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Genetic evidence for glacial refugia of the temperate tree *Eucryphia cordifolia* (Cunoniaceae) in southern South America[1]

RICARDO A. SEGOVIA[2,4,5], MARÍA F. PÉREZ[3,4], AND LUIS F. HINOJOSA[2,4]

[2] Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, [3] Departamento de Ecología, Pontificia Universidad Católica de Chile, Center of Advanced Studies in Ecology & Biodiversity (CASEB), and [4] Instituto de Ecología y Biodiversidad (IEB)

- *Premise of the study:* The temperate forests of southern South America were greatly affected by glaciations. Previous studies have indicated that some cold-tolerant tree species were able to survive glacial periods in small, ice-free patches within glaciated areas in the Andes and in southern Patagonia. Here we asked whether populations of the mesothermic species *Eucryphia cordifolia* also were able to survive glaciations in these areas or only in unglaciated coastal areas.
- *Methods:* The chloroplast intergenic spacer *trnV-ndhC* was sequenced for 150 individuals from 22 locations. Genetic data were analyzed (standard indexes of genetic diversity, a haplotype network, and genetic differentiation) in a geographical context.
- *Key results:* Two of the nine haplotypes detected were widespread in high frequency across the entire range of the species. The highest levels of genetic diversity were found around 40°S, decreasing sharply northward and more moderately southward. No differences in genetic diversity were found between Andean and coastal populations. Notably, seven haplotypes were found in a small area of the Coast Range known as the *Cordillera Pelada* (40°S). The differentiation coefficients G_{ST} and N_{ST} revealed that most of the genetic variation detected was due to variation within populations.
- *Conclusions:* The low levels of population differentiation and the high genetic diversity found in the Cordillera Pelada suggest that this area was the main refugium for *E. cordifolia* during glaciations. Nevertheless, given the high levels of genetic diversity found in some Andean populations, we cannot discount that some local populations also survived the glaciation in the Andes.

 Key words: climate change; cpDNA; *Eucryphia cordifolia* Cav.; phylogeography; Pleistocene glaciations; refugia; South America; temperate rain forest.

The present-day patterns of temperate species richness have been shaped by strong quaternary climatic oscillations (McGlone, 1996; Qian and Ricklefs, 1999; Ricklefs et al., 1999; Qian and Ricklefs, 2000). The response of the biota to the expansion of ice sheets during the glacial period has been associated with regions where modern elements may have survived glacial periods with greatly reduced abundance and distribution, the so-called glacial refugia (Hewitt, 2000; Willis and Whittaker, 2002; Bennett and Provan, 2008). Early biogeographical models proposed that biota contracted their distribution range to unglaciated, warmer areas, typically at low latitude and altitude, from whence species expanded their distribution during interglacial periods (Bennett et al., 1991; Lacourse et al., 2005). Today, however, it is known that some species also may have survived in pockets of favorable microclimates within areas at higher latitudes previously regarded as inhospitable (multiple or cryptic refugia) (Cruzan and Templeton, 2000; Bennett and Provan, 2008; Rull, 2009; Premoli et al., 2010).

Temperate rainforests of southern South America exhibit a set of phytogeographic traits that have been considered legacies of Quaternary climatic oscillations (Villagrán, 1991; Villagrán and Hinojosa, 1997; Villagrán et al., 1998; Villagrán, 2001; Villagrán and Hinojosa, 2005). Several species (mainly conifers) with disjunct distributions in the summits and higher elevations of the Andes and Coast Ranges may have resulted from the successive events of expansion and contraction associated with climatic fluctuations (Villagrán, 2001). In addition, there is a high concentration of tree species and endemism in a relatively small area in the Coast Range between 36°S and 40°S. This pattern has been attributed to the repetitive role of the Coast Range as a glacial refugium (Villagrán and Hinojosa, 1997). This hypothesis is supported by geomorphologic data that show that this area remained unglaciated during the Last Glacial Maximum (LGM, Fig. 1) (Veit, 1994; Veit and Garleff, 1996), dated between 28 and 16 kyr BP (Denton et al., 1999; Kaplan et al., 2008), as well as by palynological evidence, which indicates that many species responded to glaciations by contracting their ranges to suitable refugia in mid and low elevations of the Coast Range (Villagrán, 1991).

