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Out in the cold: genetic variation of *Nothofagus pumilio* (Nothofagaceae) provides evidence for latitudinally distinct evolutionary histories in austral South America

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Abstract

***Nothofagus pumilio* is the dominant and almost ubiquitous tree species in mountainous environments of temperate South America. We used two types of molecular markers (cpDNA and isozymes) to evaluate the effects of the Paleogene paleogeography of Patagonia and more recent climatic oscillations of the Neogene on such cold-tolerant species' genetic makeup. Phylogeographic analysis on sequences of three cpDNA non-coding regions at 85 populations yielded two latitudinally disjunct monophyletic clades north and south of *c.* 42°S containing 11 and three haplotypes, respectively. This indicates a long-lasting vicariant event due to the presence of an extended open paleobasin at mid latitudes of Patagonia. Also distribution patterns of cpDNA haplotypes suggest regional spread following stepping-stone models using pre-Cenozoic mountains as corridors. Comparable genetic diversity measured along 41 sampled populations using seven polymorphic isozyme loci provides evidence of local persistence and spread from multiple ice-free locations. In addition, significantly higher heterozygosity and allelic richness at high latitudes, i.e. in areas of larger glacial extent, suggest survival in large and isolated refugia. While, higher cpDNA diversity in lower latitudes reflects the complex orogeny that historically isolated northern populations, lower isozyme diversity and reduced F_{ST} values provide evidence of local glacial survival in numerous small locales. Therefore, current genetic structure of *N. pumilio* is the result of regional processes which took place during the Tertiary that were enhanced by contemporary local effects of drift and isolation in response to Quaternary climatic cycles.**

Keywords: ancient geological events, chloroplast DNA, glaciations, isozymes, Nothofagaceae, *Nothofagus*, South America

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Introduction

Neogene events as the last glaciations in mountainous environments are considered main drivers of genetic patterns for many woody taxa from temperate areas. However, for deep-rooted lineages with widespread ranges, distribution patterns of genetic polymorphisms may also reveal legacies from an ancient past (Premoli 1997). Distribution patterns of austral lineages including *Nothofagus* trees are a reflection of drifting and dispersal since the Paleogene when southern continents were part of Gondwana (Markgraf *et al.* 1995). However, while land masses in the Australasian sector entered the warm-temperate to tropical climatic zones leading to rapid species radiation, South America only moved 10° of latitude equatorwards which resulted in low diversification and extinction rates (Markgraf *et al.* 1995). This is reflected in the morphological stasis found in the fossil record of distinct woody genera including *Nothofagus* (Markgraf *et al.* 1996) and conifers (Del Fueyo 1996;

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Villagrán *et al.* 1996) from Patagonia. From the Neogene onwards, the influence of westerly stormtracks on southern latitudes determined relatively minor amplitude glacial-interglacial cycles which resulted in forest survival in small stands for prolonged periods (Markgraf *et al.* 1995). Also the oceanic setting of southern lands and the physical heterogeneity of the landscape, including coastal mountain ranges, provided a great diversity of habitats for tree persistence. As a result, *Nothofagus* from southern South America provides a unique opportunity to test hypothesis on the early divergence of cold-tolerant taxa under distinct geographic, geologic, and climatic scenarios shaping their gene pools.

At the early Cenozoic, the paleogeography of West Gondwana within the Patagonia-Antarctic realm differed significantly from present-day spatial configuration of oceans and land. Numerous paleobasins characterized the Paleogene geography of southern South America and the Antarctic Peninsula (Romero 1986; Lisker *et al.* 2006). Also warm climates of the Late Cretaceous progressively deteriorated at the Eocene-Oligocene boundary, and by the Late Miocene-Early Pliocene approximately 7 and 5 Ma, the oldest known glaciation occurred in Patagonia (Rabassa *et al.* 2005). Pollen diagrams that cover the last glacial-interglacial period (25 000 years ago to present) suggested that substantial ice-free areas existed throughout the austral Andes. As a result, forest taxa were able to spread from a great number of microclimatologically favourable locations where they had persisted locally during full-glacial episodes (Markgraf *et al.* 1995) in as far south as Tierra del Fuego (Heusser 1989; Markgraf 1993). Fossil pollen of *Nothofagus* is clearly distinguished and have been used to delimit four extant subgenera *Brassospora*, *Fuscospora*, *Lophozonia*, and *Nothofagus* (Hill & Read 1991), which are monophyletic and related as: (*Lophozonia* (*Fuscospora* (*Nothofagus*, *Brassospora*))) (Manos 1997). Although fossil records show continuous presence of *Nothofagus* along latitude, ecologically distinct widespread species within subgenus *Nothofagus* share the same pollen type (Hill 2001). Undistinguishable pollen, usually referred as *Nothofagus dombeyi* type, is represented in the core by *N. dombeyi*, *N. antarctica*, and *N. pumilio* (Heusser 1971). Therefore, biogeographic reconstructions for any given taxon based on pollen are difficult.

Numerous genetic studies using isozymes documented vegetation history in austral South America. These put forward for the first time the multiple refugia hypothesis in ice-free areas some of which were located towards the high-latitude range i.e. those areas of greater ice extent, of cold-hardy trees (Premoli 1998; Premoli *et al.* 2000, 2002). Therefore genetic data provided a new scenario for Southern Hemisphere plants which reflected a pre-Pleistocene evolutionary history (Premoli 1997; Moore 2000). Also, other genetic studies including isozymes and/or PCR-RFLP chloroplast DNA (cpDNA) have provided evidence of the multiple refugia hypothesis (Marchelli & Gallo 2006; Azpilicueta *et al.* 2009; Pastorino *et al.* 2009). In contrast, geographically concordant patterns by cpDNA sequences of all five species within subgenus *Nothofagus* at 10 locations in Patagonia provided evidence of chloroplast capture events due to repeated hybridization/introgression and thus suggested an ancient shared past (Acosta & Premoli 2009). Although species within subgenus *Nothofagus* may hybridize under natural conditions at particular locales which have been confirmed by genetic evidence (Premoli 1996; Stecconi *et al.* 2004; Quiroga *et al.* 2005), they occur as clearly identifiable and ecologically distinct taxa even when growing in sympatry. In addition, a molecular dating study within subgenus *Nothofagus* showed a latitudinally significant genetic structure by means of cpDNA sequences that dates back to the Paleogene suggesting that extant species consist of ancient lineages (Premoli AC, P Mathiasen, and MC Acosta, unpublished data). Similarly, a recent cpDNA analysis of the widespread *Nothofagus cunninghamii* from southeastern Australia and Tasmania reflects a phylogeographic structure older than the Pleistocene (Worth *et al.* 2009). Hence, cpDNA data of *Nothofagus* may predate Pleistocene events.

