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High genetic variation in marginal fragmented populations at extreme climatic conditions of the Patagonian Cypress *Austrocedrus chilensis*

María Verónica Arana [a], Leonardo A. Gallo [a], Giovanni G. Vendramin [b], Mario J. Pastorino [a,c], Federico Sebastiani [b], Paula Marchelli [a,c,*]

[a] Unidad de Genética Ecológica y Mejoramiento Forestal, Instituto Nacional de Tecnología Agropecuaria (INTA), EEA Bariloche, CC 277, 8400 Bariloche, Argentina
[b] Istituto di Genetica Vegetale, CNR, 50019 Sesto Fiorentino (Firenze), Italy
[c] CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), Argentina

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ABSTRACT

Knowledge about current patterns of genetic structure of populations together with the evolutionary history of a species helps to understand and predict the adaptation of populations to future climate change. We assayed variation at nuclear microsatellite markers among peripheral vs. continuous populations of the temperate South American species *Austrocedrus chilensis*, to investigate the role of historical vs. demographical forces in shaping population genetic structure. This species occurs in continuous populations in the west and central distribution range, but becomes highly fragmented at the eastern limit, which comprised ice-free areas during Quaternary glaciations and has extreme climatic conditions at present times. Bayesian analysis methods identified two contrasting patterns of genetic structure; (I) populations from humid, mesic and peri-glacial regions formed a single deme with relatively low genetic differentiation and high admixture levels whereas (II) a highly heterogeneous genetic structure with low level of admixture was found in the steppe, towards the east and northeast limit of the distribution range. In the steppe, population fragmentation, restricted gene flow and isolation-by-distance were also inferred. In addition, several small steppe populations showed high genetic diversity and divergent gene pools, suggesting that they constitute ancient refuges from pre-Holocene glaciations with just a subgroup of them contributing significantly to post-glacial spread. These results are discussed in relation to patterns of genetic variation found for other temperate species and the contribution of the particular southern Andes topography and climate to post-glacial spread.

1. Introduction

Habitat conditions strongly determine population size and distribution. On a regional scale, it is expected that in heterogeneous environments the abundance of a species becomes highest at the geographical centre of its range and less abundant at the margins of its distribution area, where habitat conditions are less favourable (e.g. Brown et al., 1995; Lawton 1993; Vucetich and Waite 2003). This concept represents the basis of the "abundant centre" model (Sagarin and Gaines 2002), and its extension suggests that both effective population size and rate of gene flow should be highest at the centre and lowest at the edges of the species distribution range (Eckert et al., 2008). The increased isolation at the range limits and the low population size may contribute to the loss of genetic variation through drift and reduced gene flow (Lesica and Allendorf, 1995; Vucetich and Waite, 2003). Moreover, influx of "center" alleles might swamp local adaptations (Bridle and Vines 2006; Kirkpatrick and Barton 1997); although under some circumstances gene flow from the centre range can prevent the extinction of edge populations (Holt and Gomulkiewicz 1997). It is generally expected that geographically peripheral populations should exhibit lower within-population genetic diversity and higher genetic differentiation among populations than central ones, but may often be better-adapted to unfavourable conditions (Blows and Hoffmann 2005; Eckert et al., 2008; Hoffmann and Blows 1994), although several exceptions were reported (Lesica and Allendorf, 1995; Vucetich and Waite, 2003). On a broad geographical scale, patterns of genetic variability and differentiation may be shaped by past climate fluctuations, thus restricting the "abundant centre" model to the context of range modifications driven by stochastic climatic changes (Hampe and Petit, 2005). For example, the drastic reduction of temperate forest areas during Quaternary glaciations and the subsequent post-glacial expansion into new territories strongly affected the distribution range of several temperate species, with important consequences for the geographic structuring of their genetic diversity (Hewitt, 2000). Their effects on present-day genetic variation are considered to be particularly relevant

* Corresponding author. Fax: +54 2944 424991.
E-mail address: pmarchelli@bariloche.inta.gov.ar (P. Marchelli).

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in temperate trees (Hu et al., 2009), given that their long life cycles led to the occurrence of relatively few generations since the end of the last glacial period, which dates at about 14,000 ^{14}C years BP (Lowell et al., 1995). Long-term isolation of populations in ice-age refuges could have led to their genetic differentiation (Bennet et al., 1991; Petit et al., 2003). In addition, populations constituting glacial refuges are expected to harbour larger levels of genetic diversity within populations than those located in the re-colonised regions, because of allele loss and increase in homozygosity during post-glacial expansion as a consequence of successive founder events (Brewer et al., 2002; Hewitt, 2000; Petit et al., 2002). However, admixture of divergent lineages during re-colonization might yield complex distribution patterns of diversity outside refuge areas (Petit et al., 2003; Terrab et al., 2008). Most of the best molecular evidence of the legacy of quaternary ice in the spatial distribution of genetic diversity comes from studies of European tree species (Comes and Kadereit 1998; Hampe and Petit 2005). However, the consequences of glaciations and post-glacial migration on genetic structuring cannot be generalised (Grivet et al., 2006; Magni et al., 2005).