Recent genetic evidence indicates that some dominant tree species were able to survive glacial periods in many localities within glaciated areas (Markgraf, 1995; Marchelli et al., 1998; Premoli, 1998, 2004; Marchelli and Gallo, 2006). Areas with high levels of genetic diversity suggesting long-lasting persistence have been identified at high latitudes in southern Patagonia, as well as in the foothills of the Andes Range (Marchelli
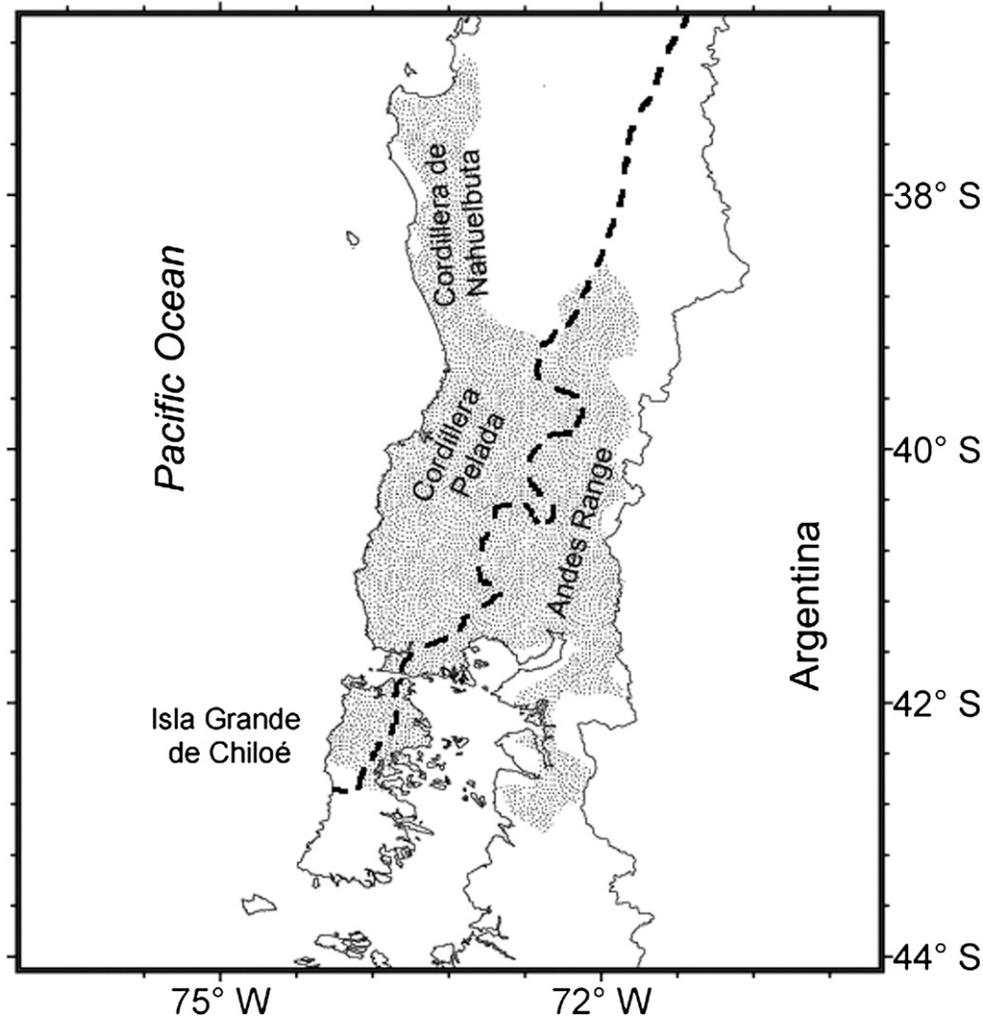
Fig. 1. Present distribution of *Eucryphia cordifolia*. The dashed line indicates the extent of the ice sheet during the Last Glacial Maximum according to Holling and Schilling (1981).

and Gallo, 2006; Mathiasen and Premoli, 2010; Premoli et al., 2010). Likewise, high levels of population divergence and phylogeographic structure attributed to isolation in multiple refugia have been detected. These studies have focused on cold-tolerant species such as the conifers *Fitzroya cupressoides* (Allnutt et al., 1999; Premoli et al., 2000; Premoli et al., 2003), *Pilgerodendron uviferum* (Premoli et al., 2001; Premoli et al., 2002; Allnutt et al., 2003), *Araucaria araucana* (Bekessy et al., 2002), and *Podocarpus nubigena* (Quiroga and Premoli, 2010) and the angiosperms *Nothofagus alpina* (Marchelli et al., 1998; Marchelli and Gallo, 2006), *N. pumilio* (Premoli, 2004; Mathiasen and Premoli, 2010; Premoli et al., 2010), and *Embothrium coccineum* (Souto and Premoli, 2007; Vidal-Russell et al., 2011). Only one study focused on a less cold-tolerant tree species. Núñez-Avila and Armesto (2006), using RAPD, showed that the phylogeography of the mesothermic *Aextoxicon punctatum* agrees with the coastal refugium hypothesis, suggesting expansion southward and eastward from coastal populations. Thus, the responses of mesothermic species of southern South American temperate forests to Pleistocene glaciations, including range shifts, migration routes, and population divergence, are still poorly understood.

Eucryphia cordifolia (Cunoniaceae) is an endemic tree of the southern South American temperate rainforest that may be an indicator of climate warming in palynological studies (Villagrán, 1988; Heusser et al., 1999; Moreno and León, 2003; Abarzúa et al., 2004; Moreno, 2004). At present, this species grows principally in Chile between 36.8°S and 43.3°S at elevations from sea level to 700 m (Fig. 1). This species is capable of colonizing disturbed habitats (Veblen and Ashton, 1978) and has showy flowers pollinated by more than 70 species of hymenopterans and dipterans (Smith-Ramírez et al., 2005). The fruit is a capsule with small, winged seeds dispersed by wind. Pollen grains of *E. cordifolia* are indistinguishable from those of *Caldcluvia paniculata*, which is distributed farther south between 38°S to 45°S at elevations from sea level to 1000 m.a.s.l. (Rodríguez et al., 1983) The absence of *Eucryphia/Caldcluvia* pollen records from the Longitudinal Valley in the Lake District at 41°S (Moreno, 1997; Heusser et al., 1999; Moreno et al., 1999; Moreno and León, 2003) and Isla Grande de Chiloé (Villagrán, 1988; Abarzúa et al., 2004; Heusser and Heusser, 2006) during the LGM suggests these species contracted north of the ice-sheet line. Nevertheless, the possibility that species survived glaciations locally in small populations (undetectable in the

pollen record) cannot be completely discarded. The maximum abundance of *Eucryphia/Caldcluvia* was recorded in the early Holocene at lowland sites from the Lake District (41°S) and Isla Grande de Chiloé (42°S–43°S), when conditions were presumably warmer and drier than today (Heusser, 1966, 1984; Villagrán, 1985, 1988; Abarzúa et al., 2004; Moreno, 2004). Moreno (2004) documented a decrease in the abundance of *Eucryphia/Caldcluvia*, accompanied by a re-expansion of conifers and other cold-tolerant elements, that has occurred since the Mid-Holocene, when conditions became cooler and wetter in northwest Patagonia (40°S–43°S). Finally, the present distribution of *E. cordifolia* would have been established about 3000 yr ago (Moreno, 2004).

