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Abstract	Fragmentation of the habitat due to glaciations, fires and human activities affected the distribution range of *Araucaria araucana* in southern South America. On the borders of the Argentinean Patagonian steppe, the species is restricted to isolated patches without natural regeneration. Our objective is to test the hypothesis that these populations are relicts of pre-Pleistocene origin. A total of 224 individuals from 16 populations were sampled. Twenty chloroplast microsatellites, 19 non-coding chloroplast DNA regions and eight mitochondrial DNA fragments were screened for polymorphisms. A low transferability rate of universal primers from Pinaceae and also a low variation were detected for this ancient species. Only one non-coding region of the chloroplast DNA showed polymorphism allowing the identification of five haplotypes. A low genetic differentiation (G_{ST} = 0.11; G'_{ST} = 0.267) and lack of geographic structure was found. Allelic richness was lower and genetic differentiation higher among the eastern isolated populations, suggesting a long lasting persistence. Conservation guidelines are given for these relictual populations, which are located outside the limits of the National Parks.

Keywords (separated by '-')	Geographical genetic structure - Chloroplast DNA - Mitochondrial DNA - Patagonian temperate forests - Monkey puzzle tree - Fragmentation
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RESEARCH ARTICLE

Biogeographic history of the threatened species *Araucaria araucana* (Molina) K. Koch and implications for conservation: a case study with organelle DNA markers

P. Marchelli · C. Baier · C. Mengel · B. Ziegenhagen · L. A. Gallo

Received: 16 July 2008 / Accepted: 28 April 2009

Abstract Fragmentation of the habitat due to glaciations, fires and human activities affected the distribution range of *Araucaria araucana* in southern South America. On the borders of the Argentinean Patagonian steppe, the species is restricted to isolated patches without natural regeneration. Our objective is to test the hypothesis that these populations are relicts of pre-Pleistocene origin. A total of 224 individuals from 16 populations were sampled. Twenty chloroplast microsatellites, 19 non-coding chloroplast DNA regions and eight mitochondrial DNA fragments were screened for polymorphisms. A low transferability rate of universal primers from Pinaceae and also a low variation were detected for this ancient species. Only one non-coding region of the chloroplast DNA showed polymorphism allowing the identification of five haplotypes. A low genetic differentiation ($G_{ST} = 0.11$; $G'_{ST} = 0.267$) and lack of geographic structure was found. Allelic richness was lower and genetic differentiation higher among the eastern isolated populations, suggesting a long lasting persistence. Conservation guidelines are given for these relictual populations, which are located outside the limits of the National Parks.

Keywords Geographical genetic structure · Chloroplast DNA · Mitochondrial DNA · Patagonian temperate forests · Monkey puzzle tree · Fragmentation

Introduction

Habitat fragmentation could be considered as one of the main causes of population and species loss and has become a key issue in conservation biology (Eriksson and Ehrlen 2001). The species autecology is usually altered and several genetic processes are affected when the populations are drastically reduced and the landscape is fragmented (Hartl and Clark 1988; Hanski and Simberloff 1997). Isolation among fragmented populations might generate genetic differentiation, inbreeding and increased levels of genetic drift (Templeton et al. 2001). Such effects may differ depending on the degree of fragmentation and the biology of the species (Young and Boyle 2000). Gene flow can be restricted if distances among extant populations are large, but it can also be favoured in some circumstances due to the opening of the landscape (e.g. Robledo-Arnuncio et al. 2004).

The most severe fragmentation in South American temperate forests occurred during the Quaternary when glaciers occupied most of the current distribution range and forests were restricted to small refugia. In addition to the drastic overall reduction in species' geographic range, the

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remaining patches probably experienced a severe bottleneck. However, the type of glaciation in the southern Hemisphere which was mostly restricted to valleys, especially north of 41°S (Flint and Fidalgo 1964, 1969; Rabassa and Clapperton 1990; Markgraf et al. 1996) led to the suggestion of the persistence of forests scattered in several small refugia. Unfortunately, no continuous palynological records exist linking the Late Tertiary with the Quaternary. Although some records extend back to 30,000 or 40,000 BP, most continuous fossil pollen data begin at 14,000 BP when the last full-glacial period had come to an end (Markgraf et al. 1996). The lack of detailed pollen maps and precise locations of possible refugia is a constraint to make comparisons with or to complement genetic information. However, at the same time, it increases the relevance of using genetic markers as a powerful tool to shed light on the Quaternary history. Several studies have suggested the existence of multiple refugia for species of the region, both based on highly conserved DNA markers such as maternally inherited chloroplast DNA (Marchelli et al. 1998; Marchelli and Gallo 2006; Azpilicueta et al. 2009; Pastorino et al. 2009), and also with nuclear markers (e.g. Premoli et al. 2000; Bekessy et al. 2002; Pastorino and Gallo 2002; Marchelli and Gallo 2004). These refugia might have been located at the Coastal Mountains, in Chile, and also at both sides of the Andes Mountains. Recolonization began about 14,000 years BP (Heusser et al. 1996; Moreno 1997), but the current vegetation structure was established only about 3,000 years ago (Villagran 1991; Heusser et al. 1999; Bennett et al. 2000).

Since aborigine settlement, some 11,000 years ago (Montané 1968), forests begin to be altered by human activities. However, the impact was more significant during the Twentieth century when the frequency and intensity of intentional fires increased in order to establish agricultural and livestock activities. A dramatic reduction of 40% in forest surface occurred in the first half of the past century (Lara et al. 1999). The situation is even worse at the eastern border of the forest distribution area, in Argentina, where extreme environmental conditions due to drought stress in association with high human impact restrict natural regeneration of forests.