In South America, three main glaciations were identified in the austral region of the Andes Mountains, which were found to be correlated with the last major glaciations of northern Europe (Caldenius, 1932; Rabassa and Clapperton, 1990). Patterns of genetic diversity within and among populations of several native tree species such as *Araucaria araucana, Austrocedrus chilensis*, *Fitzroya cuppressoides*, *Nothofagus nervosa* and *Nothofagus obliqua* were affected by the Quaternary ice, as revealed by nuclear or chloroplast markers (Allnutt et al., 1999; Azpilicueta et al., 2009; Bekessy et al., 2002; Marchelli and Gallo 2004, 2006; Pastorino and Gallo 2002; Premoli et al., 2002). In contrast to glaciations in Northern Europe, which occurred mainly as a continuous ice-cap (Hewitt 2000), ice distribution in the southern Andes showed a remarkably scattered pattern to the north of latitude 42°S. This region was characterised by broad ice bodies located in the Andes Mountain valleys which run transversely to the line of mountains in a west–east direction (Flint and Fidalgo 1964). This pattern of ice distribution may have left ice-free areas, providing a wide range of microhabitats suitable as refuges for forest taxa (Markgraf et al., 1995). On the other hand, south of latitude 42°S the ice was homogeneously distributed over the Andes (Flint and Fidalgo 1969), bordering in the east with the steppe which probably did not offer a favourable environment for species refuges because of the extreme climatic conditions (e.g. Pastorino et al., 2009). In addition, some climatic characteristics of the southern Andes may have shaped the effect of the Quaternary climatic oscillation in the structuring of South American temperate species genomes. The strong winds with predominant eastward direction during pollination and seed dispersal seasons are factors that may greatly affect gene flow patterns of forestry species. Moreover, the rain shadow effect, causing one of the most abrupt west–east precipitation gradients in the world (Prohaska 1976), may have imposed strong selection pressure. Frequent volcanism also played an important role in species' distribution patterns and colonization routes, (Millerón et al., 2008) and more recently, habitat disturbance due to natural and artificial fires was shown to affect the genetic variation patterns of native species (e.g. Pastorino and Gallo 2002; Premoli and Kitzberger 2005).

Austrocedrus chilensis (D. Don) Pic. Ser. et Bizzarri is a wind-pollinated and seed dispersed dioecious conifer with a wide distribution range, in latitudinal terms, in both Chile and Argentina. It is most common in the eastern Andes, in Argentina, where its distribution range covers a wide precipitation gradient, ranging from 2500 mm per year in the west, at the border with Chile, to 400 mm per year in the east, in the Patagonian steppe (Pastorino and Gallo, 2002). Forest types vary along the rainfall gradient, occurring as continuous populations in the western, humid region and in the mesic zones, while occurring in small, fragmentary groups towards the arid zones in the steppe (Gallo et al., 2004). Previous studies using allozyme markers in the natural Argentinean range showed that the highest diversity is located in some of the northern and eastern populations of the steppe, with non significant correlation between genetic and geographic variation (Pastorino and Gallo, 2002). These populations are located in regions that remained free of ice sheets during the Quaternary glaciations and it is suggested that they are relict populations (Pastorino and Gallo, 2002).

The peripheral populations of *Austrocedrus chilensis* are assumed to be adapted to extreme climatic conditions, similar to those predicted for the whole region under the foreseeing global climatic change (Breda et al., 2006; IPCC, 2007; Meehl and Tebaldi, 2004). Therefore, they represent a key material for conservation and management purposes. The recent development of highly polymorphic nuclear microsatellites for *A. chilensis* (Arana et al., 2008) provides the opportunity to identify multi-locus genotypes with a much higher precision than allozymes. Additionally, microsatellites posses the advantage of containing phylogenetic information in the distribution of allele sizes (Hardy et al., 2003), and therefore can contribute to elucidate the post-glacial re-colonization. Phylogeographical inferences have in earlier works typically relied on organelle markers representing single gene histories. To fully address the population history of an organism, several distinct genealogies from independent genetic markers are needed (e.g. Ballard and Whitlock, 2004). By combining mitochondrial or chloroplast DNA sequences with nuclear markers, demographic processes acting on different time scales will be captured due to different modes of inheritance, effective population size and mutation rate (e.g. Hewitt, 2001; Semerikov and Lascoux, 2003). A number of recent studies contrasting genetic population structures at organelle and nuclear loci have gained an improved understanding of past and present population demographic events for many species (e.g. Gamache et al., 2003; Heuertz et al., 2004a,b; Magri et al. 2006; Tollefsrud et al., 2009). A extremely low rate of transferability of organelle universal primers to Southern Hemisphere conifers (Marchelli et al., 2009) and the absence of polymorphism of the few that transferred in *Austrocedrus chilensis* (Fallour and Gallo, personal communication) did not allow comparisons between uniparentally and biparentally inherited markers, but stressed the relevance of the use of highly informative nuclear markers as microsatellites.

The general objective of this work was to contrast patterns of genetic variability at nuclear microsatellite markers in continuous versus fragmented *A. chilensis* populations to infer the role of historic and demographic forces in shaping population genetic structure. We evaluated the possibility that steppary populations were isolated since pre-Holocene times therefore constituting ancient glacial refugia. We also considered the hypothesis of post-glacial re-colonization from these multiple scattered refuges in the Patagonia steppe. For these purposes, we analysed patterns of genetic variation of 14 populations of *A. chilensis* along the limits of the species, using eight polymorphic nuclear microsatellite markers in combination with classical and Bayesian genetic analysis. In addition, interactions of post-glacial re-colonization with stochastic climatic disturbances and the particular South Andes topography and climate in the determination of patterns of genetic structure are discussed.

2. Materials and methods

2.1. Plant material

The sampled material consisted of mature *A. chilensis* leaves from 30–32 trees per population, from a total of 14 populations.

A minimum distance of 50 m between selected trees was maintained in order to avoid sampling of relatives. Population sampling was directed to mostly cover the peripheral range of the species, with emphasis on the analysis of fragmented steppe populations which could constitute relict populations adapted to conditions foreseeing under global climatic change. In order to study possible patterns of genetic structure associated with post-glacial expansion of the species from putative eastern refuges, populations located at two east–west transects at about latitude 40° and 41° were included, named Traful and Bariloche transects, respectively. These transects included populations situated in areas which were covered by ice during glaciations at the humid and mesic locations, and a section of the steppe which was located outside the ice limits. The Traful transect was made up of Chacabuco (marginal), Paloma Araucana and Pedregoso populations while Bariloche transect comprised Llao-Llao, Otto, San Ramón (marginal), Fragua (marginal), Pilcañeu North (relictual) and Pilcañeu South (relictual). In addition, two populations located at the northern extreme (Cañada Molina and Rahueco; relictual) and one located at the southern extreme (Corcovado, marginal) were included in the analysis, thus representing both latitudinal limits of the species distribution range (Pastorino et al., 2006). Populations were classified according to the forest type as *continuous* or *peripheric*. Leaves were conserved at −80 °C until DNA extraction.