The aim of this study is to evaluate the levels and distribution patterns of genetic polymorphisms of *Nothofagus pumilio* (Poepp. et Endl.) Krasser in order to reconstruct its biogeographic history. We hypothesize that historical factors at different spatial and temporal scales have shaped the genetic makeup of such widespread and cold-hardy tree. *Nothofagus pumilio* is the dominant component of high-elevation and high-latitude forests inhabiting along *c.* 3000 km of continuous mountainous chain of the southern Andes. We used two different types of molecular markers that allowed us to differentiate historical and contemporary patterns of genetic drift and gene flow. These were cpDNA sequences which are maternally inherited, and thus genetically conserved, and isozymes which are codominant nuclear markers with biparental, and thus reflect current gene flow rates (Ennos 1994). Isozyme polymorphisms were previously used in all species within subgenus *Nothofagus* including *N. pumilio* (Premoli 1997, 2003; Premoli & Kitzberger 2005; Premoli & Steinke 2008; Steinke *et al.* 2008). Although microsatellites for *Nothofagus* were developed for some species within subgenus *Lophozonia* (Azpilicueta *et al.* 2004; Jones *et al.* 2004; Marchelli *et al.* 2008) their transferability to *N. pumilio* on analyses of 500 individuals from 10

populations have yielded a comparable number of alleles to those found using isozymes (M. Arbetman and P. Mathiasen, Universidad Nacional del Comahue).

We assume that genetic structure by cpDNA sequences will reflect the early evolution of *N. pumilio* as a result of regional processes of dispersal and vicariance. On the other hand, isozyme polymorphisms will show local responses to long-term glacial regimes which have differed latitudinally. While in the north the landscape was dominated by erosive warm-based glaciers from high-elevation catchments and remained confined to valleys (Rabassa & Clapperton 1990), in the south cold-based low erosional efficiency and/or slow moving ice sheets resulted in protective glaciation (Thomson *et al.* 2007). As a result, we predict that in the north numerous relatively small ice-free areas may have allowed local persistence of tree taxa whereas in southerly areas of maximum ice extent either forests were eliminated or persisted within isolated remnants.

Study species

Nothofagus pumilio (Nothofagaceae, 'lenga') is broad-leaved winter-deciduous which together with *N. antarctica* is the most widely distributed species in the southern South American temperate forests from 35 to 55°S latitude in Argentina and Chile. *Nothofagus pumilio* is the principal component of high-elevation and high-latitude forests of Patagonia. It mostly occurs as pure forests or mixed with *N. antarctica* in the south whereas in the north also grows with *N. betuloides* – *N. dombeyi* and the conifer *Araucaria araucana*. It is found from sea level in the south to the treeline along its entire latitudinal range, which in the north can attain *c.* 2000 m a.s.l. Striking plant architectural and ecological differences exist with elevation (Barrera *et al.* 2000; Cuevas 2000; Premoli 2004) some of which have a genetic basis (Premoli 2003; Premoli & Brewer 2007; Premoli *et al.* 2007). *Nothofagus* species are monoecious, wind pollinated, self-incompatible (Riveros *et al.* 1995), and maintain high outcrossing rates (Premoli 1996). However, seed dispersal occurs mainly by gravity resulting in local movements (Rusch 1987; Cuevas 2000).

Materials and methods

Sampling schemes at regional spatial scale

For the cpDNA analyses, *N. pumilio* samples were analyzed from a total of 85 sites. Fresh tissue was collected from 78 locations distributed across the entire species range and the other seven sites consisted of dry material from herbarium specimens (Table S1, Supporting information). Isozyme analyses were performed on a total of 1183 samples randomly collected from 41 populations. All sampling sites were located at the lower altitudinal limit where the species has a tree-form growth habit, thus avoiding genetic differences due to elevation (Table S1, Supporting information).

Sample processing

Chloroplast DNA. Fresh foliage collected at each site was frozen with liquid nitrogen and stored at −70 °C. DNA extraction of frozen foliage and herbarium specimens was performed using the DNeasy Plant-Mini-Kit (Qiagen) following the manufacturer's instructions, and the CTAB method (Doyle & Doyle 1990) modified for plants with high concentration of polysaccharides. DNA concentrations and fragment size were assessed by electrophoresis on agarose gels and comparisons with 1-kb DNA ladder (Fermentas). Non-coding regions of cpDNA were amplified by polymerase chain reaction (PCR) using 10 universal primer pairs described in Taberlet *et al.* (1991), Demesure *et al.* (1995), Dumolin-Lapègue *et al.* (1997), and Hamilton (1999). The intergenic spacer regions separating *psb*B-*psb*H (BH), *trn*L-*trn*F (LF) and *trn*H-*psb*A (HA) were the most variable and selected for full analysis. To facilitate DNA release a reaction with 2 μL (10 ng) of DNA extract and 6 μL of GeneReleaser® (BioVentures) was performed previous to PCR in the thermocycler (conditions: 15 min at 85 °C; hold at 32 °C). The PCR reaction mix contained 2 μL of DNA extract (~10 ng), 5 μL of 5 × Green GoTaq® Reaction Buffer (Promega), 0.25 mM of each dNTP, 0.3 μM of each primer, and 1.25 U of GoTaq® DNA polymerase (Promega), in a total volume of 25 μL. The PCR amplification conditions were: 4 min at 95 °C, 35X (1 min at 94 °C; 1 min annealing at 57 °C for BH, 54 °C for LF and 56 °C for HA; 1.5 min at 72 °C), and 6 min at 72 °C. The PCR products were purified by a reaction using 2.5 U of Exonuclease I (USB) and 0.25 U of Shrimp Alkaline Phosphatase (USB) for 10 μL of PCR product (conditions: 15 min at 37 °C; 15 min at 85 °C). The amplified DNA was sequenced with an ABI PRISM 3100 *Avant* Genetic Analyzer (Applied Biosystems) using a BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems). The cycle sequencing reactions were performed following the manufacturer's protocols. The three selected regions were sequenced in both directions for at least one individual from each population to confirm sequence polymorphisms. Sequences of each sampled haplotype were deposited in GenBank (accession numbers: BH: GU152886-GU152893 and GQ863397-GQ863405; LF: GU152870-GU152877 and GQ863379-GQ863387; HA: GU125878-GU152885 and GQ863388-GQ863396).

Sequencing data were aligned with MEGA4 (Tamura *et al.* 2007) and concatenated manually into a single combined dataset for posterior analyses. Chloroplast DNA haplotypes were determined from both nucleotide substitutions and indels. We calculated different diversity parameters including variation in alignment size, number of haplotypes (H), C/G content (%CG), number of variable sites, number of transitions (ti), transversions (tv), indels (I), and nucleotide (π) and haplotype (h) diversity with ARLEQUIN v2.0 (Schneider *et al.* 2000). Demographic analyses were conducted by means of Tajima's *D* (Tajima 1989), Fu and Li's *F* (Fu & Li 1993), and observed and expected mismatch distribution graphics, using the program DnaSP v4.10.9 (Rozas *et al.* 2003).

