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The effect of different glaciation patterns over the current genetic structure of the southern beech *Nothofagus antarctica*

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Abstract Different regional patterns of glaciation are expected to have brought about a differential effect on the present genetic structure of natural tree populations in the temperate regions. The aim of the present study is to test this hypothesis in *Nothofagus antarctica*, a key tree species of the temperate forests of southern South America. An almost continuous ice layer characterized the region of the Andes south of 41°S, while towards northern latitudes the pattern was more fragmented. Therefore, a higher chance for the location of larger or more numerous glacial refuges in the north of the Argentinean range, leads us to predict a higher genetic diversity in this region. Twelve natural populations of *N. antarctica* were sampled along the northern half of its Argentinean range, including six above 41°S and six below that latitude. Sampled populations were genetically characterized through cpDNA and isozyme gene markers. Both groups of populations were compared by means of several diversity and differentiation parameters. A genetic structure analysis was conducted with isozyme data through clustering and Bayesian approaches. Based on three polymorphic chloroplast regions, only two haplotypes were distinguished, one corresponding to the nine northernmost sampled populations and the other to the two southernmost ones. Only the population located between those two groups resulted polymorphic. AMOVA analyses also revealed a latitudinal genetic structure for the populations surveyed, and higher levels of genetic variation were recognized in the northern populations.

Keywords cpDNA · Genetic variation · Glacial refuges · Isozymes · Ñire · Patagonia

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Introduction

The last glaciation has acted as a major force responsible for modelling the current distribution and genetic structure of natural tree populations in the temperate regions. Numerous studies account for this evolutionary relation; most of them correspond to the northern hemisphere, where glaciation has been much more extensive and continuous than in the south.

Donat (1933) was the first to suggest differentiation processes determined by glacial influence in southern South America. He postulated the existence of a glacial barrier between 48°S and 52°S in order to explain the bicentric distribution of several taxa. Other latitudinal differences in the Patagonian biota were more recently proposed based on the reconstruction of paleo-climates by Markgraf et al. (1996), who suggested a latitudinal division for the evolutionary history of Patagonian vegetation into three areas: south of 51°S, between 51°S and 43°S and north of 43°S.

Different regional patterns of glaciation are expected to have brought about a differential effect on the ecosystems under their influence. A single key species distributed over different glaciation patterns could give the possibility to test this hypothesis. Such a scenario can be found in southern South America.

Based on geological and geomorphologic observations, Flint and Fidalgo (1964, 1969) delineated maps showing

the eastern limit of the ice sheet during Last Glacial Maximum (LGM) between 39°10′ S and 43°10′ S. Glaciated and ice-free areas are distinguished in detail; this allows us to recognize two different glaciation patterns in the north of Argentine Patagonia, roughly delimited by the 41°S latitude.

As described by Flint and Fidalgo (1964, 1969), north of 41°S the water divide follows a clear north–south direction coinciding with a high mountain axis, which forms the political division between Chile and Argentina. The mountain ice sheet nourished from the west by the Pacific humidity exceeded the Andes range through the transverse valleys as single tongues that extended eastward from the high mountains down main valleys, now occupied by lakes. These ice tongues became more sharp-pointed northward, leaving in this direction more ice-free areas between them.

South of 41°S, instead, the water divide is constituted by several mountain ranges with north–south orientation located from 25 to 75 km east of the high mountain axis, thus constituting a broader Cordillera. Those eastern mountains acted as barriers to eastward flow of the mountain ice sheet, which was not thick enough as to surmount most of them. Consequently, the eastern border of the ice followed mainly a north–south direction determined by the water divide. Beyond it, the dry steppe probably did not offer great suitable places for the establishment of forests. On the contrary, the ice-free areas between the "glacial tongues" in the northern region were not as dry as the arid steppe, thus providing better chances for the localization of bigger or more numerous glacial refuges.

Nothofagus antarctica (G. Forster) Oersted, locally called Ñire, is a tree species of the temperate forests of southern South America that ranges along both glaciation patterns. It has a broad ecological range, covering almost 20 latitudinal degrees, in altitudes from the timberline to the sea level, and in extremely opposite humidity conditions, from peat bogs to semiarid areas with less than 500 mm annual precipitation (Ramírez et al. 1985).

Particularly the northern Argentinean range of *N. antarctica* is fragmentary, with pure forest patches of variable dimension scattered in a steppe matrix or in combination with patches of other forest tree species. Mixed patches with *Austrocedrus chilensis* (D.Don.) Pic. Ser. et Bizzarri, *Araucaria araucana* (Molina) K. Koch. and other *Nothofagus* species are common in this region. In contrast, a more continuous pure forest characterizes the region southward of 41°S.

