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Retracing the evolutionary history of *Nothofagus* in its geo-climatic context: new developments in the emerging field of phylogeology

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ABSTRACT

Phylogeographic studies have made a significant contribution to the interpretation of genetic lineage distribution in response to climate changes, such as during glaciation events of the Neogene. However, the effects of ancient landscapes associated with global sea level rises, tectonic processes, and climatology driving lineage evolution have been largely overlooked. These effects can be tested in widespread lineages of cold-tolerant species that have endured cooling, and thus, phylogeographic patterns may reflect large-scale processes that were not reset by the ice ages. We hereby combine geological evidence from marine sedimentary basins, Andean orogeny, and climatology with molecular dating and statistical phylogeography to infer how geological and climatic processes affected the distribution of lineages in cold-tolerant *Nothofagus* species during the Cenozoic. A total of 239 populations along the entire range of all species within the genus *Nothofagus* (*N. antarctica*, *N. betuloides*, *N. dombeyi*, *N. nitida*, and *N. pumilio*) were sampled and analyzed by sequencing three non-coding regions of the chloroplast. We found 30 chloroplast DNA haplotypes that were geographically structured. Molecular dating calibrated with fossils revealed that ancestral lineages appeared in Eocene/Oligocene, whereas most divergences took place during the Miocene; in turn, Bayesian skyline plots showed that population expansion occurred in the Early Pleistocene (1.5–1 million years ago). Lineage divergence from all wide-ranging *Nothofagus* was spatially and temporally concordant with episodic marine transgressions and warmer times in Patagonia during Eocene/Miocene Epochs. Long-lasting stable raised areas preserved haplotype diversity throughout Patagonia, from where cold-tolerant taxa expanded their ranges during pre-Quaternary times. The detailed study of such ancient divergences is novel and allows us to infer the effects of geological processes on distribution patterns of ancient lineages, that is, phylogeology.

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INTRODUCTION

Phylogeographic studies use coalescent principles to trace biogeographic history based on DNA gene genealogies of multiple populations and species (Avise *et al.*, 1987). Such approaches have made a significant contribution to the interpretation of lineage distribution in response to climate changes, such as during glaciation events of the Neogene. As a result, they provided support for the existence of refugial areas and defined migration routes after glacial retreat for different taxa around the world (Hewitt, 2001). While abundant evidence supports the existence of refugia in ice-free areas located in warmer latitudes during the Last Glacial Maximum (LGM), other studies in temperate latitudes of South America have shown that cold-tolerant taxa probably survived *in situ*, where post-glacial population expansion occurred without major range shifts (see review in Sérsic *et al.*, 2011). Molecular data confirmed early stud-

ies based on fossil pollen (Markgraf *et al.*, 1995; Barreda *et al.*, 2007; Markgraf & Huber, 2010; Quattrocchio *et al.*, 2011), which in combination with ecological niche modeling provided conclusive evidence of long-term survival of cold-hardy species through the ice ages in multiple glacial refugia (*sensu* Premoli, 1998; Jakob *et al.*, 2009; Premoli *et al.*, 2010; Cosacov *et al.*, 2013). Species persistence throughout their current ranges implies that the genetic structure of such cold-hardy taxa would result in the conservation of ancient polymorphisms that were not reset by Quaternary glaciations (Premoli *et al.*, 2012). As a consequence, phylogeographic and phylogenetic patterns yielded by molecular data have probably overlooked the effects of alternative barriers to gene flow other than the presence of glaciers and unsuitable climates that affected the long-term persistence and divergence of the associated biota within a particular area. For example, while molecular diversification within the Amazon Basin was first linked to refugial areas, it was later agreed that regional diversification events are the result of complex geological scenarios such as orogeny that mostly preceded the ice ages (Antonelli *et al.*, 2009; Hoorn *et al.*, 2010).

The ancient genus *Nothofagus* has been an important focus for the discussions of biogeographical history. This was mainly due to its southern distribution in Gondwana, the fact that nuts have limited dispersal movement and contains an extensive and distinctive fossil record, particularly pollen with the oldest record dated in the uppermost Santonian (Hill & Read, 1991). Pollen diversity consists of clearly distinct morphological groupings (Dettmann *et al.*, 1990) that match the four extant clades *Fuscospora*, *Lophozonia*, *Nothofagus*, and *Trisyngyne* (ex *Brassospora*) (Hill & Read, 1991; Heenan & Smissen, 2013). By the Oligocene at the latest, the four clades were present and, since then, the amount of change, particularly for microfossils, has been relatively minor (Hill, 1991). Currently, species occur in austral forests of southeast Australia and Tasmania, New Caledonia, New Guinea, New Zealand, and southwestern South America. Monophyly and deep divergences, together with morphological concordance, has been recently claimed as an argument in favor of these four clades to be considered at the primary rank of the genus (Heenan & Smissen, 2013). Thus, the current genus *Nothofagus* is at present restricted to temperate areas of South America, although it was widespread among Gondwanan continents in the Cenozoic (Dettmann *et al.*, 1990). Most species within *Nothofagus*, although ecologically and genetically distinct, are similarly widespread and encompass the entire distributional range of temperate forests in southern South America, including the deciduous *N. antarctica* (G. Forst.) Oerst. and *N. pumilio* (Poepp. & Endl.) Krasser, as well as three evergreen species [*N. betuloides* (Mirb.) Oerst., *N. dombeyi* (Mirb.) Oerst., and *N. nitida* (Phil.) Krasser; Veblen *et al.*, 1996]. They share a similar pollen type, which allows hybridization between almost all species pairs (Premoli, 1996; Stecconi *et al.*, 2004; Quiroga *et al.*, 2005). Cycles of hybridization–introgression and chloroplast capture among extant and ancestral taxa result in concordant cpDNA phylogeographic patterns, whereas nuclear DNA (ITS) illustrates relationships among delimited species (Acosta & Premoli, 2010). As species divergence is younger than haplotype divergence found in *Nothofagus*, the chloroplast DNA (cpDNA) phylogeny can be used to trace the biogeographic history of lineages in complex landscapes (Premoli *et al.*, 2012).

Abundant marine deposits show that previous to the Andean uplift, Patagonia was characterized by a complex topography that significantly impacted on the configuration of suitable areas for plants (Ramos, 1982; Bechis *et al.*, 2014). However, to date, no effort has been made to analyze and integrate geological and molecular data to test the archipelago-like hypothesis (Premoli *et al.*, 2012) at small spatial scales, considering that the presence of marine transgressions and inner basin formation acted as effective barriers to gene flow. We hereby combine geological evidence from marine and lacustrine sedimentary basins, Andean orogeny, and climatology with molecular dating and statistical phylogeography based on a large dataset of DNA sequences of the chloroplast genome from the widespread South American cold-tolerant genus *Nothofagus*. We aim to infer how geological and climatic processes affected the distribution of *Nothofagus* lineages during the Cenozoic.

