al*. (2007). The percentage of loci successfully transferred was calculated for each pair source-target species (CSA% Cross Species Amplification; Table 2). We classified the transference according to the quality of the amplified product as 1: excellent amplification product, 2: no specific amplification, 3: multiple bands, smear or faint amplification product, 4: no amplification.

Genetic distances (p) and amplification rates were compared by Pearson's correlation (r) using R 2.10.0 (R-Development-core-team. 2008). Matrices of genetic distances (p) and Jaccard distances (dissimilarity, Jd) were compared by Mantel test using the program NTSYS (Rohlf, 2001). To confirm the presence of the microsatellite stretches in *A. araucana,* the sequences were aligned and compared against the sequences reported for the source species (obtained from NBCI Genbank), using the program Bioedit (Hall, 1999).

In *A. araucana*, number of alleles per locus (N_A), effective number of alleles (N_e), exclusion probability (P_E) [following equation 2a in Jamieson and Taylor (1997)], expected (H_e) and observed (H_o) heterozygosities (Nei, 1971) and deviations from Hardy Weinberg

Table 2. Upper part. p: genetic distance for *18S, 26S, rbcL, matK, rps4, cp16S, coxI* and *atpI* gene sequences respectively. Lower part. Pearson correlation's coefficients and respective p values between genetic distance at each gene and CSA %

Source species	Target species	p genetic distance to source species								CSA %	NM	N	References
		18S	*26S*	*rbcL*	*matK*	*rps4*	*cp16S*	*coxI*	*atpI*				
A. angustifolia	*Agathis alba*	0.757	0.739	0.736	0.699	0.014	0.004	0.023	0.021	36.4	22	3	Present study
A. angustifolia	*A. bidwilli*	0.002	0.006	0.011	0.004	0.000	0.001	0.001	0.002	45.5	22	3	Present study
A. angustifolia	*A. heterophylla*	0.004	0.005	0.016	0.015	0.010	0.002	0.018	0.018	45.5	22	3	Present study
A. angustifolia	*A. cunninghamii*	0.004	0.003	0.015	0.013	0.013	0.004	0.020	0.017	31.8	22	3	Present study
A. angustifolia	*A.araucana*	0.001	0.004	0.005	0.003	0.003	0.004	0.002	0.001	77.3	22	3	Present study
		0.001	0.004	0.005	0.003	0.003	0.004	0.002	0.001	100.00	6	6	Salgueiro *et al.*, 2005
A. cunninghamii	*A. heterophylla*	0.000	0.005	0.008	0.003	0.003	0.004	0.006	0.001	81.3	16	2	Scott *et al.*, 2003
A. cunninghamii	*A. bidwilli*	0.004	0.006	0.017	0.017	0.013	0.003	0.021	0.019	47.8	23	2	Scott *et al.*, 2003
A. cunninghamii	*Agathis robusta*	0.007	0.006	0.033	0.023	0.016	0.005	0.032	0.020	45.5	22	2	Scott *et al.*, 2003
A. cunninghamii	*A.araucana*	0.003	0.004	0.016	0.013	0.013	0.007	0.022	0.018	20.00	10	6	Salgueiro *et al.*, 2005
		0.003	0.004	0.016	0.013	0.013	0.007	0.022	0.018	20.00	5	3	Present study
A. cunninghamii	*A. angustifolia*	0.004	0.003	0.015	0.013	0.013	0.004	0.020	0.017	20.00	10	60	Salgueiro *et al.*, 2005
A. bidwilli	*A. heterophylla*	0.000	0.005	0.008	0.003	0.003	0.004	0.006	0.001	80.00	5	2	Scott *et al.*, 2003
A. bidwilli	*A. cunninghamii*	0.004	0.006	0.017	0.017	0.013	0.003	0.021	0.019	100.00	9	2	Scott *et al.*, 2003
A. bidwilli	*Agathis robusta*	0.007	0.006	0.033	0.023	0.016	0.005	0.032	0.020	77.8	9	2	Scott *et al.*, 2003
Pearson's correlation (r)		–0.3457	–0.3414	–0.3525	–0.3474	–0.2693	–0.1318	–0.2841	–0.2554				
p		0.2811	0.2872	0.2765	0.2858	0.5414	0.5865	0.5964	0.7575				

CSA %: microsatellite Cross-Species Amplification success between source species and target species (according to the quality as defined in Table 1). Origins of data used to estimate the percentages are specified in the column references. NM: number of microsatellite loci analyzed in each referenced work. N: number of analyzed samples.

expectations were estimated using GenAlEx 6.3 (Peakall and Smouse 2006). The same genetic parameters were calculated for three loci in *A. angustifolia* using the allele frequencies reported by Patreze and Tsai (2010). For the other loci, allele frequencies were not available. The presence of null alleles at each locus was verified with MICRO-CHECKER version 2.2.0.3 (Van Oosterhout *et al.,* 2004).