Araucaria araucana (Molina) K. Koch (Pehuen, also known as Monkey puzzle tree) is a conifer endemic to the northern region of the temperate forests of Argentina and Chile, with a current distribution between 37°20′S and 40°20′S. Towards the eastern extreme, in the ecotone between the forests and the Argentinean steppe, the distribution pattern can be described as discontinuous, and is mainly determined by the topography and the climate of the region. In this area, fragmented populations are the consequence of overexploitation, replacement by exotic conifers, large forest fires of anthropogenic origin and introduced livestock that impedes the natural regeneration and leads to a physical erosion of the soil (Gallo et al. 2004). In addition, *A. araucana* forests are currently used by the Mapuche communities who live within the forest since pre-historic times. The present situation of extreme poverty led to an exceeded increment of livestock which is fed with the edible seeds of *Araucaria* and which also provokes soil erosion that excludes natural regeneration (Sanguinetti et al. 2002; Bekessy et al. 2002). Besides, seeds are collected for human consumption and sale. *A. araucana* is currently on risk of extinction (Farjon and Page 1999). The threat is increased due to its restricted present distribution, its slow growth and its limited dispersal ability. For all these reasons, it was included in the Appendix I of CITES (http://www.cites.org/eng/app/appendices.shtml) and listed in the 2008 IUCN Red List of Threatened Species (http://www.iucnredlist.org) as a vulnerable species. Still, significant genetic variation within and among populations was detected in this species when analysed with nuclear genetic markers (Bekessy et al. 2002), the variation being higher within the eastern more fragmented populations (Gallo et al. 2004).

The high genetic diversity encountered at the longitudinal margin of the species distribution range highlights the importance of these populations for conservation. Moreover since they are the most seriously affected by fragmentation and human activities. Therefore our main concern in this study is to focus on the eastern distribution of the species in Argentina. Assuming the described type of glaciation in the current distribution range of *A. araucana* we would like to test the hypothesis that the species persisted in the area in scattered and fragmented populations located towards the east of the glacial margins, in this case possibly representing 'rear edge' populations. Rear edge populations are stable relicts usually isolated and much older than any populations from the rest of the range (Hampe and Petit 2005). Moreover, eastern populations of *A. araucana* are characterized by a higher proportion of clonal growth, and a lower impact of fires than western (humid) continuous forest. Considering the long life span with specimens that could reach more than 500 years, the relic populations could be composed of ancient genotypes. Consequently, we might expect a higher genetic differentiation among marginal populations because they could have kept their ancient genetic differences longer. Therefore eastern isolated populations would have both more diversity and differentiation at organelle DNA loci than western larger and continuous populations assumed to be the result of a recolonisation process. We have chosen organelle DNA markers since they proved to perform best in studies on historic biogeography or phylogeography, respectively (Petit and Vendramin 2006). Besides, chloroplast and mitochondrial DNA are supposed to be paternally

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inherited in Araucariaceae according to cytological evidences (Kaur and Bhatnagar 1984). We will try to demonstrate this mode of inheritance by means of the same organelle molecular markers used in the population genetic study. Since *A. araucana* is wind-pollinated and paternal inheritance of the organelles is assumed we want to test a second hypothesis of connectivity between small refugia due to extensive pollen movement.

Materials and methods

Sampled populations

Sixteen populations distributed over the whole eastern geographic range of *Araucaria araucana* were sampled (Fig. 1; Table 1). Between ten and thirty individuals per population were collected. In order to avoid the sampling of related trees a minimum distance of 50 m between individuals was always maintained. Leaves were kept at −80°C until DNA extraction.

DNA extraction

DNA was extracted from leaves following the protocol of Dumolin et al. (1995) or using the Qiagen DNA Extraction kit. DNA concentration was estimated either on agarose gels or with a photometer, and working dilutions of 5 ng/µl prepared.

Amplification of chloroplast DNA microsatellites (cpSSRs)

Twenty chloroplast microsatellites (cpSSRs) designed for members of the Pinaceae by Vendramin et al. (1996) were checked for PCR products on two individuals per population. PCR amplifications were performed in a total volume of 25 µl containing dNTPs (each 0.2 mM), 2.5 mM $MgCl_2$, 0.2 µM of each primer, 1× reaction buffer, 25–100 ng template DNA, and 1 U of *Taq* polymerase (Invitrogen or Promega). PCR amplifications were performed using a Biometra thermal cycler with the following profile: 5 min denaturing at 94°C, followed by 30 cycles of 1 min