The reconstruction of the recent history of natural populations of *N. antarctica* is one of the objectives of the present study, and palynological antecedents are crucial for this purpose. Unfortunately, such information is scarce and present only in specific studies for scattered locations in Patagonia. In addition, pollen morphology makes it difficult to draw a distinction among the *Nothofagus* species (Heusser 1984). However, some authors can clearly identify six South American *Nothofagus*, among them *N. antarctica* (Markgraf and D'Antoni 1978).

The scattered pollen records suggest the persistence of *Nothofagus* forests in several locations west of the Andes during the last glaciation, mainly along the Pacific Coastal Range (Villagrán 1991), but also along the Central Valley and foothills of the Andes north of the Lake District (42°S) (Heusser 1983, 1984; Villagrán 1991), and even at latitudes between 43°S and 51°S (Markgraf et al. 1996; Ashworth et al. 1991, cit. in Markgraf et al. 1996).

East of the Andes the information between 43°S and 51°S is scarce and, according to one study at Perito Moreno Glacier (50°S) (Mercer and Ager 1983, cit. in Markgraf et al. 1996) apparently there were no trees. On the other hand, local studies from north of 41°30′ S showed evidence to support the presence of *N. antarctica* in the eastern side of the Andes shortly after glaciation, about 14,000 years BP (Markgraf 1984, 1987; Markgraf et al. 1986; Markgraf and Bianchi 1999). Accordingly, several genetic studies support the existence of multiple ice-age refuges east and west of the Andes of different native tree species [e.g. *Nothofagus nervosa* (Phil.) Dim. et Mil. (Marchelli et al. 1998; Marchelli and Gallo 2006), *Fitzroya cupressoides* (Mol.) Johnst. (Premoli et al. 2000), *Austrocedrus chilensis* (Pastorino and Gallo 2002), *Pilgerodendron uviferum* (Don.) Flor. (Premoli et al. 2002)].

The hypothesis of the present study is that the forest ecosystems northward and southward of 41°S have differentially been affected by Last Glacial Maximum, and our prediction is that the degree and distribution of the genetic variation of *N. antarctica* will show a different pattern on either side of this line. We additionally expect a higher genetic diversity and differentiation among the northern populations due to the likely occurrence of multiple glacial refuges there. On the contrary, the southern populations are supposed to be less variable due to post-glacial re-colonization from northern refuges and consequent loss of genetic variants after several founder events.

Our first approach will be based on the screening of polymorphisms on the chloroplast genome through PCR-RFLP. Due to their low mutation rate and maternal inheritance in most angiosperms (Birky 1995), cpDNA markers have been utilized to reconstruct recent life history of several forest tree species, particularly to determine the most likely localization of glacial refuges and to infer post-glaciation migration routes (e.g. Petit et al. 2002). Different cpDNA lineages in natural populations are evidence of their different origins. Nuclear genes will also be surveyed by using isozyme markers, which due to their bi-parental inheritance, higher variability and higher mutation rate, are

expected to reflect the impact of processes suffered during the last generations after deglaciation. The bi-parental inheritance implies that the influence of pollen-mediated gene flow, which is usually much more extended than the exchange of genes through seeds, can be detected in the patterns of the genetic variation observed with these markers.

Materials and methods

Sampling

Twelve natural *Nothofagus antarctica* populations of the northern half of its Argentinean range were sampled (Table 1), six located northward and six southward of 41°S. Buds from a total of 674 trees equally distributed between both groups were collected in winter. In each population, trees were chosen keeping a minimum distance of 30 m among them in order to reduce the risk of sampling related individuals. Samples were kept at −18°C until laboratory analyses.