MATERIALS AND METHODS

Sampling of natural populations

Leaf material was collected from 474 individuals at 239 locations, each one representing one population, belonging to the five *Nothofagus* species (*N. antarctica*, *N. betuloides*, *N. dombeyi*, *N. nitida*, and *N. pumilio*) distributed throughout their entire geographic range in southern South America (Table S1, Fig. 1A). We emphasized sampling of numerous populations to obtain a complete geographic pattern of genetic variation, as the same chloroplast DNA haplotypes were present in many nearby populations from large geographic areas. To avoid errors due to misidentification of *Nothofagus* specimens and consequently erroneous sequences from GenBank (discussed by Acosta & Premoli, 2010), all samples used in this study were sequenced at Laboratorio Ecotono of Universidad Nacional del Comahue. The outgroup consisted of samples of *Betula pendula* Roth (MCA 221) and other *Nothofagaceae* species belonging to different clades. These included one individual of each *Fuscospora alessandri* Espin. (MCA 224), *F. fusca* (Hook. fil.) Oerst. (MCA 183), and *F. solandri* (Hook. fil.) Oerst. (MCA 184) and one individual of each *Lophozonia glauca* (Phil.) Krasser. (MCA 222), *L. menziesii*

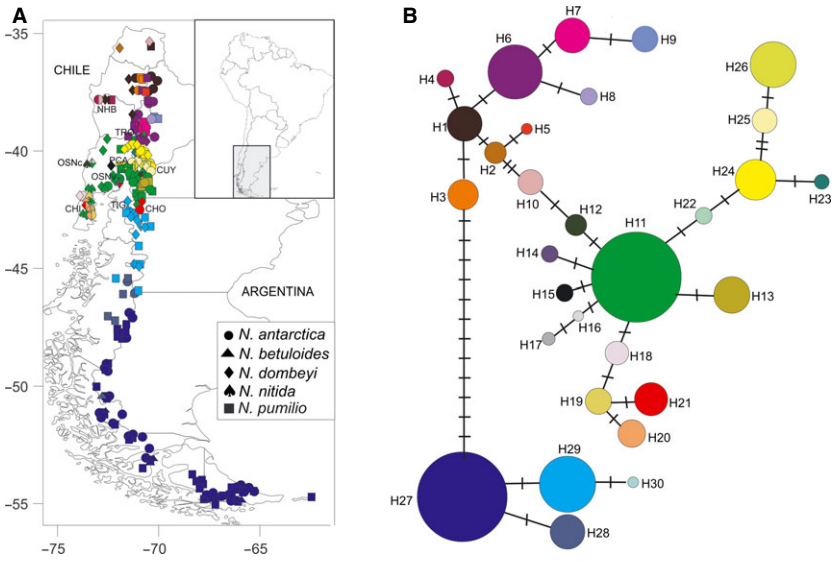

Fig. 1 Geographical distribution and genealogical relationships of the chloroplast DNA haplotypes found in *Nothofagus* in southern South America. (A) Symbols depict location map of the sampled *Nothofagus* populations of each species; their chloroplast DNA haplotypes (H) are indicated in colors. Codes of localities: NHB: Nahuelbuta National Park, TRO: Tromen Lake, PCA: Cardenal Samoré International Pass, CUY: Cullín Manzano, OSNc: Osorno city, OSNv: Osorno Volcano, CHI: Chiloé Island, TIG: Tigre River, CHO: Cholila. (B) Median-joining network of chloroplast DNA haplotypes.

(Hook. fil.) Oerst. (MCA 186), *L. moorei* (Muell.) Krasser. (MCA 223), *L. nervosa* (Phil.) Dim. and Mil. [= *L. alpina* (Poepp. & Endl.) Oerst.] (MCA & EK 136), and *L. obliqua* (Mirb.) Oerst. (MCA & EK 140), and two individuals of *L. cunninghamii* (Hook. fil.) Oerst. (MCA 185). Voucher specimens were deposited in the herbarium of Centro Regional Universitario Bariloche, Argentina (BCRU).

DNA extraction, amplification, and sequencing

Fresh tissue was kept in a portable cooler until arrival at the laboratory at Universidad Nacional del Comahue, Bariloche, Río Negro, Argentina. Total DNA was extracted with a DNeasy plant mini kit (Qiagen, Hilden, Germany). A reaction of 2 μL of DNA extract (10 ng) and 6 μL of GeneReleaser® (BioVentures Inc., Murfreesboro, TN, USA) was performed for 15 min at 85 °C (followed by a hold at 32 °C) prior to PCR to facilitate DNA release. Three noncoding regions of the chloroplast genome were amplified using primer pairs *psb*B-*psb*H (Hamilton, 1999), *trn*L-*trn*F (Taberlet *et al.*, 1991), and *trn*H-*psb*A (Hamilton, 1999). The PCR mix contained 2 μL of template DNA (10 ng), 0.625U GoTaq DNA polymerase (Promega, Madison, WI, USA), 5 μL 5× Green GoTaq® reaction buffer (Promega), 0.25 mM of each dNTP, and 0.3 μM of each primer in a total volume of 25 μL. The PCR cycling scheme consisted of an initial denaturation step at 95 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C, 54 °C, or 56 °C (for BH, LF, and HA, respectively) for 1 min, extension at 72 °C for 1:30 min, a final extension at 72 °C for 7 min and a final hold at 15 °C. All reactions were carried out on an Applied Biosystems 2720 thermocycler (Applied Biosystems, Foster City, CA, USA). Amplification products were separated by electrophoresis on a 1% agarose gel, stained with Syber Safe (Invitrogen, Eugene, OR, USA), and visualized with a UV transilluminator. PCR products were cleaned with Exonuclease I (Fermentas, Burlington, ON, Canada) and Shrimp Alkaline Phosphatase (USB, Cleveland, OH, USA). Cycle sequencing was performed using Big Dye terminator chemistry (Applied Biosystems) following the manufacturer's manual. Automated sequencing using both forward and reverse amplification primers was conducted on an ABI PRISM 3100 AVANT (Applied Biosystems) at the sequencing facility of Laboratorio Ecotono, Universidad Nacional del Comahue. All unique sequences were deposited in GenBank (Accession numbers: **BH:** GQ863274–GQ863275, GQ863285–GQ863286, GQ863367–GQ863370, GQ863397–GQ863399, GQ863401–GQ863402, GQ863405, GU152886–GU152887, GU152889, GU152891–GU152893, JN247414–JN247418; **LF:** GQ863302–GQ863303, GQ863313–GQ863314, GQ863371–GQ863374, GQ863379–GQ863381, GQ863383–GQ863384, GQ863387, GU152870–GU152871, GU152873, GU152875–GU152877, JN247419–JN247423; **HA:** GQ863330–GQ863331, GQ863341–GQ863342, GQ863375–GQ863378, GQ863388–GQ863390, GQ863392–GQ863393, GQ863396, GU152878–GU152879, GU152881, GU152883–GU152885, JN247424–JN247428). For all subsequent analyses, three chloroplast regions were concatenated for each individual.