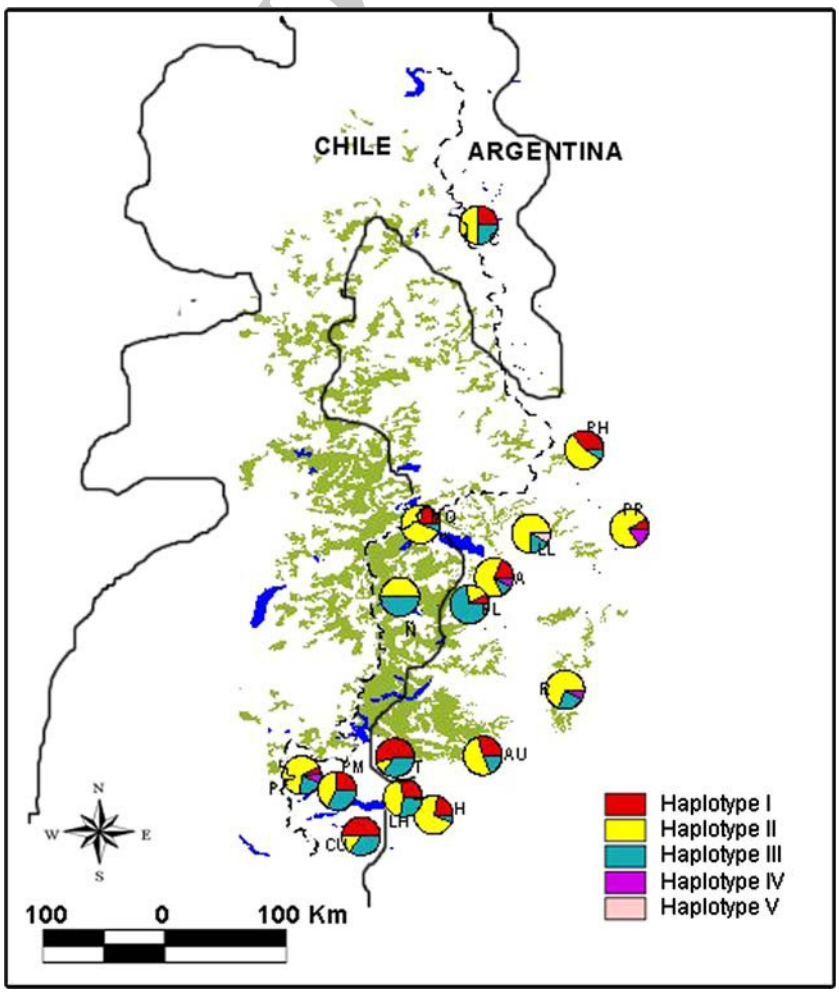

Fig. 1 Distribution range of *Araucaria araucana* and locations of the analysed populations. The five haplotypes are shown in different colours and their frequencies in each population are represented by the pie charts. The *dotted line* is the international border between Chile and Argentina. The *filled line* shows the limit of the ice cap during the Last Glacial Maximum according to Holling and Schilling, 1981

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Table 1 Geographic location, type of forest and allelic richness for the sixteen analysed populations of *A. araucana*

Population	Latitude	Longitude	Altitude	Type of forest	N	N_H	r (10)	Cr_T	Cr_S	Cr_D	G_{ST}	G'_{ST}
Ñorquinco (Ñ)	39°5′ 44.3″	71°19′ 24.9″	1083	Continuous	10	2	1.000	−1.8	−2.8	1.1		
Paimún (P)	39°40′ 30″	71°38′ 30″	1050	Continuous	29	4	2.101	−0.1	0.5	−0.6		
Pulmarí (PL)	39°7′ 10.8″	71°05′ 51.9″	1099	Continuous	11	3	1.909	−0.8	−0.1	−0.7		
Moquehue (MQ)	38°51′ 33.5″	71°15′ 27.4″	1302	Continuous	18	3	1.533	−2.7	−1.2	−1.4		
				Mean for the group			1.636				0.195	0.459
Caviahue (C)	37°53′ 14.9″	71°04′ 08.2″	1721	Fragmented	12	3	2.000	−1.7	0.2	−1.9		
Pino Hachado (PH)	38°37′ 3.1″	70°43′ 31.8″	1451	Fragmented	11	3	1.909	−1.6	−0.1	−1.5		
Tromen (T)	39°37′ 02″	71°20′ 23″	984	Fragmented	11	3	1.909	0.0	−0.1	0.1		
Aucapan (AU)	39°36′ 40″	71°3′ 16.5″	1300	Fragmented	17	3	1.945	−2.2	0.0	−2.2		
Lonco Luan (LL)	38°53′ 22.1″	70°53′ 55.2″	1567	Fragmented	12	3	1.818	−0.9	−0.4	−0.5		
Río Aluminé (A)	39°01′ 56″	71°01′ 05″	1090	Fragmented	11	4	2.818	1.4	2.7	−1.3		
Los Helechos (LH)	39°44′ 52″	71°18′ 47″	981	Fragmented	13	4	2.955	2.2	3.1	−0.9		
Piedra Mala (PM)	39°43′ 28″	71°31′ 38″	985	Fragmented	12	3	2.000	−1.5	0.2	−1.7		
				Mean for the group			2.169				0.038	0.114
Primeros Pinos (PP)	38°52′ 21.6″	70°34′ 45″	1453	Isolated	12	3	1.818	2.5	−0.4	2.8		
Huechulafquen (H)	39°48′ 12.5″	71°12′ 51.3″	846	Isolated	18	3	1.533	−2.7	−1.2	−1.4		
Rahue (R)	39°23′ 49.6″	70°47′ 17.6″	1450	Isolated	13	3	1.766	−0.9	−0.5	−0.4		
Currhue (CU)	39°52′ 20.2″	71°26′ 55.5″	970	Isolated	14	3	1.934	−0.5	−0.0	−0.5		
				Mean for the group			1.763				0.202	0.482

N number of individuals analysed, N_H number of haplotypes detected, r allelic richness, Cr_T contribution to total allelic richness, Cr_S contribution due to diversity, Cr_D contribution due to differentiation

denaturing at 94°C, 1 min annealing at 55°C and 1 min extension at 72°C, with a final extension step at 72°C for 8 min and a final soak at 4°C. The same program was tried with a lower annealing temperature (52°C) for all the primers that did not amplify with 55°C. PCRs for primers pairs Pt110048 and Pt26081 were optimised using a gradient between 50°C and 64°C.