Chloroplast DNA

DNA was extracted from buds following the protocol described by Dumolin et al. (1995) with slight modifications as mentioned in Marchelli and Gallo (2006). A total of 60 individuals was analysed (five from each of the 12 populations). Twenty trees originating from 10 populations (two individuals per population) were employed to screen chloroplast DNA for polymorphisms. Different combinations of universal primers (Heinze 2007) were used to amplify 11 intergenic non-coding regions: y*cf*3-*ccmp*6 (Heinze 2007; Weising and Gardner 1999), *atp*H-*atp*I (Grivet et al. 2001), *trn*D-*trn*T (Demesure et al. 1995); *trn*S-*rps*4 (Demesure et al. 1995; Souza-Chies et al. 1997), *ucp*-a/*trn*T-*ucp*-b/*trn*L (Taberlet et al. 1991), *rpo*C1-f5-*rpo*C1 exon2 (Grivet et al. 2001; Liston 1992), *atp*I-*ccmp*5 (Grivet et al. 2001; Weising and Gardner 1999), *trn*K1-*trn*K2 (Demesure et al. 1995), *mat*Kf2-*ccmp*1 (Heinze 2007; Weising and Gardner 1999), *trn*K2-*trn*Q (Dumolin-Lapègue et al. 1997), *ccmp*5-*rpo*C2-r5 (Weising and Gardner 1999; Grivet et al. 2001). Ten chloroplast DNA microsatellites (cpSSRs) were additionally tested: *ccmp*1, *ccmp*2, *ccmp*5, *ccmp*6, *ccmp*7 (Weising and Gardner 1999), *mdt*1, *mdt*3, *mdt*4, *mcd*4, *mcd*5 (Deguilloux et al. 2003). Amplifications were carried out in a total volume of 25 µl containing about 15 ng of template DNA, 1.6 mM of $MgCl_2$, 100 µM of each dNTP, 0.2 µM of each primer and 1 U of *Taq* polymerase (Invitrogen-Gibco) with the respective 1× PCR buffer. A common PCR program (Heinze 2007) was used for all the intergenic non-coding regions and for five of the cpDNA SSRs (Weising and Gardner 1999): 3 min at 94°C, followed by 10 cycles of 50 s at 94°C and 1 min at 70°C, then 35 cycles of 50 s at 94°C, 50 s at 55°C and 2 min at 70°C, with a final extension step of 10 min at 70°C. For the remaining cpDNA SSRs the PCR conditions were as proposed by Deguilloux et al. (2003) and the following cycling temperatures were employed: 5 min at 94°C, 25 cycles of 1 min 94°C, 1 min at 45°C and 1 min at 72°C, with a final extension step of 8 min at 72°C. The reactions were performed either in a Biometra Uno-Thermo Block or in a MJResearch PT-200 thermo cycler. In those cases where amplification of the intergenic regions was positive, 8 µl of PCR product were digested by 5 U of restriction endonuclease *Hin*fI or *Taq*I in a total volume of 23 µl. Digestion was performed at 65°C for 3 h in the case of *Taq*I and at 37°C overnight in the case of *Hin*fI. The total digestion volume was loaded into 8% non-denaturating polyacrylamide gels. Electrophoresis was carried out in TRIS borate EDTA (1×) at 300 v for 4 h. Visualization of the fragments was done under UV light after staining with ethidium bromide. Gel documentation was obtained with a digital camera, and images were analysed with the BioDoc Analyse version 2.0 (Biometra).

Three chloroplast regions showed polymorphism in the subsample: *atp*H-*atp*I; *trn*D-*trn*T, both after digestion with *Hin*fI, and y*cf*3-*ccmp*6 digested with *Taq*I. These regions were then screened in all the individuals ($N = 60$). One individual of each variant for each chloroplast region (six in total) was sequenced in an ABI3100 facility (Applied Biosystems) in order to confirm the polymorphisms. The regions were amplified as mentioned above and purified using the Wizard Kit of Promega. Products were sequenced using the Big Dye 3.1 terminator cycle sequencing kit

Table 1 Location of the sampled *Nothofagus antarctica* populations ordered from north to south

Population	Latitude S	Longitude W	Altitude (m a.s.l.)
Northern group			
Roblecillos (R)	36°40′	70°48′	1,400
Caviahue (Ca)	37°50′	71°01′	1,700
Tromen (T)	39°36′	71°27′	850
Quilanlahue (Q)	40°08′	71°28′	950
Correntoso (Co)	40°38′	71°39′	850
Córdoba (C)	40°39′	71°09′	850
Southern group			
Guillelmo (G)	41°25′	71°29′	850
El Foyel (Fo)	41°40′	71°29′	750
Ternero (Te)	41°57′	71°22′	800
Cholila (Ch)	42°32′	71°32′	550
Futalaufquen (F)	42°56′	71°35′	850
Trevelin (V)	43°10′	71°24′	650

(Applied Biosystems) and analysed with MEGA 3.1 (Kumar et al. 2004).

Polymorphic fragments were labelled by decreasing order of fragment size as visualized in the polyacrylamide gels. Point mutations in the restriction endonuclease motif were denoted with a 9. Haplotypes were defined according to different combinations of length variants, following the procedure of Demesure et al. (1996). The average within-populations gene diversity (h_S), the total gene diversity (h_T) and the gene differentiation over all populations (G_{ST}) were estimated according to Pons and Petit (1995) using the program HAPLODIV available at http://www.pierroton.inra.fr/genetics/labo/Software/.