Results

Transferability was successful for 7, 7, 10, 10 and 17 SSRs in *A. cunninghamii*, *Agathis alba*, *A. bidwilli, A. heterophylla* and *A. araucana*, respectively (Table 2, upper part). Cases classified in category 3 (faint amplification products) were considered as a successful transference. On the other hand, the screening of polymorphisms was performed using only the primers classified in category 1. Jaccard similarity coefficients between target species and *A. angustifolia* (source species) were 0.368, 0.421, 0.450, 0.450 and 0.800 for *A. cunninghamii*, *Agathis alba*, *A. bidwilli, A. heterophylla* and *A. araucana*, respectively. The relationships between *p* genetic distances for the eight genes and amplification rates were non significant (Table 2, lower part). No significant correlations between *p* genetic distance and Jaccard distances (Jd) for any of the eight genes studied were observed. The highest correlation was obtained for the gene *18S* ($r = 0.393$, $p = 0.074$).

In *A. araucana,* loci Aang 01, Aang 03, Aang 18, Ag 23 and Ag 56 showed high quality of amplification; the locus Aang 15 displayed a high number of alleles in the source species and in the Andean species and therefore an effort was made to improve the amplification quality (Table 1). These six loci, together with locus CRCAc 1 (reported as transferable by Salgueiro *et al.*, 2005) were sequenced. All the sequenced fragments contained the same repetitive motif that was reported originally in the source species (See Annex 1 for details). Due to the difficulty in obtaining adequate PCR products for primers of quality 3 (smear, faint or multiple bands) we limited the screening for polymorphism to those primers of high quality, except when the numbers of alleles obtained were high. In *A. araucana*, the five successfully tested microsatellites (Ag23, Ag56, Aang15, Aang18 and CRCAc1) segregated in a Mendelian way, as evidenced by comparing embryos with the parental genotype assessed from megagametophytes (data not shown). The number of alleles ranged between 3 and 22 and the effective number of alleles between 1.45 and 8.73. Significant deviations from Hardy-Weinberg expectations were detected at three loci (Ag 23, Aang 15 and CRCAc1). At locus Aang 15, significant evidence for the presence of null alleles (Fisher combined probability test $p < 0.001$) was observed. Observed and expected heterozygosity ranged from 0.307 to 0.685 and from 0.282 to 0.890, respectively (Table 3). Exclusion probability (Jamieson & Taylor 1997) was 0.816.

Discussion

For a successful cross-species amplification of microsatellite markers, the repeat sequence and the flanking regions containing the selected primer sites must be conserved across taxa. Therefore a higher ge-

Table 3. Loci transferred from *Araucaria angustifolia* to *Araucaria araucana,* and estimated genetic parameters

Locus	*A. araucana*							*A. angustifolia* (source species)					
	Size range (bp)	N	N_A	N_e	H_O	H_E	Frequency of null alleles (%)	N	N_A	N_e	H_O	H_E	Author
Ag 23*	240-250	73	6	3.50	0.685	0.727	1.6	101	18	5.42	0.782	0.819	Patreze and Tsai, 2010
Ag 56	150-170	75	3	1.45	0.307	0.282	0	103	9	4.01	0.747	0.754	Patreze and Tsai, 2010
Aang 15*	190-250	66	22	8.73	0.576	0.89	16.7	101	16	8.83	0.891	0.613	Patreze and Tsai, 2010
Aang 18	200-320	52	4	—	0.423	0.448	1.5	12	12	—	0.500	0.930	Schmidt *et al.,* 2007
CRCAc 1*	210-240	74	5	—	0.514	0.546	0	60	4	—	0.025	0.127	Salgueiro *et al.,* 2005 (from Scott *et al.,* 2003)

* Hardy-Weinberg disequilibrium expectations ($P < 0.05$). N: Number of individuals. N_A: numbers of alleles per locus. N_e: effective number of alleles. H_o: observed heterozygosity. H_e: expected heterozygosity.

nomic homology is likely to translate into greater conservation of SSR flanking regions and, as a result, in higher transferability of primer pairs (Rossetto, 2001). Studies within the Araucariaceae family showed a very low genetic variability among and within species (Peakall *et al.,* 2003; Scott *et al.,* 2005). Moreover, after sequencing, similar structure of the microsatellite stretches and a high sequence conservation of the flanking regions was observed among several species of Araucariaceae (Scott *et al.,* 2003). These results suggest the possibility of a successful transferability of SSRs within the family.