Isozyme electrophoresis. Fresh leaf material collected was kept refrigerated until processing in the laboratory. Enzyme extractions, protein electrophoresis, and enzyme staining protocols followed Premoli (1996, 1998, 2003). Six enzyme systems coding for 8 putative isozyme loci were resolved as follows: alcohol dehydrogenase (*Adh*1), aldolase (*Ald*1), isocitric dehydrogenase (*Idh*1, *Idh*2), and malate dehydrogenase (*Mdh*1, *Mdh*2, *Mdh*3) in the morpholine-citrate buffer by Ranker *et al.* (1989), whereas phosphoglucoisomerase (*Pgi*2) in the modified system B by Conkle *et al.* (1982). Heterogeneity in gene frequencies across populations was evaluated by chi-square tests following Workman & Niswander (1970). Genetic variation parameters were calculated for each population by the mean number of alleles per locus (A), number of alleles per polymorphic locus (A_P), effective number of alleles (A_E), percent of polymorphic loci under the *sensu stricto* criterion (*P*), observed and expected heterozygosity (H_O and H_E, respectively) using PopGene v1.32 (Yeh *et al.* 1999). Since allelic richness may be more useful than other parameters to identify historical processes such as bottlenecks and population admixture (Comps *et al.* 2001; and references therein), we calculated total allelic richness (A_R) following Comps *et al.* (2001) using the program CONTRIB v1.02 (Petit *et al.* 1998). We compared allelic richness after rarefaction to a common sample size of 20 individuals per population (40 gene copies). Therefore, populations with less than 20 individuals were not used to compute allelic richness. We performed a multiple regression analysis to investigate the relationship between latitude and different within-population genetic diversity parameters. Wright's (1931) fixation Index ($F = 1-[H_O/H_E]$) was calculated for each population, and departures from Hardy–Weinberg expectations were tested by chi-squared tests for each polymorphic locus. Genetic structure of populations was analyzed by hierarchical *F*-statistics with the program FSTAT (Goudet 2001) which computes non-biased estimations for F_{ST} as the degree of among-population divergence between all population pairs (Wright 1965). The degree of significance of estimations was analyzed by means of 95% confidence intervals that were computed by permutations over all loci following Weir & Cockerham (1984). Total genetic diversity (H_T) and the mean within-population diversity (H_S) was calculated according to Nei (1973).

Analyzed populations by isozymes were classified a priori into groups accordingly to the two main clades yielded by the phylogenetic cpDNA analysis: North (between 36–42°S), and South (between 42–55°S). This resulted in 11 and 30 populations that were analyzed within each group encompassing a latitudinal range of 6 and 13 degrees, i.e. two populations/degree of latitude along the entire distribution, which resulted in an average of 31 and 28 individuals/population analyzed in the North and South, respectively. Genetic diversity parameters were compared by groups of populations using *t* test or Mann–Whitney *U* tests when data did not fit a normal distribution. Also degree of genetic divergence among populations within each group was analyzed by pairwise F_{ST}.

Phylogenetic analyses

The phylogenetic relationships among cpDNA haplotypes were inferred by maximum parsimony (MP) and maximum likelihood (ML) methods using PAUP 4.0b10 (Swofford 2002) and Bayesian inference (BI) analysis using MrBayes v.3.1.2 (Ronquist & Huelsenbeck 2003). Indels of more than 1-bp were treated as single characters resulting from one mutational event. Gaps were coded as presence/absence only for the MP analysis and were not considered for the ML and BI analyses. ML and MP analyses were performed using a heuristic search with 100 random replicates, based on branch swapping with tree-bisection-reconnection (TBR), and unordered and equally weighted character states. The BI analysis was performed using four replicates with four chains (three heated and one cold) that were run for two million generations sampling every 100 generations with a burn-in period of approximately 10% of the trees. Nodal support was determined by performing a MP and ML bootstrap analysis (Felsenstein 1985) with 1000 replicates in PAUP*; and by using Bayesian posterior probabilities as implemented by MRBAYES. The most appropriate models of nucleotide substitution identified by ModelTest v3.7 (Posada & Crandall 1998) and MRMODELTEST v2.3 (Nylander 2004) under the Akaike information criterion (AIC) were implemented in the ML and BI analyses, respectively. The best-fit model for ML was K81uf+I, and GTR+I for BI. Sequences of

Nothofagus obliqua, *N. nervosa*, and *N. glauca* within subgenus *Lophozonia* were used as outgroup. Furthermore, we generated a Median-Joining network (Bandelt *et al.* 1999) with the program Network v4.201 (available from http://www.fluxus-engineering.com) to visualize relationships among haplotypes.

Landscape analyses

Patterns of genetic divergence among sampling areas across the range of *N. pumilio* were investigated by analysis of molecular variance (AMOVA) using cpDNA and isozyme genetic distances among populations using the program GenAlEx version 6.2 (Peakall & Smouse 2006). Also hierarchical AMOVA was used to test for genetic structure at various geographic scales: among geographic regions following the two main clades yielded by the phylogenetic cpDNA analysis (north, between 36–42°S and south, between 42–55°S), among populations within regions, and within populations. The significance of global, hierarchical, and pairwise Φ_{ST} values was evaluated by permutations based on 9999 replicates. To identify groups of populations at geographically adjacent sampling areas that were maximally differentiated from each other we performed a spatial analysis of molecular variance SAMOVA v 1.0 (http://web.unife.it/progetti/genetica/Isabelle/samova.html, Dupanloup *et al.* 2002) based on 100 simulated annealing steps and examined maximum indicators of cpDNA differentiation (F_{CT} values) setting the number of groups of sampling sites from $K = 2$ through $K = 14$. To portray Genetic Landscape Shapes (GLS) by means of 3-D surface plots of genetic distance patterns (Z coordinates) across the full landscape analyzed in this study (X and Y coordinates) we used the program Alleles in Space (Miller 2005) on cpDNA and isozymes using different grid sizes (20×20, 50×50, 100×100) and with a range of distance weight values ($a = 0.5$ through $a = 2$) to avoid misinterpretations of the data.