Isozymes

Isozyme genotypes were determined by subjecting buds of each of the 674 sampled trees to horizontal starch gel electrophoresis. Laboratory procedures followed those described by Stecconi et al. (2004) with slight modifications. Vegetative extraction buffer I from Cheliak and Pitel (1984) and a discontinuous buffer system was utilized on routine: 0.3 M boric acid/0.06 M NaOH till pH 8.2 (Poulik 1959); gel: 0.07 M Tris/0.008 M citric acid till pH 8.5; starch 10.5% w/v, sucrose 2% w/v; for 6 h at 80 mA. Two isozymes with their genetic control previously determined (Pastorino et al. 2008) were revealed with the staining solutions proposed by Cheliak and Pitel (1984): phosphoglucose isomerase (PGI, E.C.5.3.1.9) and phosphoglucomutase (PGM, E.C.2.7.5.1).

Allelic and genotypic frequencies were calculated for each population by means of GSED 1.1d (Gillet 1997), and subsequently the following diversity parameters were estimated: allelic richness r_g, with CONTRIB 1.02, available at http://www.pierroton.inra.fr/genetics/labo/Software (Petit et al. 1998), effective number of alleles A_e, with GSED 1.1d, expected heterozygosity H_e and observed heterozygosity H_o, using Arlequin 3.0 (Excoffier et al. 2005). Allelic richness was calculated after rarefaction to a common sample size (g, the smallest) in order to standardize and avoid the bias of the uneven sample sizes of the different populations. A corrected value was expressed by subtracting one from that calculated after the Hulbert (1971) formula (El Mousadik and Petit 1996).

Differences in allelic frequencies among populations were tested by means of an exact test with Arlequin 3.0. Once homogeneity was denied, differentiation was expressed by means of Gregorius' (1985) D_j and δ, calculated with GSED 1.1d and considering all the populations as infinite, and with the more widely-known F_{ST} of Wright (1978) obtained with Arlequin 3.0.

Genetic structure of the sampled populations was analysed through different approaches. First, a cluster analysis based on Gregorius' (1974) genetic distances between populations (d_0) was made using the UPGMA linkage procedure (Sneath and Sokal 1973) with SAS software (1989). A second approach was achieved by utilizing the software package BAPS 4.14 (Corander et al. 2003), based on Bayesian inference. Geographic coordinates of each population were added in this case. In order to transform geodesic in geocentric coordinates, the program Transcoord TS was utilized (Olondriz and Brunini 1998).

Subsequently, a partitioning of the molecular variance among and within groups (Excoffier et al. 1992) was performed in each of the three groupings proposed [i.e. the original hypothesis (northern and southern groups), the result of the cluster analysis based on d_0 genetic distance, and the structure resulting from the Bayesian approach], by means of locus-by-locus AMOVA analyses using Arlequin 3.0.

In order to test the hypothesis that northern populations are more variable than southern ones, two groups were formed by pooling the trees of the populations northward and southward of 41°S in each of them. Thus, each group contained a similar number of trees from six populations. A homogeneity G test (Sokal and Rohlf 1981) of the allelic frequencies was performed between these two groups with the help of GSED 1.1d, and their diversities were characterized through the parameters described above.

However, this procedure implied a certain bias since sample sizes of each population were neither equal nor proportional to the corresponding population sizes. Thus the over-represented populations brought about a greater weight on the calculated diversity parameters. As an alternative, the average value among the populations of each group was calculated for each of the parameters.

Results and discussion

Chloroplast DNA

The three polymorphic cpDNA fragments scored in all the populations consisted in one insertion/deletion in fragment *ycf3-ccmp6* and two restriction site mutations, one in each of the other two analysed fragments. These polymorphisms allowed the identification of two haplotypes separated by three mutations. The analysis of the DNA sequences confirmed the presence of an insertion/deletion of 14 bp in fragment *ycf3-ccmp6*.

The distribution of these two haplotypes was geographically structured in a latitudinal way, since the nine northernmost sampled populations bore one haplotype and the two southernmost ones held the other. The only polymorphic population was Cholila (42°32′ S), which is situated at a latitude between those two groups of

populations (Fig. 1). Consequently, the genetic diversity calculated at the species level resulted obviously very low ($h_s = 0.033$, s.e. $= 0.033$; $h_t = 0.324$, s.e. $= 0.141$), and the differentiation among populations very high ($G_{ST} = 0.897$, s.e. $= 0.113$). This was also the case for other *Nothofagus* species of these forests [$G_{ST} = 0.93$ for *N. nervosa* (Marchelli and Gallo 2006); $G_{ST} = 0.757$ for *N. obliqua* (Azpilicueta et al., submitted.)].