PCR products were checked for positive amplification on 1% agarose gels run in 0.5× TBE buffer at 60 V for 30 min and at 90 V for 1 h, and visualised under UV light after staining with ethidium bromide.

For those primers where amplification was positive, ten individuals per population in 13 populations were screened for polymorphism in a 6% standard denaturing polyacrylamide gel. PCR products were mixed with 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and 10 μM NaOH and denatured at 94°C for 6 min. Gels were run at 2,500 V and 90 Watt for 1 h and 15 min at 54°C and silver stained following the protocol by Bassam et al. (1991).

Amplification of intergenic spacer regions and introns within the chloroplast and the mitochondrial DNA

Nineteen primer pairs for analyzing chloroplast intergenic spacer regions and introns were checked: *trn*F-*trn*Vr, *trn*V-*rbc*Lr (Dumolin-Lapegue et al. 1997); *trn*T-*trn*F (Taberlet et al. 1991); *trn*Q-*trn*G, *rpo*C1-*trn*Cr, *rpl*20-*trn*W, *trn*V-*trn*H, *psb*D-16S, *trn*L-*trn*V (Parducci and Szmidt 1999); *trn*S-*psb*C, *trn*K1-*trn*K2, *trn*H-*trn*K, *trn*D-*trn*T, *psa*A-*trn*S, *trn*S-*trn*fM, (Demesure et al. 1995); *trn*Q-*trn*S, *trn*S-*trn*R (Dumolin-Lapegue et al. 1997; Grivet et al. 2001). For the region *trn*C-*trn*D three different primer pairs were tested: those described by Demesure et al. (1995) and Parducci and Szmidt (1999) and a primer pair designed exclusively for *Araucaria araucana* based on the sequence obtained after amplification with primers *trnC-trnD* from Demesure et al. (1995). The sequences of these primers were: 5′-AGACAATTTGTGCTGCTCCA-3′ (F) and 5′-TTCTTCCTCGATTTCCGGAT-3′(R). Therefore a total of 21 primer pairs were checked. Due to problems with amplifications several PCR conditions were tried with most of the primers. The general PCR mix was the same described above for the cpSSRs. In addition, different $MgCl_2$ concentrations were tested from 1.5 to 3.2 mM, as well as several DNA concentrations from 15 to 60 ng of template DNA, and 1 or 1.5 U of *Taq* polymerase (Invitrogen). Besides, addition of 0.1 μg/μl BSA, polyvinylpyrrolidone (PVP) (in concentrations between 0.38 mM and 1.53 mM) and/or polyethylene glycol (PEG) (9.2 mM and 18.4 mM) were added in order to improve PCR conditions.

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Table 2 Chloroplast and mitochondrial DNA primer pairs with positive amplification in *Araucaria araucana*

Genome	Primer pair	T° annealing	$MgCl_2$ (mM)	Fragment size (bp)	Restriction	N
Chloroplast (cpSSRs)	Pt26081	57	2.5	108	NA	130
	Pt36480	55	2.5	ND	NA	130
	Pt63718	52	2.5	100	NA	130
	Pt71936	55	2.5	134	NA	130
	Pt87268	55	2.5	137	NA	130
	Pt110048	56	2.5	ND	NA	130
Intergenic regions	CD	57	2.0	~2600	*Taq*I	224
					*Hinf*I	11
					*Hae*III	15
	DT	48	2.0	~1200	*Alu*I	13
					*Hae*III	11
	K1K2	51	1.6	~2600	*Alu*I	8
					*Hae*III	9
	QS	54	2.0	~2000	*Taq*I	11
					*Hae*III	11
	SR	54	2.0	~2000	*Taq*I	11
	SC	55	1.5	~1500	*Hae*III	18
					*Hinf*I	18
					*Taq*I	18
Mitochondrial	nad1-2	55	1.6	~220	–	13
	nad5-4	54	2.0	~800	*Taq*I	19
					*Hae*III	66
					*Alu*I	8
					*Hinf*I	73
	Cox3	57	1.8	~400	*Hae*III	10
					*Alu*I	10

Reaction conditions and restriction analysis. Restrictions were done at 65°C for 3 h for *Taq*I and at 37°C overnight for the other enzymes
N number of individuals tested belonging to at lest 8 populations, *NA* not applicable

PCR was carried out in a Biometra thermal cycler with the following profile: 4 min denaturing at 94°C, followed by 30, 35 or 40 cycles for 1 min denaturing at 94°C, 1 min annealing temperature (Table 2) and 2 min 50 s extension at 72°C, with a final extension step of 72°C for 10 min and a final soak of 4°C. The optimal PCR conditions for the fragments with positive amplification are presented in Table 2. PCR products were checked in agarose gels as described in the preceding section.

Eight universal primer pairs for amplifying mitochondrial DNA were screened in *A. araucana*: *nad*1 exon2, *nad*4 exon1 (Demesure et al. 1995); *nad*4 exon3, *nad*5 exon1, *nad*5 exon4 (Dumolin-Lapegue et al. 1997); *nad*3 exon2, *nad*3 *rps*12 (Soranzo et al. 1999), *cox*3 (Duminil et al. 2002). PCR amplifications were performed in a total volume of 25 µl containing dNTPs (each 0.2 mM), 1.8 mM $MgCl_2$, 0.2 µM of each primer, 1× reaction buffer, 30 ng template DNA, and 1 U of *Taq* polymerase (Invitrogen or Promega). PCR amplifications were performed using a Biometra thermal cycler with the following profile: 4 min denaturing at 94°C, followed by 30 cycles of 1 min denaturing at 92°C, 1 min annealing temperature (Table 2) and 2 min extension at 72°C, with a final extension step of 72°C for 10 min and a final soak of 4°C. PCR products were checked in agarose gels as described in the preceding section.