Results

Geographical distribution of genetic variation

Chloroplast DNA. We analyzed a total of 194 individuals with an average of two individuals per sampled site (range 1–6) (Table S1, Supporting information). The sequence analysis of 194 individuals from 85 *N. pumilio* populations yielded 14 different geographically structured cpDNA haplotypes (Fig. 1). The length of the aligned cpDNA sequences varied between 776–791 bp (BH), 416–437 bp (LF), and 419–445 bp (HA). All regions were concatenated totaling a matrix of 1673 characters with 25 variable sites (Table 1). Four most frequent haplotypes (H2, H8, H12, and H14) had non-overlapping latitudinal distributions in geographically contiguous regions of Argentina and Chile. Haplotype 2 was found from 37 to 40°S, H8 was found between 40–42°S, H12 was constrained to 42–46°S, and finally H14 was widely distributed in the south from 47–55°S in the Austral Patagonian Andes. More than one haplotype was detected at four northern locations. Haplotypes H1 and H5 were found in NHB population on the Pacific Coastal Range at 38°S while the rest were located in the Andes Cordillera and consisted of haplotypes H2 and H7 in MOQ population at 39°S, H2 and H8 in RCH population at 39°S, and H8 and H10 in PUY population at 41°S (Fig. 1). While populations NHB, MOQ, and PUY contain haplotypes from a given lineage, population RCH consists of haplotypes from distinct lineages (for population names and locations see Table S1, Supporting information).

Neutrality tests by Tajima's D (2.57, $P < 0.05$) and Fu & Li's F (2.17, $P < 0.02$) showed positive and significantly different from zero values, indicating a decrease in population size in the northern clade. The mismatch distribution analysis showed a bimodal distribution indicating that sampled populations throughout the entire range have been historically isolated (Fig. 2a). The analysis made for the northern clade showed a unimodal distribution of pairwise differences, indicating that populations have suffered a severe bottleneck followed by population expansion (Fig. 2b).

Isozyme analyses. A total of 1183 individuals from 41 populations were genotyped. Seven of the eight analyzed loci were polymorphic in at least one population. These were *Adh*1, *Ald*1, *Idh*1, *Idh*2, *Mdh*2, *Mdh*3, and *Pgi*2. Analyzed populations were highly heterogeneous in their allelic frequencies (chi-square test, $P < 0.001$). Patterns of genetic diversity were heterogeneous along latitude. Multiple regression analysis yielded a significant increase with increasing latitude ($P < 0.001$) of the effective number of alleles (A_E, $r^2 = 0.46$), allelic richness (A_R, $r^2 = 0.61$), and observed and expected heterozygosity (H_O, $r^2 = 0.40$ and H_E $r^2 = 0.43$). North and south groups of populations were genetically distinct. Allelic frequencies differed significantly ($P < 0.05$) among populations within the two groups for five of the six possible tests in the North group, and for all seven loci in the South group (data not shown). South populations had significantly higher total allelic richness A_R and observed heterozygosity H_O than the North ones (Mann–Whitney U test: $Z_{(11,30)} = -2.31$, $P = 0.019$; $Z_{(11,30)} = -2.25$, $P = 0.023$, respectively) (Table 2). On average fixation indexes (F) yielded significantly positive estimates. Departure from

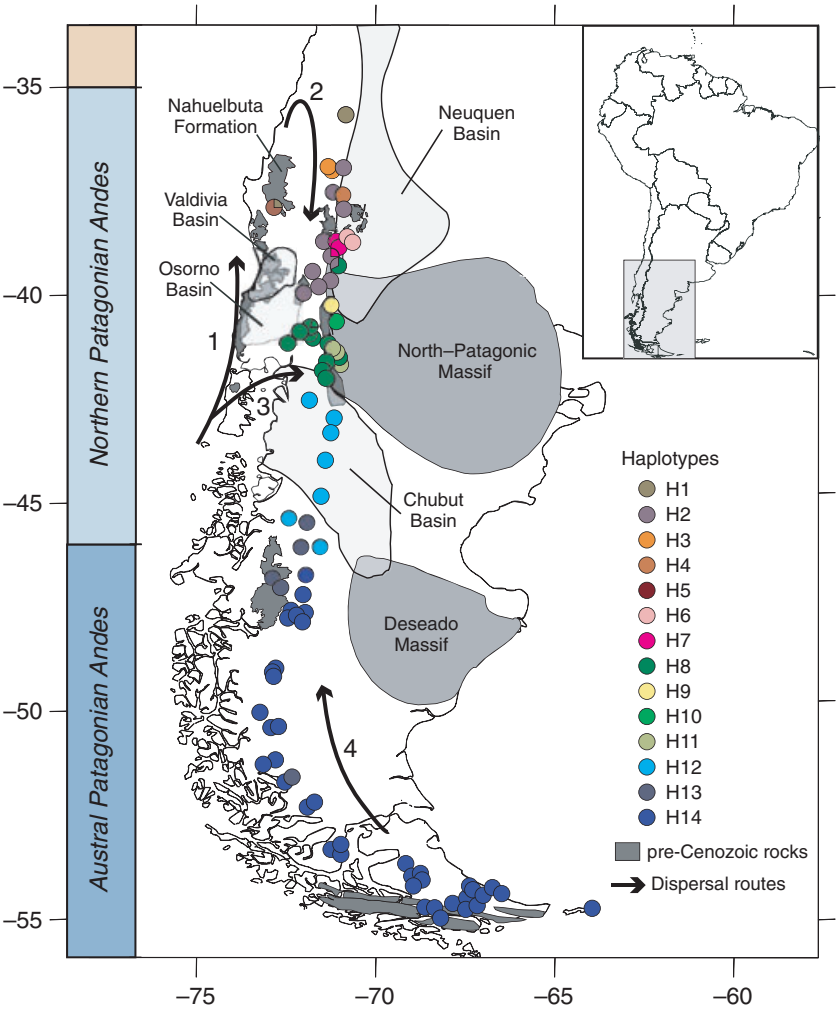

Fig. 1 Map of southern South America showing the distribution of 14 cpDNA haplotypes based on the combined sequences of *psb*H-*psb*H, *trn*L-*trn*F and *trn*H-*psb*A intergenic spacers for 85 sites of *Nothofagus pumilio*. Black arrows indicate hypothetical dispersal routes during the Paleogene.

Table 1 Molecular diversity indices calculated for each cpDNA marker separately (BH, LF, and HA) and concatenated (BH-LF-HA), and for the two major clades (North and South) identified for *Nothofagus pumilio* populations

Region	*P*	*N*	Size (bp)	Range (bp)	H	CG%	Ps	ti	tv	I	h	π
BH	85	194	791	776–791	2	36.9	4	0	3	1	0.4834 (0.0155)	0.0110 (0.0056)
LF	85	194	437	416–437	6	33.9	5	0	0	5	0.7648 (0.0127)	0.0173 (0.0090)
HA	85	194	445	419–445	11	26.5	16	0	8	8	0.7949 (0.0194)	0.0277 (0.0139)
BH-LF-HA	85	194	1673	1611–1673	14	33.4	25	0	11	14	0.8686 (0.0127)	0.0171 (0.0083)
North	34	108	1671	1630–1671	11	33.4	12	0	5	7	0.8343 (0.0216)	0.0119 (0.0059)
South	51	86	1634	1632–1634	3	33.6	2	0	0	2	0.5473 (0.0483)	0.0004 (0.0003)

BH, LF, and HA correspond to cpDNA *psb*H-*psb*H, *trn*L-*trn*F and *trn*H-*psb*A intergenic spacers. Number of analyzed populations (*P*) and individuals (*N*), alignment size and range are indicated in base pairs (bp), number of haplotypes (H), CG content (%CG), number of polymorphic sites (Ps), transitions (ti), transversions (tv), indels (I), haplotype (h) and nucleotide (π) diversity. Standard errors are in parentheses.

