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Chloroplast DNA markers reveal a geographical divide across Argentinean southern beech *Nothofagus nervosa* (Phil.) Dim. et Mil. distribution area

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Abstract *Nothofagus nervosa* is one of the most important species of the temperate forests of southern South America. On the eastern slope of the Andes Mountains (Argentina) it has a very small and narrow natural distribution area which follows the valleys of the numerous west-east lake basins. Re-colonisation after the last glaciation is assumed to have originated from refugia located in the western pacific coast or even in free interglacial patches of the Andes Mountains. Sixteen pairs of primers were used to amplify 16 non-coding regions of chloroplast DNA in 11 populations from over the entire distribution area in Argentina. After restriction analysis two polymorphic fragments were found which defined two haplotypes and allowed a differentiation among populations. A clear geographic divide occurs separating populations distributed south and north of a west-east mountain chain. This suggests that after the last glaciation the species spread out from at least two different refugia. Hypotheses about the possible location of these refugia are discussed.

Key words Chloroplast DNA · Intraspecific variation · Geographical differentiation · Glacial refugia · *Nothofagus* · *Fagaceae*

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Introduction

The temperate forest in southern South America lies on both sides of Andes Mountains covering a broad latitudinal range (33°–55° S). The genus *Nothofagus*, with its ten species, is the most important component of this forest.

During the Quaternary this area was affected by glacial-interglacial cycles. By the time of the last glaciation, 18 000–20 000 years ago, glaciers covered most of the region. However, and in contrast with glaciations in the Northern Hemisphere, in the Southern Hemisphere glaciation occurred in patches leaving many ice-free areas which provided potential refugia for forest taxa, even at high latitudes (55°S) (Markgraf 1983; Markgraf et al. 1995). At central latitudes (36°–42°S), on the west side of the Andes (Chile), relatively wet and cold glacial climates allowed forests to persist. On the east side (Argentina), the steppe expanded towards the Andes and forests were greatly reduced (Markgraf 1983). Refugia for *Nothofagus* species were probably in the pacific coastal mountains and on both sides of the Andes Mountains. The deciduous species *Nothofagus obliqua* and *Nothofagus nervosa* seemed to have survived the glaciations within these refugia (above 41°S) and after the ice retreated they expanded to the south along the Andes Mountains (Villagran 1991).

Our study is on *Nothofagus nervosa* (Phil) Dim. et Mil. (Lennon et al. 1987) (= *N. alpina* = *N. procera*) a deciduous, wind pollinated, anemochorous angiosperm tree species. In Argentina it has a very small distribution area. It covers only 55 000 hectares in a narrow fringe of about 120 km in length and about 40 km in maximum width, following the numerous west-east lake basins. This particular distribution is probably related to the pattern of the last glaciation. Re-colonisation after the ice retreated could have occurred either from western or eastern Andes refugia, but mostly following the west-east glacial valleys. Therefore latitudinal genetic differentiation is likely to be expected.

Despite the slow rate in sequence evolution of chloroplast DNA (cpDNA) (Palmer 1987; Clegg et al. 1991) intraspecific variation has been reported for cpDNA in many forest tree species (Ferris et al. 1993; Tsumura et al. 1994; Ziegenhagen et al. 1995; Demesure et al. 1996; Dumolin-Lapègue et al. 1997 b). Ferris et al. (1993) found a clear east/west division across European oaks. Since the inheritance of cpDNA is uniparental, and predominantly maternal in angiosperms, its gene flow is reduced compared to nuclear gene flow. Therefore, differences in cpDNA sequence can persist allowing for population differentiation and the reconstruction of post-glaciation migration routes (Kremer and Petit 1993; Petit et al. 1993; Dumolin-Lapègue et al. 1997 b).

The small distribution area in Argentina, its overexploitation in the past due to its high wood quality, and its relatively fast growth, make *N. nervosa* an important species for conservation and breeding programs. The study of the genetic variation and evolutionary history of the species will greatly contribute to orientate such programs. We present here an analysis of the geographic differentiation among 11 populations of *N. nervosa* situated along the natural distribution area of the species in Argentina as revealed by cpDNA polymorphisms.