Phylogenetic analysis

Sequences were aligned using MEGA 3.1 (Kumar *et al.*, 2004) with manual adjustments as needed. One sequence of each haplotype was used in the phylogenetic analyses. Phylogeny reconstruction under parsimony was conducted using the program TNT (Goloboff *et al.*, 2003). Heuristic searches included 1000 random addition replicates, TBR branch swapping, and gaps were coded following the 'simple indel coding' method (Simmons & Ochoterena, 2000). Length variation at the mononucleotide repeat (poly T) in the *trn*H-*psb*A sequence was not coded due to the uncertain homology of the sequence positions. Support for monophyly was determined by jackknife resampling of 1000 replicates using the same criteria as those used for the regular parsimony searches. Bayesian analysis was conducted using MRBAYES v. 3.1.2 (Huelsenbeck & Ronquist, 2001) with a model of sequence evolution generated by MRMODELTEST v. 2.2 (Nylander, 2004) that implemented the hierarchical likelihood ratio test (hLRT) and the Akaike Information Criterion (AIC). The evolution model that best fit chloroplast datasets was GTR + G (Rodríguez *et al.*, 1990). The analysis consisted of two independent runs of 3×10^6 generations with four chains each (three heated and one cold) and trees were saved every 100 generations in each run. Approximately, 10% of the trees (corresponding to the burn-in period) were discarded and a 50% majority rule consensus tree was constructed from the remaining trees.

Molecular dating

The split of the stem lineage *Nothofagus* from the outgroup *B. pendula* was considered as the root of the tree. Common sources of error when using fossils to estimate lineage divergence times include incompleteness, age estimation, taxonomic misidentification and/or invalid placement into the phylogeny of the fossil record (Near & Sanderson, 2004). To reduce these sources of error, we used the calibration points revised by Sauquet *et al.* (2012), which verify the phylogenetic placement and geochronological data of *Nothofagus* fossil record (Table S2). Divergence times were estimated using BEAST version 1.6.2 (Drummond & Rambaut, 2007). Three distinct calibration scenarios using safe but late and/or early but risky fossil age constraints were used, following the test suggested by Sauquet *et al.* (2012). The input file was prepared in BEAUTI version 1.6.2 (provided in the BEAST package). The substitution model was GTR with a Gamma site heterogeneity model with four categories, following the Mr. Modeltest result; the clock model was set as an uncorrelated log-normal relaxed model; and the Yule or Birth-death process was selected as a prior for the distribution of divergence dates. Fossil age constraints were all implemented as hard minimum bounds using uniform priors with arbitrarily large maximum bounds (1000 Ma), except for the maximum age constraint on the root, implemented using a normal distribution prior. The Monte Carlo Markov Chain was set to run for 1.5×10^7 generations, sampling every 100 cycles.

Population analyses

To examine genetic relationships among populations, a median-joining network was constructed using NETWORK v. 4.1.1.2 (Bandelt *et al.*, 1999). Ambiguous connections (loops) in the network were resolved using predictions from coalescent theory (Crandall & Templeton, 1993), principally the geographical criterion. A Mantel test (Mantel, 1967) was performed using GENALEX 6.2 (Peakall & Smouse, 2006) to evaluate the significance between genetic and geographic distances of populations across a landscape. To identify groups of populations, a spatial analysis of molecular variance SAMOVA v. 1.0 (Dupanloup *et al.*, 2002) was performed based on 100 simulated annealing steps. This program defines geographically homogeneous groups of populations maximizing the differences from each other, using the proportion of total genetic variance (FCT). Several runs were performed to evaluate different numbers of groups (K) from $K = 2$ to $K = 20$, to find the number of K where FCT reaches a plateau and the obtained groups of populations have different haplotypes.

Molecular diversity indices (Table 1) for each cpDNA marker separately (BH, LF, and HA), each species studied, and groups of populations defined by the SAMOVA analysis were calculated in ARLEQUIN v. 2.0 (Schneider *et al.*, 2000). Due to the unequal sample size, we estimated the number of haplotypes by adjusting the sample size to 10 individuals for each species and 20 individuals for each group (the smallest sample size was 21 individuals for species and 39 individuals for groups) with a rarefaction analysis using the program CONTRIB v1.02 (Petit *et al.*, 1998). A 'mismatch distribution' analysis was performed to distinguish between models invoking past exponential growth versus historical population stasis (Rogers & Harpending, 1992; Excoffier, 2004). A multimodal distribution of differences between haplotypes is usually found in samples taken from populations at demographic equilibrium, whereas the distribution is usually unimodal in populations that have passed through a recent demographic expansion (Excoffier, 2004). The goodness-of-fit of the observed mismatch distribution to that expected under a sudden expansion model was evaluated using parametric bootstrapping with the sum of squared deviations (SSD). A significant SSD ($P \leq 0.05$) indicates deviation from the null model of population expansion. In addition, Tajima's D (Tajima,

Table 1 Molecular diversity indices calculated for each cpDNA marker separately (BH, LF, and HA), each *Nothofagus* species, and each SAMOVA group identified for *Nothofagus* populations. Haplotype (*h*) and nucleotide (π) diversity, Tajima's D, Fu's F_S, sum of squared deviations (SSD), and estimated expansion time (ka) are shown