Hardy–Weinberg expectations was rejected in 40% of the 108 performed tests. North populations presented higher percentages of loci with significant positive fixation indexes than South populations (42 and 35%, respectively), which indicate a heterozygote deficit for the analyzed loci in those populations.

Hierarchical analysis of population structure by *F* statistics yielded on average significantly positive

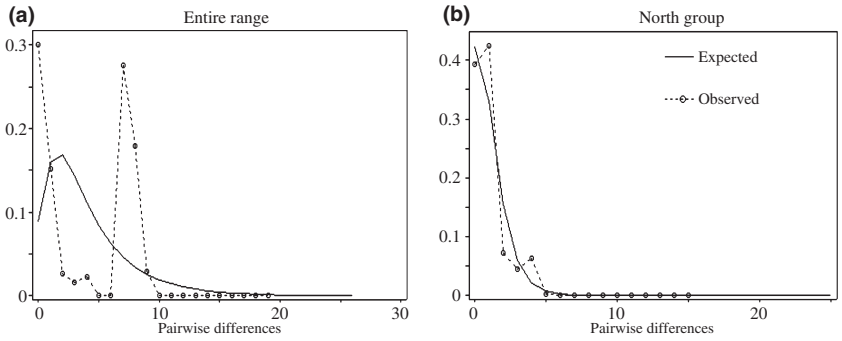

Fig. 2 Mismatch distribution graphics of pairwise comparisons of haplotypes for all (a) and for North populations (b) of *Nothofagus pumilio*. Expected frequencies of pairwise differences in a growth/decline population model are indicated by solid line. Observed frequencies of pairwise differences between haplotypes are indicated by dash line.

Table 2 Measures of within-population isozyme variation parameters averaged for the two major clades (North and South) identified for *Nothofagus pumilio* populations along the latitudinal gradient

Group	*P*	*N*	*A*	A_P	A_E	A_R*	*P*	H_O*	H_E
North	11	342	1.35 (0.11)	1.40 (0.12)	1.06 (0.03)	0.43 (0.14)	29.6 (8.4)	0.032 (0.015)	0.044 (0.017)
South	30	841	1.41 (0.19)	1.46 (0.22)	1.13 (0.12)	1.06 (0.74)	34.2 (17.0)	0.055 (0.033)	0.080 (0.061)
Population	41	28.9	1.40 (0.18)	1.45 (0.20)	1.11 (0.11)	0.89 (0.70)	32.9 (15.3)	0.049 (0.031)	0.070 (0.055)
Species	41	1183	2.75 (0.89)	3	1.11 (0.08)	2	87.5	0.052 (0.044)	0.091 (0.070)

P, number of populations; *N*, number of individuals; A, mean number of alleles per locus; A_P, number of alleles per polymorphic locus; A_E, effective number of alleles; A_R, corrected allelic richness after rarefaction to a common sample size of 20 individuals; *P*, proportion of polymorphic loci under the *sensu stricto* criterion; H_O and H_E, observed and expected heterozygosity, respectively. Standard deviations are in parenthesis.*$P < 0.05$, Mann–Whitney *U*-test.

among-population divergence (F_{ST} = 0.200, 95% CI = 0.138–0.232). Significant F_{ST} values were also found among populations within each latitudinal group. Southern populations attained mean greater values (F_{ST} = 0.217, 95% CI = 0.166–0.243) than those in the north (F_{ST} = 0.059, 95% CI = 0.019–0.159) which was confirmed by significant differences between pairwise F_{ST} values between populations from distinct groups (Mann–Whitney *U* test: Z = −3.83, $P < 0.001$). Overall total genetic diversity measured by Nei's statistics was low (H_T = 0.089) most of which was distributed within populations (H_S = 0.072). Higher total genetic diversity was measured in the South (H_T = 0.103) than in the North group (H_T = 0.047) (N–S: t_7 = 0.46, P = 0.662; paired *t* test by locus).

Phylogenetic analyses

The MP analysis revealed two most parsimonious trees which required 74 steps (CI = 0.9189, RI = 0.9589). The topology of the strict consensus was identical to one of the two most parsimonious trees. The ML analysis yielded one most-likely tree (−ln*L* = 2543.1) similar to those obtained by MP. The BI tree was congruent with the MP and ML trees, the topologies differing only with respect to nodes with low bootstrap support in either tree. All phylogenetic analyses recovered the same two monophyletic clades with strong bootstrap support. The northern clade (north of 41° 59′S) contains 11 haplotypes (H1–H11) from northern Patagonia of Chile and Argentina. The southern clade (south of 42° 51′S) is represented by only three haplotypes (H12–H14) that are widely distributed in southernmost Patagonia (Fig. 3). The northern clade had higher average nucleotide and haplotype diversity than the southern clade (Table 1). Results of the median-joining network analysis yielded one network including all 14 haplotypes with no internal reticulations (Fig. 4). We identified four groups of haplotypes that suggested a non-random distribution based on geography (Fig. 1). The haplotypes of northern-most populations and those on the Pacific Coastal Range formed a group, hereafter coastal-north, had the most internal position on the network and therefore were considered ancestral. The other three groups, hereafter Andean-north, middle-north, and south, containing high-frequency variants had non-overlapping distribution across *N. pumilio* latitudinal range. In particular, southern