In this study, we used the intergenic cpDNA region *trnV-ndH* to explore the genetic diversity and geographic structure of 22 populations of *E. cordifolia*. We asked whether populations of this species were able to survive glacial periods only in lowlands of coastal areas between 36–40°S, as has been suggested by the palynological record, or also in small, ice-free patches within glaciated areas, as has been documented for cold-tolerant species. If populations of *E. cordifolia* were derived post glaciation from coastal areas, southern and Andean populations should be genetically similar to coastal populations. Furthermore, if this is the case, within-population genetic diversity should be lower in Andean populations than in coastal populations, and it should decrease with latitude because of founder effects. Similarly, genetic diversity should be greater in low- and mid-elevation populations than at higher altitudes in the Coast Range.

MATERIALS AND METHODS

Sampling and DNA extraction—We sampled 150 individuals of *E. cordifolia* from 22 populations in Cordillera de Nahuelbuta (in the Coast Range to the north), Cordillera Pelada (located farther south), Longitudinal Valley, Andes Range, and the Isla Grande de Chiloé (Fig. 1, Table 1). We collected foliar tissue

from six to 10 individuals per population. Selected trees were at least 50 m apart to avoid sampling from clones or closely related individuals. Total genomic DNA was isolated from silica-dried foliar tissue with the DNAeasy Plant Mini Kit from QIAGEN (Valencia, California, USA).

DNA extraction, amplification, and sequencing—We screened six individuals from three populations for polymorphism in several cpDNA regions, including the intergenic spacers *trnT-trnF* (Taberlet et al., 1991), *trnD-trnT*, *trnC-trnD* (Demesure et al., 1995), *trnG-trnS* (Wakasugi et al., 1994), *orf184-petA*, *trnS-trnR* (Grivet et al., 2001), *trnV-ndhC*, *trnQ-rps16*, *ndhF-rpl32R*, *trnL-rpl32F* (Shaw et al., 2007), and the cpDNA microsatellites ccmp4, ccmp5, ccmp6, ccmp7, and ccmp10 (Weising and Gardner, 1999). We selected the intergenic spacer *trnV-ndhC* because of its higher level of polymorphism. Polymerase chain reactions (PCR) were carried out in 25-μL reaction volumes containing 7 μL of DNA template, 1 mM of dNTP mix, 0.5 U of *Taq* polymerase, 1× reaction buffer supplied with the enzyme, 0.1 μm of each primer, and 5 μg bovine serum albumin. Amplifications were carried out using the following conditions: 94°C (4 min), 30 cycles of 94°C (45 s), 50°C (45 s), 72°C (3 min), and a final extension at 72°C for 10 min. The fragments were purified and sequenced at the Macrogen sequencing center (Seoul, South Korea).

Genetic data analyses—The cpDNA sequences were edited and aligned with the program Bioedit v. 7.0.9 (Hall, 1999). Two multibase indels were found and were coded as binary characters. The method of parsimony was employed to evaluate the relationships among haplotypes and to construct a haplotype network (TCS 1.21; Clement et al., 2000)). In the analysis, both site mutations and indels were hypothesized to be equally likely to evolve, and each indel was assumed to have originated independently from other indels. The following indices of genetic diversity were estimated for each population and also for each region separately using the program DNASp v. 5 (Librado and Rozas, 2009): number of haplotypes (K), haplotype diversity (H), and nucleotide diversity (π). To examine possible founder effects and explore the latitudinal migration northward and southward from potential refugia, we analyzed the variation of within-population genetic diversity with latitude using a nonlinear regression model. To avoid the effect of altitude, we included only populations located at low and middle elevations. In addition, we grouped the *E. cordifolia* populations into three equal-sized units: North (36.8–38.8°S), Center (38.9–40.8°S), and South (40.9–42.8°S) (Table 1). Then we compared the within-population genetic parameters among groups using Student's *t* test. To examine the migratory process eastward, we compared within-population genetic measures between Andean and coastal populations. We estimated the

TABLE 1.   Geographic location, altitude (Alt), sample size (n), geographic region, and measurements of genetic diversity of 22 population of *Eucryphia cordifolia* in Chile. K = number of haplotypes; H = haplotype diversity; π = nucleotide diversity. The Roman numerals after the names of the localities indicate administrative regions of Chile.