Taking into consideration the very low mutation rate of the chloroplast genome (Clegg et al. 1994), the existence of two groups of populations whose haplotypes are differentiated by three mutations suggests a long lasting isolation in at least two different glacial refuges from which the analysed populations of *N. antarctica* could have spread out after the last glacial period. The larger distribution of the northern haplotype might be related with the two different glaciation types. The northern, less glaciated region could have been re-colonized first, since glacial retreat could be assumed to have been faster. In agreement with this suggestion, the few, local registers of fossil pollen reported that forest expansion was delayed by about 3,000 years east of the Andes at latitudes south of 43°S (Markgraf et al. 1996). Thus, a refuge located in the northern region between the "glacial tongues", as suggested for other studied species (Marchelli and Gallo 2006; Azpilicueta et al., submitted), would have expanded after glaciation retreat by re-colonizing the valleys now free of ice. Southward of 41°S, instead, a refuge could only have been situated in the steppe, just in front of the glacier. This idea has been already proposed for other three drought tolerant tree species of Patagonia: *Austrocedrus chilensis* (Pastorino and Gallo 2002), *Nothofagus obliqua* (Azpilicueta et al., submitted) and *Araucaria araucana* (Marchelli et al., submitted).

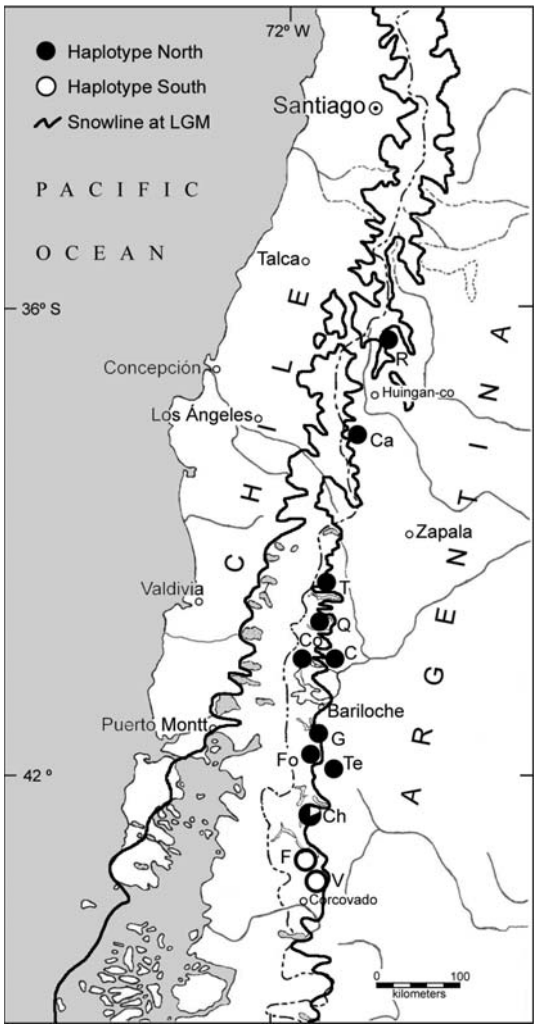

Fig. 1 Snowline at Last Glacial Maximum (LGM) according to Hollin and Schilling (1981) and *Nothofagus antarctica* sampled populations showing the distribution of the two cpDNA haplotypes found (full circle corresponds to the northern haplotype, empty circle to the southern haplotype)

The presence of the two haplotypes at Cholila could be indicating a secondary contact zone with the admixture of migratory routes. Meeting points usually result in transition zones of highly diverse populations (e.g. Vendramin et al. 1998; Mátyás and Sperisen 2001). This possible admixture of two migratory routes seems to have been relatively recent, since only one population turned out to be polymorphic, which is precisely located between the two groups of monomorphic populations.

Latitudinal variation in the distribution of haplotype variants and low levels of polymorphisms were also observed in other *Nothofagus* species (Marchelli and Gallo 2006; Millerón et al. 2008; Azpilicueta et al., submitted) suggesting a general trend in these forests. The low levels of cpDNA variation observed for the *Nothofagus* species analysed so far contrast with those of other forest trees where several haplotypes were usually identified (see review in Petit et al. 2005). Regarding our samples, Cholila is the only population which presented evidence of seed-mediated gene flow among populations of both groups.

Latitudinal differentiation among cpDNA variants was also observed in plant species from North America (Soltis et al. 1997; Magni et al. 2005). Moreover, a marked difference in the historical colonization between oak species from North America and Europe was interpreted as the response of the species to different glacial scenarios. Whereas in Europe oaks were separated from the ice sheet by a widespread tundra, in North America oaks grew close to the ice sheet and therefore movement after LGM was moderate (Magni et al. 2005; Grivet et al. 2006). Evidence in South America seems to support a scenario similar to that of North America.