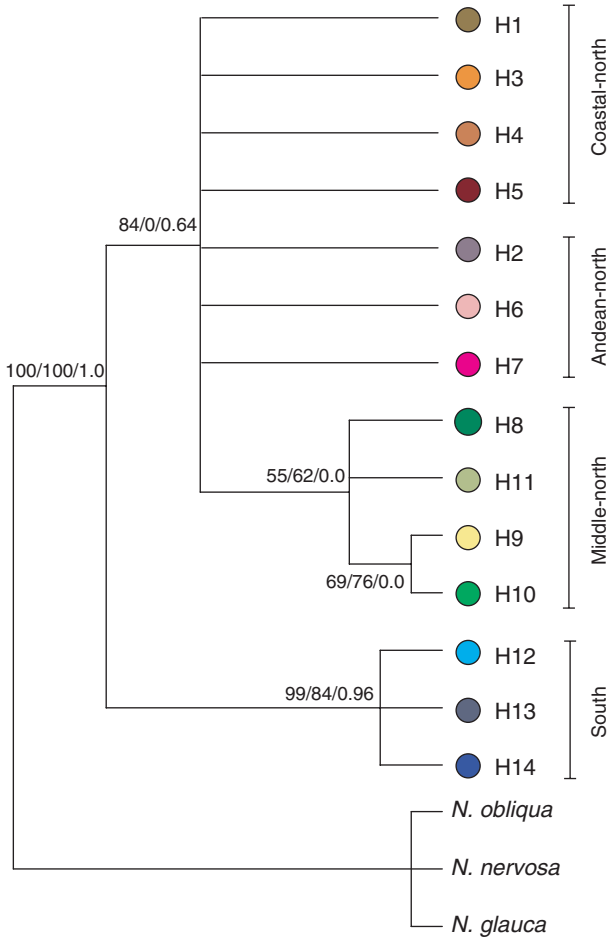

Fig. 3 Phylogenetic tree showing the relationships among cpDNA haplotypes based on the combined cpDNA sequences of *psb*H-*psb*H, *trn*L-*trn*F and *trn*H-*psb*A intergenic spacers for 85 sites of *Nothofagus pumilio*. Numbers indicate bootstrap values for MP, ML, and posterior probabilities for BI analyses.

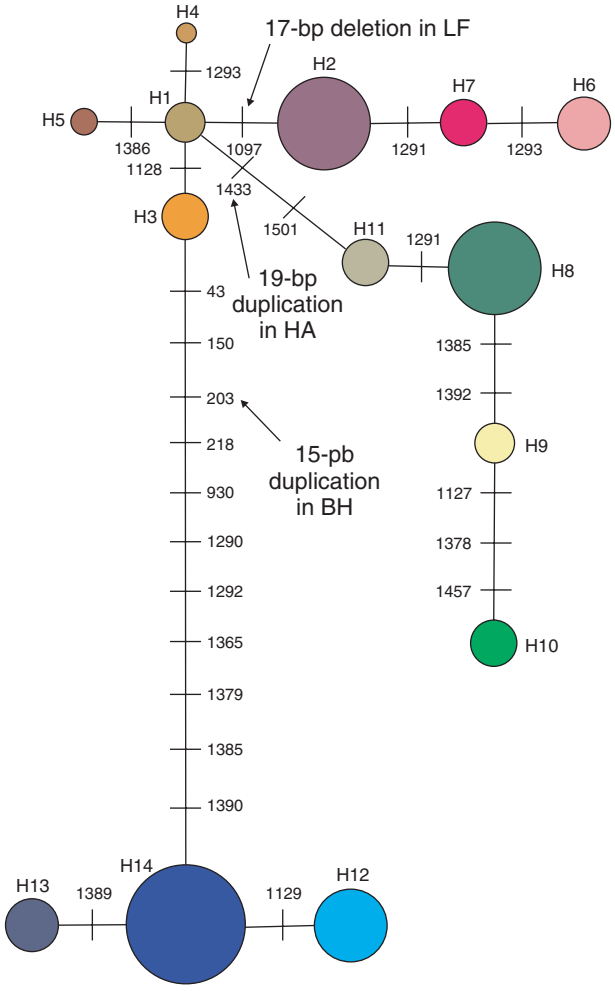

Fig. 4 Median-Joining network showing the relationships among 14 cpDNA haplotypes of *N. pumilio* based on the combined cpDNA sequences of *psb*H-*psb*H, *trn*L-*trn*F and *trn*H-*psb*A intergenic spacers. Circle size is proportional to the haplotype frequency.

haplotypes were clearly distinct and separated by 11 mutational steps (Fig. 4).

Landscape analyses

Results from AMOVA yielded a significant genetic structure within *N. pumilio*. Chloroplast DNA analyses indicated the presence of substantial among-population genetic structure (Φ_{ST} = 0.978; P = 0.001). Pairwise Φ_{ST} values ranged from 0.000 to 0.997 and 188 out of 1431 total pairwise Φ_{ST} values where significantly different than zero. Also hierarchical AMOVA yielded significant (P = 0.001) partitioning of cpDNA at all hierarchical levels, i.e. between north and south regions (Φ_{RT} = 0.737), among populations within regions (Φ_{SR} = 0.947), and among populations (Φ_S = 0.986). SAMOVA analyses on cpDNA sequences suggest the existence of genetically distinct groups. Analyses with K = 2, identified two latitudinal groups separated at 42°S (F_{CT} = 0.741; P < 0.001), which correspond to the north and south lineages from the phylogenetic analyses (see Fig. 3). Analyses with K = 3 and K = 4 yielded similar results which provided additional groups within the northern area. Four groups of populations consisted of coastal-north populations, Andean-north located between 38–40°S, middle-north between 40–42°S, and populations south of 42°S (F_{CT} = 0.967; P < 0.001). The F_{CT} values did not increase substantially in the K = 5 through K = 7 relative to the value found at K = 4.

AMOVA results for the isozyme analyses yielded a significant genetic structure. The overall Φ_{ST} value for isozymes was 0.338 (P = 0.001) with pairwise Φ_{ST} ranging from 0.000 to 0.733, and the 82% of those values were

significantly different than zero. Hierarchical AMOVA using isozymes was significant ($P = 0.001$) at all hierarchical levels. However, genetic structuring between north and south regions ($\Phi_{RT} = 0.021$) was an order of magnitude lower than $\Phi_{SR} = 0.331$ and $\Phi_{ST} = 0.346$ values which stand for subdivision among populations within regions and among populations, respectively. The level of subdivision between north and south regions was also an order of magnitude greater for cpDNA $\Phi_{RT} = 0.737$ than that for isozymes $\Phi_{RT} = 0.021$. Similarly, SAMOVA procedure failed to partition isozyme data. Most probably, higher gene flow rate by means of isozymes in *N. pumilio* than cpDNA (see Discussion) prevented the use of SAMOVA analyses which in turn maximize genetic differentiation when gene flow rates within groups are at least 1000 times larger than that among groups (Dupanloup *et al.* 2002).

Genetic landscape shapes interpolation analyses produced surface plots that qualitatively supported results from SAMOVA (Fig. 5). A major genetic differentiation zone indicating a probable important barrier to gene flow was observed at mid-latitudes (42–43°S) for both, the isozyme and the cpDNA analyses (Fig. 5a,b). Discontinuities for cpDNA were also found at 38, 40, 46, and 48°S (Fig. 5a). In addition, major genetic differentiations were obtained for isozymes at 46 and 50°S (Fig. 5b). Additional features of the landscape shape show that patterns of cpDNA genetic distances decreased with increasing latitude. On the other hand, isozyme genetic distances were higher towards the centre of the range and decreased gradually to both northern and southern distributional extremes. Similar surface plots were obtained using different grid sizes and distance weighting parameters.