Grouping	*P*	*N*	Size	Range	*H*	CG%	Ps	ti	tv	*I*	*h* [after rarefaction]	π	*D*	F_S	SSD	Expansion time (confidence interval)
BH	239	474	791	776–791	2	36.94	18	0	3	15	0.427 (0.016)	0.010 (0.005)	3.011	10.122	0.368	
LF	239	474	437	417–437	8	34.02	23	1	1	21	0.707 (0.015)	0.011 (0.006)	0.146	−1.458	0.019	
HA	239	474	472	420–446	23	26.41	60	0	8	53	0.846 (0.010)	0.031 (0.015)	2.465	−1.175	**0.038**	
N. antarctica	89	133	1700	1632–1670	21	33.42	99	0	11	89	0.890 (0.016) [0.890 (0.016)]	0.016 (0.008)	2.371	0.160	**0.051**	1463 (46–11423)
N. betuloides	13	22	1674	1632–1670	5	33.44	55	0	10	46	0.797 (0.049) [0.797 (0.049)]	0.016 (0.008)	2.530	6.687	0.103	924 (120–5293)
N. dombeyi	48	94	1675	1632–1670	19	33.36	74	1	12	63	0.909 (0.015) [0.906 (0.016)]	0.010 (0.005)	0.098	−3.332	**0.011**	152 (9–413)
N. nitida	7	21	1671	1669–1670	4	33.32	7	0	3	4	0.595 (0.107) [0.595 (0.107)]	0.001 (0.001)	−0.592	1.203	0.458	1 (0–25)
N. pumilio	82	204	1673	1632–1670	14	33.44	72	0	11	62	0.860 (0.012) [0.860 (0.012)]	0.017 (0.008)	3.100	6.592	0.097	1071 (9–5332)
Group 1	17	39	1671	1649–1670	6	33.45	24	0	1	23	0.783 (0.036) [0.791 (0.032)]	0.004 (0.002)	0.297	−0.492	**0.002**	95 (56–143)
Group 2	24	59	1650	1632–1649	5	33.39	20	0	1	19	0.593 (0.057) [0.593 (0.057)]	0.001 (0.001)	−0.153	−0.576	**0.002**	56 (27–92)
Group 3	21	39	1670	1669–1670	4	33.35	5	0	3	2	0.548 (0.077) [0.548 (0.077)]	0.001 (0.001)	0.052	1.165	0.399	0
Group 4	79	192	1675	1632–1670	18	33.32	74	1	12	63	0.690 (0.035) [0.685 (0.035)]	0.003 (0.002)	**−1.783**	**−9.913**	**0.005**	71 (13–129)
Group 5	98	145	1659	1632–1659	4	33.55	27	0	0	27	0.530 (0.034) [0.530 (0.034)]	0.001 (0.000)	0.175	0.257	0.018	45 (30–69)

P, number of analyzed populations; *N*, number of analyzed individuals; *H*, number of haplotypes; %CG, CG content; Ps, number of polymorphic sites; ti, transitions; tv, transversions; *I*, indels. Alignment size without outgroups and range are indicated in base pairs (bp).Standard errors are indicated in parentheses. Results consistent with demographic expansion are shown in bold.

1989) and Fu's F_S (Fu & Li, 1993) tests of neutrality were calculated to detect range expansions. Significant negative values of Tajima's D and Fu's F_S indicate population expansion. The significance of both values was calculated from 1000 simulated samples using a coalescent algorithm. Neutrality tests and mismatch distribution analyses were performed using ARLEQUIN, and the latter were graphed in DNASP v.4.10.9 (Rozas *et al.*, 2003).

The change in effective population size of SAMOVA's grouping and the time as the beginning of an expansion was estimated through Bayesian skyline plots (Drummond *et al.*, 2005) as implemented in BEAST. The analysis used the optimal substitution model for each data subset using MRMODELTEST and a strict clock with a uniform distributed prior clock rate. Root height and the clock mean rate for each group were set according to the chronogram obtained with BEAST. A randomly generated tree was used as the starting tree with a coalescent Bayesian Skyline tree prior with 10 groups and a piecewise-constant skyline model. The MCMC was run for 50 million generations, sampling every 1000 generations. The Bayesian skyline plot was produced in TRACER v. 1.5 (available from the BEAST site) using the log file from the analysis in BEAST and discarding 10% of the runs as burn-in. In addition, to estimate the time since the beginning of an expansion, we used $\tau = 2ut$, where t is the time elapsed (in generations) between the initial and current population sizes, and $u = 2\mu k$, where μ is the mutation rate and k is the length of the sequence (Rogers & Harpending, 1992). We assumed a mean generation time of 25 years (Donoso, 2006).

RESULTS

Chloroplast DNA data

The sequence analyses of 474 individuals sampled from 239 *Nothofagus* populations yielded 30 distinct cpDNA haplotypes (Tables 1 and S1, Fig. 1A). Some haplotypes (H2, H5, H8, H14, H15, H16, H17, H22, H23, and H30) were unique to one population. *Nothofagus antarctica* and *N. pumilio* had two unique haplotypes each;

therefore, *N. dombeyi* was the species with the highest number of species-specific haplotypes (seven). The size of the *psb*B-*psb*H intergenic spacer was 791 bp in haplotypes H1–H26 and 776 bp in haplotypes H27–H30. The length of *trn*L-*trn*F varied from 417 in haplotypes H6–H9 to 437 bp in haplotypes H29–H30, whereas the intergenic spacer *trn*H-*psb*A was more variable than the others and ranged in size between 420 (H28) and 446 bp (H30). Alignment of the final matrix included 1700 bp and required the introduction of 17 gaps, ranging from 1 to 25 bp in length. Haplotypes H1–H26 had one insertion of 15 bp in *psb*B-*psb*H, haplotypes H6–H9 had one deletion of 17 bp in the *trn*L-*trn*F spacer, and haplotypes H10–H20 and H22–H26 had one insertion of 19 bp in *trn*H-*psb*A. Only haplotype H30 had one insertion of 25 bp. In addition, single-base deletions were found in a poly-T region at *trn*L-*trn*F and *trn*H-*psb*A, as well as in a poly-A region of the latter.

Phylogenetic analysis

Parsimony analyses of the aligned chloroplast data yielded 190 most parsimonious (MP) trees of length 118, with a consistency index (excluding autapomorphies) (CI) of 0.797, and a retention index (RI) of 0.948. The entire data matrix contained 83 parsimony informative characters. Bayesian inference (BI) and BEAST trees had the same in-group topology. The MP consensus tree has less resolution (more polytomies in the clade H1–H26) than BI and BEAST trees. The phylogenetic tree (Fig. 2) from cpDNA sequences of *Nothofagus* populations yielded two major divergent clades at 42° S latitude with high support values: a northern group (H1–H26; jk (jackknife support) = 94%, BPP (Bayesian posterior probability) = 1.00) and a southern group (H27–H30; jk = 73%, BPP = 1.00), which diverged at approximately 46 (95% confidence and highest posterior density intervals = 28–62) million years ago (Ma).