2.2. DNA extraction

Total DNA was extracted from 100 mg of fresh tissue, using either QIAGEN DNAesy Plant Kit (Qiagen, Cat. No. 69106) according to the manufacturer's instructions or the protocol described by Doyle and Doyle (1987). In the latter, an adjustment of the original protocol was performed for DNA extraction from *Austrocedrus chilensis* leaves, which consisted of two extra dichloromethane extractions before DNA precipitation with isoamylic alcohol. Before DNA extraction, leaves were frozen with liquid nitrogen and ground using an automatic grinding mill (Mixer Mill, Resch). Pellets were resuspended in 50 μL TE 1X with a final treatment of 2 μl/mg of RNAsa at 37 °C.

2.3. Microsatellite markers and data analysis

Seven highly polymorphic tetranucleotide microsatellite markers (Achi3-9) (Arana et al., 2008) and one dinucleotide microsatellite designed from a clone belonging to the original tetranucleotide library used for isolating the other described loci (Achi10; forward primer: 5′ TGATTCAACATGCATTCAATTACA 3′ and reverse primer: 5′ TGCCCTATCTACATAGTCACAAAGA 3′) were selected for genotyping *A. chilensis* individuals. Following a sequential Bonferroni correction, the eight markers showed no significant ($P = 0.05$) linkage disequilibrium using Fisheŕs exact test calculated by F-STAT software package version 2.9.3 (Goudet, 2002). PCR amplification was performed as described in Arana et al. (2008). For PCR reaction one of each primer pair was end-labelled with FAM, HEX or TAMRA, and multiplexed PCR products were run on a MegaBACE 1000 (GE Healthcare). Electropherograms were analysed using the MegaBACE fragment profiler version 1.2 software (GE Healthcare).

Genetic diversity at nuclear SSR loci in each population was quantified in terms of number of alleles per locus (A), percentage of private alleles (AP), allelic richness (r_g) after rarefaction to the smallest sample size (g) and correction by subtracting one, according to El Mousadik and Petit (1996), allelic diversity or effective number of alleles (v) (Gregorious, 1978), and the mean observed and expected heterozygosity (*Ho* and *He*) calculated using F-STAT software package version 2.9.3 (Goudet, 2002)and GSED software (Gillet, 1997). The possible effect of recent bottlenecks was evaluated following Cornuet and Luikart (1996). We run the program BOTTLENECK v. 1.2.02 (Piry et al., 1999 available at http://www.ensam.inra.fr/URLB) under the Two-phased model of mutation (TPM) recommended for microsatellites by the authors, and also under the Stepwise mutation model (SMM). Deviation from the equilibrium gene diversity (*Heq*) was tested using the Wilcoxon signed rank test. According to Luikart and Cornuet (1998) populations that have recently experienced a reduction of their effective population size exhibit an excess of Hardy–Weinberg equilibrium heterozygosity (*He*) with respect to the mutation – drift equilibrium heterozygosity (*Heq*), what is caused by a faster loss of alleles than a reduction in heterozygosity during a bottleneck.

The inbreeding coefficient (F_{IS}) in each population was estimated according to Wright (1978). The presence of a phylogeographical signal (i.e. alleles within the same population are more related on average than alleles sampled further apart), can be tested by comparing F_{ST}, with $R_{ST.}$ This comparison was performed according to Hardy et al. (2003) by constructing a F_{ST} analogue based on allele size randomization, with computation of R_{ST} after each allele size random permutation (pR_{ST}). The expected value after such permutation is equal to an F_{ST} because only the structure due to allele identity remains (Hardy and Vekemans, 2002). The statistical significance of each estimate was calculated by permutation tests with 10,000 random permutations using the software SPAGeDI v. 1.2 (Hardy and Vekemans, 2002). Isolation-by-distance was investigated to infer spatial genetic structure following Rousset (1997). A Mantel Test with 10,000 permutations was performed to evaluate the correlation between the matrix of pairwise genetic differentiation between populations ($F_{ST}/(1 - F_{ST})$) and the matrix of natural logarithm of the linear geographic distance between populations (in kilometres). F_{ST} may be overestimated in the case of null alleles (Chapuis and Estoup 2007). We controlled for the potential effect of null alleles on genetic differentiation by calculating F_{ST} values using the 'excluding null allele' (ENA) method by Chapuis and Estoup (2007) implemented in FREENA. Additionally, we estimated the genetic differentiation of each population with respect of the others (D_j; Gregorius and Roberds (1986), using GSED (Gillet, 1997). We analysed the association between the natural logarithm of each geographical coordinates (latitude or longitude), the genetic differentiation of each populations with respect to the other (D_j), and the genetic diversity after rarefaction (r_g) through respective multiple regression analysis. For the genetic differentiation we applied an arcsin squared root transformation, as recommended for data ranging between 0 and 1. Both multiple regressions were performed with the statistical package R 2.8.1 (R Development Core Team, 2008), and normality was verified through the Shapiro–Wilk normality test.

A non-metric multidimensional scaling (NMDS) of pairwise F_{ST}–distances was used to quantify the actual genetic distances between populations. NMDS was performed following Kruscaĺs non-metric multidimensional scaling using the isoMDS function implemented in the package MASS (Venables and Ripley, 2002) of R v. 2.8.1 (R Development Core Team, 2008). An iterative algorithm was used which usually converges at around 10 iterations.