The lack of detailed palynological information in southern South America is a constraint to further argue the possible location of glacial refuges. Moreover, the low

levels of polymorphisms detected at the chloroplast DNA avoid further speculations on the recolonization history with this sole source of information.

Isozymes

Allelic frequencies and the genetic variation parameters of the analysed populations are shown in Table 2. Regarding the mean values of those parameters among the 12 populations, the intra-population variation should be considered as moderate at the species level. Quilanlahue population presented the lowest values of intra-population variation (Table 2). An effective number of alleles per locus (A_e) of only 1.028 is evidence of virtual monomorphism in the two sampled genes. Contrasting, Correntoso population, which is near to it, is the most diverse, as seen through A_e (1.446) and H_e (0.307).

Significant differences in allelic frequencies were observed between the northern and the southern groups of pooled populations for both surveyed loci ($P_{PGI} = 0.0018$ and $P_{PGM} < 0.0001$). Both the pooling and the average-among-populations alternatives to compare northern and southern populations showed a higher genetic diversity in the north regarding the number of effective alleles (A_e) and expected (H_e) and observed (H_o) heterozygosities (Table 2). However, the results diverged between both alternatives when the allelic richness was considered. The pooling procedure threw a higher richness in the north but the average-among-populations showed the opposite. This result is mainly caused by the unexpectedly rather frequent presence of the rare allele *Pgi2-120* in the southern population El Foyel (9%), and the virtual monomorphism of both markers in the northern population Quilanlahue. In any case, one should consider that all alleles present in the southern group are also present in the northern group, while one of the alleles of the northern group could not be found in the south (*Pgm2-96*).

Genetic differentiation was moderate at the species level ($\delta = 0.082$; $F_{ST} = 0.109$), being the northern populations the most differentiated (Table 2). The average value of D_j among northern populations (0.106) is quite larger than among the southern ones (0.065), in agreement with the fragmentary condition of this part of the range. Restricted gene flow among fragments would explain larger differentiation. In the south portion of the region sampled, instead, lower differentiation seems to respond to the expectation for a widespread species (Hamrick et al. 1992), in which pollen-mediated gene flow is typically extensive.

Table 2 Number of analysed trees (No. t.), isozyme allelic frequencies and intra-population variation and differentiation parameters of the sampled *Nothofagus antarctica* populations and the northern and southern groups

Populations	*Pgi2*						*Pgm2*					Variation parameters				
	No. t.	Alleles					No. t.	Alleles								
		100	82	78	60	120		100	90	75	96	r_g	A_e	H_e	H_o	D_j
Roblecillos (R)	55	0.945	0.027	0.027	0.000	0.000	54	0.676	0.324	0.000	0.000	1.200	1.372	0.274	0.305	0.104
Caviahue (Ca)	65	0.969	0.000	0.008	0.008	0.015	54	0.537	0.463	0.000	0.000	1.017	1.386	0.279	0.197	0.189
Tromen (T)	58	0.983	0.000	0.000	0.017	0.000	23	0.739	0.261	0.000	0.000	0.763	1.265	0.214	0.148	0.089
Quilanlahue (Q)	51	0.990	0.010	0.000	0.000	0.000	28	0.982	0.018	0.000	0.000	0.498	1.028	0.027	0.028	0.100
Correntoso (Co)	52	0.894	0.019	0.087	0.000	0.000	18	0.694	0.306	0.000	0.000	1.279	1.446	0.307	0.300	0.115
Córdoba (C)	53	0.962	0.009	0.028	0.000	0.000	20	0.850	0.100	0.025	0.025	1.928	1.204	0.173	0.163	0.038
Guillelmo (G)	57	0.930	0.009	0.053	0.000	0.009	51	0.784	0.167	0.049	0.000	1.714	1.322	0.246	0.168	0.042
El Foyel (Fo)	89	0.820	0.034	0.056	0.000	0.090	76	0.980	0.020	0.000	0.000	1.593	1.215	0.178	0.171	0.141
Ternero (Te)	50	0.930	0.010	0.060	0.000	0.000	33	0.924	0.076	0.000	0.000	1.141	1.157	0.137	0.146	0.056
Cholila (Ch)	43	0.930	0.012	0.047	0.012	0.000	31	0.935	0.065	0.000	0.000	1.351	1.145	0.128	0.111	0.061
Futalaufquen (F)	46	0.957	0.011	0.033	0.000	0.000	45	0.856	0.144	0.000	0.000	1.085	1.198	0.167	0.121	0.015
Trevelin (V)	48	0.958	0.010	0.031	0.000	0.000	40	0.962	0.038	0.000	0.000	0.987	1.083	0.076	0.079	0.073
Mean	55.6	0.939	0.013	0.036	0.008	0.010	39.4	0.827	0.165	0.006	0.002	1.213	1.235	0.185	0.161	0.082
Pool of northern populations	334	0.958	0.011	0.024	0.005	0.003	197	0.707	0.288	0.003	0.003	3.380	1.333	0.250	0.206	–
Pool of southern populations	333	0.908	0.017	0.048	0.002	0.026	276	0.909	0.082	0.009	0.000	2.794	1.203	0.169	0.139	–
Average northern populations	55.7	–	–	–	–	–	32.8	–	–	–	–	1.114	1.284	0.212	0.190	0.106
Average southern populations	55.5	–	–	–	–	–	46.0	–	–	–	–	1.312	1.187	0.155	0.133	0.065