Unit	Code	Locality	Latitude S	Longitude W	Alt	n	Geographic group	K	H	π
North	U1	Hualpén (VIII)	36.788	73.158	0	6	Cordillera de Nahuelbuta	1	0.000	0.0000
	U2	Escuadrón (VIII)	36.936	73.089	450	6	Cordillera de Nahuelbuta	1	0.000	0.0000
	U3	Llico (VIII)	37.224	73.583	300	10	Cordillera de Nahuelbuta	2	0.200	0.0010
	U4	Contulmo (VIII)	38.01	73.185	373	9	Cordillera de Nahuelbuta	3	0.639	0.0031
Center	U5	Huife (IX)	39.224	71.654	498	6	Andes Range	3	0.600	0.0021
	U6	Pelada Poniente 4 (XIV)	39.978	73.649	258	6	Cordillera Pelada	3	0.600	0.0030
	U7	Los Ulmos (XIV)	40.034	72.988	150	10	Longitudinal Valley	4	0.778	0.0038
	U8	Pelada Poniente 3 (XIV)	40.089	73.566	616	6	Cordillera Pelada	3	0.733	0.0023
	U9	Pelada Poniente 2 (XIV)	40.157	73.61	212	7	Cordillera Pelada	4	0.810	0.0047
	U10	Pelada Poniente 1 (XIV)	40.167	73.545	705	7	Cordillera Pelada	1	0.00π0	0.0000
	U11	Pelada Oriente (XIV)	40.263	73.331	354	6	Cordillera Pelada	3	0.600	0.0030
	U12	Huaquecura (XIV)	40.188	71.933	300	8	Andes Range	3	0.607	0.0030
South	U13	Petrohué (X)	41.126	72.412	272	6	Andes Range	3	0.733	0.0031
	U14	Valle Los Ulmos (X)	41.29	72.578	533	7	Andes Range	2	0.476	0.0023
	U15	Cayetue (X)	41.319	72.272	313	6	Andes Range	2	0.533	0.0026
	U16	Camino a Ralun (X)	41.331	72.414	184	6	Andes Range	3	0.733	0.0036
	U17	Puelo (X)	41.701	72.405	73	6	Andes Range	3	0.600	0.0021
	U18	Puelche (X)	41.734	72.634	7	7	Andes Range	2	0.286	0.0014
	U19	Puerto Montt (X)	41.781	73.447	13	6	Longitudinal Valley	2	0.533	0.0026
	U20	Senda Darwin (X)	41.881	73.83	24	8	Isla Grande de Chiloé	3	0.750	0.0024
	U21	Quemchi (X)	42.146	73.701	147	6	Isla Grande de Chiloé	3	0.733	0.0028
	U22	Detico (X)	42.877	73.568	36	6	Isla Grande de Chiloé	2	0.600	0.0030
Totals						150		9	0.6626	0.0032

TABLE 2. Polymorphic sites in *Eucryphia cordifolia* for the intergenic spacer region *trnV-ndhC* of cpDNA. Gaps are indicated by "—". Dots represent invariable sites with respect to the haplotype H1.

Haplotype	Nucleotide position																	
	47	48	49	50	51	52	54	146	251	264	332	333	334	335	336	337	338	390
H1	—	—	—	—	—	—	A	T	T	C	G	A	A	T	T	T	A	G
H2	—	—	—	—	—	—	.	.	C	.	—	—	—	—	—	—	—	.
H3	—	—	—	—	—	—	.	.	C	.	.	.	.	.	.	.	.	A
H4	—	—	—	—	—	—	C	.	C	.	—	—	—	—	—	—	—	.
H5	—	—	—	—	—	—	C	.	C	.	.	.	.	.	.	.	.	.
H6	—	—	—	—	—	—	.	.	C	.	.	.	.	.	.	.	.	.
H7	T	G	G	A	A	T	.	.	C	.	.	.	.	.	.	.	.	A
H8	—	—	—	—	—	—	.	.	.	T	.	.	.	.	.	.	.	.
H9	—	—	—	—	—	—	.	G	C	.	—	—	—	—	—	—	—	.

correlation between genetic diversity and altitude of populations of the Cordillera Pelada. In addition, we tested for evidence of expansion and contraction by calculating Tajima's D and Fu and Li's F statistics using the program DNASp v. 5 (Librado and Rozas, 2009):

Genetic differentiation among populations was evaluated using the G_{ST} and N_{ST} coefficients (Pons and Petit, 1995, 1996). Both coefficients estimate the ratio between the mean within-population genetic diversity (υ_s and h_S) and total genetic diversity (υ_T and h_T), but whereas N_{ST} takes into account the genetic distances between haplotypes (ordered alleles), G_{ST} ignores them (unordered alleles). A test of 1000 permutations was used to evaluate whether N_{ST} was significantly greater than G_{ST}. A greater N_{ST} means that more closely related haplotypes occur in the same population, indicating phylogeographic structure (Pons and Petit, 1996). A permutation test was conducted in the program PERMUT (Pons and Petit, 1996; Burban et al., 1999). To visualize spatial patterns of genetic distances over the study area, a genetic landscape shape was constructed with the program Alleles in Space, version 1.0 (Miller, 2005). This procedure uses a Delaunay triangulation connectivity network based on each sampling location and assigns a genetic distance at the geographic midpoint of each sample pair. Genetic distances are then interpolated uniformly across the entire geographic sampling area, and a 3-dimensional surface plot is constructed, with the surface heights representing genetic distance. Analysis was performed with a grid size of 50×50 and a range of interpolation parameters (a = 0.1–3.0).

Genetic differentiation among populations was also explored with a spatial analysis of molecular variance implemented in the program SAMOVA v. 1.0 (Dupanloup et al., 2002). This method defines partitions of local populations, maximizing the proportion of genetic variance explained by differences between groups of populations (F_{CT} coefficient of the analysis of molecular variance, AMOVA; Excoffier et al., 1992). Analyses were performed with 2–5 groups, and the significance of F_{CT} was tested for each partition in the program Arlequin, using AMOVA (Excoffier et al., 1992).