Population genetic structure was inferred using two Bayesian methods: BAPS (Bayesian Analysis of Population Structure) version 3.2 (Corander et al., 2003) and STRUCTURE version 2.2.3 (Pritchard et al., 2000). BAPS was used for estimating hidden population substructure, given information about the population origin of each sampled individual. We performed two independent analyses in which geographical coordinates of populations were included or omitted. For each condition, BAPS mixture clustering was run ten times, setting the parameters to default values (Corander et al., 2003) and for each repetition, the order of the populations randomised in the input file. Bayesian clustering STRUCTURE method

was also used to detect population-hidden sub-structures, assigning individual multi-locus genotypes probabilistically to a K number of clusters which should represent ancestral groups based on allele frequencies, achieving linkage equilibrium within clusters. Grouping was performed using the admixture model. The number of genetically distinct clusters K was set to vary from 1 to 10. The model was run with three independent simulations for each K to confirm that patterns did not vary between runs, using a burn-in length of 10,000 and run length of 10^6 iterations. Other parameters were set to default values as suggested by Pritchard and Wen (2003). The ΔK statistic, based on the rate of change of log likelihood of data [$L(K)$] between successive K values was used to determine the optimal K, selecting the lowest K number that yielded [L(K)] constant, and was able to capture the larger structure of the data in a biologically sensible way (Pritchard and Wen 2003).

3. Results

3.1. Intra-population genetic diversity

The analysis of the 8 microsatellite loci in 448 individuals belonging to 14 populations of *A. chilensis* located across the eastern side of the Andes showed a total number of alleles per locus ranging from 8 to 51, yielding a total of 280 alleles scored in the species.

At the population level, the mean number of alleles per locus ranged from 9.5 (Population Pilcañeu North, eastern extreme) to 14 (Population Rahueco, northern extreme). Values of *Ho* ranged from 0.61 to 0.82, showing little variation across populations (CV = 13.43). Allelic richness (r_{36}) and diversity (v) were relatively high, ranging from 7.09 to 10.57 and from 2.98 to 5.46, respectively. While Corcovado showed the smallest values of r_{36} and v, San Ramón harboured the largest values for both parameters. However, r_{36} and v were not necessarily associated in each population (Table 1). No evidence of recent population bottlenecks was observed among the analysed populations, under both the Two-phased mutation (TPM) and the Stepwise mutation (SMM) models ($P > 0.05$).

As a general feature, the cumulative action of inbreeding within populations was moderate to low ($F_{IS} = 0.007$ up to 0.195; CV = 1.47) and significantly different from zero in eleven out of fourteen populations, in accordance with the general observation for woody species (Hamrick et al., 1992).

3.2. Genetic differentiation and isolation-by-distance

The differentiation among populations ($F_{ST} = 0.074$) showed similar values as those reported for conifers (Hamrick et al., 1992; Petit et al., 2003) and no significant differences were detected when allele sizes are considered ($R_{ST} = 0.080$, $P = 0.382$). Correction for null alleles only marginally decreased absolute F_{ST} values (0.075 versus 0.073), which indicates that null alleles were not strongly biasing the differentiation index. The Mantel test revealed a significantly high correlation between the geographic and genetic distances of the populations ($r = 0.54$; p [random $Z \leqslant$ observed Z] = 0.9992 out of 10,000 permutations) (Fig. 1). A

Table 1
Genetic variation in *A. chilensis* in 14 populations analysed. No alleles: total number of alleles; AP (%): percentage of private alleles calculated as the percentage of the number of private alleles in the population over the total number of alleles in the population. *Ho*: observed heterozygosity; *He*: expected heterozygosity; r_g: allelic richness after rarefaction to 36 genes,ν:: allelic diversity; D_j: differentiation of each population with respect of the others; F_{IS}: Wrighťs inbreeding coefficient with its associated probability. Values in brackets for F_{IS} indicate probability (P) of deviation from equilibrium.

Population	ID	Latitude	Longitude	Situation of the population	Location related to quaternary ice zones	No Alleles	AP (%)	*Ho*	*He*	*rg*	ν	F_{IS}	D_j
Fragua	F	41°5́S	70°57́W	Peripheral/ steppe	Peri-glacial	97	3.09	0.76	0.78	9.31	4.47	0.036 (0.115)	0.322
Chacabuco	CH	40°39́S	71°0́W	Peripheral/ steppe	Out of ice limits	94	2.12	0.61	0.72	8.47	3.51	0.165 (0.0001)	0.421
San Ramón	SR	41°3́S	71°5́W	Peripheral/ steppe	Peri-glacial	111	9.00	0.68	0.82	10.57	5.46	0.176 (0.0001)	0.377
Pilcañeu North	P I	41°13́S	70°42́W	Peripheral/ steppe	Out of ice limits	76	2.63	0.64	0.67	7.09	2.98	0.057 (0.0313)	0.457
Pilcaniyeu II	P II	41°14́S	70°41́W	Peripheral/ steppe	Out of ice limits	90	6.60	0.76	0.79	8.37	4.72	0.051 (0.0371)	0.434
Rahueco	Ra	37°9́S	70°35́W	Peripheral/ steppe	Out of ice limits	112	9.82	0.70	0.79	10.18	4.85	0.136 (0.0001)	0.586
Riscos Bayos	Rba	37°58́S	70°47́W	Peripheral/ steppe	Out of ice limits	96	18.75	0.68	0.77	9.06	4.39	0.134 (0.0001)	0.649
Molina	Mo	37°7́S	70°36́W	Peripheral/ steppe	Out of ice limits	100	18.00	0.67	0.82	9.53	5.40	0.195 (0.0001)	0.643
Mean ± SD of the group						97.00 ± 11	9.02 ± 6.59	0.67 ± 0.05	0.77 ± 0.05	9.07 ± 1.10	4.47 ± 0.86		0.486 ± 0.12
Corcovado	Co	43°31́S	71°27́W	Continuous/ mesic	Into ice limit	77	2.59	0.69	0.74	6.97	3.80	0.083 (0.0086)	0.460
Pedregoso	G	40°37́S	71°35́W	Continuous/ humid	Into ice limits	97	4.12	0.73	0.79	9.03	4.80	0.101 (0.0005)	0.383
Llao-Llao	LL	41°3́S	71°32́W	Continuous/ humid	Into ice limits	109	0.91	0.72	0.79	10.33	4.77	0.103 (0.0001)	0.285
Otto	O	41°8́S	71°19́W	Continuous/ mesic	Into ice limits	99	4.00	0.82	0.81	9.29	5.21	0.007 (0.3751)	0.335
Maitén	M	42°2́S	71°12́W	Continuous/ mesic	Into ice limits	88	4.54	0.70	0.71	8.17	3.5	0.04 (0.0792)	0.372
Paloma Araucana	PA	40°40́S	71°21́W	Continuous/ mesic	Into ice limits	107	1.86	0.72	0.81	10.11	5.13	0.123 (0.00012)	0.363
Mean ± SD of the group						96.17 ± 12	2.96 ± 1.44	0.73 ± 0.04	0.77 ± 0.04	8.98 ± 1.25	4.54 ± 0.71		0.366 ± 0.05