Allelic richness (r_g)for a rarefaction of 36 genes in the single populations and 392 genes in the groups, effective number of alleles (A_e), expected heterozygosity (H_e), observed heterozygosity (H_o) and gene-pool differentiation (D_j)

The northern population Caviahue was the most differentiated one, as measured by D_j (0.189), probably due to the good allele frequency balance in *Pgm2* locus (it is the population with the most even structure in this marker), and the presence of four alleles in *Pgi2*. The southern population El Foyel followed ($D_j = 0.141$), in this case due to the relative high frequency of the rare allele *Pgi2-120* (Fig. 2).

The region surveyed roughly coincides with that of a population genetic study of another native tree species: *A. chilensis*, also based on isozyme markers (Pastorino et al. 2004). In the case of this conifer, the northern populations also turned out to be the most variable.

Through the homogeneity exact test among populations significant differences were revealed ($P < 0.0001$). Two clear groups can be recognized in the UPGMA dendrogram: the smaller one constituted by four of the northern populations, and the other by Quilanlahue, Córdoba and those of the southern half of the sampled region (Fig. 3). A single population clearly separated in each group: Caviahue and Foyel, in the northern and southern groups respectively.

The best structure from the Bayesian analysis resulted in three groups: one with the northern populations Roblecillos, Tromen and Caviahue, other with Foyel solely, and the third one constituted by the rest [log (marginal likelihood) of the optimal partitioning $= -868.044$]. Thus, besides the explicit separation of the northern and the southern populations of the original hypothesis, in the other two structures proposed (those resulting from the dendrogram and the Bayesian approach), a northern group could also clearly be seen. This evidenced a general latitudinal trend.

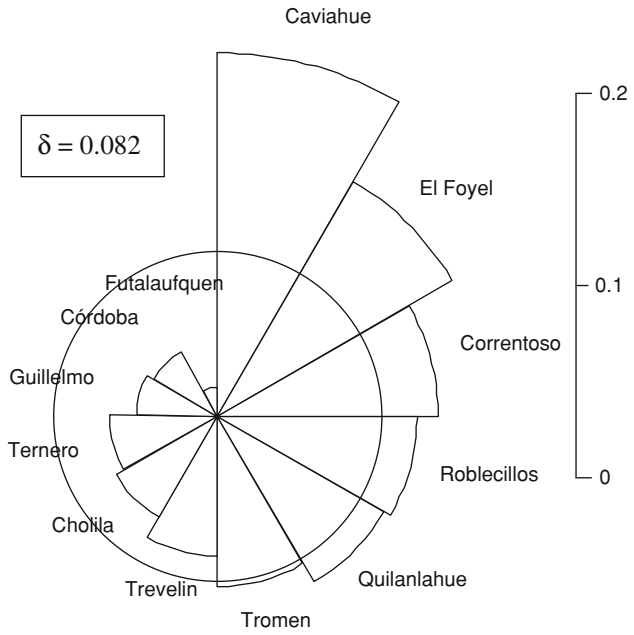

Fig. 2 Allelic differentiation snail for the gene pool in *Nothofagus antarctica* sampled populations. Length of the radii of each "pie portion" is proportional to D_j, and radius of the circle is proportional to δ

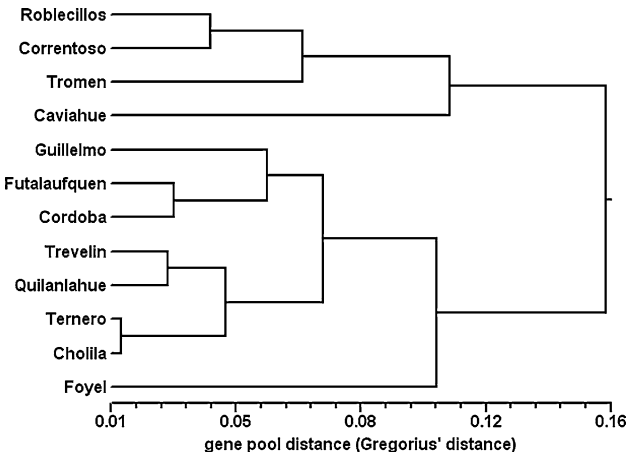

Fig. 3 UPGMA dendrogram with gene pool distances at two isozyme loci between 12 *Nothofagus antarctica* natural populations