RESULTS

The *trnV-ndhC* alignment was 418 bp in length with five substitutions and two indels of six and seven bp. Nine haplotypes
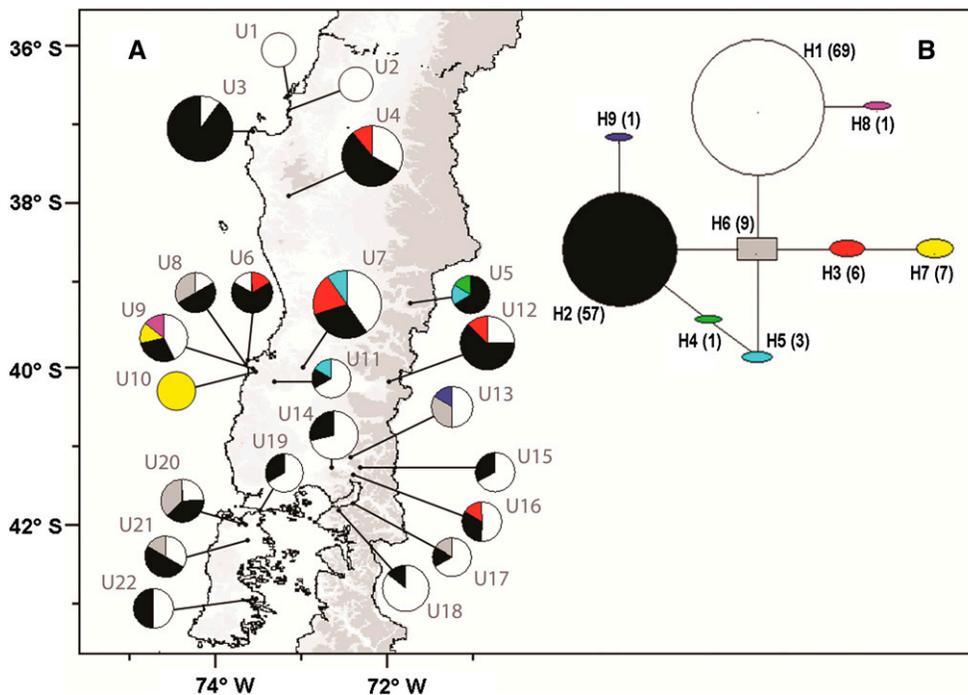

Fig. 2. Geographic distribution and genealogical relationships of the nine cpDNA haplotypes found in *Eucryphia cordifolia*. (A) Pie charts reflect the frequency of occurrence of each haplotype in each locality. Haplotype colors correspond to those shown in the network. Pie chart sizes are proportional to the number of individuals analyzed in each population. (B) Statistical parsimony network linking the nine haplotypes. Circle sizes are proportional to haplotype frequencies. Each line represents one mutational step. The number of individuals found for each haplotype is shown in parentheses.

were detected (Table 2). Two of them (denoted H1 and H2) were present in high frequency across the entire distribution range of *E. cordifolia* (Fig. 2A). The most common haplotype (H1) was detected in 20 of the 22 localities and the second (H2) in 19. The other seven haplotypes (denoted H3–H9) were present in fewer than 20% of the total individuals and were found principally in the center of the latitudinal range of *E. cordifolia* (39–40°S).

The haplotype network (Fig. 2B) suggested that the widespread haplotypes H1 and H2, along with H3 and H5, were all derived from haplotype H6 by only one mutation step. The high number of connections observed for H6 suggests that it corresponds to the ancestral haplotype. This haplotype was found in only five localities situated in Cordillera Pelada (U8), the northern edge of Isla Grande de Chiloé (U20, U21), and the Andean localities of Petrohue (U13) and Puelo (U17). The other haplotypes (H4, H7, H8, and H9) were separated from ancestral haplotype by only two mutational steps (Fig. 2b). Two of them (H7 and H8) were restricted to the western slopes of the Coast Range in the Cordillera Pelada, and the other two were restricted to the Andean localities of Huife (H4) and Petrohue (H9).