The northern group comprises two clades (H1–H9; BPP = 1.00) containing the northernmost analyzed populations (north of 39°S) and another clade (H11–H26, south of 39°S; BPP = 0.87) sister to H10 haplotype which, interestingly, is geographically located near clade (H1–H9). Most haplotype divergences took place between 30 Ma (95% confidence and highest posterior density intervals = 15–48 Ma) and 14 Ma (2–34 Ma). Other more recent divergences occurred at 11 Ma (1–33 Ma)–4 Ma (0.02–14 Ma), that is, haplotypes H6-H9 distributed from 36° 49′ S to 39° 36′ S (jk = 20%, BPP = 0.70), haplotypes H18-H21 (BPP = 0.77), which are found mainly in Chiloé Island, H22-H26 from 39° 36′ S to 40° 55′ S (jk = 28%, BPP = 0.99), and H29-H30 from 42° to 45° (BPP = 0.96). The most recent divergences were dated at 2 Ma (0.34–8 Ma) (H16–H17 and H25–H26) (jk = 65%, BPP = 0.97 and jk = 85%, BPP = 1.00).

Population analyses

In general, the configuration of the haplotype network (Fig. 1B) is consistent with the clades predicted by phylogenetic trees that recovered two main lineages. The haplotype H10 connects the two northern clades (H1–H9 and H11–H26). Within the northern group, H11 was the most frequent and widespread haplotype ($n = 104$) with the greatest number of mutational connections, from which other related haplotypes derive (H12–H26). Similarly, within the southern group, H27 was the most common haplotype ($n = 92$), and haplotypes H28–H30 derived from it.

A Mantel test suggested that genetic distances for cpDNA haplotype data were significantly and strongly correlated with geographic distance ($r = 0.617$, $P = 0.001$), suggesting a pattern of isolation by distance. SAMOVA was used to identify the subdivision that most likely explains the cpDNA haplotype structure observed in *Nothofagus* populations. The F_{CT} value increased asymptotically with increasing number of groups, leveling out at $K = 5$ (F_{CT} 0.931, $P < 0.0001$). The five groups of populations were latitudinally structured. Groups 1 and 5 consisted of northern-most and southernmost populations located north of 37°49′S (H1–H5 and H10) and south of 42°S (H27–H30), respectively (Table S1). The other three groups lay on both slopes of the Andes. Group 2 included northern populations located predominantly between 37°49′–39°36′°S (H6–H9); group 3 encompassed populations between 40°07′–40°55′S (generally H25–H26), and group 4 contained widespread populations in lowland areas between 39°14′ and 42°42′ S (H11–H24) including populations from Chiloé Island (Table S1).

The highest haplotype diversity was found in *N. dombeyi* and *N. antarctica*, followed by *N. pumilio* (Table 1). The northernmost populations (group 1) and those from the widespread group 4 showed the highest haplotype and nucleotide diversity (Table 1). Populations of highest haplotype diversity were on the western Coastal Cordillera (Nahuelbuta National Park and Coastal Range near Osorno); the Andes (Cardenal Samoré International Pass and Osorno Volcano) and nearby sites (Cullín Manzano); and Chiloé Island (between Castro and Ancud, and Huillinco). Secondary Contact zones between divergent lineages were found in Lanín National Park (near Tromen Lake) and further to the south, in Río Tigre and Cholila regions (Fig. 1A).

The sum of squared deviations (SSD) values (Table 1) indicated that *N. antarctica* and *N. dombeyi* have expanded recently. This range expansion was not supported by the other estimated parameters as Tajima's D and Fu's F_S. Nonetheless, *N. dombeyi* has a negative Fu's Fs (but not significant), which may suggest that this species has had a major range expansion. The combined mismatch analysis