In our study, successful cross-species transferability varied between 31.8 to 77.3% (CSA % in Table 2), similar to what is reported in others Araucariaceae (45.5-100%, Scott, 2004). Although the amplification rate decreased with phylogenetic distance, no significant correlations were detected. The highest percentage of transferability occurred among the American species of the genus *Araucaria* (*A. angustifolia* and *A. araucana*), in spite of the closer phylogenetic relationships between *A. angustifolia* and *A. bidwilli* (Liu *et al.,* 2009). In general, the primer transferability from the American to Australian and New Zealand species of the family (or vice versa) was among the lowest. The highest transferability rates between species were observed within each tribe of the Araucaria genus: 77.3-100% and 81.3% within tribe Columbea (Araucaria) and Eutacta, respectively (Salgueiro *et al.*, 2005 and Scott *et al*, 2003). On the other hand, the genus *Agathis* (which originated during the late Jurassic/early Cretaceous) showed a higher degree of transferability (36.4% in our study, 45.5% in Scott 2004) compared to other families with lower divergence time: 22-35.3% in *Quercus* (Isagi and Suhandono, 1997; Steinkellner *et al.,* 1997), 10.2% in *Picea* (Ven and McNicol, 1996), 20% in *Pinus* (Fisher *et al.,* 1998), 38.3% in *Melaleuca* (Rossetto *et al.,* 2000) and 20% in *Eucalyptus* (Brondani *et al.,* 1998).

The presence of the repetitive motifs was verified by sequencing, concluding than these microsatellite loci are orthologous in *A. araucana* (see Annex 1). The homology of sequences between the analyzed species was variable, although the same repetitive motif was identified in each case. Locus Aang 15 presented a high number of alleles in *A. araucana*, although the effective number of alleles was similar to that in the source species *A. angustifolia* (8.73 and 8.83 respectively, Table 3).

Salgueiro *et al.* (2005) reported a complete transferability of six markers developed for *A. angustifolia* testing six individuals of *A. araucana*. However, in our study only two of them could be amplified, with a high rate of amplification failure for the rest of the primers when screened on a large sample size. Similar results were obtained by Scott (2004) in *A. cunninghamii* where some loci required significant optimization of the PCR conditions. The amplification failure could be due to point mutations (SNPs) in the annealing site, which could therefore reduce the transferability rate. On the other hand, technical limitations reducing SSR transferability rate in *A. araucana* cannot be completely ruled out. In fact, although the south American Araucarias share a common ancestor (estimated divergence times by fossil records is 98.9-142 MYA), the process of speciation restricted *A. araucana* to the cold Andes region and *A. angustifolia* to the moist regions of the southern Brazilian highlands (Stefenon, 2007). These differences in the ecological niches are reflected in their types and contents of foliar terpenes (Schmeda-Hirschmann *et al.,* 2005). Csaikl *et al.* (1998) reported that terpenes, polyphenols and polysaccharides are abundant in the foliage of perennials, and are co-extracted with DNA. The higher amplification failure and the lower number of loci with high amplification quality in *A. araucana* could be in part due to the presence of secondary metabolites that inhibit amplification, and the same could occur within other species of this family.

Sequencing these fragments allowed us to confirm unequivocally the presence of the microsatellite repeats in *A. araucana* and to verify their structure. In those species of the family Araucariaceae in which transferability was verified, it would be important to determine if polymorphism exists and if effectively the repeats are conserved, before using them as molecular markers for population genetics studies. In the case of *A. araucana*, we obtained a set of five microsatellite primers showing a high exclusion probability suitable for population genetic analysis that are currently being applied in pollen and seed flow studies.

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Annex

Annex 1. Comparison of sequences between the source species (*A. angustifolia* and *A. cunnighamii)* and *A. araucana* (target species). Repetitive motifs are underlined. Locus name is mentioned before each alignment of sequences