Restriction fragment length polymorphisms

A PCR-RFLP analysis was performed with those cpDNA and mtDNA fragments that gave a positive amplification. Digestion of 7 µl of the PCR product was done in a total volume of 22 µl by including 5 U of restriction endonuclease with the respective manufacturers' buffer. Between one and four enzymes were used for each amplified primer. Temperature and reaction conditions for each enzyme are given in Table 2. Digested fragments were separated in 8% non-denaturing polyacrylamide gels run at 300 V for 3–6 h and visualised under UV light after staining with ethidium bromide. Gel documentation was obtained with a digital camera the image analysed with BioDoc Analyse version 2.0 (Biometra).

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Mode of transmission of the cpDNA

Due to the lack of controlled crosses, mothers and seeds were collected within one of the most diverse populations in terms of the detected cpDNA variation. This should assist to indirectly determine the mode of inheritance of the chloroplast DNA. DNA was extracted from each of five mothers and five embryos per mother. DNA from embryos was obtained with the protocol of Stefenon et al. (2004). PCR-RFLP of the polymorphic fragment was done as described above. Since we only found variation in the cpDNA but did not find any variation in the mitochondrial loci under study, this kind of indirect inheritance analysis was tried only for the plastid DNA.

Data analysis

Polymorphic fragments were labelled by decreasing order of fragment size as visualised in the polyacrylamide gels and as described by Demesure et al. (1996). Haplotypes were defined according to different combinations of length variants. Allelic richness (r_g) was calculated according to El Mousadik and Petit (1996) setting a rarefaction number of 10 (the smallest sample size) in order to compare the diversity among populations without the bias that is introduced by uneven sample sizes. The contribution of each population to total allelic richness (Cr_T), the contribution due to diversity (Cr_S) and that due to differentiation (Cr_D) were estimated according to Petit et al. (1998). Calculations were made using the program CONTRIB (Petit et al. 1998). The average within-population gene diversity (h_S), the total gene diversity (h_T) and the gene differentiation over all population (G_{ST}) were estimated according to Pons and Petit (1995) using the program HAPLODIV (the software is available at http://www.pierroton.inra.fr/genetics/labo/Software/). Additionally, Hedrick's standardized genetic differentiation (G'_{ST}) was calculated. This parameter standardizes the observed value of G_{ST} by the maximum level that it can obtain for the observed amount of genetic variation, and therefore corrects for different h_s values (Hedrick 2005).

For analyzing the spatial genetic structure we decided to use two approaches, namely a stratified one where we *a priori* grouped the populations according to different forest types (1) and an approach without any a priori assumption (2).

(i) In view of the different forest types, populations were divided into three groups, and genetic parameters were calculated within each of them. *Continuous forests* were those located to the western more humid region and composed by large and dense populations. *Isolated forests* form the eastern edge of *Araucaria araucana* in Argentina and populations are small and probably relicts from much older than pre-Holocene times. *Fragmented forests* are located at intermediate longitudinal positions between the former two and could be considered as the result of a recent fragmentation, mainly by fires and volcanism. The genetic parameters were calculated for each group and we used nested AMOVA (Excoffier et al. 1992) to estimate the significance of the genetic differentiation among regions (ϕ_{RT}), among populations within regions (ϕ_{PR}) and within populations (ϕ_{PT}).

(ii) Two numerical analyses were used to unravel the geographical population structure. First, we conducted a Mantel test (Mantel 1967), to look for the existence of a correlation between geographic and genetic distances using GenAlEx (Peakall and Smouse 2006). The geographic distance matrix was constructed from the latitudes and longitudes given in Table 1. The genetic distance matrix was derived from the coefficients of gene differentiation between all pairs of populations (G_{ST}) using the program DISTON (Petit 2000, http://www.pierroton.inra.fr/genetics/labo/Software/). Second, we performed a spatial analysis of molecular variance (SAMOVA) to define groups of populations that are maximally differentiated from each other (Dupanloup et al. 2002). An initial arbitrary partition in K groups was made, setting K-values between 2 and 10. The F_{CT} index associated with genetic differentiation among the K groups was computed after repeating the iterative simulated annealing process 10,000 times using the software SAMOVA 1.0 (Dupanloup et al. 2002).

Results

Chloroplast SSRs

Six out of 20 primers for chloroplast SSR loci amplified in *Araucaria araucana* (Table 2), however, no polymorphism was detected among the analysed populations. Evidence of repetitive units was given confirming the presence of a microsatellite motif since the typical slippage patterns were observed in the polyacrylamide gels, which are indicative for PCR of small repetitive units.

Intergenic spacer regions and introns within the chloroplast and the mitochondrial DNA

Among the 21 chloroplast primers checked in *A. araucana*, six gave reliable amplification products, the latter having been subsequently analysed by PCR-RFLP (Table 2).

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Table 3 Definition of the five haplotypes found in *A. araucana*

Haplotype	CD/TaqI 1	CD/TaqI 2	CD/TaqI 3
I	1	1	1
II	2	2	1
III	1	2	1
IV	2	1	2
V	2	1	1

Polymorphism was detected only within the chloroplast fragment amplified by primers *trnC-trnD*, obtaining the best amplifications with the primer pairs described by Parducci and Szmidt (1999). Three polymorphic regions within the amplified fragment allowed the identification of five haplotypes (Table 3). The other five chloroplast DNA regions showed no variation after digestion with one to four endonucleases (Table 2).