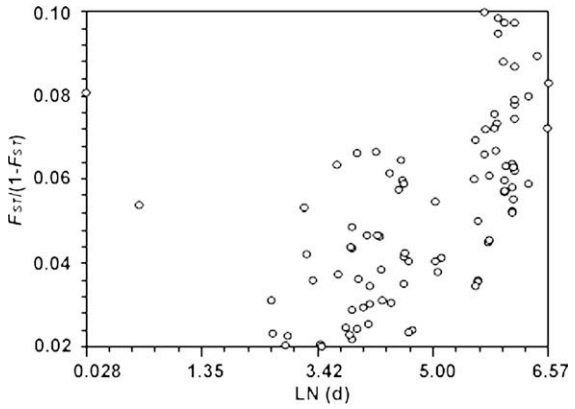

Fig. 1. Correlation between mean $F_{ST}/(1 - F_{ST})$ and the logarithm of the geographical distance (in km) of the analysed populations of *A. chilensis* in the eastern Andes.

significant association between genetic differentiation and geographic coordinates was detected (Multiple *R*-squared: 0.633, $P = 0.040$), attributed mainly to the longitude ($P_{long} = 0.0029$; $P_{lat} = 0.056$). In the case of allelic richness, a less pronounced, but still significant association was estimated (Multiple *R*-squared: 0.455, $P = 0.035$), due to correlation with latitude ($P_{long} = 0.0568$; $P_{lat} = 0.0117$). Non-metric multidimensional scaling of F_{ST}-distances among populations separates the northern most populations. In general, the peripheral populations are situated at the margins of the plot (Fig. 2). The final stress value was 10.87% deviation with respect to the initial distance matrix.

3.3. Population genetic structure

Overall, results indicated that *A. chilensis* has large genetic variability, mostly within populations. In addition, fragmented populations located in the steppe, at the eastern and northeastern limits of the natural distribution area of the species displayed the highest differentiation (Figs. 1 and 2) and showed a significant correlation between genetic differentiation and longitude. In order to gain information about possible structuration of genetic variation in the species, we combined the information of the eight SSR data for the 14 populations with the aid of Bayesian approaches which, unlike the classical F_{ST} analysis, have the advantage of merging information from several loci into a single probability model (Corander et al., 2003).

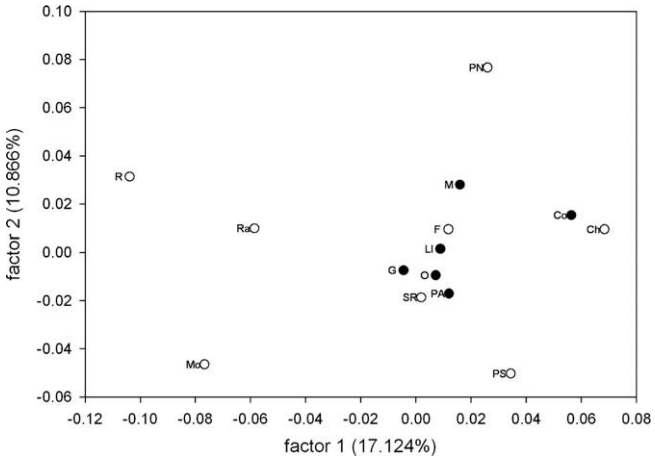

Fig. 2. Non-metric multidimensional scaling (NMDS) based on F_{ST}-distances among populations. Black dots correspond to continuous populations and circles to peripheral populations.