Aang 01

```
                 ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                         10          20          30          40          50
A. angustifolia  CCTGACGGGT  TCACTCCTAC  CT-TACGGTA  ATTGCATTAC  ATATCAGTCA
A. araucaria     -TTGAGGGGG  GCGCTCCCAC  CCGTGCGGGG  AGTGCGTGAC  ATATCAGTGA

                 ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                         60          70          80          90         100
A. angustifolia  ACAATCCTGC  TCAAATATCT  CCTAGAACAC  TGTCTACACA  AACATATCTA
A. araucaria     GCAANNNCAC  TCAAATATCT  CCTAGAGCAC  TGTCTACACA  AACATATCTA

                 ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                        110         120         130         140         150
A. angustifolia  CTCTCTCTCT  CTCTCTCTCT  CTCTCTCTCT  CTCTCTCTCT  CTCT-----A
A. araucaria     CTCTCTCTCT  CTCTCTCTCT  CTCTCTCTCT  CTCTCTCTCT  CTCTCTGCTA

                 ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                        160         170         180         190         200
A. angustifolia  TGTCTATAGT  CTTCAAT-AC  ATTACATAGT  ACAATACCAT  ATACATGATA
A. araucaria     TGTCTATAGT  CT-CAATTAT  AT-ACATAGC  AAAAGANAAT  ATACGAGATG

                 ....|....|  ....
                        210
A. angustifolia  TCTGCCCAAA  TGAA
A. araucaria     TGNGCGNAAG  TGG-
```

Aang 03

```
                 ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                         10          20          30          40          50
A. angustifolia  -CCGCCTACC  TCAATCACTG  GTAAGTTGCT  CACCATTGTG  TACAACAAGG
A. araucana      T-CGCCTACC  TCAATCACTG  GTAAGTTGCT  CACCATTGTG  TACAACAAGG
```

Annex 1 (cont.). Comparison of sequences between the source species (*A. angustifolia* and *A. cunnighamii)* and *A. araucana* (target species). Repetitive motifs are underlined. Locus name is mentioned before each alignment of sequences

	60	70	80	90	100
A. angustifolia	CTTCCTACAC	CTCAAGTTTG	CGCTGGAGGA	AAATGGAGAA	TGTTGGATGT
A. araucana	CTTCCTACAC	CTCAAGTTTG	CGCTGGAGGA	AAATGGAGAA	TGTTGGATGT

	110	120	130	140	150
A. angustifolia	TTTGTGCTCG	TTCTCTCTCT	CTCTCTCTCT	CTCTCTCCAT	ATAAAATGTC
A. araucana	TTTGTGCTC–	–TCTCTCTCT	CTCTCTCTCT	CTCTCTCCAT	ATAAAATGTC

	160	170	180	190	200
A. angustifolia	CGAGGGTTGA	TTCCGAAAGG	GGGTAGGCTT	GCATCCCATA	CTTCAGTATT
A. araucana	CGAGGGTTGA	TTCCGAAAGG	GGGTAGGGTT	GCATCCCATA	CTTCAGTATT

	210	220	230	240	
A. angustifolia	TGGAGTTTAG	GTGGATAAAC	ATGTATTGGA	TAAGCACATT	GTCCCAT
A. araucana	TGGAGTTTAG	GTGGATAAAC	ATGTATTGGA	TAAGCACATT	GTCCAAA

Ag 23

	10	20	30	40	50
A. angustifolia	TTTGCTA–GT	GAG–––––––	–––CTCCACT	CTGTTTNAGT	TCTTGGTGTA
A. araucana	TNNGCNNAGT	GAGAGAGCAA	GAGCTCCACT	TTGTTGTAGC	CCTTGGGGTA

	60	70	80	90	100
A. angustifolia	TTT–TGTATG	AACAAAGACA	CAAACATTGT	–TGTTATTTT	TTAGCATTAC
A. araucana	TTTATGNATG	AACAGAGACA	CAAATATTTA	CTATTATTTT	TTAGCACTAC

	110	120	130	140	150
A. angustifolia	TTTTATATAT	ATA–––––––	GTGTGTGTAG	TGTAGTGTGT	GTGGATTTAT
A. araucana	TTATATATAT	ATATATATAT	––––GTGT–G	TGT–GTGTGT	GTGGCTTTAT

	160	170	180	190	200
A. angustifolia	TATTTCATGT	TGGCATCTTG	GTGTGTAGTN	TACATAATAG	TATGTATTGA
A. araucana	TATTTCATGT	TGGAATCTTG	NTGTGTTNTT	TAGAT––––G	TNTGTGTTGA

	210	
A. angustifolia	TGCCTCATTG	A
A. araucana	TGCCTCATTG	A

Ag 56

	10	20	30	40	50
A. angustifolia	CCACACTCAA	AACAATAGCA	GTTCATTTTA	ACACGTTACA	ACATGCATGC
A. araucaria	CCACACTCAA	AACAATAGCA	GTTCATTTTA	ACACATTACA	ACATGCATGC