Material and methods

Plant material

N. *nervosa* seeds were harvested as a bulked collection in 11 populations selected from all over its small natural distribution area in Argentina. Analyses were carried out on embryos as well as on germinated seedlings. Embryos were shock-frozen in liquid nitrogen and stored at −20°C until DNA extraction was performed. Cotyledons and leaves were cut from the seedlings and shock-frozen just before DNA extraction. Ten embryos or ten seedlings from each of 10 of the 11 populations were analysed for two different cpDNA regions (*trn*D-*trn*T and *psa*A-*trn*S, Demesure et al. 1995). As no within-population variation was found, sample size was reduced down to two embryos or two seedlings per population for analysing another 14 different cpDNA regions. Moreover, four embryos from an additional population (population no. 11) were analysed only for the polymorphic fragments.

DNA extraction

Embryos

Embryos were separated from the seeds and total DNA was extracted using a DNeasy Plant Kit (QIAGEN, Hilden, Germany, see instructions of the manufacturer) and a mini-preparation procedure as described by Ziegenhagen et al. (1993).

Cotyledons and leaves

Total DNA was extracted according to Dumolin et al. (1995) with slight modifications including a final treatment with 0.5 µg RNaseA (Boehringer Mannheim, Germany) at 37°C for 30 min.

PCR amplification

All primer pairs used in this study were described by Demesure et al. (1995) and Dumolin-Làpegue et al. (1997 a). Table 1 presents the primers, the annealing temperatures and restriction enzymes. PCR amplification was carried out in a total volume of 25 µl containing about 20 ng of template DNA, 2.5 mM of $MgCl_2$, 100 µM of each dNTP, 0.2 µM of each primer and 0.25 U of *Taq* polymerase with the respective 1 × PCR buffer (*Taq* polymerase and 10 × PCR buffer purchased from Eurogentec, Ougree, Belgium) following the cycle profile as described by Demesure et al. (1995). PCR was run in the Touch-Down™ Thermal System (Hybaid Limited, Teddington, UK).

Digestion

For restriction analysis, 5 µl of PCR products were digested by 5 U of restriction endonuclease according to the primer/enzyme combinations shown in Table 1, in a total volume of 20 µl. Digestion was performed at 65 °C for 3 h in the case of *Taq*I and at 37 °C for 4 h to overnight in the case of the other enzymes. The total digestion volume was loaded into the gel.

Visualisation of PCR products and restriction fragment patterns

PCR products were checked by electrophoresis in 0.8% agarose gels run in TRIS borate EDTA (0.5 x) at 80 V for about 2 h.

Table 1 Primer pairs, annealing temperatures and restriction enzymes used on cpDNA of *N. nervosa*

Primer pair	Abbreviations	Annealing temperature (°C)	Restriction enzyme
*trn*D-*trn*T	DT	50	*Hae*III
			*Taq*I
*psa*A-*trn*S	AS	57	*Hin*fI
*trn*F-*trn*Vr	FV	57.5	*Taq*I
*trn*T-*trn*F	TF	57	*Taq*I
			*Hin*fI
*trn*C-*trn*D	CD	58	*Taq*I
			*Hin*fI
*trn*M-*rbc*L	ML	58	*Taq*I
			*Hin*fI
*trn*H-*trn*K	HK	62	*Taq*I
			*Hae*III
*rpo*C1-*trn*Cr	rpoCC	47.5	*Taq*I
			*Hin*fI
trnK_1-*trn*K_2	K_1K_2	52.5	*Taq*I
			*Hin*fI
*trn*V-*rbc*Lr	VL	57.5	*Taq*I
			*Hin*fI
*trn*Q-*trn*Rr	QR	56.5	*Taq*I
			*Hin*fI
trnK_2-*trn*Qr	K_2Q	47.5	*Taq*I
		52	–
		57	*TaqI*
*trn*S-*trn*T	ST	57	*Taq*I
		54	*Taq*I
*trn*S-*trn*fM	SfM	62	*Taq*I
		57	–
*trn*T-*psb*Cr	TC	52.5	–
		50	*Taq*I
*trn*fM-*psa*Ar	fMA	47.5	*Taq*I
		50	*Taq*I

Restriction fragments were separated by electrophoresis in 8% non-denaturating polyacrylamide gels run in TRIS borate EDTA (1 x) at 300 V for 3–4 h.