According to the AMOVA analyses, the structure revealed by the UPGMA dendrogram based on Gregorius' genetic distance appeared to be the best. It showed that almost all of the variation not explained by the intrapopulation variation lay on the variation among groups (15.59%), and additionally revealed the lowest percentage of variation among individuals within populations (81.81%) (Table 3).

The AMOVA analyses showed a good structure for the three proposed groupings, since the variation among-groups resulted in the three cases higher than the variation among-populations (all covariance components associated with the different possible levels of genetic structure were significant, see Table 3). Namely, the AMOVA analyses serve as additional evidence to support a latitudinal structure of the sampled populations.

Final considerations

The combination of two marker types characterized by very different evolutionary dynamics, such as cpDNA and isozymes, is desirable in order to get a complete picture of the genetic pattern of the natural populations of a species. The results of both marker types are not necessarily coincident but complementary (Avise 1994), and both sources of information have been recommended in order to identify evolutionary significant units (Moritz 1994).

Two groups of populations with a latitudinal distribution were identified by the cpDNA markers, which could be assumed as being of different origin. A merging region resulting from seed-mediated gene flow was also detected. This result rules out the hypothesis of the southern populations deriving from a northern refuge. However, it serves

Table 3 Results of the locus-by-locus AMOVA analyses showing the partitioning of the variation among hierarchical components according to the different genetic structures proposed (in percentage, with *P* values between brackets)

	Among groups	Among populations	Within populations
Without grouping	–	10.99 (<0.001)	89.00 (<0.001)
North/South hypothesis	8.02 (0.031)	6.25 (<0.001)	85.72 (<0.001)
Dendrogram on genetic distance	15.59 (0.003)	2.60 (<0.001)	81.81 (<0.001)
Bayesian analysis (BAPS)	12.33 (0.008)	2.60 (<0.001)	85.07 (<0.001)

to corroborate a latitudinal pattern of genetic variation. Likewise, the isozyme approach allowed the identification of other latitudinal groups undetected with chloroplast markers, therefore providing additional insights. These nuclear markers were also useful to recognize the higher genetic variation of the northern populations. Different genetic variation patterns between north and south are hence evident, although not with a strict division at 41°S. We can nevertheless confidently sustain a general latitudinal trend in the genetic structure of *N. antarctica*, in agreement with the latitudinal pattern of glaciation.

To sum up, in the eastern foothills of the Andes range, along the northern half of its current distribution area, *N. antarctica* would have survived last glaciation in at least two refuges, one situated somewhere north of 42°30′ S, and the other somewhere south of that latitude. On the other hand, the higher differentiation among the northern populations as revealed by the nuclear markers, would be evidence of several patches with restricted gene flow among them (even pollen-mediated gene flow). However, those patches would have a unique origin (due to be carrier of the same cpDNA haplotype), and thus two alternative hypotheses arise as possible: either (1) migration effectively occurred after glaciation from a unique refuge located in any of the transversal valleys, as to re-colonize the previously glaciated areas, and thus the isolation among those patches is only a feature of the last generations, or (2) the patches located in different valleys derived through a fragmentation process caused by glaciation from a somehow continuous forest existing before LGM, thus creating several refuges but with a unique haplotype. The second alternative matches the already proposed multiple refuges model (Marchelli and Gallo 2006), and sounds more plausible. In any of both scenarios, and closer to the present days, the northern refuge (or refuges) would have extended its (their) influence up to 42°30′ S, where the northern genes would have merged with the southern ones.

These results, although matching the previous information on other Patagonian tree species, should be confirmed with additional information. First of all, more nuclear genes are desirable to support the derived conclusions. Likewise, additional polymorphic cpDNA fragments could be the key to identify different haplotypes among the northern populations of *N. antarctica*. Also the screening of more southern populations, and even those of Tierra del Fuego Island, will cast light on the evolutionary relations between LGM and the genetic patterns of the main forest trees of Patagonia. The consideration of the widespread *Nothofagus pumilio* (Poepp. et Endl.) Krasser, which has a roughly coincident latitudinal range with our object of study, would be relevant in this regard. Ongoing research lines will give insights into the evolutionary history of these widespread species.

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