	60	70	80	90	100
A. angustifolia	ATGATTATAC	TCTTAACCCT	ATTCTATATC	AAAATTTGTA	TTCTCTCTCT
A. araucaria	ATGATTATAC	TCTTAACCCT	ATGCCATAGC	AAATTTTGTA	TTCTCTCTCT

	110	120	130	140	150
A. angustifolia	CTCTCTCTCT	CTC–––––AT	CAAATTACCT	TCTTGTATCTG	ATTGGCCAAC
A. araucaria	CTCTCTCTCT	CTCTCTCATC	AAATTACCTT	CTTGTATCTG	ATTGGCCAAC

Annex 1 (cont.). Comparison of sequences between the source species (*A. angustifolia* and *A. cunnighamii)* and *A. araucana* (target species). Repetitive motifs are underlined. Locus name is mentioned before each alignment of sequences

```
                  ....
A. angustifolia   TTCA
A. araucaria      TTCA
```

CRCAc 1

```
                  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                          10          20          30          40          50
A. cunninghamii   ----------  ---------G  AGAGAGAGAG  AGAGAGAGAG  AGAGAGAGAG
A. araucana       TANAACATGG  ANAACATAT-  ----------  -GAGAGAGAG  AGAGAGAGAG

                  ....|....|  ....|....|  ....|....|
                          60          70          80
A. cunninghamii   AGAGAGATAG  TGCTTGGAAA  GAAT------
A. araucana       AGAGAG-TAG  TGCTTGGAAA  -AATGAATAA
```

Aang 15

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                  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                          10          20          30          40          50
A. angustifolia   TCAACATCTG  ATCA--CATT  CTCAAGAGTT  GG--ATCA-A  GAAAT---GA
A. araucana       -----GTGTG  ATTGGTCATT  TTTATTA-TT  GGCAATGAGA  GAAAACAGGA

                  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                          60          70          80          90         100
A. angustifolia   GTAAACCCAC  AGAGAGAGAG  AGAGAGAGAG  AGAGAGAGAG  AGAGAGAGAG
A. araucana       GNAAA---AN  AGAGAGAGAG  AGAGAGAGAG  AGAGAGAGAG  AGAGAGAGAG

                  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                         110         120         130         140         150
A. angustifolia   ----------  ----------  ----------  ----------  --------GC
A. araucana       AGAGAGAGAG  AGAGAGAGAG  AGAGAGAGAG  AGAGAGAGAG  AGGAGAGAGA

                  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                         160         170         180         190         200
A. angustifolia   ACAGTAGAGC  CCTAAGCTCT  GGCATTCAAT  AAAGACAGGA  GGAGAGGTAC
A. araucana       AAAGT-GAGN  GNGTGTGTGT  G-TGGGNNNT  NNNNNNNNNG  GGGGGAGAGA

                  ....|....|  ....|....|  ....|....|
                         210         220         230
A. angustifolia   AGTAATTAGG  ATATAATATT  TATTTA-TGT
A. araucana       -GAGAGG-GCG CGCGCNCA-C  ACACA----
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Aang 18

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                  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                          10          20          30          40          50
A. angustifolia   TTATTTGCAC  A-CATA-CAG  ATGTATGTTT  GTGCATCTCT  TTGTGTGGGC
A. araucana       TTTCTGGCGC  CGCGTTTCAG  CTTCATCT--  GCGCTTCT-T  NNNNNNNNGC

                  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                          60          70          80          90         100
A. angustifolia   AGGCATGCAT  GTTGAACACA  GGTATGTTAC  ATCTCTCTCT  CTCTCTCTCT
A. araucana       ---CCTTC--  --TGAACTCA  ----TCTTTC  --CTCTCTCT  CTCTCTGGCC

                  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                         110         120         130         140         150
A. angustifolia   CTCTCTCTCA  CACACACACA  CACGCGCGCG  CACACGTG-   ----T-AAAT
A. araucana       CA-TGTCTGA  -AGAAGCGCA  GATGAA-GCT  GA-ACGTGGC  GCCATGAAAG

                  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                         160         170         180         190         200
A. angustifolia   GCGTAC--AG  ATACAGATGC  ATGTT--CGT  GCAC-ATGTG  AGTGTGCATG
A. araucana       GCGGGCTCAG  CAGCAGG-GA  AGGTGGCCGC  GATCCATCCG  A-TGAGGAAG

                  ....|....|  ....|....|  ..
                         210         220
A. angustifolia   TCTTTGTGTG  GGCAGGCATG  CA
A. araucana       TTGCTGAAAA  AGGTGGCATA  -A
```