PCR products, as well as restriction fragment patterns, were visualised by UV fluorescence after staining with ethidium bromide (0.25 µl/ml staining solution).

Analysis of variation

In our study, polymorphism occurred as length variation of the restriction fragments. 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.

Results

DNA extraction

N. nervosa is generally assumed to be a difficult subject for DNA extraction, particularly from embryos, due to high levels of polysaccharides, and from buds, because of the presence of resins. Few reports on DNA extraction from this species exist [e.g. Manos (1997) from leaf material]. In a preliminary study one of us succeeded in extracting DNA from buds of *N. nervosa* and *N. obliqua* using the procedure described by Ziegenhagen et al. (1993) (Gallo 1996, internal report). In the present study different procedures were tried with different results. DNA from leaves and cotyledons was successfully extracted following the procedure of Dumolin et al. (1995). From embryo tissue, DNA was extracted using a DNeasy Plant Kit (QIAGEN, Hilden Germany) and also by using the mini-preparation procedure described by Ziegenhagen et al. (1993); both of them gave good results.

PCR products

Among the 16 pairs of chloroplast primers employed in this study, ten (DT, AS, FV, CD, ML, HK, rpoCC, K_1K_2, VL, and QR) yielded distinct amplification products, although in some cases the lengths were slightly different to those described by Demesure et al. (1995) and Dumolin-Làpegue et al. (1997) (Table 2). In the case of the primer pair *trn*Q-*trn*Rr two PCR products were obtained, while using the primer pair *trn*K_2-*trn*Qr three amplification products were formed. The remaining four (ST, SfM, TC, and fMA) gave poor amplification products.

As far as could be judged from agarose gels, the lengths of the amplification products did not differ between individuals or populations.

Restriction analysis

Restriction analysis revealed a length polymorphism in two PCR products (DT and FV, Table 3). Figure 1

Table 2 Lengths of amplification products as detected in agarose gels

Primer pair	Length of amplification products in bp
*trn*D-*trn* T	1100
*psa*A-*trn* S	3000
*trn*F-*trn*Vr	3000
*trn*T-*trn*F	1600
*trn*C-*trn*D	3000
*trn*M-*rbc*L	3000
*trn*H-*trn* K	1800
*rpo*C1-*trn*Cr	4500
trnK_1-*trn*K_2	2500
*trn*V-*rbc*Lr	3800
*trn*Q-*trn*Rr	3100/1900
trnK_2-*trn*Qr	3000/2000/1400
*trn*S-*trn* T	1300
*trn*S-*trn*fM	1600
*trn*T-*psb*Cr	3500
*trn*fM-*psa*Ar	5000/1200/1100

Table 3 Polymorphic fragments, labelled in decreasing order of fragment size, and defined haplotypes

Population number	DT *Taq*I band 2	DT *Hae* III band 2	FV *Taq*I band 2	Haplotype
5	1	2	1	I
6	1	2	1	I
10	1	2	1	I
16	1	2	1	I
17	1	2	1	I
19	1	2	1	I
21	1	2	1	I
24	1	2	1	I
27	2	1	2	II
28	2	1	2	II
30	2	1	2	II