Three mitochondrial introns could be amplified in *A. araucana*. The amplification with primers located at the second and third exons of the mitochondrial gene *nad1* (Demesure et al. 1995) gave a very short fragment (220 bp). After sequencing (AY286496) the lack of the second intron in the *nad1* gene was verified, as was also detected for other members of the Araucariaceae (Gugerli et al. 2001). This short fragment was monomorphic among the analysed individuals. The amplification of intron 4 of the *nad5* gene gave a product of about 900 bp which showed inconsistent or no variation after digestion with four different restriction endonucleases (*Taq*I, *Hae*III, *Alu*I and *Hinf*I). Finally, no restriction sites were detected within the product obtained with primer *cox3* (ca. 400 bp) both with *Hae*III and *Alu*I.

Inheritance of the cpDNA

The comparison of mothers and their offspring from open-pollination revealed the presence of offspring haplotypes different from that of the mother (Table 4). Thus, the indirect method employed allowed us to strengthen the cytological evidence of paternal inheritance of the chloroplast genome in *A. araucana*.

Table 4 Analysis of mothers and offspring to infer the mode of inheritance of the chloroplast DNA

Mother	Mother haplotype	Offspring haplotype		
		Haplotype I	Haplotype II	Haplotype III
T2	III	0	1	4
T31	II	0	5	0
T17	II	0	4	1
T10	II	0	1	1
T13	II	0	2	0

Genetic diversity and geographic distribution

The length variants detected within *trn*C-*trn*D allowed the identification of five haplotypes with a relatively high level of diversity among the analysed populations ($h_s = 0.572$ (s.e. = 0.027) and $h_t = 0.642$ (s.e. = 0.030)). On the contrary, low levels of genetic differentiation were observed ($G_{ST} = 0.110$, s.e. = 0.041). The standardized genetic differentiation was larger, but still low for an organelle DNA ($G'_{ST} = 0.267$). Allelic richness varied between 1.000 and 2.955, being the most diverse two *fragmented* populations (LH and A) (Table 1). Moreover, the mean allelic richness (r_{10}) was highest for the group of *fragmented* populations, although not significantly different from the other two groups (Table 1). The lowest values for r_{10} were detected in two *continuous* and one *isolated* populations (N, MQ and H, respectively). On the contrary, genetic differentiation was lower for the intermediate group of *fragmented* populations as compared to the *continuous* and the *isolated* groups (Table 1). The analysis of molecular variance (AMOVA) showed nosignificant differences among regions ($\phi\phi_{RT} = 0.002$, $P = 0.311$), but considerable structure among populations within regions ($\phi_{PR} = 0.101$, $P = 0.001$) and within populations ($\phi_{PT} = 0.103$, $P = 0.001$).

The partition of the contribution to total allelic richness in the components of diversity and differentiation showed that three populations (two *fragmented* and one *isolated*) contributed most: Río Aluminé (A), Los Helechos (LH) and Primeros Pinos (PP). The contribution was attributable to diversity in the former two populations and to differentiation in the latter (Table 1).

The geographic distribution of haplotypes was not structured and the Mantel test did not reveal a correlation between geographic and genetic distances ($P > 0.05$).

For the spatial analysis of molecular variance (SAMOVA) we repeated the analysis increasing the number of groups (K) from 2 up to 10. F_{CT} should increase with K because of the reduction of the proportion of variance due to differences between populations within each group (F_{SC}, Dupanloup et al. 2002). However, *Araucaria araucana* populations showed the reverse tendency and the highest index ($F_{CT} = 0.2846$, $P < 0.01$) was obtained for $K = 2$. With higher K-values some groups were made of only one population, which indicates that the geographical structure disappeared (Heuertz et al. 2004). The results showed that one group of two populations is retained, and formed by populations "Tromen" (T) and "Curruhue" (CU), being all the other populations in the second group.

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Discussion

Transferability of organellar "universal" primers: dealing with an ancient genome

The highly conserved structure and linear arrangements of cpDNA from very distant plant taxa (Palmer and Stein 1986) allowed for the design of universal and consensus primers for the amplification of intergenic regions (e.g. Taberlet et al. 1991; Demesure et al. 1995; Dumolin-Lapegue et al. 1997; Grivet et al. 2001; Heinze 2007). A high transferability rate was usually reported for these primers which were used in several tree species from different families (see review and references in Petit et al. 2005). To a lesser extent, chloroplast SSRs were also described as being universal, at least at higher taxonomic levels. Firstly described by Powell et al. (1995) in pines, universal primers were designed for *Pinus thunbergii* (Vendramin et al. 1996) which exhibited a considerable rate of transferability to other Pinus species (Morgante et al. 1998; Vendramin et al. 1998; Ribeiro et al. 2001; Echt et al. 1998) six *Abies* species (Vendramin and Ziegenhagen 1997; Vendramin et al. 1999; Ziegenhagen et al. 1997) and also *Picea abies* (Vendramin et al. 2000).