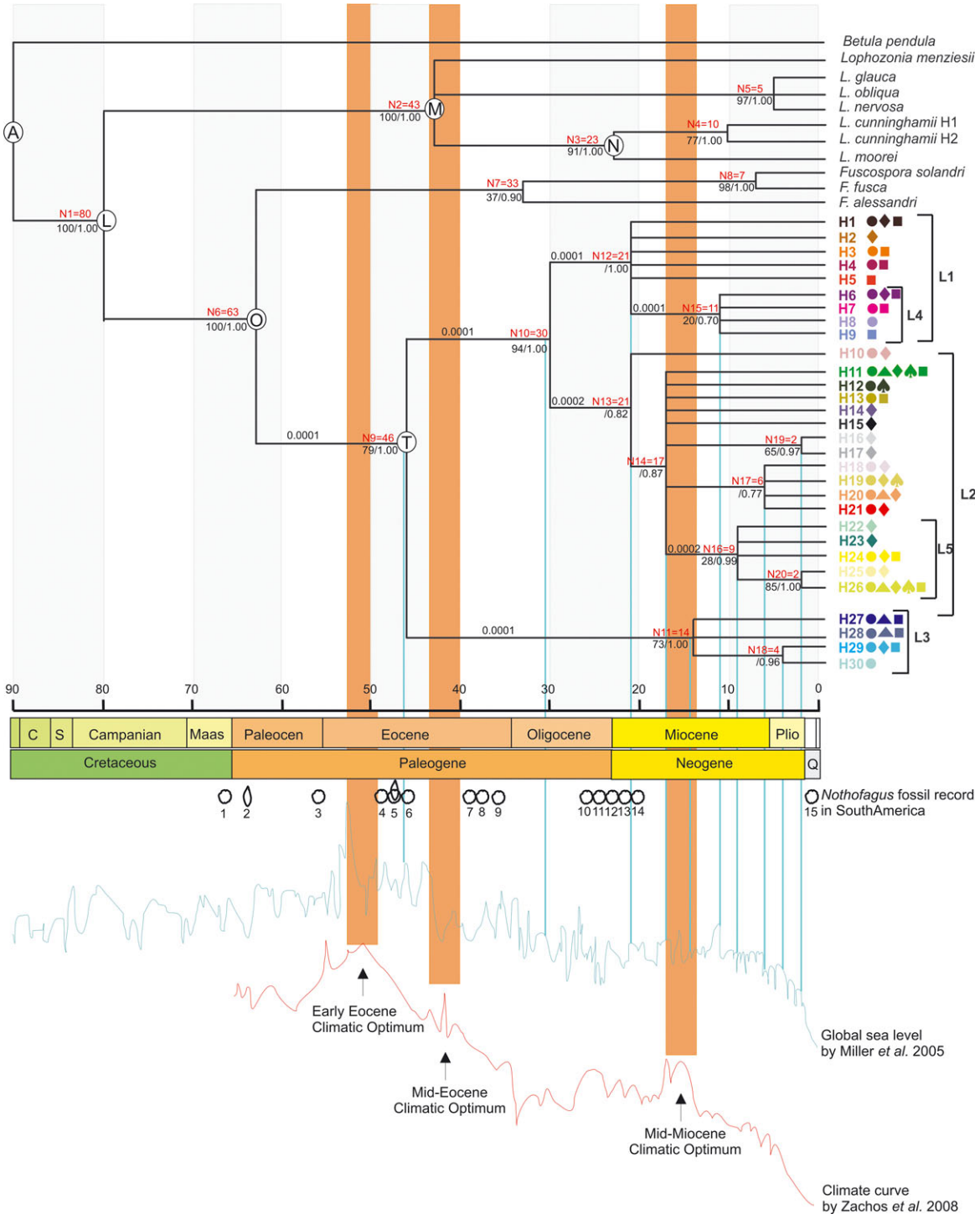

Fig. 2 Chronogram indicating the evolutionary relationships among chloroplast haplotypes (H) and lineages (L) found within *Nothofagus* in southern South America. Numbers above nodes indicate the age obtained from the molecular dating analysis. Mean substitution rate is indicated along each branch. Bootstrap (BS)/posterior probability (BPP) values for maximum parsimony and Bayesian inference analyses, respectively, are shown below branches. Letters in circles correspond to time constraints used by Sauquet *et al.* (2012). Symbols following haplotype names indicate species where they were found (symbol legend in Fig. 1A). C, Coniacian; H, Holocene; Maas, Maastrichtian; P, Pleistocene; Plio, Pliocene; Q, Quaternary; S, Santonian. Fossil record references: 1 = Cerro Dorotea Formation (Leppe *et al.* 2012); 2 = Salamanca Formation (Wilf *et al.* 2013); 4 = Río Pichileufu, Volkheimer com pers. (Wilf *et al.*, 2005); 5 = Ligorio Márquez Formation (Okuda *et al.*, 2006) 3, 6–9, 12 = Río Chico, La Huitrera, Río Turbio, Slogget, San Julián, and Centinela Formations, respectively (Quattrocchio *et al.*, 2011); 10 = Lileo Formation (Leanza *et al.*, 2002), 11 = Ñirihuau Formation (Bechis, 2004), 13 = Chenque Formation (Barreda, 1997); 14 = Valdivia Basin (Le Roux & Elgueta 2000); 15 = Markgraf & Huber (2010).

and significant SSD values showed a sudden expansion for groups of populations 1, 2, and 4, as defined by SAMOVA according to genetic and geographic affinities (Fig. 3A and Table 1). This expansion was generally supported by neutrality tests (in general negative Tajima's D and Fu's F_S), although these tests were not always statistically significant. The Bayesian skyline plot showed a pronounced increase in effective population size of all population groups by SAMOVA (Fig. 3B). Groups 1, 2, and 3 (northernmost populations) expanded about 1.5 Ma after a long phase of only a slight population size increase; and group 5 (southern populations) have expanded more recently, at 1 Ma. Noteworthy, the widespread group 4 with SSD, Fu's FS, and Tajima's D supporting expansion, showed a great population expansion at *ca.* 200 Ka. Estimates of demographic expansions based on the location of the crest of the unimodal mismatch distribution (see expansion time and confident intervals in Table 1) ranged from 45 Ka (group 5, belonging to southern populations) to 95 Ka (group 1, belonging to northern populations).

DISCUSSION

Deep genetic divergences with haplotype diversity throughout the current range of temperate forests support the idea that geological process, different from those of the glacial

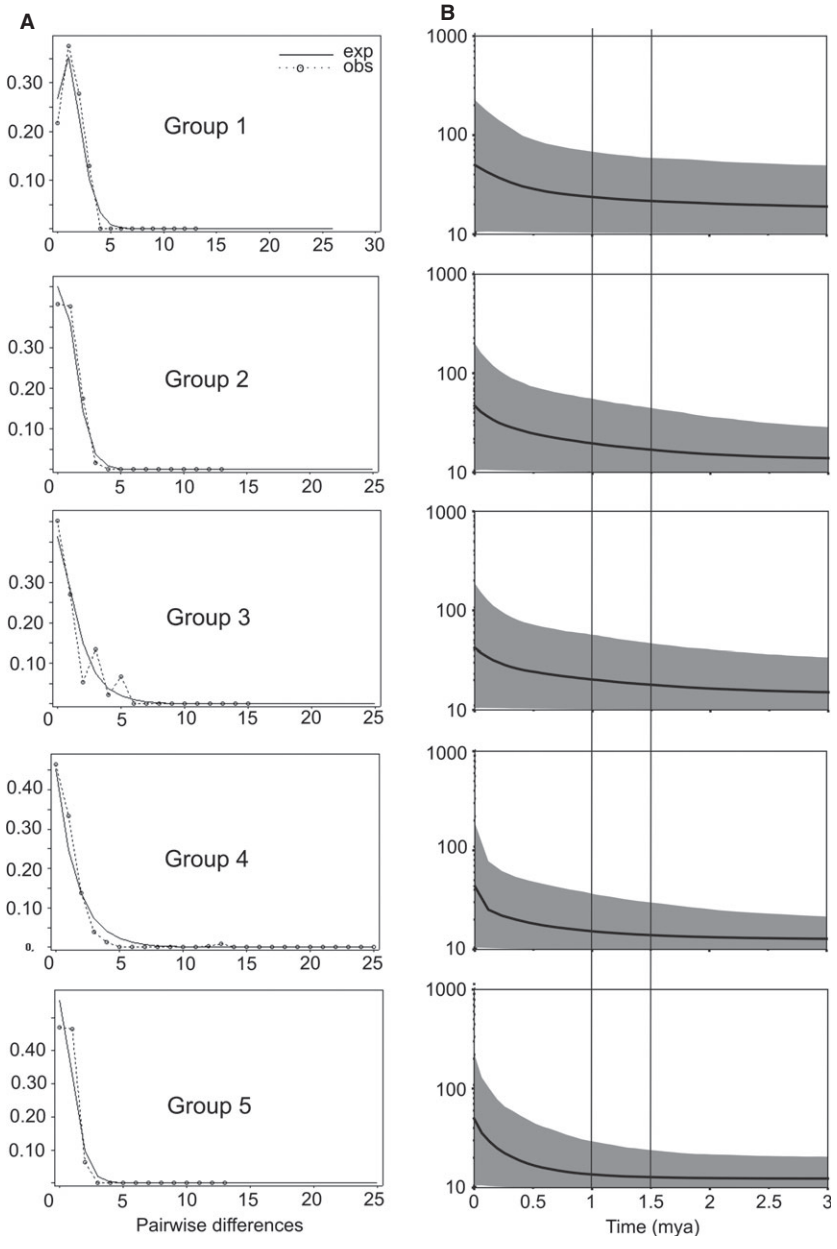

Fig. 3 Estimates of historical population changes. (A) Mismatch distribution graphs of pairwise comparisons of chloroplast DNA haplotypes in groups of *Nothofagus* populations. Expected and observed frequencies of pairwise differences in a growth/decline population model are indicated with solid and dashed lines, respectively. (B) Bayesian skyline plot.

history of the Neogene, shaped chloroplast *Nothofagus* lineages. The divergence between north and south clades at approximately 46 Ma is consistent with the early presence of *Nothofagus* fossils in Patagonia (Fig. 2; Wilf *et al.*, 2005; Okuda *et al.*, 2006; Leppe *et al.*, 2012; Wilf *et al.*, 2013). Until the Middle Eocene, *Nothofagidites* (*Nothofagus* pollen fossil) was scarce in the South American fossil record (Leanza *et al.*, 2002), most likely due to geological processes and climates that favored other warm-loving lineages (Zachos *et al.*, 2008). As a result, cold-tolerant *Nothofagus* ancestors probably existed as small and isolated populations.