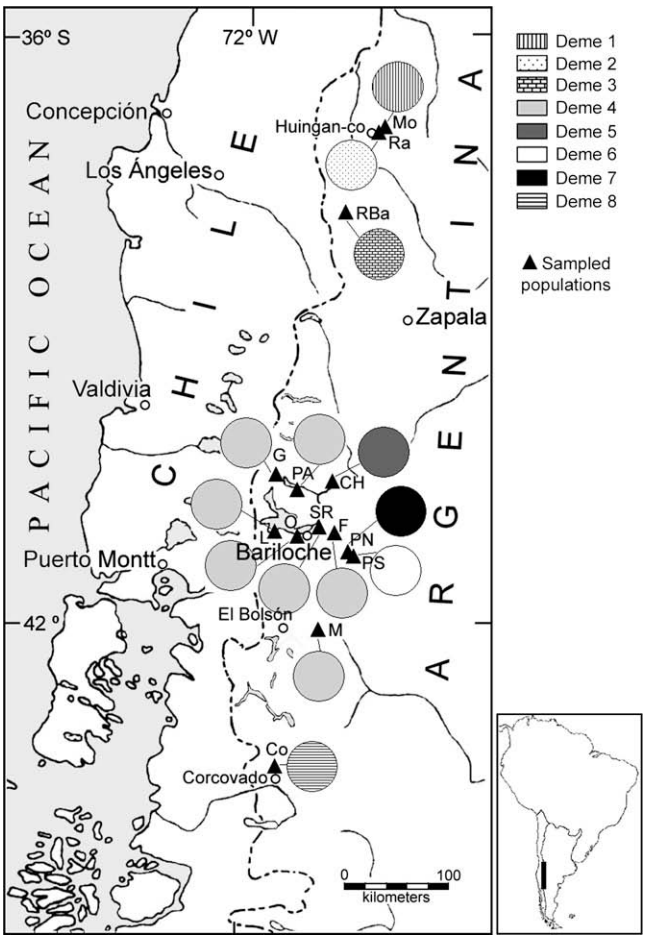

Fig. 3. Geographical distribution of *A. chilensis* populations in the demes, indicated by different graphic patterns, obtained by the Bayesian Analysis of Population Structure (BAPS). Population names are given in Table 1. Triangles indicate location of each population.

We performed 10 independent BAPS runs, which showed consistent grouping of populations between repetitions, with the best grouping in eight different demes. While humid and mesic continuous populations constituted a single deme together with the populations San Ramón, Fragua and Maitén, each of the remaining populations constituted individual demes, indicating a marked genetic structure for the species in the steppe area (Fig. 3). Moreover, BAPS analyses performed considering the spatial coordinates of each population showed similar results, with the difference that Chacabuco grouped in deme1 (data not shown), indicating that the structure found using this approach is strong and mostly independent of the geographical localization of the populations.

Results using the clustering method STRUCTURE confirmed the existence of heterogeneity in the spatial distribution of genetic variation of *A. chilensis* (Fig. 4). Grouping of individuals at different "gene clusters" *K* showed a strong increment in the log likelihood for the data; $\ln(X/K)$, as *K* was increased for $1 \leqslant K \leqslant 4$. However, for $K \geqslant 5$, $\ln(X/K)$ increased slightly and progressively (data not shown). Therefore, we chose the smallest *K* value with biological significance that captures the major structure in the data (Pritchard and Wen, 2003). Under these conditions, the best grouping of individuals was found for $K = 4$ and the admixture model indicated the existence of four ancestral gene pools. Interestingly, three out of four gene pools were mostly represented in populations located at the extreme limits of the distribution range of the species, which were outside the ice during glaciations. Gene pool 1 included the populations Cañada Molina, Rahueco and

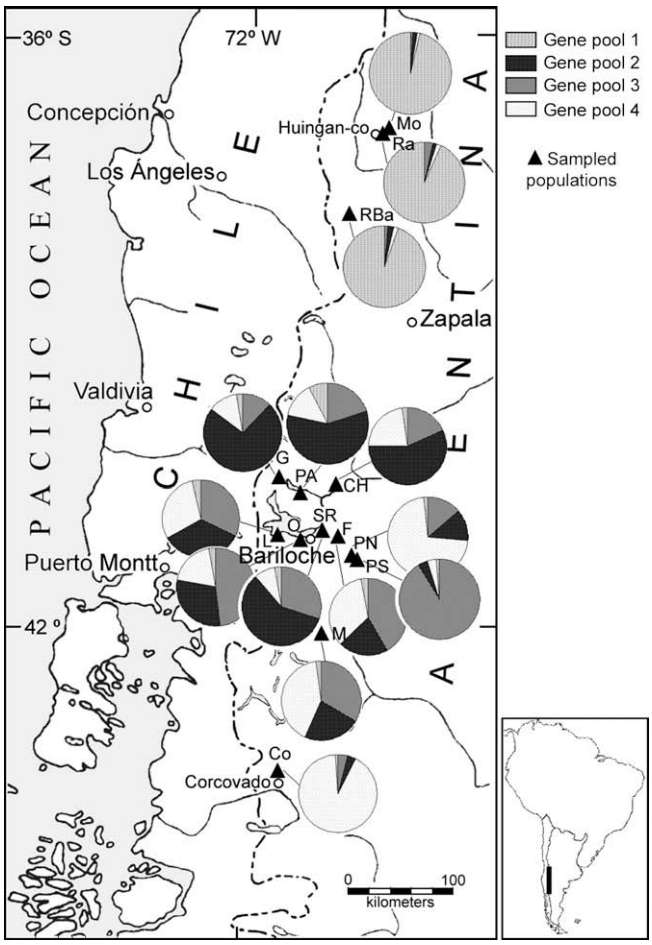

Fig. 4. Distribution of ancestral gene pools identified using STRUCTURE in *A. chilensis* eastern Andes populations. Different graphic patterns indicate different gene pools. Population names are given in Table 1. Triangles indicate location of each population.

Riscos Bayos, located at the northeastern extreme of the range. On the other hand, gene pool 3 was strongly represented in Pilcañeu South, located at the extreme east, while gene pool 4 was most abundant in Pilcañeu North and Corcovado, located at the eastern and southern extremes of the distribution area, respectively. Gene pool 2 was present predominantly in the populations that constituted deme 4 in BAPS, being more abundant in populations of the Traful west–east transect, as well as in the steppe population San Ramón. Though gene pool 2 was clearly predominant in the populations that constituted deme 4 in BAPS, these populations also contained a relatively large proportion of the other three ancestral pools.