The number of haplotypes per population (K), haplotype diversity (H), and nucleotide diversity (π) varied substantially among populations (Table 1). The highest levels of diversity were found in two populations situated around 40°S: one of them in the lowlands of the Cordillera Pelada (U9) and the other 50 km eastward in the Longitudinal Valley (U7). Genetic diversity decreased gradually with increasing altitude in the Cordillera Pelada ($N = 5$, r = –0.89, $P < 0.05$), reaching its lowest value at the upper limit of the distribution of *E. cordifolia* at 705 m.a.s.l. (U10). The number of haplotypes per population decreased northward to the Cordillera de Nahuelbuta, where two populations were fixed for haplotype H2, as well as southward. A significant quadratic correlation between genetic diversity and latitude was detected considering populations located at low and mid elevations ($N = 21$, $r^2 = 0.57$, $P < 0.001$ for K; Fig. 3). The same tendency was detected when populations were grouped by latitude. Thus, populations located in the center of the species' range at low or middle elevations (38.9°–40.8°S) had a significantly greater number of haplotypes than those located north ($P < 0.01$) and south of 40°S ($P < 0.01$) (Table 3). Central populations also had a significantly greater haplotype diversity (H) and nucleotide diversity (π) than northern populations ($P < 0.01$), but they were not significantly greater than southern populations ($P = 0.10$ for H, and $P = 0.08$ for π) (Table 3). Nevertheless, when all central populations are considered together, this region has H and π values 18% and 38% higher, respectively, than those of all sampled southern populations, even though sampling effort was lower. No significant difference between Coast and Andes Range populations were found for any genetic diversity measure ($N = 17$, $P = 0.25$ for K; $P = 0.13$ for H, and $P = 0.15$ for π; Table 4). However, it is notable that seven of the
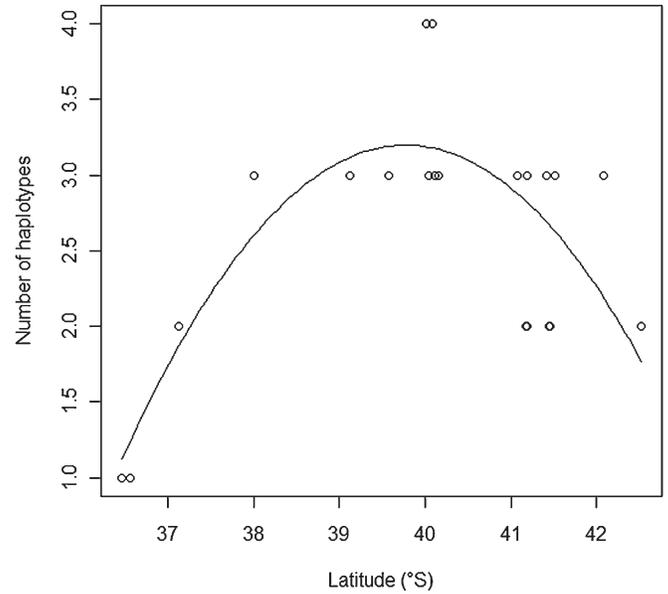

Fig. 3.   Latitudinal variation (L) in the number of haplotypes (N) for *Eucryphia cordifolia* population. A quadratic regression function was adjusted (N = –297 + 15.1 * L – 0.2 L_2; $r^2 = 0.57$, $P < 0.001$).

nine haplotypes detected for *E. cordifolia* were found in the Cordillera Pelada, two of them restricted to the western slope. Considering all populations together, this small area presented the same number of haplotypes and H and π values 50% greater than all sampled Andean populations. Values of Tajima's *D* (0.08, P = not significant) and Fu and Li's *F* (–0.49, P = not significant) did not differ significantly from zero; thus, the hypothesis of neutral variation was not rejected.

The coefficients of differentiation G_{ST} (0.214) and N_{ST} (0.274) were significantly different from zero ($P < 0.001$) but were lower than 30% (Table 5), indicating that most of the variation was found within populations. The permutation test showed that N_{ST} was significantly greater than G_{ST} ($P = 0.04$), suggesting the existence of phylogeographic structure. This pattern, however, was explained principally by the high divergence of population U10, located at the upper altitudinal limit of *E. cordifolia*. This population is fixed for the rare haplotype H7, which was found only in a nearby population located 200 m downward. In fact, no significant difference between G_{ST} and N_{ST} was detected when population U10 was removed from the data set ($G_{ST} = 0.134$ and $N_{ST} = 0.139$; P = ns). Genetic landscape analysis was congruent with these results and showed the strongest genetic separation among populations in the Cordillera Pelada (Fig. 4). Genetic distances consistently decreased toward the southern and northern ends of the range. Accordingly, SAMOVA results did not show a

TABLE 3.   Mean within-population diversity and total diversity in three latitudinal regions (north of 39°S, between 39–41°, and south of 41°S). K = number of haplotypes; H = haplotypic diversity; π = nucleotide diversity. Standard deviations are indicated in parentheses.

Unit	Individuals	Populations	Mean within-population diversity			Total diversity		
			K	H	π	K	H	π
North	31	4	1.75 (0.96)	0.21 (0.30)	0.0010 (0.001)	3	0.546 (0.038)	0.00268
Center	49	7	3.29 (0.49)	0.68 (0.09)	0.0031 (0.001)	8	0.706 (0.047)	0.00339
South	64	10	2.50 (0.53)	0.60 (0.15)	0.0026 (0.001)	5	0.592 (0.045)	0.00245

TABLE 4. Mean within-population diversity and total diversity in the Andes and Coast Ranges. K = number of haplotypes; H = haplotypic diversity; π = nucleotide diversity. Standard deviations are indicated in parentheses.

Range and unit	Individuals	Populations	Within-population diversity			Total diversity		
			K	H	π	K	H (SD)	π
Andes Range								
North	14	2	3 (0)	0.604 (0.01)	0.003 (0.001)	5	0.593 (0.14)	0.0027
South	38	6	2.5 (0.55)	0.56 (0.17)	0.003 (0.001)	5	0.529 (0.08)	0.0023
Total	**52**	**8**	**2.625 (0.52)**	**0.571 (0.15)**	**0.003 (0.001)**	**7**	**0.63 (0.05)**	**0.0029**
Coast Ranges								
Cordillera de Nahuelbuta	31	4	1.75 (0.96)	0.210 (0.3)	0.0010 (0.001)	3	0.55 (0.04)	0.0026
Cordillera Pelada	25	4	3.25 (0.5)	0.686 (0.1)	0.0032 (0.001)	7	0.73 (0.06)	0.0034
Total	**56**	**8**	**2.5 (1.1)**	**0.448 (0.3)**	**0.0021 (0.002)**	**7**	**0.63 (0.04)**	**0.0030**

clear geographic grouping of populations. With k = 2, populations U2 and U1, located in the northern extreme of the distribution and both fixed for the widespread haplotype H1, formed a group. At increasing values of k (from 3 to 5), each new delimited group was represented by only one population.