Discussion

cpDNA genetic structure

Latitudinally divergent cpDNA structures suggest that widespread *N. pumilio* consists of deep-rooted and separate northern and southern lineages with distinct evolutionary histories. Many phylogeographic studies particularly on Northern Hemisphere plant taxa have provided evidence of significant genetic structures by means of cpDNA markers. These studies showed that most lineages inhabiting colder northern areas were derived from warmer refugia further south which have been interpreted as a result of glacial history (Petit *et al.* 2002; Soltis *et al.* 2006). Nevertheless, a growing body of evidence suggests that cold-tolerant tree species were able to survive in refugia within colder regions (Stewart & Lister 2001; Rendell & Ennos 2002; Tsuda & Ide 2005; Anderson *et al.* 2006; Bhagwat & Willis 2008; Tollefsrud *et al.* 2008) as early hypothesized (Moore 2000; Premoli *et al.* 2000). Under glacial scenarios of long distance dispersal and given that relatively greater glacial extent existed towards the cold south in South America, austral populations should have been derived from those located under warmer northern areas (Villagrán & Hinojosa 2005). The lack of common as well as intermediate haplotypes along more than 2000 km provide unequivocal evidence that north and south clades of *N. pumilio* have been historically isolated and that neither of them has derived from the other (Figs 1 and 4). On the other hand, hypotheses of either extinction of common haplotypes along the range or the loss of northern haplotypes in the south and *vice versa* seem unlikely given that our study comprises the entire latitudinal and longitudinal distribution of *N. pumilio*. In addition, geographically concordant phylogeographic patterns among all five species within subgenus *Nothofagus* support the hypothesis that cpDNA polymorphisms reflect an early evolutionary history (Acosta & Premoli 2009).

The results of hierarchical AMOVA and SAMOVA clearly indicate that cpDNA and isozyme structures reflect contrasting temporal signals. Lower Φ_{RT} values and the lack of geographic structure by SAMOVA for isozymes indicate higher contemporary than historical gene flow rates. This is relevant in *N. pumilio* because it has relatively low fecundity and restrictions for establishment exist particularly at northern latitudes.

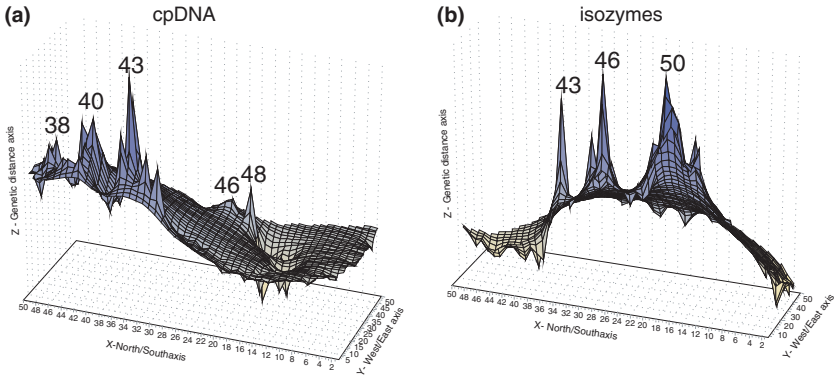

Fig. 5 Genetic Landscape Shape interpolation using a 50×50 grid and a distance weighting parameter of $a = 1$. *X* and *Y* axes correspond to geographic locations within the overall physical landscape examined in this study. Surface plot heights reflect genetic distances among *Nothofagus pumilio* populations and numbers above peaks indicate approximate latitudes.

Therefore, genetic differences, particularly between north and south cpDNA lineages, have probably accumulated before LGM that occurred *c.* 25 000 years ago in Patagonia. Undetected geographic structure by isozymes reported here may be biased and wide-range studies using higher-resolution markers may help to elucidate glacial history of *N. pumilio*. Nevertheless, the observed cpDNA structure suggests that ancient processes have shaped *N. pumilio* gene pool which has been reinforced by glacial history. For example, the Coastal Cordillera has been suggested as a glacial refugia based on fossils (Villagrán & Hinojosa 2005) and genetic evidence for distinct taxa (Allnutt *et al.* 1999; Premoli *et al.* 2000; Marchelli & Gallo 2006; Azpilicueta *et al.* 2009; Vidal-Russell *et al.* 2009). Therefore, ice-free areas such as the Coastal Range have probably conserved ancestral cpDNA haplotypes in *Nothofagus*.

Paleogeographic features of Patagonia may explain genetic patterns. We hypothesize that the presence of the western Chubut open paleobasin at mid latitudes about 43°S (Suárez & Márquez 2007) produced a vicariant event between *N. pumilio* populations. Geographically concordant wide-ranging disjunction at 43°S was also observed using isozyme polymorphisms in the widespread ancient conifer *Podocarpus nubigena* (Quiroga & Premoli 2009). The opening of a late-Tertiary open Pacific Valdivia paleobasin at *c.* 40°S latitude (le Roux & Elgueta 2000) is concordant with the spatial split of the northern group in two lineages as shown by SAMOVA analysis with $K = 3$. One of these comprises the coastal-north that includes ancestral haplotypes together with the Andean-north, and the other contains haplotypes of the middle-north group (Figs 1 and 3). Also, the mismatch distribution analysis showed that in the north a bottleneck occurred followed by range expansion throughout two routes following a stepping stone model: along the Pacific Coastal Range in the west and the Northern Patagonian Massif in the east (Fig. 4). We therefore hypothesize that ancestral haplotypes migrated north (Fig. 1, route 1) along the Pacific Coastal Range before the opening of the Valdivia paleobasin as far as favourable climatic and topographic conditions allowed. From that point, colonization took place towards the south (Fig. 1, route 2) flanking the western margins of the Neuquén interior paleobasin (Suárez & Márquez 2007). On the other hand, middle-north lineage migrated north (Fig. 1, route 3) surrounding the eastern margins of the Osorno interior paleobasin (le Roux & Elgueta 2000). Available land for dispersal during that time was scarce due to the presence of several basins (Lisker *et al.* 2006) and sea level was higher than present-day (Miller *et al.* 2005). We hypothesize that suitable land for plant dispersal in northern latitudes consisted of numerous fragmented geologic units of at least Mesozoic age. In particular, the Nahuelbuta formation, which consists of Paleozoic rocks, shelters the unique haplotype H5 (see Fig. 1). Also the distribution of haplotypes H6, H7, H9, H10, and H11 is correlated with the presence of old geologic units that have been reported in different parts of the Patagonian region (Giacosa & Heredia 2004; Glodny *et al.* 2008; Cembrano & Lara 2009). In contrast, genetically homogenous populations at austral-most latitudes suggest *in situ* evolution within large units of emergent terrain (Drewry 1976; Pankhurst *et al.* 1999, 2006; Rosello *et al.* 2004) allowing long-lasting persistence. Moreover, the fact that haplotype H12 is derived from southern most haplotype H14 suggests that colonization of mid latitude areas most probably took place from south to north. This probably occurred once the Chubut paleobasin was drained at the onset of the Miocene uplift of the main Andes Cordillera (Fig. 1, route 4). Similarly, phylogeographic scenarios reflecting events of the Tertiary were also suggested for Mediterranean trees by Magri *et al.* (2007).