In addition to the Bayesian analyses that indicated relatively high homogenization among central populations (deme 4 in BAPS), we calculated the diversity and differentiation among populations within groups and also between groups in order to capture differences due to restrictions in gene flow. If peripheral populations did not extensively contributed to gene pools of continuous populations, then differences between groups should be significant. Group 1 included the seven populations identified as deme 4 in Fig. 3 whilst group 2 was made up of the seven populations forming seven different demes. Differentiation was significantly larger among peripheral and fragmented populations of group 2 compared to the continuous and homogeneous populations of group 1 ($F_{ST1} = 0.029$ and $F_{ST2} = 0.114$, $P = 0.005$; $R_{ST1} = 0.026$ and $R_{ST2} = 0.124$; $P = 0.005$), reflecting the higher homogenization within populations of the continuous forests. No significant differences were detected when allele sizes were considered. On the other hand, in contrast to that predicted by the abundant centre model, there were no significant differences in diversity between small fragmented and continuous groups (data not shown).

4. Discussion

4.1. Genetic structure: peripheral versus continuous populations

Peripheral fragmented populations at the limits of a species distribution range are usually characterised by low genetic intra-population diversity and high differentiation among them (Blows and Hoffmann, 2005; Eckert et al., 2008; Hoffmann and Blows, 1994; Lesica and Allendorf, 1995; Vucetich and Waite, 2003). However, this was not a strict rule for the *A. chilensis* populations analysed in our study. Overall, peripheral populations showed slightly lower values for some diversity parameters (*rg* and ν) than continuous populations, with some small fragmentary populations (e.g. Rahueco, ca. 50 individuals) displaying large variation (Table 1). Moreover, within-population diversity was heterogeneous along the periphery of the range, with populations from the northeast (Rahueco, Riscos Bayos, Molina), San Ramón and Fragua exibiting higher values than those from the east, such as Pilcañeu North and Chacabuco. Demo-stochasticity may have impacted differentially on within-population diversity, having a lesser effect on populations located in the northeast and the immediate limits of the ice sheets, but having a greater impact on populations located at the extreme eastern limits of the range.

Three main evolutionary forces are responsible for genetic variation in neutral markers like SSRs: *genetic drift*, which decreases genetic variation within populations, and *mutation* and *gene flow,* which have the opposite effect (Wright, 1978). The high mutation rates of microsatellites (Li et al., 2002) may generate new variants and maintain the levels of within-population diversity on the periphery of the studied specieś range. However, the distribution pattern of genetic variation in *A. chilensis* should not be strictly associated with the marker, since allozymes showed a similar trend (Pastorino and Gallo, 2002). Clonal growth through vegetative propagation is common in peripheral populations of *A. chilensis* and is a possible mechanism to overcome adverse conditions in sites where seedling recruitment is almost absent (Gallo et al., 2004). The extended generation time gained through clonal growth might reduce the impact of genetic drift (Ellstrand and Roose, 1987). Local scale genetic processes related to life traits of a species, like mating systems, also contribute in shaping the genetic structure of populations (Templeton et al., 2001; Darling et al., 2008). *A. chilensis* is dioecious, and therefore strictly out-crossing, a life-trait that contributes to maintain high levels of within-population diversity.

Despite displaying high diversity, peripheral populations showed greater genetic differentiation than continuous populations (Table 1), which might reflect restricted levels of gene flow, as supported by the large proportion of private alleles in some of these border populations. Even though wind-pollinated species are supposed to maintain high levels of gene flow (e.g. Hamrick et al., 1992), fragmentation might result in restrictions to gene movement (e.g. Provan et al., 2008; Sork et al., 2002). The strong winds in a predominantly eastward direction present during pollination and seed dispersal seasons might restrict gene flow among the extreme eastern populations. Accordance to this, among-population differentiation is correlated with geographical distance (Fig. 1) and mostly associated with longitude. In addition, the application of Bayesian methods such as BAPS (Corander et al., 2003) showed two contrasting distribution patterns of within-species genetic variability, in which the humid and mesic continuous

populations located to the west of the range composed a single genetic deme together with steppe populations Fragua and San Ramón, while the peripheral populations constituted, each one, a single deme (Fig. 3). Additionally, the Non-metric multidimensional scaling (NMDS) among pairwise F_{ST}-distances clearly separates the peripheral populations. This supports the observation of restricted gene flow and the presence of divergent gene pools towards the limit of the specieś distribution range.

Ongoing evolutionary forces such as those described above, but also historical stochastic events like Quaternary glaciations, contribute to the general patterns of diversity observed in relictual populations of different species (e.g. Tang et al., 2008; Marchelli et al., 2009). Steppe populations of *A. chilensis* are located in areas which constituted ice-free regions during Quaternary glaciations. The high diversity and differentiation values found in some of their small, fragmented populations, in conjunction with the presence of divergent gene pools towards the east and northeast of the range, indicate that Quaternary glaciations may have contributed to the distribution of genetic variability across the specieś eastern Andes range.

4.2. The genetic footprint of stochastic climate change during the Quaternary

As a general feature, refuge tree populations which persisted through multiple glacial cycles show highly divergent gene pools (Jansson and Dynesius, 2002). The presence of divergent gene pools in the steppe coinciding with high among-population differentiation and a relatively high number of private alleles suggests that they are refuges of the species in the eastern Andes. Therefore, steppe populations would constitute "stable edges" according to Hampe and Petit (2005), which persisted *in situ* in a narrow region between the ice sheets and the arid environment of the steppe. In contrast to the Northern Hemisphere, where a latitudinal shift in species distribution occurred (e.g. Hu et al., 2009; Petit et al., 2008), several authors reported the persistence of forests even at high latitudes in the southern hemisphere during full glacial times (Markgraf, 1993), with topography playing a relevant role (Markgraf et al., 1995). In addition to *A. chilensis*, survival of forests in multiple refuges has been postulated for other southern tree species (Allnutt et al., 1999; Azpilicueta et al., 2009; Bekessy et al., 2002; Marchelli and Gallo 2004, 2006; Pastorino and Gallo 2002; Premoli et al., 2000).