DISCUSSION

The mesothermic species *E. cordifolia* showed relatively low levels of genetic diversity and population differentiation in the chloroplast genome compared with other temperate tree taxa of southern South America more tolerant to cold conditions, such as *Podocarpus nubigena* (Quiroga and Premoli, 2010), *N. alpina* (Marchelli et al., 1998; Marchelli and Gallo, 2006), and *Fitzroya cupresoides* (Allnutt et al., 1999; Premoli et al., 2000; Premoli et al., 2003). The differentiation coefficients G_{ST} and N_{ST} revealed that most of the genetic variation detected in *E. cordifolia* was due to variation within populations. Accordingly, SAMOVA analyses did not show a clear geographic grouping of populations, which probably was hindered by the existence of frequent and widespread haplotypes and by the
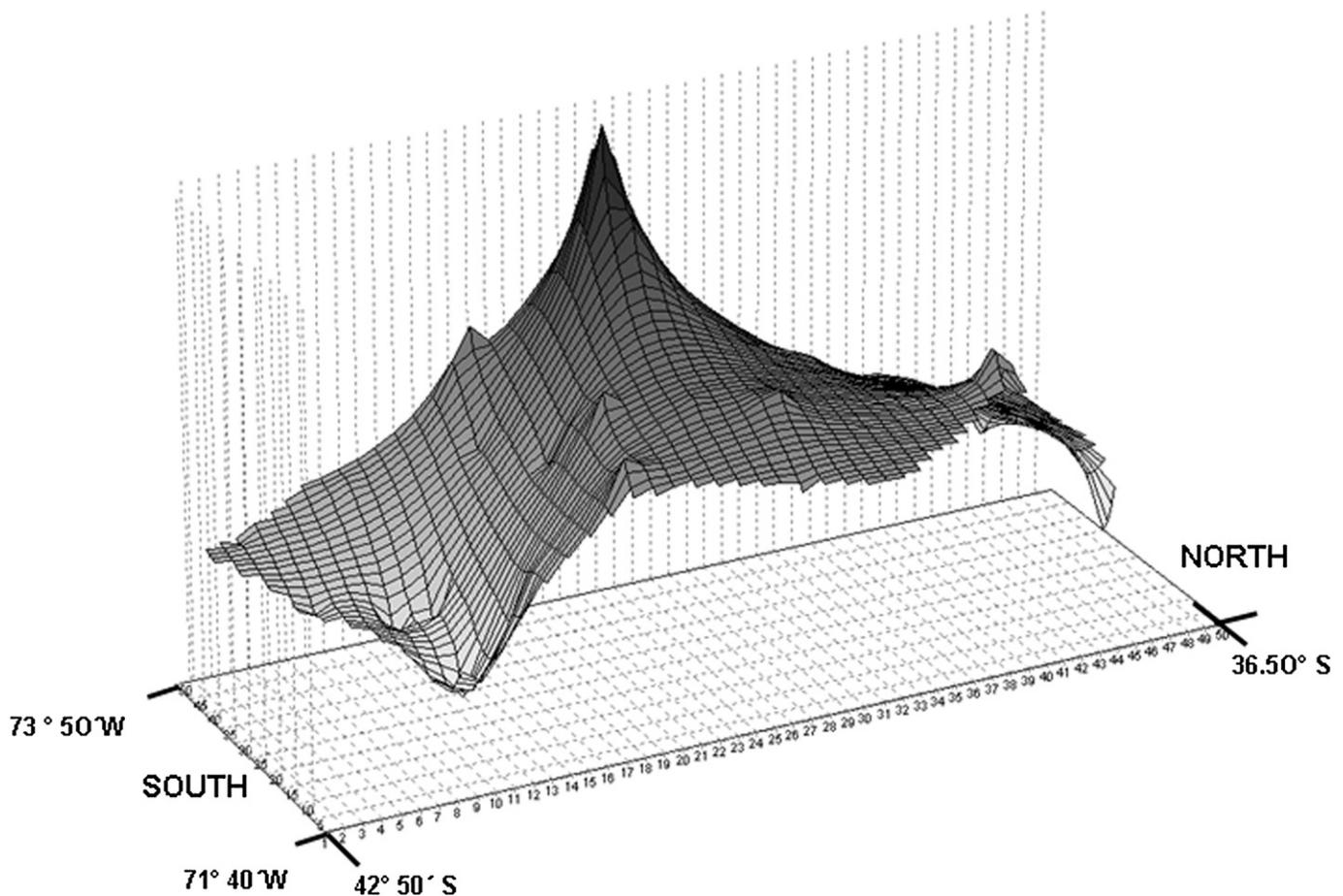

Fig. 4. Genetic landscape of *Eucryphia cordifolia*. The landscape is standardized with a grid of 50 × 50 cells, comprising the geographic range covering all localities collected. The peaks and depressions of the surface indicate the magnitude of genetic distance.

TABLE 5. Mean within-population genetic diversity (hS, vS), total genetic diversity (hT, vT), and differentiation coefficients (Gst, Nst) for all population of *Eucryphia cordifolia*. Standard errors are indicated in parentheses.