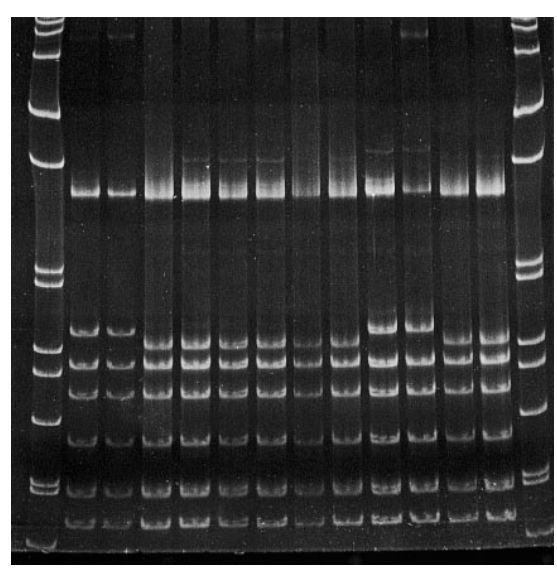

Fig. 1 Restriction patterns observed for FV cpDNA region digested with *Taq*I. *Lanes 1* and *14* kb ladder; *2 and 3:* population 16; *4–7* population 30; *8 and 9* population 27; *10 and 11* population 24; *12 and 13* population 28

shows the restriction patterns observed for primer pair FV and subsequent digestion with *Taq*I. Digestion of the PCR product obtained by primer pair DT resulted in length polymorphism for both enzymes (*Hae*III and *Taq*I, data not shown). The results obtained for DT were consistent with those for FV. Thus, these two polymorphic fragments defined two different haplotypes (Table 3). The two haplotypes allow a clear differentiation between populations. An unambiguous geographic divide occurs separating the eight populations distributed south, from those three populations distributed north, of a mountain chain including a high volcano (3776 m) (Fig. 2).

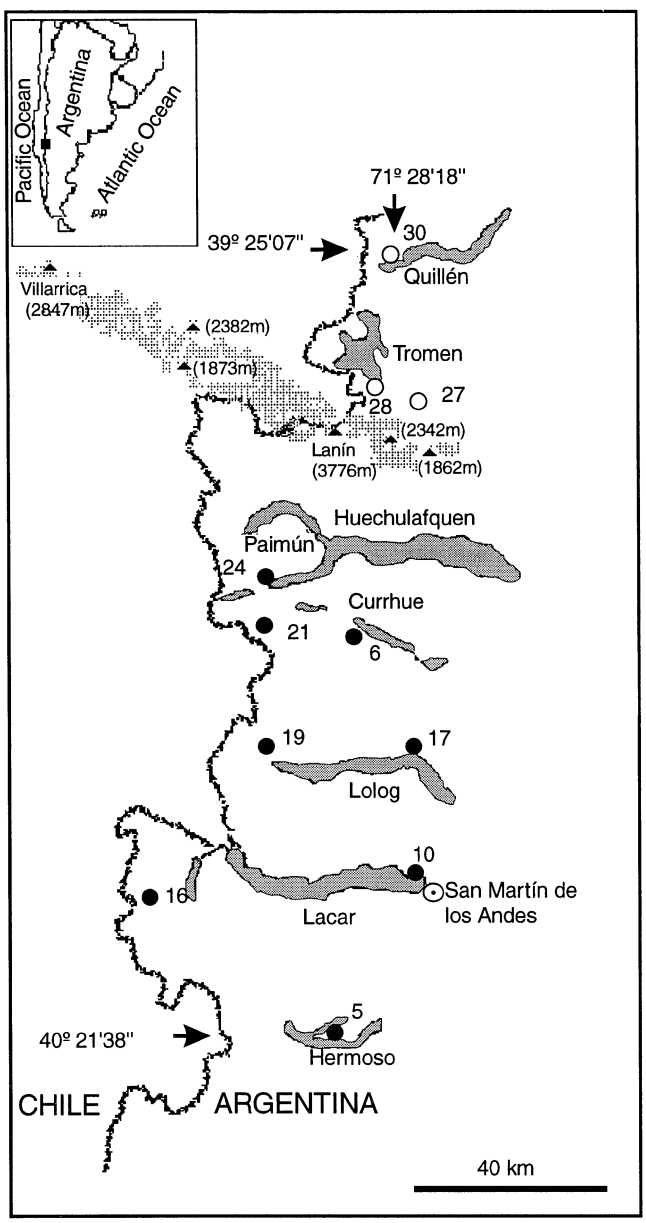

Fig. 2 Geographical location of the two haplotypes found in this study. ○ Haplotype I; ● Haplotype II