Notwithstanding, our results in *Araucaria araucana* revealed a low transferability of universal primers for amplifying both organelle intergenic spacer regions and chloroplast SSRs. It is possible that DNA quality played an important role, and the possible presence of inhibitors cannot be discarded. Although we tried different DNA extraction protocols (data not shown) and addition of substances known to improve PCR results like PVP (Koonjul et al. 1999) and PEG (De Castillo et al. 1995) the amplification was not successful with most of the primers. However, the most likely explanation for the low transferability of universal primes is the occurrence of sequence divergence between the younger taxa from which the primers were designed and the evolutionary old *Araucaria araucana.*

A possible explanation regarding cpSSRs is the phylogenetic and respectively evolutionary distance between Araucariaceae and Pinaceae, since successful transfer is expected to be more efficient the closer the relationship between the source and the target species is (Peakall et al. 2003). In *Araucaria araucana* only six out of 20 loci did amplify, but none of them showed any variants, at least in length. Besides, the amplified cpSSR loci were comparably smaller in size in *A. araucana* than the homologous sites in the members of the Pinaceae (e.g. Pt71936 exhibits 148 bp vs. 134 bp in *Pinus thunbergii* and *A. araucana* respectively or even more pronounced Pt87268 exhibits 165 bp vs. 137 bp in *Pinus thunbergii* and *A. araucana*, respectively). We cannot exclude sampling errors for low transferability and lack of variation, since only six loci could effectively be analyzed and polymorphism might be detected in other loci. Besides, we screened a relatively small number of individuals (10 per population, for 13 populations) and this could have prevented the detection of genetic variation. Another alternative to explain the lack of variation is the possibility that others than microsatellite sequences were amplified by the primers in *A. araucana*. However, even though of bad quality, sequences suggested the presence of a repetitive motif (data not shown) and the characteristic slippage of microsatellites in polyacrylamide gels was also observed. Therefore, we can assume that we are dealing with cpDNA SSRs that displayed no variation, at least in length. Different reasons could be proposed for the shorter and monomorphic microsatellites: (1) *A. araucana* experienced a deletion in the neighboring sequences of the microsatellite motif, (2) and/or *A. araucana* posses short stretches of SSRs, (3) and/or problems of size homoplasy (Liepelt et al. 2001). When attempting direct sequencing of the loci for counting the explicit number of repeats we experienced common problems with sequencing microsatellite loci (Liepelt et al. 2001), and could not provide evidence to discern between the possible reasons. A prediction says that only with a certain size of the microsatellite stretch variation through slippage may occur (Messier et al. 1996; Rose and Falush 1998), although contradictory evidence was presented in yeast (Pupko and Graur 1999). Another alternative might be that *A. araucana* could have accumulated repeats along its long evolutionary history (Amos et al. 1996), but then reached a threshold and contraction mutations increased exponentially (Xu et al. 2000). No definite conclusions can be made with the current information. Highly effective new sequencing technology is expected to allow thorough insights in sequence variation of organelle DNA comparing large sets of species and/or populations.

Among the intergenic chloroplast regions, 32% of the tested primers gave an amplification product, but only one fragment showed polymorphism. A general sequence divergence could be the main cause of the amplification failure, in spite of the conservative structure and linear arrangement of the chloroplast DNA (Palmer and Stein 1986).

Concerning the mitochondrial DNA, 37.5% of the tested primers amplified. It is assumed that the degree of conservation of this genome among land plants is relatively high (Dumolin-Lapegue et al. 1997), in spite of the variation in size and gene arrangement (Palmer 1992). The loss of the second intron of the *nad*1 gene was verified as in other conifers (Gugerli et al. 2001), hence suggesting a generally similar structure. However, low levels of primer transferability were observed and inconsistent variation was detected only at the *nad*5 gene, and therefore not included.

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To sum up, alternatively to possible technical problems, low levels of genetic variation and differences in the chloroplast and mitochondrial genomes with respect to other younger taxa could be assumed for the evolutionary old *Araucaria araucana*. Similar results were also obtained for the endemic Cupressaceae *Austrocedrus chilensis* (Fallour and Gallo, pers. com.), native to the South American forests. Low levels of genetic diversity were reported in other members of Araucariaceae. An extreme case is *Wollemia nobilis* which exhibits no variation at 13 isozyme loci, more than 800 AFLP loci and 20 SSR loci, and could represent the only living clone of an extinct species (Peakall et al. 2003). But low levels of variation were also encountered for *Araucaria cunninghamii* (Scott et al. 2005), *Agathis robusta* and *Agathis borneensis* (Peakall et al. 2003), suggesting an evolutionary trend in the family. However, considerable levels of genetic diversity were detected in *A. araucana* using RAPDs and isozymes (Bekessy et al. 2002; Gallo et al. 2004; Ruiz et al. 2007), which could be related to a higher mutation rate of the nuclear genome compared to the chloroplast (Wolfe et al. 1987).

Distribution of cp DNA genetic diversity and glacial history

Maternally inherited markers are the most suitable for phylogeographic reconstructions since they allow the investigation of seed movement. However, paternally inherited plastid DNA polymorphism have also proved to be useful markers and were applied in several studies among conifer species (e.g. Vendramin et al. 1998; 1999; 2000; Gomez et al. 2005; Bucci et al. 2007) distinguishing the same populations from the same geographic regions as maternally inherited organelle markers (e.g. Vendramin et al. 2000; Sperisen et al. 1998) in *Picea abies*. Therefore, when studying species without maternally inherited organelles, the use of paternal lineages could provide insights into past genetic processes as well.