Alleles	Within-population genetic diversity	Total genetic diversity	Differential coefficients
Unordered	$hS = 0.525 \ (0.0552)$	$hT = 0.668 \ (0.0400)$	$Gst = 0.214 \ (0.0932)$
Ordered	$vS = 0.486 \ (0.0539)$	$vT = 0.669 \ (0.0748)$	$Nst = 0.274 \ (0.1293)$

concentration of rare haplotypes in the center of the distribution of the species. The lack of phylogeographic structure in *E. cordifolia* contrasts with the high levels of population divergence documented for cold-tolerant taxa and attributed to the isolation in multiple refugia during glacial periods.

The concentration of rare haplotypes and the high levels of genetic diversity found around 39–40°S suggest that *E. cordifolia* populations contracted to these latitudes during glacial periods. The coastal areas located between 36–40°S were previously proposed by Villagrán and Hinojosa (1997) as glacial refugia on the basis of the presence of Quaternary nitisols (Veit, 1994; Veit and Garleff, 1996) and the concentration of species richness and endemism with narrow distributions. Accordingly, we found high levels of genetic diversity in the Cordillera Pelada. Considering all populations, this small area presented seven of the nine haplotypes detected for *E. cordifolia* and had values of haplotype and nucleotide diversity 50% greater than all sampled Andean populations. Landscape analyses showed that this region also is characterized by high levels of differentiation. This pattern is concordant with the predictions for glacial refugia in mountain regions by Hewitt (1999), who proposed that altitudinal migrations induced by glacial–interglacial changes might have been rather slow and therefore may have promoted population divergence at a regional scale. In fact, the Cordillera Pelada is particularly steep and reaches altitudes greater than 1000 m, where there are conifers and Magellanic moorlands. The complex topography of the Cordillera Pelada probably restricted gene flow between the western and eastern slopes and among valleys during glacial times. This process is reflected today in the presence of haplotypes exclusive for each slope.

We detected a remarkable decrease in within-population genetic diversity northward to Cordillera de Nahuelbuta, as predicted by a series of founder effects in which successive population bottlenecks during range expansion reduce diversity (Hewitt, 1996; Comps et al., 2001; Petit et al., 2002). This result is consistent with the scenario proposed by Villagrán (1994), who suggested a southward contraction of the Valdivian Rainforest during the Mid and Late Holocene (between approximately 10 000 and 6000 yr BP), when conditions were drier than today, followed by a re-expansion northward that probably started about 3000 yr BP, when modern climatic conditions were established. We also detected a reduction of genetic diversity southward, but it was less pronounced. This pattern is consistent with the classic scenario of contraction to lower latitudes and posterior colonization. However, we detected no significant Fu and Li's *F* nor Tajima's *D* indices that would indicate expansion. It is possible that the genetic footprint of postcolonization expansion was eroded by the posterior contraction that *E. cordifolia* experienced during the Mid-Holocene. Indeed, the fossil record shows a rapid expansion of *E. cordifolia* in northern Patagonia (40–42°S) during the Holocene between 10 000 and 8000 yr BP, but it also shows a significant reduction in pollen abundance after 6900 yr BP (Abarzúa et al., 2004; Moreno, 2004).

The lack of significance of Fu and Li's *F* and Tajima's *D* indices also could indicate long-lasting persistence in multiple refugia. The exclusive haplotypes in the Andean localities of Huife (H4) situated at 39°S and Petrohue (H9) situated southern at 41°S suggest that some populations of *E. cordifolia* survived glaciations locally in ice-free Andean areas due to a discontinuity in the ice cover (Markgraf, 1995). This hypothesis is also supported by the ancestral haplotype H6 in Petrohue. A connection between both northern Andes and Coast Range refugia would have been established early, as revealed by the presence of haplotype H4 in Huife (U5) and by the presence of the closely related haplotype H5 in Huife, Longitudinal Valley (U7), and the eastern slope of the Cordillera Pelada (U11). Whether migration was westward or eastward between both areas is a problem that cannot be addressed here because the relation between H4 and H5 was not completely established (Fig. 2).

Potential glacial refugia in Andean localities have been documented for some cold-tolerant taxa, including, for example, *N. alpina* (Marchelli and Gallo, 2006) and *N. obliqua* (Azpilicueta et al., 2009). This hypothesis contrasts with palynological records from Andean areas that show a dominance of the *Eucryphia/Caldcluvia* pollen type around 9000 yr BP during the Holocene, when the temperature was 6–7°C higher than during the LGM (Markgraf, 1991; Villagrán, 1991, 2001). However, traces of *Eucryphya/Caldcluvia* pollen type have been recorded before this optimum, which could reflect the presence of *E. cordifolia* or *C. paniculata* in small populations growing under limiting environmental conditions. These traces probably correspond to *Caldcluvia paniculata*, which grows 300 m higher than *E. cordifolia* and therefore is more tolerant to cold conditions. The discrepancy between pollen record and genetic data should be tested with leaf fossil remains (e.g., Astorga and Pino, 2011) that allow a higher taxonomic resolution.

Overall, the lack of phylogeographic structure and the concentration of genetic diversity in the center of the latitudinal range of *E. cordifolia* suggest that this mesothermic species probably survived Quaternary climatic oscillations in these latitudes, from where it began the postglacial colonization both northward and southward. Considering the high genetic diversity found in the Cordillera Pelada (40°S, approximately), together with the geomorphological, palynological, and phytogeographic evidence, we propose this area was the main glacial refugium for *E. cordifolia*. Notwithstanding, we cannot discount that some populations of *E. cordifolia* also survived glaciations locally in the Longitudinal Valley and Andean areas because of a discontinuous ice cover. Our results highlight the importance of studying species with different ecological tolerance to explain individual responses to climatic changes (Stewart, 2008; Stewart et al., 2010).

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