Discussion

PCR-RFLP anaylsis of the chloroplast genome provides an efficient tool for the detection of cpDNA polymorphisms as was demonstrated by Demesure et al. (1996) and Dumolin-Làpegue et al. (1997 b) who efficiently explored non-coding cpDNA regions for intraspecific variation in European beech and oak. Despite the slight differences in the length of the PCR products obtained, 10 of the 16 pair of primers developed by Demesure et al. (1995) and Dumolin-Là$^{+}$pegue et al. (1997 a) successfully amplified ten cpDNA regions of *N. nervosa*, confirming the universality of the tool and the high conservation of the chloroplast genome. Length polymorphisms were detected in two of the cpDNA regions analysed: *trn*F-*trn*V and *trn*D-*trn*T. The very small difference in length found among the polymorphic restriction fragments, especially those produced after digestion of the DT PCR product, suggests the possibility of conformational differences due to an inversion. Nevertheless, an indel (insertion/deletion) cannot be discarded (Dumolin-Làpegue and Petit, personal communication). Restriction by both enzymes (*Hae*III and *Taq*I) determined a length polymorphism in the DT cpDNA region that divides the 11 populations into two groups. Moreover, for each restriction enzyme these groups are opposite in respect of the length of the polymorphic band (Table 3). It may be that two mutations are involved. Sequencing of the fragment to determine the type and number of mutations would be of great interest.

To our knowledge, this study is the first molecular approach towards an analysis of the genetic differentiation between populations of the southern beech *N. nervosa*. In spite of its extremely small natural range of distribution there is an evident polymorphism in the chloroplast genome which could be detected by our PCR-RFLP approach. The two polymorphic fragments observed defined two haplotypes which allowed a clear differentiation among the populations (Table 3). These two haplotypes are geographically polarised; haplotype I occurs exclusively south, while haplotype II only occurs north, of a mountain chain that includes a high volcano (Lanín volcano, 3776 m) and which runs west-east dividing the narrow distribution area of the species. This mountain chain extends to the west, into Chile, with a series of high mountains also including a high volcano (Villarrica volcano, 2847 m) (Fig. 2). At this latitude the distribution area is very narrow (about 6 km). This area was affected not only by glaciations but also by the post-glaciation eruptions responsible for the actual shape of the volcano (Rabassa et al. 1990).

Geographic structure of cpDNA polymorphisms has been reported for different species and related to post-glaciation migration routes. In *Abies mariesii* a geographical cline in a small distribution area was found

(Tsumura et al. 1994). Also in *A. alba* a possible geographic cline of two cytotypes was reported (Ziegenhagen et al. 1995). Two lineages also divide the range of *Argania spinosa* in Morocco (El Mousadik and Petit 1996). Ferris et al. (1993) reported an intraspecific polymorphism in two European oaks distributed east and west in Europe. The divide runs down the centre of Europe, an area with hybrid zones where western and eastern taxa meet and also the line that divides many birds which migrate to and from the south each year taking westerly and easterly routes.

In the Southern Hemisphere the glaciations occurred in patches, leaving many possible refugia for plants and animals (Markgraf et al. 1995). The occurrence of two different haplotypes in *N. nervosa* populations suggests the existence of at least two different refugia from where the species spread out. These refugia could be located either in the coastal mountains or in the Andes Mountains (Villagran 1991). The coastal mountains are about 250 km away from the nearest extant population of *N. nervosa* in Argentina. Fruit dispersal in *Nothofagus* is very poor (Hill 1992) and in *N. nervosa* it can only reach between 50 and 100 m per year (Donoso 1993). Thus, even under the most favorable conditions, the species would have needed between 30 000–100 000 years to cross from coastal mountains to Argentina. This far exceeds the time that has elapsed since the last glaciation (18 000–20 000 years) and even more so for the time cited for its expansion (9500–3000 years) (Markgraf 1983; Villagran 1991). This has led us to suggest that *N. nervosa* populations from Argentina spread from refugia situated in the Andes Mountains. It would be of great interest to also analyse populations from Chile in order to verify our hypothesis.