The formation of sedimentary basins characterized the landscape of Patagonia since the Paleogene (Ramos, 1982). Marine ingressions that took place between 47.8 and 28.4 Ma north and south of 42°40′S, together with the extensive plateau basalts at mid-latitudes of Patagonia (Ramos, 1982; Asensio *et al.*, 2010; Malumián & Náñez, 2011), are probably related to a major latitudinal split (north and south clades) within *Nothofagus* forests (Premoli *et al.*, 2012; Fig. 4A). *Nothofagus* pollen fossils are known from geographically distant locations, such as Río Chico, La Huitrera, Slogget, and San Julián Formations from Middle Eocene to Early Oligocene (Fig. 4A; Quattrocchio *et al.*, 2011). This barrier may have impacted on the gene pool of other dominant Andean lineages, such as *Podocarpus* (Quiroga & Premoli, 2010), and may explain intraspecific disjunctions of several plant species at midlatitudes of Patagonia (Villagrán & Hinojosa, 2005).

Several depocenters are known from the Late Oligocene and Early Miocene of the Patagonian Andes (Fig. 4B, Asensio *et al.*, 2010; le Roux & Elgueta, 2000; Folguera *et al.*, 2003; Giacosa & Heredia, 2004; Charrier *et al.*, 2007; Paredes *et al.*, 2009; Hervé *et al.*, 1995; Marensi *et al.*, 2005). Recently, Bechis *et al.* (2014) constrained the age of this marine transgression within the Late Oligocene-Early Miocene until Middle Miocene. This extensive marine transgression could have produced the split between lineages L1 and L2 at 39°36′S from H10, and the divergence within lineages L1, L2, and L3 at 21, 17 and 14 Ma, respectively (Fig. 4C). Pollen fossils of *Nothofagus* were found in Lileo, Valdivia, Ñirihuau, Chenque, and Centinela Formations from the late Oligocene/Miocene boundary, suggesting a mountain forest environment of high humidity and cold climate (Barreda, 1997; le Roux & Elgueta, 2000; Leanza *et al.*, 2002; Bechis, 2004; Quattrocchio *et al.*, 2011). Ancestors of haplotypes H6–H9 diverged at 11 Ma, probably due to the Cura-Mallín basin from Middle Miocene (Zapata & Folguera, 2005). More recent haplotype divergences that occurred at 9, 6, and 4 Ma (Fig. 4D) may have been favored by marine transgressions at the late Middle Miocene, Late Miocene, and Early Pliocene. These ages have been assigned recently to Chilean formations near the Pacific coast and along the

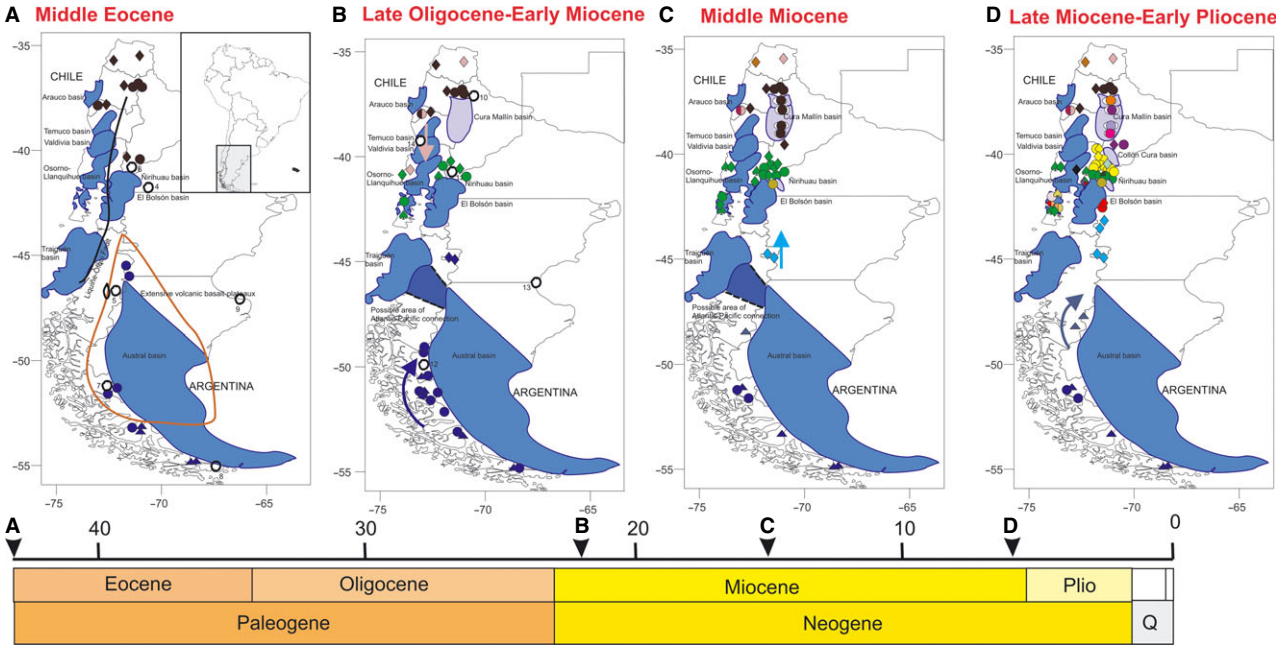

Fig. 4 Paleolandscape reconstruction of southern South America showing the Oligocene-Miocene (some from Eocene) boundary basins and the hypothetical distribution of populations and haplotype *Nothofagus* lineages. In (A), the Basalt plateau and Liquiñe-Ofqui fault are indicated with brown and black lines, respectively. In (B–D), colonization routes are shown following the color of haplotype lineage expansion. Pollen or leaves indicate the location of fossil records; references in legend of Fig. 2. Light and dark blue shaded areas depict lacustrine and marine environments, respectively. White areas within basins indicate mountain that became islands when marine transgression occurred.