Landscape analyses by means of cpDNA yielded three genetic discontinuities along latitude at 38, 40, and 43°S (Fig. 5). These could be interpreted as suture zones between long-term divergent genetic sources because of the presence of the Neuquen, Valdivia, and Chubut paleobasins, respectively. In contrast, two genetically distinct groups of populations by means of PCR-RFLP of cpDNA markers on 12 populations, and thus a reduced portion of the range of the widespread *Nothofagus antarctica*, was interpreted as a long lasting isolation in at least two different glacial refuges north and south *c.* 43°S (Pastorino *et al.* 2009). Moreover, polymorphic areas by means of PCR-RFLP using cpDNA markers in the warm-temperate *Nothofagus nervosa* of subgenus *Lophozonia* were interpreted as spread from distinct glacial refugia north and south of a west-east mountain chain at *c.* 40°S (Marchelli *et al.* 1998). Within this area, the watershed draining to the Pacific Ocean was indicated as having a glacial origin (Marchelli & Gallo 2006). A recent molecular dating analysis yielded a 40 Ma divergence between north and south lineages at 43°S and a 20 Ma disjunction within the northern lineage at 40°S for all species within subgenus *Nothofagus* including *N. pumilio* and *N. antarctica* which in turn confirms pre-Pleistocene vicariant events due to paleobasins (AC Premoli, P Mathiasen, MC Acosta). At present, these paleobasins correspond to river basins that drain into the Pacific Ocean and therefore besides of potential effects of glaciations on such watersheds, our data suggest that ancient tectonic controls within these areas have affected vegetation history and species' gene pools.

Novel neogene trends

Isozyme diversity in the widespread *Nothofagus pumilio* differs latitudinally. Significant heterogeneity of allelic frequencies and F_{ST} values, especially in such genetically impoverished species, provides evidence of a long-lasting divergence such that mutations were accumulated within latitudinally distant populations. Parameters of within-population genetic variation increased towards colder southern latitudes, including allelic richness A_R which is expected to be affected by bottlenecks. The higher overall genetic diversity in the south may suggest that *N. pumilio* populations may have persisted locally through time. Given the limited seed dispersal of *N. pumilio*, such high genetic diversity in the south as to be the result of long distance migration from northern sources after the LGM is unlikely. Estimations of pollen/seed flow for *N pumilio* as means of degree of population differentiation from nuclear (isozymes) and maternally inherited markers (cpDNA) following Ennos (1994) indicated that pollen migration rates were as much as 100 times greater than those by seed. We estimated the rate of postglacial colonization from northern refugia to southern areas. We considered that the LGM occurred about 20 000 years ago and north and south populations were *c.* 1000 km apart. We took into account the following parameters which are biased towards optimum conditions for *N. pumilio* regeneration. These were: seed dispersal distances of 100 m from the mother tree, a 50 years generation time, and production of abundant viable seeds which occur during masting events every *c.* 5 years. We found an estimate of annual migration by seed of 0.4 m and by pollen of 40 m. As a result, pollen of *N. pumilio* was able to reach dispersal distances of at most 800 km since the LGM which is far less than the range occupied by the species. Therefore genetic structures arising from glacial scenarios consisting of population extinction due to an enlarged ice cover in southern areas, and wide-ranging postglacial colonization from warmer ice-free northern areas are not supported by our data on *N. pumilio*.

We hypothesize that numerous ice free locations existed in the north that maintained continuous gene flow which in turn supports the multiple refugia hypothesis (Premoli 1998). Nevertheless, *N. pumilio* in northern refugia was impacted by drift and inbreeding due to reductions in population size because of the effects of valley glaciers on such mountain species. The absence of geographic barriers by means of landscape analysis in the north suggests either that substantial gene flow has been maintained at lower latitudes or that barriers to migration are too recent to be detected by isozyme patterns (Latta 2006). In the south, glacial activity is thought to have had low-erosional efficiency and even the presence of non-glaciated frozen ground was suggested (Thomson *et al.* 2007). Hence, we can speculate that southern populations have probably persisted in large pieces of available land and thus consisted of relatively big and panmictic populations that remained isolated among each other through time. Landscape analysis show genetic discontinuities in three locations at 43, 46, and 50°S (Fig. 5b) suggesting that multiple refugial areas may have existed in the south which have became into contact more recently. For example, ecological niche modelling of LGM distribution of *N. pumilio* shows significant suitable areas east of the ice north and south 43°S (AC Premoli and T Kitzberger, unpublished data) that confirms such secondary contact area. Also such discontinuity may be a reflection of the ancient genetic structure evidenced by cpDNA (Fig. 5a).

Significantly lower F_{ST} values in the north may reflect relatively continuous gene flow among refugial populations surviving in numerous and relatively close ice-free locations. Thus, for such self-incompatible tree populations in the north maybe more resilient to future local fragmentation due to either climate change or human activities, since greater opportunities have existed in the past for deleterious alleles to be removed by natural selection. Therefore, southern populations maybe prone to suffer from modifications on gene flow rates and population size which should be taken into consideration for the design of management and conservation actions on *N. pumilio* which is considered a key tree species under current logging practices.

Conclusion

In this study we propose that cpDNA variation patterns of *N. pumilio* were controlled by ancient paleogeography rather than more recent Neogene events such as Pleistocene glaciations. Hence, latitudinal cpDNA structure reflects vicariant events due to paleobasins and early dispersal routes of *N. pumilio* in South America. Present-day continuous forests at *c.* 40 and 43°S in areas where distinct haplotypes belonging to divergent clades were found represent suture zones between temporarily isolated lineages. Complex cpDNA genetic structure in the north suggests an evolutionary history of isolation and drift, whereas low cpDNA differentiation in the south indicates absence of restrictions for historical gene flow. Elevated isozyme diversity at high latitudes reveals long-term persistence within large areas and significant among-population divergence suggests that genetic differences have accumulated through recent times. In contrast, genetically similar and impoverished northern populations reflect local survival in several

relatively small ice-free locales. We hereby put forward a new scenario for discussions on plant phylogeography from temperate high-latitude forests. Major genetic differentiation reflected by cpDNA polymorphisms in *N. pumilio* at regional scales occurred mainly due to Paleogene forces significantly controlled by geology. In addition, the evidence presented here suggests that cold-tolerant ancient taxa as *N. pumilio* have responded vigorously to climatic oscillations of the Quaternary and persisted locally over large geographic areas.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Geographic location of sampling sites and number of individuals (*N*) for cpDNA and isozyme analyses on *Nothofagus pumilio*

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