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References

Clegg MT, Learn GH, Golenberg EM (1991) Molecular evolution of chloroplast DNA. In: Solander RK et al. (eds) Evolution at the molecular level Sinauer Assoc., Sunderland, Massachusetts, USA

Demesure B, Sodzi N, Petit RJ (1995) A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. Mol Ecol 4:129–131

Demesure B, Comps B, Petit RJ (1996) Chloroplast DNA phylogeography of the common beech (*Fagus sylvática* L.) in Europe. Evolution 50:2515–2520

Donoso, C (1993) Bosques templados de Chile y Argentina. Editorial Universitaria, Chile

Dumolin S, Demesure B, Petit RJ (1995) Inheritance of chloroplast and mitochondrial genomes in pedunculate oak investigated with an efficient PCR method. Theor Appl Genet 91:1253–1256

Dumolin-Làpegue, S, Pemonge MH, Petit RJ, (1997a) An enlarged set of consensus primers for the study of organelle DNA in plants. Mole Ecol 6:393–397

Dumolin-Lapègue S, Demesure B, Fineschi S, Le Corre V, Petit RJ (1997b) Phylogeographic structure of white oaks throughout the European continent. Genetics 146:1475–1487

El Mousadik A, Petit RJ, (1996) Chloroplast DNA phylogeography of the argan tree of Morocco. Mol Ecol 5:547–555

Ferris C, Oliver RP, Davy AJ, Hewitt GM (1993) Native oak chloroplasts reveal an ancient devide across Europe. Mol Ecol 2:337–344

Hill RS (1992) *Nothofagus*: evolution from a southern perspective. TREE 7:190–194

Kremer A, Petit RJ (1993) Gene diversity in natural populations of oak species. Ann Sci For 50 (Suppl 1):186–202

Lennon JA, Martin ES, Stevens RA, Wigston DL (1987) *Nothofagus nervosa* (Phil.) Dim. et Mil. the correct name for raulí, Chilean southern beech (*N. procera*). Arboricult Jour 11:323–332

Manos PS (1997) Systematics of *Nothofagus* (Nothofagaceae) based on rDNA spacer sequences (ITS): taxonomic congruence with morphology and plastid sequences. Am J of Bot 84:1137–1155

Markgraf V (1983) Late and postglacial vegetational and paleoclimatic changes in subantarctic, temperate, and arid environments in Argentina. Palynology 7:43–70

Markgraf V, McGlone M, Hope G, (1995) Neogene paleoenvironmental and paleoclimatic change in southern temperate ecosystems – a southern perspective. TREE 10:143–147

Palmer JD (1987) Chloroplast DNA evolution and biosystematic uses of chloroplast DNA variation. Am Nat 130:6–29

Petit RJ, Kremer A, Wagner DB (1993). Geographic structure of chloroplast DNA polymorphisms in European oaks. Theor Appl Genet 87:122–128

Rabassa, J, Evenson EB, Clinch JM, Schlieder G, Zeitler P, Stephens GC (1990) Geología del cuaternario del valle del Río Malleo, provincia del Neuquén. Asociación Geológica Argentina, Rev. XLV (1–2):55–68

Tsumura Y, Taguchi H, Suyama Y, Ohba K, (1994) Geographical cline of chloroplast DNA variation in *Abies mariesii*. Theor Appl Genet 89:922–926

Villagran C (1991) Historia de los lagos templados del sur de Chile durante el Tardiglacial y Postglacial. Rev Chilena de Hist Nat 64:497–460

Ziegenhagen B, Guillemaut P, Scholz F (1993) A procedure for mini-preparations of genomic DNA from needles of silver fir (*Abies alba* Mill.). Plant Mol Biol Rep 11:117–121

Ziegenhagen B, Kormuták A, Schauerte M, Scholz F (1995) Restriction site polymorphism in chloroplast DNA of silver fir (*Abies alba* Mill.) Forest Genetics 2(2):99–107