In spite of the low levels of polymorphism and the low transferability rate of universal primers in *Araucaria araucana*, the variation detected in the chloroplast DNA allowed the identification of five haplotypes. As expected for a paternally inherited plastid that moves with pollen grains, genetic differentiation was very low ($G_{ST} = 0.11$), even when correcting for the different h_s values ($G'_{ST} = 0.267$). Similar estimations were obtained for other species like *Abies alba* (Vendramin et al. 1999), *Pinus pinaster* (Vendramin et al. 1998), *Picea abies* (Vendramin et al. 2000) and preliminary results in the congener *A. angustifolia* (Schlögl et al. 2007). The low level of differentiation implies that gene flow via pollen in *Araucaria araucana* might be extensive and therefore counterbalancing the divergence among populations (Gallo et al. 2004). The genetic differentiation is significantly increased both among the *continuous* and also among the *isolated* groups of populations ($G_{ST} = 0.195$; $G'_{ST} = 0.459$ and $G_{ST} = 0.202$; $G'_{ST} = 0.482$, respectively, compared to the much lower value obtained for the *fragmented* group, $G_{ST} = 0.038$; $G'_{ST} = 0.114$). Moreover, the *continuous* and *isolated* groups were characterized by lower levels of allelic richness, as compared to the *fragmented* populations. Differences were not significant among the mean allelic richness for the three groups, therefore suggesting that pollen flow could be balancing the effects of genetic drift in the small populations.

During the Last Glacial Maximum, about 20,000–18,000 years BP, glaciers within the current distribution range of *A. araucana* were mainly confined to the valleys (Flint and Fidalgo 1964; Rabassa and Clapperton 1990). Many of the sampled populations were located beyond the limits of the ice cap (Hollin and Schilling 1981; Fig. 1) and could therefore be considered as remnants of pre-Holocene origin. Thus, a scenario of numerous small patches of forests in favourable microhabitats throughout full glacial times could be envisaged. The genetic structure observed at the eastern marginal populations (reduced allelic richness and increased genetic differentiation) is compatible with a long-lasting isolation and the effects of stochastic processes on small populations. Relict populations of *Abies ziyanensis* showed similar patterns (Tang et al. 2008) and also peripheral compared to central populations in several reviewed species (Eckert et al. 2008). If the pre-Pleistocene distribution of *Araucaria araucana* was only partially reduced because of the type of glaciation at these latitudes, then we could expect multiple refugia without strong genetic differentiation among them. Moreover, pollen flow could have existed among the refugia as was the case in *Abies alba* (Liepelt et al. 2002). Then, after glacials retreated, diffusive colonisation from multiple eastern refugia would have led to the current lack of geographic structure, and higher diversity at those intermediate populations due to admixture with lineages coming from the west. Eventually, some long distance dispersal events might have taken place, which could be supported by the distribution of the two rare haplotypes. The eastern group could then be considered as relictual and isolated for a long time, while the western might either have originated in Andean refugia or be the result of a recolonisation from the east. In that case, genetic differences would be the result of drift and founder events. Multiple refugia for the species were suggested by Bekessy et al. (2002) based on variation detected with RAPDs among populations from Chile and Argentina and also by Ruiz et al. (2007) among Chilean populations using isozyme markers.

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Ecological features of *A. araucana* such as high plasticity and pioneer life history traits could have favoured the stable persistence of small populations throughout full glacial times. In addition, the increased proportion of vegetative propagation at these localities (Burns 1993; Gallo et al. 2004) might have helped to preserve some diversity. Thus, genetic drift due to isolation could have been counteracted by gene flow via pollen and "frozen" genetic structures due to clonal persistence. Unfortunately, a poor pollen representation of *Araucaria* which left hardly a trace (Kershaw and McGlone 1995) avoids genuine comparison of molecular and paleobotanic data.

Human impact and conservation considerations

The clustering of populations Tromen (T) and Curruhue (CU) is not fully supported by geographic proximity. The grouping of heterogeneous provenances may be interpreted either as intermediate populations diverging by drift or as non-autochthonous stands (Vendramin et al. 2000). The Curruhue population is of questionable origin and possibly the result of an aborigine settlement. Moreover, Tromen and Curruhue are situated along a commercial route highly used by the original communities to travel between current Chile and Argentina. Therefore, our results could be providing some extra evidence to anthropological studies in Patagonia.

Araucaria araucana has several peculiarities that stress the importance of conserving its genetic diversity. First, the species is included in the Appendix I of CITES (http://www.cites.org/eng/app/appendices.shtml) and listed in the 2008 IUCN Red List of Threatened Species (http://www.iucnredlist.org) as a vulnerable species. Red Appendix of CITES. Second, although not logged at present times, it is highly affected due to human activities. The severe erosion evidenced in the eastern populations as the result both of human impact and natural desertification processes calls for an urgent action. Rear edge populations were declared as "disproportionately important for the long-term conservation of genetic diversity, phylogenetic history and evolutionary potential of species" (Hampe and Petit 2005). The small and scattered populations at the easternmost edge are located outside National Parks, and are the most prone to extinction. Several of these populations belong to private owners and legislation is not clear in this concern. Consciousness on the high allelic richness of these patches of forests and therefore on their high conservation priorities should be given to authorities.

To face the foreseeing global climatic change and the future variation of vegetative conditions, conservation of genetic diversity from natural populations is required as a preliminary step for preserving adaptive potential of the species (Gregorius 1991). Therefore, information about the history of a species like number and types of glacial refugia as well as migration routes are essential for conservation activities (Vendramin et al. 2000). Our results provide evidence of the relictual condition of eastern populations, related with glacial history, and stress the higher genetic differentiation among populations of this area. Similarly, previous studies with isozyme markers revealed higher genetic diversity within eastern populations (Gallo et al. 2004). A combination of differentially inherited genetic markers is highly important in the definition of conservation units (Petit et al. 1998). Inclusion of adaptive traits in addition to neutral markers is also relevant when establishing conservation priorities given the lack of congruence between the distribution of the variation at both levels (Bekessy et al. 2003). For *Araucaria araucana* we are also gathering information on adaptively significant traits provided by field trials (progeny and provenance tests). Besides, morphological variation on seed traits and early seedling growth is currently under study (Izquierdo and Gallo, unpublished).

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