Chilean Central Depression (Encinas *et al.*, 2007; Finger *et al.*, 2007). Noteworthy, H13 is currently found between Ñirihuau and El Bolsón basins, indicating the possible separation area (Fig. 4C). Toward the south, haplotype H28 probably diverged, flanked by the Traiguén and Austral Basins to the north and south, right in the area where the possible connection between Atlantic and Pacific oceans probably existed (Bechis *et al.*, 2014); then, this haplotype expanded from the south to the north (Fig. 4D).

If inner basins and marine transgressions became isolated and promoted lineage divergence, *Nothofagus* populations may have persisted in emerged areas, as evidenced by their great haplotype diversity (which is coincident with the ages of mountains from Fig. 3 in Folguera *et al.*, 2011), thus supporting the archipelago hypothesis (Premoli *et al.*, 2012). Relatively stable Coastal Cordillera emerged by the Late Triassic, whereas the southern Andes were developed as discrete phases in latest Cretaceous, late Eocene, latest Oligocene to Miocene, and late Quaternary (Glodny *et al.*, 2008; Folguera *et al.*, 2011). Ancestors of lineage L1 probably subsisted in stable areas toward the northern range of the Coastal Range, such as at the Nahuelbuta Formation, where it became isolated from lineage L2. In addition, fragmented raised inland areas on the main current Andean Range at 40°S (Gonzalez Díaz, 1979; le Roux & Elgueta, 2000), the mid-latitude Coastal Range (Ramos, 1982), and positive areas of northern Chiloé Island (Ramos, 1982; Duhart & Adriasola, 2008), could have favored the long-lasting persistence of relatively widespread haplotypes of lineage L2.

Once marine transgressions receded probably as a result of the uplift of the Andes Mountain during the Miocene, two major routes followed the expansion of north and south haplotype lineages. These colonization routes may also be linked to the uplift, timing and direction, of the southern Andes rise. Northern lineage L1 expanded to the south (Fig. 4B) from stable areas of the Coastal Range at Nahuelbuta Formation. Southern haplotypes derived from lineage L3 expanded to the north (Fig. 4B) from stable areas, such as Río Turbio and Slogget Formation (Palazzesi & Barreda, 2007) and highlands between Traiguén and Austral Basin (Fig. 4D). In addition, the uplift of the main Andes Range during Miocene times originated a series of small intermontane depocenters within the Cura Mallín Basin (García-Morabito & Ramos, 2008). The presence of these elevated terrains could explain the divergence of lineage L4 (Flynn *et al.*, 2008; González Díaz & García Morabito, 2010). The split in lineage L5 probably occurred because of flooded areas between 39°–40°S (Ramos, 1982; Zapata & Folguera, 2005) during Miocene times. The west-to-east distribution of H21 throughout the Andes could indicate that Chiloé Island may have been part of the continent in the past, at 6 Ma or less. This may have occurred during periods of lower sea level, such as glaciations, when the coastline extended further out into the sea, producing the connection of the flora (Rabassa & Coronato, 2009).

Cold-loving *Nothofagus* lineages have shown to be a good indicator of climatic events. They have been historically less affected by low temperatures and high mountains may have also acted as refuges in warmer periods. The highest haplotype divergence coincides with the Mid-Miocene Climatic Optimum (see Fig. 2; Zachos *et al.*, 2008). These warmer temperatures probably forced small *Nothofagus* populations to find refuge in cooler areas in high mountains. On the contrary, major changes in climate at the Eocene/Oligocene boundary from 'glasshouse' to 'icehouse' conditions (Huber & Nof, 2006) resulted in the development of rather cooler trends. Fossil pollen data from Patagonia shows the irruption of *Nothofagus* forests by Middle Eocene-Early Oligocene, suggesting a marked cooling trend at this time (Barreda & Palazzesi, 2007; Palazzesi & Barreda, 2007). Furthermore, while the uplift of the main Andean Cordillera in Oligocene/Miocene times restricted the dispersal of *Nothofagus* ancestors because of the increased aridity in the current Patagonian steppe (Barreda & Palazzesi, 2007), mountain areas created new cooler habitats for species colonization. Surprisingly, the ages of most cpDNA divergence (ranging from 21 to 2 Ma) obtained are in agreement with the age estimates obtained using nuclear ITS sequences of the origin of current species (ranging from 22 to 1 Ma, Premoli *et al.*, 2012). Hence, the same climatic and tectonic settings that impacted in the origin of chloroplast genetic variants probably have produced speciation in *Nothofagus*.

Nonetheless, lineage divergence time is not concordant with the last bottleneck suffered by studied populations. While most haplotype divergence generally occurred during Oligocene/Miocene times, the last bottleneck suffered by the studied lineages was about 1.5–1 Ma, which agrees with the Great Patagonian Glaciation dated 1.55–1.02 Ma (Rabassa & Coronato, 2009). In addition, estimates of demographic expansions began before the LGM occurred at *ca.* 20 Ka, particularly those of northernmost and mid-latitude groups (ranging from 95–56 Ka) and, more recently, the southern group (45 Ka). In general, environmental conditions and forest extent prior to 40 Ka were probably comparable with the present one (V. Markgraf pers. comm.), as evidenced by the high number of *Nothofagus* fossil records from 140 to 21.5 Ka (Valero-Garces *et al.*, 2005; Heusser *et al.*, 2006; Markgraf & Huber, 2010).

CONCLUDING REMARKS

Glaciations of the Quaternary have been largely used to explain phylogeographic patterns of different temperate areas of the world (Hewitt, 2001), including Patagonia

(see reviewed articles in Sérsic *et al*., 2011). While they have undoubtedly impacted on species' gene pools, for cold-tolerant taxa, such as *Nothofagus*, which endured the ice ages in multiple refugia (*sensu* Premoli, 1998), genetic variants may also reflect tectonic forces of the Eocene/Miocene Epochs. This genetic diversity was preserved over time and reinforced by recurrent cycles of hybridization/introgression (Acosta & Premoli, 2010) among extinct and current taxa, which diverged since the Oligocene (Premoli *et al*., 2012). The analysis of multiple populations by molecular dating methods and statistical phylogeography in combination with geologic, climatological, and paleontological data allow us to infer the effects of basins and marine transgressions, orogeny, and climatic changes on the distribution of ancient lineages, that is, phylogeology.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Collection sites, vouchers specimens, haplotypes, and SAMOVA groups of the studied *Nothofagus* populations.

Table S2 Fossils considered in the calibration points (adapted from Sauquet *et al.*, 2012).

Table S3 Bayesian relaxed molecular clock age estimates (Ma) for different chloroplast DNA haplotype clades within *Nothofagus*, according to different calibration scenarios and tree priors using BEAST.