In contrast to those on the periphery of the range, populations located in ice-covered zones during the Quaternary such as Pedregoso and Paloma Araucana (Traful transect), Llao-Llao and Otto (Bariloche transect) and Maitén, displayed little differentiation, which is in accordance with the homogeneity observed in re-colonised areas of the northern hemisphere (see for example Heuertz et al., 2004a,b; Magri et al., 2006). The relatively high level of gene diversity suggests the occurrence of admixture from different refuges during re-colonization, as reported for several European species, conforming "melting pots" of genetic diversity (Petit et al., 2003). In addition, the application of the Bayesian approach STRUCTURE (Pritchard and Wen, 2003) detected mixtures of several ancestral gene pools in populations of the re-colonised area which reflected proportions and composition of gene pools similar to the relict populations San Ramón, Fragua and Chacabuco (Fig. 4). On the other hand, the extreme southern Corcovado population showed both little variation and low admixture compared to the populations of the re-colonised regions over 42°S. Corcovado is situated in an area homogeneously ice covered during the Quaternary and located relatively far away from refuges. It is probable then that this population has arisen from just a few colonisers enriched in ancestral pool 4, and founder events during migration in combination with demo-genetic stochastic processes such as genetic drift could have played important roles in determining within-population genetic diversity.

The fact that (i) ancestral gene pool 2 is highly represented both in most of the re-colonised area and in the steppe populations Chacabuco, San Ramón and Fragua, and was scarcely present in other steppe populations (Fig. 4) and that (ii) deme 4 in BAPS groups San Ramón, Fragua and Chacabuco (the latter when geographical coordinates are included for the analysis) with those populations located in the presumed post-glacial migratory route of the species (Fig. 3), suggests that San Ramón, Fragua and Chacabuco are the most probable relict populations that significantly contributed to post-glacial re-colonization of the eastern Andes species range. Recent studies on temperate and boreal trees indicate that some populations apparently survived the Last Glacial Maximum in peri-glacial environments located probably tens of kilometres from the ice sheets (Anderson et al., 2006; Cheddadi et al., 2006; Shepherd et al., 2007). This is the most likely explanation for the survival of populations in locations such as Fragua or San Ramón, which are situated in areas that comprised the very range limit of the ice during the Quaternary. Even though no continuous pollen records exist from the Patagonian region extending throughout the Holocene, vegetation reconstruction was proposed based on palynological sequences from different deposits (Manzini et al., 2008). The fact that these records indicate the presence of *A. chilensis* within the present-day humid and mesic range at 70°W–71°W shortly after glaciations (Markgraf 1980, 1983, 1984, 1987; Markgraf and Bianchi 1999) points to migration from nearby refuges located in proximity to the ice-cap. This differential contribution of just a subgroup of *A. chilensis* original refuges to post-glacial expansion is in agreement with the proposed "Fragmentation-and-Reclusion-Theory" which establishes that in heterogeneous landscapes, where different temporal and spatial patterns of glaciations took place and where ice reduction was accompanied by significant changes in the environment because of other macro-disturbances such as volcanism or fires, several glacial refuges would have remained isolated with little chance of expansion when the ice retreated, and just a few refuges may have contributed to re-colonization (Gallo et al., 2008). This theory also finds relevant examples in the coastal populations of two southern beeches, (*Nothofagus nervosa and N. obliqua*) that showed endemic and ancestral haplotypes, probably as a result of prolonged isolation and reclusion (Azpilicueta et al., 2009; Marchelli and Gallo 2006). Charcoal sediments imbibed in volcanic ash layers were found in the region around 39°36'S (Heusser et al., 1988) which may have constituted a barrier for the expansion of the extreme *A. chilensis* northeastern relict populations, as reported for *N. nervosa* (Millerón et al., 2008). On the other hand, the strong winds of a predominantly west–east direction during pollination and seed dispersal might have restricted post-glacial gene flow from the easternmost populations, such as Pilcañeu North and Pilcañeu South. The evidence for the presence of glacial refuges in the east and northeast distribution of the specieś range found in this work is in agreement with previous allozyme studies (Pastorino and Gallo, 2002). However, the higher level of polymorphism detected with microsatellites, combined with the use of Bayesian classification approaches, allowed a more detailed description of the patterns of genetic structuring across the studied specieś range, as well as a clear differentiation of gene pools in refuges and their relative contribution to post-glacial spread. These last are essential features for understanding the effect of stochastic climate changes in the evolution of *A. chilensis* populations across the eastern Andes range.

To sum up, the present-day structure of *A. chilensis* across the eastern Andes seems to be shaped by several factors, with Quaternary climate oscillations playing a major role. While glaciations and post-glacial expansion are the most relevant factors shaping among-population differentiation, within-population genetic

diversity is heterogeneous throughout the distribution range with demo-stochasticity playing a minor role in the small populations located at the northeastern limit of the distribution area, whilst being apparently important to populations in the extreme east. Fragmentation due to glaciations, volcanism and forest fires left those relict populations at the eastern border of the species' natural range, which can be considered ancient refuges from pre-Holocene glaciations, but they are not necessarily the irradiation centres from where re-colonization took place. Our data indicates that re-colonization occurred from a limited number of refuges, with peri-glacial populations significantly contributing to post-glacial spread.

Rear edge populations are often considered extremely important for the survival and evolution of the biota, since they are likely to have played key roles in the maintenance of biodiversity throughout the Quaternary (Hampe and Petit, 2005). Peripheral populations of *A. chilensis* could belong to this evolutionary key populations and are therefore of the utmost importance for conservation. They should also be taken into consideration when the assisted migration of individuals for further conservation purposes is considered, in anticipation of global climatic change. The extreme arid conditions might favour a similar directional selection that could have homogenised in most cases their adaptive genetic diversity. The isolation by restricted gene flow and the small effective population sizes might additionally induced, in some of them, the action of other evolutionary important forces. Besides, marginal and relictual populations maintain privative mutations that increase their genetic diversity. All these attributes should encourage the formulation of special conservation strategies of these peripheral populations that are all located on private land, many kilometres far from the Patagonian National Parks.

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